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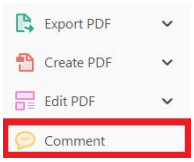

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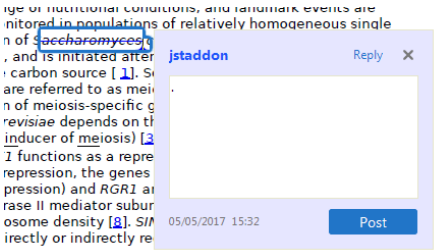

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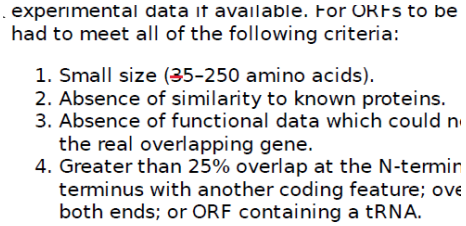

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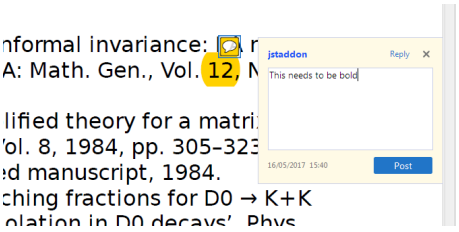

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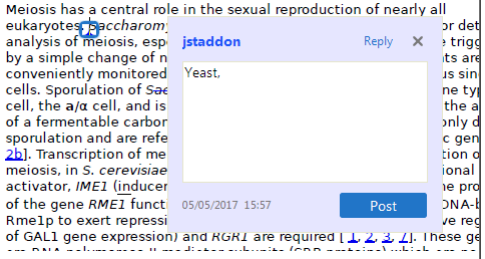

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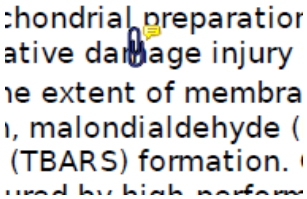

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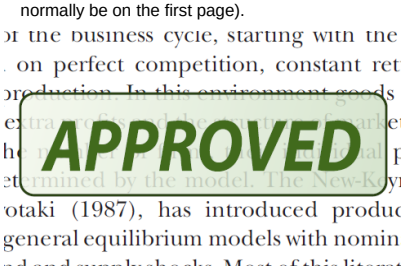

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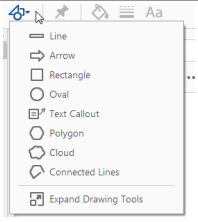

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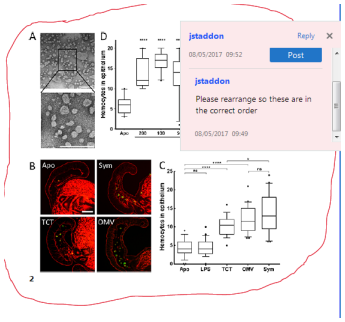

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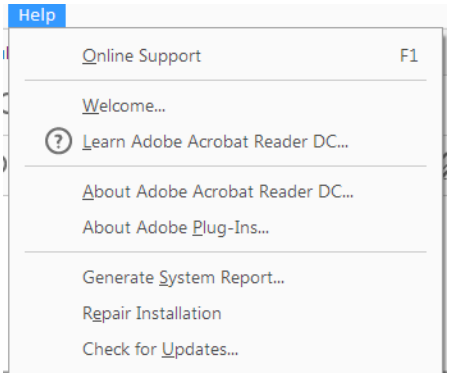

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REVIEW

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DNA vaccines for fish: Review and perspectives on correlates of protection

