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Review

A comparative review on European-farmed finfish RNA viruses and their vaccines

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ABSTRACT

The diseases causing the highest ecological and socio-economical impacts in European farmed finfish are produced by RNA viruses. Salmon, trout, sea bream, sea bass, carp and turbot, suffer viral nervous necrosis produced by *betanodaviruses* (VNNV), infectious pancreatic necrosis produced by *aquabirnaviruses* (IPNV), viral haemorrhagic septicemia (VHSV) and infectious haematopoietic necrosis (IHNV) produced by *novirhabdoviruses*, spring viremia of carp produced by *vesicular-like rhabdoviruses* (SVCV), salmon pancreas disease and trout sleeping disease produced by *alphaviruses* (SAV) and infectious salmon anaemia produced by *isaviruses* (ISAV). There are not yet any effective treatments other than destroying all fish in infected farms, avoiding fish movements to and from infected areas and, in some particular cases, vaccination. The comparative study of the molecular characteristics of those RNA viruses and the state of knowledge of their vaccines, point to the development of new DNA vaccines for some RNA viruses, design of new mass delivery methods, maternal transfer of immunity, more extensive crossprotection studies between genotypes, use of safer all-fish plasmid control elements and study of DNA plasmid distribution after vaccination, as some of the major gaps that need urgent filling. In addition, to obtain similar protection levels to those produced by viral infections in survivors, live attenuated and/or some oil-adjuvanted inactivated virus vaccines, molecular adjuvants and/or other viral components (dsRNA or viral proteins interfering with fish defences), might have to be included in new DNA vaccine formulations. Furthermore, to be approved by the corresponding European authorities, fish viral DNA vaccines would also require the study of the persistence in fish of the introduced DNA, their possible impact to the aquatic environment and the acceptance of potential consumers.

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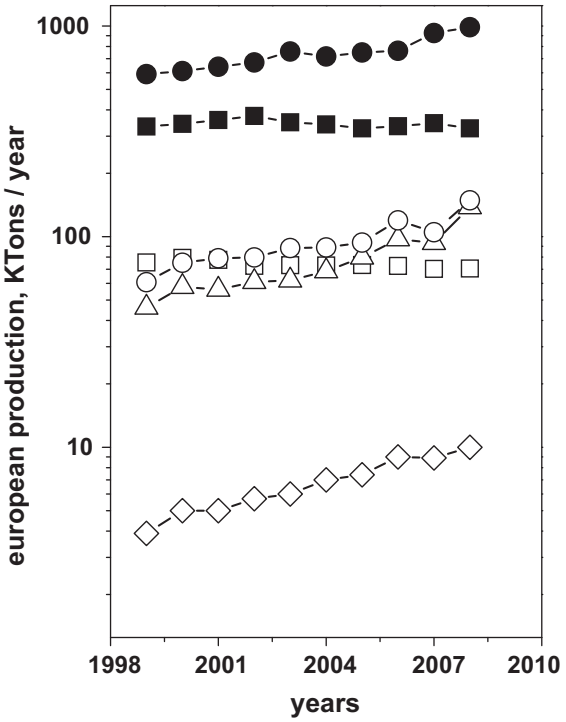

Fig. 1. Relative production during recent years of European farmed finfish species, data from the Federation of European Fish Producers (FEAP), published in their Aquamedia web page (http://www.aquamedia.org). Production levels and rates of other finfish species are much lower (not shown). (●) Salmon; (■) trout; (□) carps; (○) sea bream; (△) sea bass; (◊) turbot.

1. Major European diseases affecting farmed finfish caused by RNA viruses

The most important finfish production by European aquaculture involves 6 fish species (Fig. 1) distributed among different geographical locations (Fig. 2). Thus during the last ~10 years the highest annual productions were of Atlantic salmon *Salmo salar* (~900 kt/year), rainbow trout *Oncorhynchus mykiss* (~320 kt/year), sea bream *Sparus aurata* (~150 kt/year), sea bass *Dicentrarchus labrax* (~140 kt/year), carp *Cyprinus carpio* (~70 kt/year) and turbot *Scophthalmus maximus* (~10 kt/year). While the production of salmon, trout and carps remains stabilized, the production of sea bass, sea bream and turbot shows a tendency to continue their growth (Fig. 1). Finfish production levels and/or their rates of growth serve for an approximated estimation of the relative ecological and socio-economic impacts of the viral diseases they suffer. Because of the high-mortality viral diseases cause in farmed finfish, and the lack of specific treatments or practical prevention methods such as vaccines, many of those diseases are notifiable to the OIE (Office International des Epizooties) (http://www.oie.int) and/or to the European Union [1].