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Abstract

Recently in 2016, the European Medicines Agency (EMA) recommended granting a marketing authorization in the EU for "Clynav," a DNA vaccine against salmon pancreas disease (salmonid alphavirus-3). Generally, DNA vaccines induce both early and late immune responses in fish that may be protective against disease. Several transcriptomic approaches have been performed to map immunome profiles following DNA vaccination, but the precise immune mechanism(s) that is responsible for protection is not known, although reasonable suggestions have been made. The current review includes an overview on main transcriptomic findings from microarray experiments after DNA vaccination against VHSV, IHNV, HIRRV and IPNV—with considerations of what can be considered as correlates of protection (CoP) or merely a surrogate of protection. Identification and use of correlates of protection (COPs) may be a strategic tool for accelerated and targeted vaccine design, testing and licensure. General rules on what can be considered as CoPs can be extracted from past knowledge on protective immune responses following vaccination that induced protection. Lastly, there will be an overview on non-viral molecular adjuvants that have been exploited to obtain higher vaccine potencies and efficacies.

KEYWORDS
aquaculture, correlates of protection, DNA vaccines

1 | INTRODUCTION

As traditional oil-based vaccines show similar efficacies as the bacterial DNA vaccines (~~Holvold,~~ Myhr, & Dalmo, 2014), the need for a bacterial DNA vaccine for fish is not as urgent as antiviral ones. Despite an enormous amount of effort invested in the development of DNA vaccines to protect veterinary animal species and humans against viruses, only a few have reached the market. In fact, only three have been licensed and reached a commercial level, from over 420 different DNA vaccine candidates that have been investigated in laboratory trials over the past 25 years. A substantial number of these even entered preclinical testing (cf. ClinicalTrials.gov and www.violinnet.org/dnavaxdb) (Racz, Li, Patel, Xiang, & He, 2014).

The three veterinary DNA vaccines that have been commercialized so far are as follows:

1. "West Nile Innovator® DNA" (Fort Dodge Animal Health/Pfizer) for protection of condors and horses against West Nile virus (Chang, Davis, Stringfield, & Lutz, 2007).
2. Apex-IHN® (Aqua Health Ltd., an affiliate of Novartis Animal Health Inc.) for the protection of salmonids against Infectious Hematopoietic Necrosis virus (IHNV) (Salonius, Simard, Harland, & Ulmer, 2007).
3. The cancer DNA vaccine "Oncept" (Merial) targeting dog melanoma (Mclean & Lobetti, 2015).

More than 20 different virus DNA vaccines have been developed experimentally for prophylactic use in fish targeting viruses such as rhabdoviridae, orthomyxoviridae, togaviridae and nodaviridae. The rhabdoviridae DNA vaccines (e.g., VHSV and IHNV) have shown high levels of efficacies, whereas others have in most instances possessed moderate to low efficacies (~~Holvold~~ et al., 2014; Munang'andu & Evensen, 2015).

2 | WHAT ARE THE CORRELATES OF PROTECTION FOLLOWING IMMUNIZATION?

To find and define correlates of protection (CoPs) may be highly beneficial in terms of future vaccine development. Based on surveys (e.g., meta-analysis) of vaccine efficacies and evaluation of mechanistically relevant immune responses governing disease protection, CoPs may be defined. This would ease development of more efficacious vaccines and vaccines against related pathogens (Plotkin, 2010). A correlate of protection (CoP) is a protective immune response—an immune marker statistically correlated with vaccine efficacy. The CoP may likely be divided into mechanistic (mCoP) and non-mechanistic CoP (nCoP), where the former is causally responsible for protection and the latter is not (but may still be regarded as a CoP) (Plotkin & Gilbert, 2012). For example, an immune signature not directly causative for disease protection may be regarded as nCoP, whereas bactericidal antibodies may be mCoP. CoP has been defined for many of the currently licensed human vaccines. Following vaccination, certain concentrations (threshold units conferring protection) of specific antibodies have been shown to be CoP against several bacterial toxins and invariant viruses. The measurements of antibodies can easily be performed while the role of T-cell-mediated immunity in disease protection can be complicated to assess (Milligan & Barrett, 2015), especially in fish. In fish, no systematic effort has been made to define correlate(s) of protection; although it is widely acknowledged that both the induction of antiviral innate immunity and antibody response are vital protecting fish against disease (Anderson et al., 1996; Long, Richard, Hawley, Lapatra, & Garver, 2017; Lorenzen et al., 1998; Mclauchlan et al., 2003; Standish, Millard, Brenden, & Faisal, 2016).

The CoP may be highly dependent on the mode of vaccination (e.g., immunogen, dose, formulation, prime-boost regime), tissue-specific response to infection and vaccination, and the particular pathogen (Plotkin, 2013). It has been shown in fish that a high vaccine dose (antigen dose) induces increased protection (Dubey et al., 2016; Munang'andu, Fredriksen, Mutoloki, Dalmo, & Evensen, 2013). In these dose–response studies, the antibody responses correlated with vaccine efficacies.

Following immunization of fish, the immune response may be tissue specific or compartmentalized, as suggested by several researchers (Encinas et al., 2010; Magadan, Sunyer, & Boudinot, 2015; Salinas, 2015; Swan, Lindstrom, & Cain, 2008; Yamaguchi, Takizawa, Fischer, & Dijkstra, 2015). Whether a compartmentalization of immune response (e.g., intestinal/branchial/dermal immune response) may result in increased or decreased protection during pathogen challenge of immunized fish may not be evident per se. It may be dependent on the portal of entry of pathogens and where replication occurs. To search for a CoP during vaccination of fish, one may consider whether a compartmentalization has occurred or not.

Surrogate of protection may be defined as "immune marker that can substitute for the clinical end point and, thus, can in some instances be used to reliably predict vaccine efficacy. DNA vaccination may induce both an early innate, and a late systemic and memory response in the host—both being protective (Plotkin & Gilbert, 2012). This two-stage event should both be considered as correlates of protection following DNA vaccination (Plotkin, 2010).

No systematic effort has yet been made to search for and define CoP(s) in fish after immunization and pathogen challenge. However, there are numerous reports on gene expression after vaccination and infection that may be considered as a good starting point in the search for mCoP and nCoP. Based on several microarray experiments, it is clear that a high number of genes are up- and downregulated following DNA vaccination—evaluated after bioinformatics analysis (Table 1). Examples are as follows: IRF3, IRF7, TLR8, Mx, ISG15, ISG56, Vig-1, Vig-8 and IFN-α1. It is highly acknowledged that type I interferons and interferon-stimulated genes (ISGs) contribute to protection from viral invasion and replication (Schneider, Chevillotte, & Rice, 2014; Wong & Chen, 2016)—and it is suggested that this also is the case for fish (Chang, Robertsen, Sun, & Robertsen, 2014; Robertsen, 2017; Zhang & Gui, 2012). The immune signature (e.g., expression of Mx, IFNs) observed in fish after DNA vaccination might be statistically correlated to protection. But in most instances, no careful assessments have been performed to statistically correlate the level of signature molecule(s) with protection. It is my opinion that there may probably be present a statistical correlation between expression of certain antiviral genes after DNA vaccination with survival from pathogen challenge, but this has to be carefully assessed. An example is a study performed by McLauchlan et al., where rainbow trout at different age were injected with VHS DNA vaccine and later challenged with homologous pathogen. Immunized fish contained highly elevated expression of Mx mRNA (liver); the elevated expression was correlated with early protection after VHS DNA vaccination—although no statistical analyses were performed (Mclauchlan et al., 2003). It can be speculated that elevated expression of Mx or some of the (signature) genes listed above (Table 1), and their products, may likely be surrogates of protection.