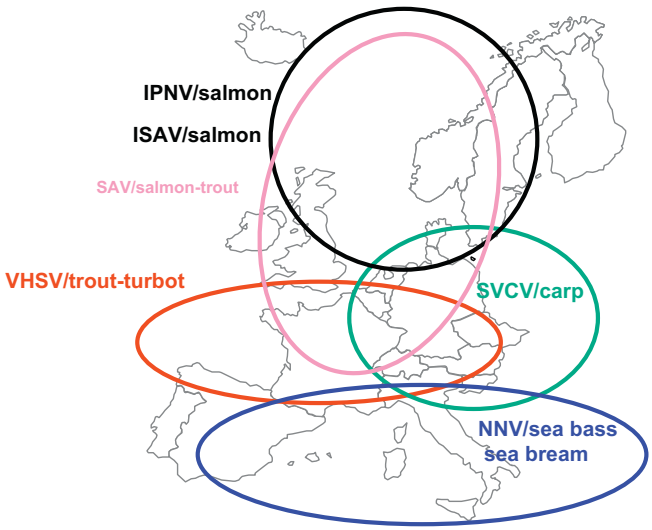

Fig. 2. Approximated geographical distribution of viral diseases of European farmed finfish. The main viruses and the finfish species affected are: VNNV (sea bream and sea bass), IPNV (salmon), VHSV and IHNV (trout and salmon), SVCV (carps), SAV (salmon and trout) and ISAV (salmon).

Viral diseases of finfish are spreading with time as suggested by the increasing number of outbreaks in farms during the last years. Furthermore, in some cases, the same viral diseases have been detected on captured wild finfish species other than those being farmed [2,3]. The most important viral diseases affecting the European farmed finfish species are caused by different genuses, belonging to 6 different families (Table 1). Thus, these viruses distribute among the following genuses and fish: *betanodavirus* (affecting sea bream and sea bass), *aquabirnavirus* (affecting most finfish species in their juvenile stages and causing severe losses during Atlantic salmon release from fresh to ocean water), *novirhabdovirus* (affecting mostly to trout but spreading to more than 50 other finfish species), *vesiculo-like rhabdovirus* (affecting carps), *alphavirus* (affecting salmon and trout) and *isavirus* (affecting salmon). These viruses have either a single (+ sense or – antisense) or double stranded RNA genome, of 5–14 kb of length present in 1–8 different RNA molecules, and coding for 2–10 different proteins. With respect to the sequence variation of their specific genes, they have 3–9 genotypes as identified by phylogenetic groupings within each viral specie (Table 1). Most relevant for the possible design of vaccines are the identifications of the specific proteins which are the targets for fish neutralizing antibodies (N-Abs) and/or some of the proteins which interfere with fish immune responses (Table 3). The RNA viruses distribute geographically in Europe according to their optimal replicating temperatures ranging from 10 to 24 °C. Finally, the present status of development of their vaccines illustrates various stages of vaccine development (Tables 4–6).

In this work, the most important RNA viruses affecting European finfish will be shortly reviewed from the point of view of their molecular characteristics and corresponding vaccines. To comparatively refer to vaccine efficacy, protection is traditionally expressed as relative percent survival (RPS) to viral challenge as calculated by the formula: [1 – (mortality in vaccinated fish/mortality in non-vaccinated fish)] × 100, however, the cumulative percent mortality (CPM) of the non-vaccinated control groups challenged under the same conditions than the vaccinated fish, must be at least of 60%, to correctly interpret the RPS value [4].

2. Viral nervous necrosis viruses (VNNV)

Fish viruses causing viral encephalopathy and retinopathy (VER), also known as viral nervous necrosis (VNN), have been isolated from more than 35 finfish species, many of them important to the European aquaculture industry such as sea bass and sea bream [5,6]. Because they can spread both horizontally and vertically from mother to offspring, early prophylaxis was focused on preventing transmission from broodstock fish to eggs/larvae [7]. Thus, in acute juvenile infections of sea bass or sea bream, these viruses might cause up to 100% mortality, nevertheless they might also produce a persistent infection, giving raise to asymptomatic carriers where no typical disease signs are detectable.

Table 1
General characteristics of the most important RNA viruses affecting European farmed finfish.

Family/genus	Viral species	Type of virion	[a]Fish Host	Temp (°C)	No. of segments/genome	Size (~kb)	Geno types	No. of coded proteins
Nodaviridae/betanodavirus	VNNV	Non enveloped Icosahedral	Sea bass Sea bream	25	2 ssRNA+	5	4–5	2
Birnaviridae/aquabirnavirus	IPNV	Non enveloped Icosahedral	Salmon Trout	10	2 dsRNA	5	7-9	4
Rhabdoviridae/Novirhabdovirus	VHSV	Enveloped Bullet	Trout Turbot	12	1 ssRNA–	11	4	6
Rhabdoviridae/Novirhabdovirus	IHNV	Enveloped Bullet	Trout	12	1 ssRNA–	11	4	6
Rhabdoviridae/Vesiculovirus	SVCV	Enveloped Bullet	Carp	16	1 ssRNA–	11	4	5
Togaviridae/Alphavirus	SAV	Enveloped Spherical	Salmon Trout	10	1 ssRNA+	12	6	4
Orthomyxoviridae/Isavirus	ISAV	Enveloped Polymorphic	Salmon	10	8 ssRNA–	14	3	10

Viral species and family/genus have been designed according to the vs7 of the International Committee on Taxonomy of Viruses [9], except for VNNV which is a general term, since other 4 species are presently described in the *betanodavirus* genus. VNNV, viral nervous necrosis viruses; IPNV, infectious pancreatic necrosis viruses; VHSV, viral haemorrhagic septicemia viruses; IHNV, infectious haematopoietic necrosis viruses; SVCV, spring carp viremia viruses; SAV, salmon alphaviruses; ISAV, infectious salmon anaemia viruses. Ss, single stranded. Ds, double stranded. Temp, optimal temperature of natural outbreaks. Sequences from European fish viral isolates are being deposited in a recent data base established for fish pathogens at the European Community Reference Laboratory for Fish Diseases at the National Veterinary Institute in Aarhus Denmark (http://www.fishpathogens.eu/)

[a] Aquacultured finfish hosts most importantly affected by the virus [1].

Viruses belonging to the VNNV group are non-enveloped with icosahedral symmetry, their genome consisting of bipartite single-stranded, positive sense RNA. Thus, their larger genomic segment, RNA1 (3.1 kb), encodes an RNA-dependent RNA polymerase, while the smaller genomic segment, RNA2 (1.4 kb), encodes the capsid protein precursor (C) (Table 1). It is during viral RNA replication, when a subgenomic RNA3 (0.4 kb) synthesized from RNA1 and encoding the non structural protein B2 is transcribed and expressed. B2 induces necrotic cell death [8] (Table 2).

All VNNV belong to the *Nodaviridae* family within the *betanodavirus* genus with the striped jack VNNV as their type specie [9]. In this review we will use VNNV as a general term to refer to this group of related viral species until they are finally classified [7,9].

According to partial sequences of their capsid (C) gene, some authors have classified *betanodavirus* isolates from Europe, Asia and Japan into 4 genotypes (19–23% sequence differences between different genotypes) [10,11]. Those different species/genotypes are: striped jack (SJNNV), barfin flounder (BFNNV), tiger puffer (TPNNV) and redspotted grouper (RGNNV). While RGNNV has been most frequently isolated from diseased marine fish from warm-water, BFNNV has been found mostly in cold-water fish [24]. Furthermore, a *betanodavirus* isolate from turbot has been suggested as a fifth genotype [12]. The above mentioned species/genotypes seem to correspond to 4 known serotypes independently of their geographical locations [10,11].

The host fish neutralizing antibodies (N-Abs) target the C protein of VNNV capsids (Table 3). On the protein C, amino acid residues at positions 1–32, 91–162 and 181–212 defined some of the fish target epitopes as mapped by pepscan binding of both neutralizing monoclonal Abs (N-MAbs) and serum samples from VNNV-infected sea bass [13]. Furthermore, the 181–212 and 254–256 positions were also recognized by other N-MAbs [14]. Those neutralizing target epitopes could be used to develop future vaccines based on their corresponding derived peptides and/or DNA sequences, however because the 223–331 region contains most of the C VNNV sequence variability, crossprotection studies among locally abundant isolates might be needed to develop practical vaccines [10].

To date different types of vaccines have been tested for VNNV, including those made with inactivated VNNV [15,16], virus-like particles (VLPs) [17–19], recombinant C protein [20,21] and synthetic peptides from the C protein [22] (Table 4). For instance, vaccination with *Escherichia coli* expressed recombinant C has given good results in different species of fish. Thus, significant protection and virus-neutralizing activity in the sera, was demonstrated in turbot juveniles and other fish species with an oil-adjuvanted intraperitoneally (i.p.) injected recombinant C fragment from striped jack VNNV [20,23]. Similar results were obtained by mixing recombinant C proteins from 3 Japanese isolates from the red spotted grouper VNNV, when challenged with an Indonesian VNNV isolate [21]. However as mentioned above and because of the differences in protection levels, a multivalent vaccine might

Table 2
Identified viral proteins which interfere with fish immune responses.

Virus	Viral component	Fish interferences	References
VNNV	B2	Necrotic cell induction	[8]
IPNV	IPNV	No apoptosis induction	[169,170]
	VP5	Anti-apoptosis	[33]
VHSV	NV	Pathogenicity?	[58,86]
	VHSV	Apoptosis induction	[60,61]
IHNV	NV	Pathogenicity?	[58,86]
	M	Apoptosis induction	[62]
SVCV	NR	NR	
SAV	SAV	Apoptosis induction	[131,171]
	E2	Apoptosis induction	[132]
ISAV	NS	Anti-IFNs	[140,141]

NV, non virion; NS, non structural; NR, not reported. Viral proteins that interfere with host defences identified to date, such as those inducing apoptosis or interfering with IFN signalling, have been included in this table because their effects might need to be counteracted to increase the efficacy of vaccines, although this possibility has not yet been tested.