It is not an easy task to define the correlate(s) of protection after DNA vaccination of fish. One may look both on the early induction of antiviral mechanisms, and a later antiviral effector phase together with the formation of specific antibodies. In addition, there may be considerable differences with respect to correlate(s) of protection against different pathogens—although there may be a certain degree of recognizable overlapping pattern among induced genes following antivirus DNA vaccination. It appears that interferons and certain ISGs may be vital for protection from number of viruses (Liu, Sanchez, Aliyari, Lu, & Cheng, 2012; Wong & Chen, 2016), and most probably also in fish (Chang, Jenssen, & Robertsen, 2016; Chang et al., 2014; Langevin et al., 2013; Purcell, Laing, & Winton, 2012). However, different ISGs may also increase virus infectivity, as shown in experiments using various cell lines (Schoggins et al., 2014). Apparently, there might be strong correlation between interferon and/or certain ISGs and disease protection, but a more comprehensive study must be performed to find out the exact correlates or surrogates of protection in vivo in fish. There may also be organ- or tissue-specific correlates or surrogates of protection (e.g., mucus

TABLE 1 Selected genes up- and downregulated, analysed by microarray experiments of fish tissues and cells, from DNA-vaccinated fish

Species	Vaccine against	Route	Analysis	Target organ(s)	Time point dpi	Central genes upregulated	Central genes downregulated	Antibody response	Ref
Turbot	VHSV	I.m	Microarray	Head kidney	72	IRF3 IRF7 TLR8 Mx IFI-56 Caspases-6, 7, 8 and 10 CD9 CD83 CD209	NF-kappa-B inhibitor ζ IKKß IFN-2	ND	Pereiro et al. (2014)
Japanese flounder	HIRRV (pHRV-G)	I.m	Microarray	Head kidney cells	7	ISG15 ISG56 Mx	ND	ND	Yasuike, Kondo, Hirono, and Aoki (2007)
	HIRRV (pHRV-N)					IgM Nephrosin NADPH oxidase factor1		ND	
Japanese flounder	VHSV	I.m	Microarray	Head kidney (ex vivo)	3	Mx C3 CD20	ND		Byon, Ohira, Hirono, and Aoki (2006)
					21	C3 TNF superfamily IgM MHC invariant chain NF-κB Transferrin		+	
Rainbow trout	IPNV	Oral	Microarray	Head kidney	7	STAT1a Type1 IFN-1 Mx1, 2 and 3 Pentraxin Vig-4 Vig-5 Vig-1 b88 CD11 TLR2 TLR5 m TNF10 TNF11 TNF14 Scya109 IL-11 IL-10 Leap2 CD163 CD98 CD166	Type 1 IFN-a Type1 IFN-a Type1 IFN-3 Type1 IFN-4 Stabilin Lect2 Scya113 Hep2 CD209a CD80/86s	ND	Ballesteros, Saint-Jean, Encinas, Perez-Prieto, and Coll (2012)

(Continues)

TABLE 1 (Continued)

Species	Vaccine against	Route	Analysis	Target organ(s)	Time point dpi	Central genes upregulated	Central genes downregulated	Antibody response	Ref
				Pyloric caeca	7	Mx3 IgM Properdin Perforin TLR5 m TLR9 TNF13 Hep2a CD2 CD163pre CD3e	i-p30 IRF1 C3 C5 TLR5 TLR8 TNFa TNFr iNOS iNOS IL-1ß IL-10 IL-22 IL-17 Cath CD276 CD79a CD33 CD80/86 m CD8 CD86	ND	
Rainbow trout	IHNV (pIHNw-G)	I.m	Microarray	Injection site	7	IFN-γ IRF-3 Mx-1 Vig-1 Vig-8 Phox p40 TCR-ß CD8-α mIgM sIgM IgT TNF-α1 TNF-α2	NS	ND	Purcell et al. (2006)
				Head kidney		IFN-a1 IFN-g IRF-3 Mx-1	TCR-ß CD8-a IL-1ß		

(Continues)

TABLE 1 (Continued)

Species	Vaccine against	Route	Analysis	Target organ(s)	Time point dpi	Central genes upregulated	Central genes downregulated	Antibody response	Ref
				Spleen		IFN-α1 IRF-3 Mx-1 Vig-1 Vig-8	NS		
				Gill		Mx-1 Vig-1 Vig-8	NS		