Table 3
Viral proteins which have been identified as targets for finfish neutralizing antibodies (N-Abs).

Virus	Short name	No. of Cys	Size (~kDa)	Supramolecular structure	~No. per virion	3D PDB no.
VNNV	C	4	35	Multimer	180	2Q23 1F8V 2Z2Q 1NOV
IPNV	VP2	5	54	Multimer	180	31DE 3FBM 2DF7 3IDE
VHSV	G[a]	16	65	Trimer	300	2J6J 2CMZ
IHNV	G[a]	16	65	Trimer	300	2J6J 2CMZ
SVCV	G[a]	15	65	Trimer	300	2J6J 2CMZ
SAV	E2[a]	18	55	Heterodimer	ND	2ALA 1Z8Y 1RER
ISAV	HE[a]	11	42	Trimer	ND	3KU3

C, capsid protein; VP2, viral protein 2; G, glycoprotein; E2, fusion protein; HE, hemaglutinin-esterase; ND, not determined. Cys, number of cysteines present in the mature protein. 3D PDB no., data from X-ray solved crystal structures of proteins from viruses of fish, human or bird hosts at the Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank (http://www.rcsb.org/pdb/home).

[a] Glycosilated proteins.

Table 4
Selected examples illustrating the use of inactivated viruses and/or recombinant/DNA vaccine types, delivery methods and challenge results with finfish RNA viruses.

Virus	Fish host	Type	Antigen	Delivery method	μg/fish	Fish weight (g)	Protection to challenge[a]			References
							HVC	RPS	CPM	
VNNV	Grouper[b]	Inact. VNNV	VNNV	i.p.	–	25	No	40–96	55–87	[24]
	Turbot	RecProt	C	i.p.	100	570	No	82	20–44	[20]
	Turbot	RecProt	C	i.p.	10	22	Yes	67	39	[27]
	Grouper	RecProt	C	i.m.	60	28	No	69–88	65–85	[23]
	Turbot	DNA	C	i.m.	20	22	Yes	−7	43	[27]
IPNV[c]	Trout	Bacu. VLP	A	Imm.	500/ml	<1	Yes	0–42	9–33	[44]
	Salmon[d]	Bacu. VLP	A	i.p.	200	55	Yes	27	77	[44]
	Salmon	DNA	A + VP2	i.m.	15	20	No	84	33	[48]
	Salmon	DNA	VP2	i.m.	25	20	No	29	33	[48]
	Trout[e]	DNA	VP2	Oral	10	1–2	No	67–84	80–90	[52]
VHSV[c]	Trout	DNA	G	i.m.	1	3–5	No	94–100	84–98	[107]
	Flounder	DNA	G	i.m.	10	3	No	93–100	73–100	[172]
	Trout	DNA	G	i.m.	10–50	13	No	94–97	93	[173]
	Trout	DNA	G	i.m.	1	2	No	96	78–83	[105]
	Trout	DNA	G	Imm.	10/ml	6	No	50	63–100	[113]
IHNV[c]	Salmon	DNA	G	i.m.	25	57–73	No	90–100	50–70	[174]
	Trout	DNA	G	i.m.	1	2	No	94	70–80	[105]
	Trout	DNA	G	i.m.	<1	2–3	Yes	100[f]	92–96	[108]
SVCV	Carp	DNA	G	i.m.	50–100	10–11	No	33–48	64	[125]
	Carp	DNA	G	i.m.	10	1–4	No	50–88	70–100	[124]
SAV	Salmon	Inact. PD	PD	i.p.	–	30	No	0	80[g]	[175]
	Trout	Rec.SAV	SAV	Inf.	–	0.5	No	100	78	[135]
ISAV	Salmon	Inact. ISAV	ISAV	i.p.	–	10–50	No	70–94	72–98	[146]
	Grouper	Inact. ISAV	ISAV	Imm.	–	0.2	No	79–95	85	[16]
	Salmon	DNA	HE	i.m.	15	20	No	39–60	41	[147]

(–) No data; RecProt, recombinant proteins derived from virus; Bacu, baculoviruses; Att, attenuated; VLP, virus-like particles; i.p., intraperitoneal injection of oil adjuvants (water–oil emulsions); i.m., intramuscular injection of aqueous DNA solutions; Imm., immersion in water solution containing the vaccine; HVC, heterologous viral challenges; RPS, relative percent survival as calculated by the formula [1 − (mortality in vaccinated fish/mortality in non-vaccinated fish)] × 100; CPM, cumulative percent mortality of the non-vaccinated control groups. CPM must be at least of 60% to correctly interpret RPS [4].

[a] Numbers are shown without decimals.

[b] Grouper, *Epinephelus coioides*.

[c] The number of published results for challenge tests with IPNV, VHSV or IHNV are very numerous [4]. Thus only some of the data with comparative purposes and representative of different approaches are shown here.

[d] Salmon, Atlantic salmon except for IHNV tests.

[e] Trout, rainbow trout, except for this case in which both rainbow and brown trout were used.

[f] RPS at 3 months after vaccination (66 RPS at 25 months after vaccination).

[g] Pathological lesions, no mortalities.

Table 5
Fish vaccines to RNA viruses that have been commercialized.

Virus	Company	Name	Country licensed	Fish host	Type	Viral antigens	Delivery method	Information[a]
VNNV	–	–	–	–	–	–	–	–
IPNV	Pharmaq AS Norway	Alpha Ject® 1000	Norway, Chile, UK	Salmon >39 g	Inactivated Monovalent	IPNV	i.p. injection	pharmac.govt.nz pharmaq.no
	Aqua Health Ltd., Novartis, Canada	Birnagen Forte	Canada	Salmon	Inactivated Bivalent	IPNV	i.p. injection	ah.novartis.com
	Centrovet Ltda Chile	IPNV	Chile	Salmon >30 g	Inactivated Mono/poly	IPNV	i.p. injection	centrovet.com
	Intervet-International BV The Netherlands	Norvax® Minova-6	?	Salmon >35 g	RecProt.	VP2	i.p. injection	[49] aqua.intervet.com
	Microtek International Inc. British Columbia, Canada	SRS/ IPNV/Vibrio	Canada Chile	Salmon >10 g	RecProt Trivalent	VP2	i.p. injection	microtek-intl.com
VHSV	–	–	–	–	–	–	–	–
IHNV	Aqua Health Ltd, Novartis, Canada	APEX-IHN	Canada	Salmon[b]	DNA	G	i.m. injection	[104] ah.novartis.com/
SVCV	Bioveta, Czech Republic	?	?	Carp	Inactivated	SVCV	i.p. injection	[121] bioveta.cf actually not offered
	Pharos, S.A., Belgian	?	?	Carp	RecProt Baculovirus	G	i.p. injection	[123] No web
SAV	Pharmaq AS Norway	PD	?	Salmon	Inactivated	SAV	i.p. injection	pharmac.govt.nz/ pharmaq.no
	Intervet-International BV The Netherlands	Norvax® Compact PD	?	Salmon >35 g	Inactivated	SAV	i.p. injection	aqua.intervet.com/
ISAV	Pharmaq AS Norway	Alpha Jects® Micro-1 ISA	Norway, Chile, Ireland, Finland	Salmon	Inactivated Monovalent	ISAV	i.p. injection	pharmac.govt.nz/
	Aqua Health Ltd., Novartis, Canada	FORTE VI	Canada	Salmon	Inactivated Multivalent	ISAV	i.p. injection	[146] ah.novartis.com/
	Microtek International Inc. British Columbia, Canada	?	Canada	Salmon	Inactivated Multivalent	ISAV	i.p. injection	microtek-intl.com/
	Centrovet Ltda Chile	ISAV	Chile	Salmon >10 g	RecProt Mono/poly	HE?	Oral[c]	centrovet.com/

Until the early 1990s, most fish vaccines were developed and commercialized by small local companies. Later on, several multinational animal health companies have acquired, or formed, joint venture companies with those smaller companies. The major commercial markets for these companies are currently the salmon and trout industries in Northern Europe, Chile, Canada and the USA. PD, pancreas disease. i.p., intraperitoneal injection of oil emulsions. According to the specification sheets of most commercial vaccines, i.p. requires fish to be anaesthetised before injection. Furthermore, injected fish might show some growth retardation and visceral adhesions. Injected vaccines are not recommended for vaccination of breeding stocks. Injected vaccines have also some risk of accidental human self-injection unless injection machines are used. Bivalent or multivalent types, refer to the addition of other inactivated antigens, usually from pathogenic bacteria. For instance, Pharmaq AS and Centrovet, produce IPN and ISA vaccines in various combinations with bacterial antigens. Microtek International is associated with the Pfizer group. (?) No data.

[a] Although, most vaccine manufacturers present data showing significant protection against the corresponding experimental viral challenge, usually publications in peer-reviewed scientific journals are absent. To obtain comparable and reliable data on commercial vaccine efficacy an independent European vaccine evaluation would be much more convenient.

[b] Salmon, are Atlantic salmon (*Salmo salar*), except in this case were it refers to the genus *Oncorhynchus*.