IRF, interferon regulatory factor; TLR, Toll-like receptor; Mx, dynamin-like large GTPases—myxovirus resistance protein; IFI56, interferon-induced 56-kDa protein; Caspases, family of protease enzymes playing essential roles in programmed cell death; CD9, a member of the transmembrane 4 superfamily, also known as the tetraspanin family; CD83, a marker molecule for mature dendritic cells (DC) and is expressed on activated B and T cells; CD209, DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin)—a marker for dendritic cells; ISG, interferon-stimulated gene; **CD20**, it is an activated-glycosylated phosphoprotein expressed on the surface of all B cells; C3, complement component C3 central in the classical and alternative complement pathways; TNF, tumour necrosis factor (TNF) superfamily refers to a superfamily of cytokines that can cause cell death (apoptosis); **MHC**, major histocompatibility complex; NF-κB: nuclear factor kappa B; Transferrin, iron-binding protein; STAT, signal transducer and activator of transcription; IFN, interferon; Pentraxin, evolutionary conserved family of proteins with a pentraxin protein domain with a pattern recognition activity; VIG, VHS virus-induced gene; B88, a VHS-induced gene; CD11, one part of the CD11/18 integrin-mediating leucocyte adhesion or complement C4 receptor; SCYA, rainbow trout chemokine; IL, interleukin; LEAP, liver enriched antimicrobial peptide; CD163, scavenger receptor often found on monocytes and macrophages; CD98, a part of the large neutral amino acid transporter; CD166, Properdin, regulator of the alternative complement cascade; Perforin, a pore-forming cytolytic protein found in the granules of cytotoxic T lymphocytes (CTLs) and NK cells; Hep, hepcidin—a antimicrobial peptide; CD2, surface cell adhesion molecule on T cells and NK cells; CD3, T-cell coreceptor helps to activate CD8 + and CD4 + T cells; i-p30, same as IFI30; C5, a part of the membrane complex after enzymatic cleavage; iNOS, inducible nitrogen oxide synthase; Cath, cathelicidin—a antimicrobial peptide; Phox p40, cytosolic protein assembled to other proteins forming the NADPH oxide complex; **Ig**, immunoglobulin; TCR, T-cell receptor; **CD8**, a transmembrane glycoprotein that serves as a coreceptor for the T-cell receptor; CD276, a protein in the immunoglobulin superfamily often associated with certain tumours; **CD79**, a transmembrane protein that forms a complex with the B-cell receptor; CD33, a transmembrane receptor expressed on cells of myeloid lineage. "Classical" adaptive genes are listed in bold. ND, Not determined. NS, Not significant.

Activated leukocyte cell adhesion

tissues) where mucus-associated antibodies mediate protection against invading pathogens (Plotkin, 2008), and in theory—commensal microflora that may regulate pathogenicity of invading pathogens (Hu & Pasare, 2013).

Downregulated genes (Table 1) may be as important as upregulated ones in terms of an immune response—as there these may represent central "checkpoints" or "controlling units" during an inflammatory response. Such checkpoint genes may be surrogates of protection. An example is regulation of IL-10, which aid to prevent excessive inflammation induced damage to cells and tissues that may help controlling bacterial load or vice versa (Brooks et al., 2006; Redford et al., 2010). Other checkpoints may include the expression and activities of T-box transcription factors T-bet and eomesodermin, where during chronic viral infection T-bet is reduced in virus-specific CD8+ cells and are dysfunctional. Eomesodermin, often elevated during chronic virus infection, may in turn induce elevated cytotoxic responses even though CD8+ cells with high eomesodermin expression produce lower amount of antiviral cytokines (Paley et al., 2012). The programmed death 1 (PD-1) and its ligands may also be considered as immune checkpoint—where exhausted CD8+ T cells display high expression of PD-1. This may also be the case during chronic virus infection (Keir, Butte, Freeman, & Sharpel, 2008).