[c] The oral vaccine based on ISAV recombinant proteins is delivered mixed with the feed by using a MicroMatrix technology developed by Advanced BioNutrition Co. (ABN) (advancedbionutrition.com).

be required for overall protection from infection with different isolates [21,23]. While most of the injection vaccines mentioned above are candidates to control the disease, a practical vaccination will still require more detailed data on various parameters, such as administration route, effective dose and duration of protection [24]. Moreover, the efficacies of those vaccines were all evaluated on juvenile or young fish and it should be taken into account that the highest mortality occurred at the larval stage, a size too small to be immunized by injection. Therefore, immunization of pre-spawning females might be a potential mean to protect larval stages, despite the fact that studies on that subject are still scarce and reported controversial results [25,26]. On the other hand, despite the protection obtained with recombinant C, the corresponding intramuscular (i.m.) injected DNA vaccine was not protective [27] (Table 4), thus indicating that DNA vaccines similar to the ones to *novirhabdoviruses* might not be the best vaccine option for VNNV viruses. Nevertheless, alternative antigens, adjuvants and/or delivery methods might be needed for each particular virus since many of those possible alternatives have not yet been tested. In addition to the technologies applied to VNNV vaccines, a reverse genetics system for the efficient recovery of infectious particles has been used to design possible experimental vaccines based on attenuated VNNV [28]. Although several of the vaccines commented above have been patented (Table 6), there are no field trials reporting their effects, nor there are any commercial vaccines being offered at this moment (Table 5).

3. Infectious pancreatic necrosis viruses (IPNV)

IPNV are one of the most widely distributed virus affecting most of the farmed finfish species, causing high mortality in recently hatched salmonids, and high worldwide economic losses in juvenile salmon when they are transferred from fresh to sea water. Furthermore, the wide presence of IPNV in asymptomatic adult car-

riers surviving the disease, contributes to their spreading, interferes with other diseases [29] and is a problem for the correct evaluation of the efficacy of vaccines during viral challenge experimentation and/or field trials.

IPNV are non-enveloped viruses with a 60 nm icosahedral structure and a bi-segmented double-stranded RNA (dsRNA A and B) of ~5 kbp. Their RNA-A encodes a polyprotein precursor and the non-structural protein VP5 (Table 1), while their RNA-B encodes the VP1 RNA-dependent RNA polymerase. After infection, the polyprotein precursor is cleaved to generate VP2 (the capsid protein), VP3 and VP4 [30]. On the other hand, IPNV interferes with type I interferon signalling [31], while contradictory reports do exist on the effects of IPNV on apoptosis [32,33] (Table 2).

IPNV belong to the *Birnaviridae* family and the *Aquabirnavirus* genus with IPNV as the type specie [9]. Comparison of the VP2 sequences from many IPNV isolates leads to the identification of, at least, 6 genogroups corresponding to 10 serotypes [34]. Thus, genogroup I corresponds to I1 (serotype A9, type strain Ja) and I2 (serotype A1, type strain WB, including most of the isolates from United States); genogroup II corresponds to serotype A3 (Ab); genogroup III corresponds to genotypes III1 (serotype A2, type strain Sp) and III2 (serotype B1, type strain TV-1); genogroup IV corresponds to serotypes A5 (type strain Te) and A6 (C1); genogroup V includes serotype A7 (strains C2) and A8 (C3) and genogroup VI corresponds to serotype A4 (He). An additional genogroup VII has been also proposed [35]. Serotypes A2–A5 are found principally in Europe.

The VP2 protein of the IPNV capsid has been identified as the main target for N-Abs (Table 3) and their positions 210–225 and 312–324 as the main epitopes [36–39]. According to the recently elucidated tridimensional structure of VP2, those main epitopes are located in the protrusions of the IPNV capsid. Because the main epitopes also contain the VP2 hypervariable region (204–344) responsible for receptor binding and for sequence differences among genotypes, crossprotection studies should evaluate the efficacy of vaccines before being practical.

One of the reasons why early attempts of vaccination to IPNV had not fulfilled many of their expected beneficial effects, might be because the highest susceptibility to IPNV infection occurs at the early stage when fish are not immunocompetent (have not reached a fully developed immune system). Therefore, it was first though that vaccines against IPNV should be designed to reduce vertical transmission of IPNV from brood stock to progeny. However, things changed and IPNV greatly increased their economic impact with the development of salmon culture since it was found that IPNV affected not only the youngest fish but also fresh-water raised salmon when it was released to the sea for further growth [30]. Other early complications to the development of IPNV vaccines were the low mortality on immunocompetent non-vaccinated fish and the generation of asymptomatic carriers among the fish surviving an IPNV infection whether the fish were vaccinated or not. Therefore, efforts were made to develop a suitable model for IPNV challenge with high mortality on non-vaccinated controls [40,41] and to test vaccinated fish by their capacity not only to resist highly virulent viral challenges but also to eliminate residual IPNV after the challenge. From the early reports on successful vaccination of adults with inactivated IPNV [42], other possible vaccine alternatives have been reported with varying degrees of success. For instance, VP2 made in recombinant yeast produced N-Abs by injection and oral deliveries [43], while VLPs obtained by expressing RNA-A in baculovirus [44] or in Semliki forest virus [45], as well as expressing VP2 on other recombinant forms [46,47] were not antigenic or not fully protective. On the other hand, only small protection levels were reported by using DNA vaccination [48] (Table 4). From all the studies mentioned above, 4 vaccine manufacturers currently offer IPNV vaccines by oil-adjuvanted i.p. injection delivery. Thus, Pharmaq AS, Aqua Health Novartis and Centrovet produce mono or multi-valent vaccines containing inactivated IPNV, whereas Intervet Norbio Norvax® Protect-IPN uses a VP2 protein fragment expressed in *E. coli* [49,50] (Table 5). However, because of problems with their efficacy in the field compared to results in the laboratory tests (the high prevalence of IPNV among fish populations might cause some of those problems), and of the presence of tissue adhesions in the fish abdominal cavity caused by i.p. injection of oil-adjuvanted vaccines [43,51], alternative vaccines and/or delivery methods are still needed. In this respect, during 2010 an 80% protection in trout was obtained by oral vaccination with VP2-coding plasmids complexed with alginates [52] (Table 4). Both the development of a possible IPNV DNA vaccine and their novel delivery method would deserve further attention in the coming years.

4. Viral haemorrhagic septicemia viruses (VHSV) and infectious haematopoietic necrosis viruses (IHNV)

Isolated from more than ~50 fish species from North America, Asia and Europe, from ~15 different commercialized fish species such as salmonids and flatfish [53] and from an increasing number of free-living marine fish species [3], VHSV cause the highest economic impact in European trout farming. Furthermore, during the last ~10 years, the VHSV-related infectious haematopoietic necrosis viruses (IHNV), originated in North America have been increasingly isolated in Europe [70,72].

VHSV and IHNV correspond to 2 different viral species of enveloped negative-stranded RNA rhabdoviruses of ~170 nm × 80 nm, having a genome of a single RNA molecule of negative polarity of ~11 kb [54,55], whose full genome sequence is known for several isolates from both VHSV [54,56] and IHNV [57] species. The VHSV/IHNV genomes show different sequences coding for 5 structural viral proteins (N, P, M and G and L proteins) and a non-virion (NV) protein (Table 1). Their genome (3′ N–P–M–G–NV–L 5′) is encapsidated by the nucleocapsid protein N associated with the RNA-dependent RNA polymerase, L and P proteins to form the replication complex. Data obtained with NV-knockout IHNV and/or VHSV, have shown that NVs are required both for optimal replication in cell culture and for *in vivo* pathogenicity, although the mechanism of their action remains to be investigated [58,59]. Finally, although the VHSV induces apoptosis, the protein causing that effect has not been identified yet [60,61], while the M protein of IHNV induces apoptosis [62] (Table 2).

VHSV and IHNV are 2 different viral species that belong to the *Rhabdoviridae* family. However, due to the common presence of a NV gene absent from other rhabdoviruses, VHSV and IHNV were placed into a new *Novirhabdovirus* genus with IHNV as the type specie [9]. Although the external protein G from VHSV/IHNV shows only ~38% of amino acid identity and there is no cross-neutralization between them, their corresponding tertiary structures [63,64] are very similar and there is some western blot cross-reactivity [65,66]. The abundant phylogenetic analyses of G [67] and/or other protein [67] sequences from different isolates have defined 4 major VHSV genotypes (I–IV) differing by ~6% in their nucleotide sequences. Similar phylogenetic analyses of American IHNV field isolates have defined 3 major genogroups (U, M, and L) and one more in Japan, differing in 4–6% in their nucleotide sequences [68]. Taken into account the classifications mentioned above, most of the European IHNV isolates belong to the M genogroup [69,70]. To facilitate comparative studies and availability to researchers, both for VHSV [71] and IHNV [72] European isolates, an European union-funded data base has been organized.

The envelope of VHSV/IHNV contains ~300 trimeric spikes of glycoprotein G, responsible for viral cell attachment and fusion. Although the G is the only targeted viral protein for fish N-Abs (Table 3), it is well known that survivors of infection do not always show high titres of N-Abs, thus suggesting that alternative mechanisms might be operating in resistance to infection [73]. Although the disulphide bonding pattern of the G protein [63], the domains participating in fusion [74–76] and the crystal structure of similar mammalian rhabdoviruses [77,78] are all known, it has been difficult to map the target epitopes for neutralization. Nevertheless, positions 140 and 433 [79] and 253 (Lorenzen, personal communication) were mapped in VHSV G and major antigenic sites I (78–81 and 230–231) and II (272–276) were identified in IHNV [80,81] by sequencing MAb resistant mutants. Therefore, the results mentioned above pointed out to the G protein as the target to develop subunit and/or DNA vaccines.

Live attenuated viruses by cell culture passages were first developed to obtain a thermoresistant VHSV strain [82] and several IHNV strains [80,83]. High levels of protection were obtained by using the attenuated strains [84,85]. However, one of the problems of those vaccines was that although they work well in the laboratory, they did not work equally well in the field. For instance, differences in fish disease susceptibility caused that safe attenuated virus for one fish strain, was virulent for another. Another problem refers to their safety. Thus, live attenuated VHSV or IHNV strains are not used commercially, mostly because their reversion frequency to pathogenic wild-type virus has not been determined. A recent alternative approach to engineer new and possible safer live recombinant VHSV/IHNV strains, including knockouts, is based on reverse genetics [86–89].