3 | THE NEED FOR STANDARDIZATION OF VACCINATION PROTOCOLS

One may expect that innate immune genes may be highly regulated at early time points post-immunization followed by adaptive immune genes some weeks after. From Table 1, the majority of the analyses were performed on tissues/cells up to 7 days post-immunization. Most of the genes may thus home to the innate immunity category —although there is a vital interplay with the adaptive immune mechanisms (Iwasaki & Medzhitov, 2015). As analyses have been performed on samples obtained from different time points and under different environment, it is hard to compare sets of results from various experiments within one fish species, and between fish species. Standardized protocols should be developed for each species, and possibly one should use "day degrees" instead of days (Standish et al., 2016)—as long as the different fish species have their own optimal environmental temperature for robust immune responses (Alcorn, Murra, & Pascho, 2002; Bowden, 2008; Cecchini & Saroglia, 2002; Magnadottir et al., 1999; Rijkers, Frederix-Wolters, & Van Muiswinkel, 1980).

The vaccine dose is another parameter of scrutiny. Any vaccine dose should be standardized with respect to fish size, that is, μg pDNA per kilo body weight, although the dose needed for protection may vary between vaccines, and from one fish species to another. This would ease any comparison between different experimental results. On the other hand, the experimental vaccines (plasmid vectors) in use in different laboratories are not often exactly similar (e.g., level of unmethylated CPGs) to each other (Williams, Carnes, & Hodgson, 2009); this would also lead to differences with respect activation and levels of gene expression.

4 | STRATEGIES TO INCREASE DNA VACCINE EFFICACY

Increased disease protection may be directly correlated with a high vaccine dose. There are, however, other ways to develop more efficacious vaccines than simply increase the dose of antigen/DNA, for example, by the introduction of genes encoding molecular adjuvants in the same DNA vaccine vector, or as a vaccine cocktail that consists the DNA vaccine together with another plasmids encoding regulatory proteins. This concept has not yet been very well explored in fish, but a few reports exist. In one study, the potential use of interferon regulatory factor-1 (IRF-1) as a vaccine adjuvant in Japanese flounder was investigated. IRF-1 has been shown to have a role in cytokine signalling and host defence against pathogens. The co-injection of IRF-1 encoding plasmid with a DNA vaccine encoding the major capsid protein (MCP) gene of red sea bream iridovirus (RSIV) resulted in elevated amount of virus neutralizing antibodies but was not significantly different from that in the fish vaccinated with the RSIV DNA vaccine alone (Caipang, Hirono, & Aoki, 2005). In another study, increased antibody and longevity responses were observed in salmon injected with plasmids encoding the molecular adjuvant IFNc (type I interferon) (Robertsen, Chang, & Bratland, 2016).

DDX4 helicase assembled with STING is a cytosolic protein capable of binding DNA that may induce type I IFN and cytokine production (Zhang et al., 2011). An experimental DNA vaccine consisting of VHSV glycoprotein G plus DDX4 was injected in olive flounder. Following immune induction of 15 and 30 days, the fish were challenged by VHSV. The improved DNA vaccine showed higher vaccine efficacy than the DNA vectors containing VHS-G gene and DDX4 gene alone did (Lazarte et al., 2017). This DDX4-adjuvanted G-protein encoded vector did, during the immune induction phase (day 14 post-injection), induce high levels of INF-1, IRF-3, ISG15 and Mx transcripts.

In another study, a plasmid encoding the pro-inflammatory cytokine IL-1ß was evaluated for its potential to boost the antibody response against BSA (bovine serum albumin) and GFP (green fluorescent protein encoded in a co-injected plasmid) in Japanese flounder. After 30 days of immune induction, the IL-1ß-encoded plasmid induced higher antibody response against BSA and GFP, albeit statistically non-significant, against BSA and GFP compared to "empty" plasmid or BSA alone (Taechavasonyoo, Hirono, & Kondo, 2013).

Interleukin 8 (IL-8) is a CXC chemokine produced by many cell types in mammals (e.g., macrophages, monocytes and fibroblasts) following infection, or stimulation by other cytokines such as IL-1ß and tumour necrosis factor alpha (TNF-α). In mammals, chemokines have been widely used as adjuvants in vaccines against viral infections, as they attract leucocytes to the site of inflammation and regulate the immune functions of the recruited cells. In fish, IL-8 has been characterized in rainbow trout among other species, and its chemo-

attractant properties established (Harun, Zou, Zhang, Nie, & Secombes, 2008). In this species, a vaccine plasmid encoding for the glycoprotein gene of VHSV was co-injected with a plasmid encoding IL-8 to explore its potential adjuvant effect (Jimenez, Coll, Salguero, & Tafalla, 2006; Sanchez, Coll, & Tafalla, 2007). When the plasmid encoding IL-8 (pIL-8$^+$) was administered together with the VHSV vaccine, an increase in IL-1ß in the spleen was found together with a higher level of cellular infiltration at the site of injection. Furthermore, fish injected with pIL-8$^+$ alone showed a significantly higher expression of TNF-α, IL-11, TGF-ß and IL-18 in the spleen (Jimenez et al., 2006). The transcription of different inducible CC chemokines was studied in rainbow trout in response to both the viral haemorrhagic septicaemia virus (VHSV) DNA vaccine and/or pIL8$^+$. This study demonstrated that pIL-8 modulated expression of other chemokines such as CK5A, CK6, CK7 and CK5B (Sanchez et al., 2007). The concept of DNA vaccination of fish may be considered quite mature compared to other veterinary animal species—given the high degree of knowledge, but why is there not more focus on molecular adjuvants increasing vaccine potency and efficacy against hard-to-combat viruses? One might consider strategies to co-inject plasmid DNA with immunostimulants of PAMP nature to induce more robust antiviral responses.

Transient overexpression and gene "knockout" systems, such as described above, may also indicate which immune molecules or mechanisms that may be considered as correlates of protection, or surrogates of protection. The concept and strategy using molecular adjuvants may anyway pave the way for renewed effort in research and development to yield more efficacious DNA virus vaccines. One may tailor virus species-specific DNA vaccines—based on prior knowledge on the correlates or surrogates of protection. Any unwanted non-target effects due to the molecular adjuvants, such as inducing exaggerated levels of, for example, cytokines, must be properly addressed.

5 | FUTURE VACCINE DEVELOPMENT

To meet the challenge to develop efficacious vaccines, systems vaccinology approach using both transcriptomics, epigenetic, proteomics and metabolomics platforms together with bioinformatics may be necessary (Hagan, Nakaya, Subramaniam, & Pulendran, 2015). Such approach should be highly conceivable as many institutions have the proper infrastructure and expertise ensuring such a holistic advancement. Following whole-genome sequencing projects for major aquaculture fish species, there are now better opportunities to analyse transcriptomic and proteomic responses following vaccination. The new next-generation sequencing (NGS) technology has not yet been used in vaccine research and development for fish. The detailed information that can be achieved from NGS, might in theory, speed the vaccine development significantly to yield high-efficacious vaccines. NGS may also be used to investigate epigenetic modifications following vaccination—that may be useful to add knowledge on how and how much individual fish (e.g., non-responders) and families respond to vaccines, and how vaccines might induce epigenetic changes resulting in modulated gene expression.

6 | CONCLUSION

An optimal vaccine must be able to induce innate mechanisms, a sufficient antibody response, induce T-cell response(s) and generate specific immune memory in the host fish species. In this respect, Apex-IHN DNA vaccine has proved to be very successful, while other DNA vaccines against other piscine viruses are in the advanced pipeline, for example, "Clynav" being developed by Elanco (formerly Novartis Animal Health) against pancreas disease virus. To define correlates of protection is a significant challenge towards the development of vaccines against current and emerging viruses. Transcriptomic, proteomic, metabolomic and epigenetic profiling during immune induction and infection would be the so-called "untapped goldmine" (Flanagan, Noho-Konteh, Ghazal, & Dickinson, 2013) that would provide a solid foundation for a rational vaccine development against the "hard-to-combat" infectious pathogens.

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