Several attempts were then made to obtain an efficacious and safer vaccine against VHSV and/or IHNV by using inactivated viruses [90,91]. Some of those vaccines (for instance, those inactivated with β-propionolactone) were very efficacious [92]. However, they have not been produced in large scale, most probably due to the difficulties and dangers associated with the requirement for production of large amounts of live virus by cell culture techniques.

The advent of genetic engineering was another technological alternative to obtain large amounts of antigenic viral subunits, such as the protein G. However, although it is well known that the G of *novirhabdoviruses* induces N-Abs and is responsible for high levels of protection, recombinant G has shown very limited and irreproducible protection. Thus, the injection of finfish with recombinant G proteins produced in *E. coli* [93,94], *Caulobacter crescentus* [95], *Aeromonas salmonicida* [96], yeast [93], and/or baculovirus [97,98], did not obtain good protection, despite the induction of some levels of N-Abs to either VSHV [93,99] or IHNV [100]. All those failures might be due to the complex post-translational processing of conformationally immunogenic G in the fish host cells, which is difficult to mimic in other organisms, specially in prokaryotics. On the other hand, synthetic peptide vaccine alternatives, such as those consisting in linear peptides corresponding to known antigenic epitopes of the G protein, also failed to elicit a significant Ab response [101].

Anderson et al. [102] were the first to report the successful use of a DNA vaccine to protect rainbow trout against IHNV challenge. Since then, DNA vaccines containing the G gene of IHNV and/or VHSV have shown a 70–100 RPS reproducible protection against their respective viral challenges after i.m. injection [102,151,113,106,159,152]. Furthermore, DNA vaccines using their corresponding G genes have been effective for any *Novirhabdovirus* tested (VHSV, IHNV and the hirame rhabdovirus HIRRV) [4,103] and there is a DNA vaccine against a marine strain of VHSV that has shown protection in flounder [172]. For each of these *novirhabdoviruses*, protection has been shown with, at least 2 different plasmid constructs, and in, at least 2 different laboratories. The success of these vaccines has allowed the 2005 approved APEX-IHN DNA vaccine (Table 5), manufactured by Vical-Aqua Health Ltd. of Canada (Novartis) [104] to be tested in the field in Canada during last years. Nevertheless, safety considerations have not allowed yet their commercialization in Europe.

Numerous reports including dual vaccination for VHSV/IHNV [105], dose–responses/time course [106], temperature dependence [107], long lasting immunity up to 2 years [108], and non specific early protection [103,109], have been described for novirhabdoviral DNA vaccines. However, for maximal vaccine efficacy in the field, DNA vaccines must provide broad protection against the different genotypes abundant in each particular geographical area and up to now the majority of publications using novirhabdoviral vaccines described homologous challenges. Thus, cross-genotype challenges assessing efficacy against VHSV strains from European genotypes II and III or the North American/flounder genotype IV have not been reported yet. Among the few reports studying heterologous challenges, significant but lower RPS in heterologous than in homologous challenges has been observed in the M genogroup of IHNV [110,111], thus arguing that although DNA novirhabdoviral vaccines provide some protection to heterologous challenges, their level of protection might depend on the relatedness of the strains. More studies are needed to reach definitive conclusions in these practical aspects of a new technology.

As indicated before, novirhabdoviral DNA vaccines are not yet being used in Europe mainly because of the possible consideration of DNA vaccinated fish as genetic modified organisms (GMO). In addition, other safety concerns about the use of the human cytomegalovirus promoter, and/or difficulties in the administration of the vaccine at a large scale in small fish (mass vaccination methods), might also contribute to augment their practical problems. In this respect, alternative delivery methods such as oral vaccination by using polyethylene-VHSV [112] or ultrasound aided immersion [113], have been described but they are not practical yet (Table 4). On the other hand, because of their small size and abundance of molecular tools, the use of a novel experimental vaccination model using zebrafish/VHSV to investigate some of the above mentioned problems might also contribute to further improve some practical aspects of the new novirhabdoviral DNA vaccines [114,115].

5. Spring viremia of carp viruses (SVCV)

SVCV affect all farmed carp species in Europe, where it causes significant morbidity and mortality typically at spring [116,117].

SVCV contain a negative-stranded RNA genome coding for L polymerase, N nucleocapsid, G glycoprotein and P and M matrix protein genes but they have no NV protein gene [118] (Table 1).

As VHSV and IHNV, SVCV belong to the *Rhabdoviridae* family but because they lack the NV gene they have been classified among the *Vesiculovirus* genus with the vesicular stomatitis Indiana virus as the type specie [9]. Furthermore, cross-neutralization studies revealed no antigenic relationships with *novirhaboviruses* [116]. On the other hand, phylogenetic analysis of a partial G-gene region of SVCV isolates resulted in genotypes Ib, Ic, and Id (isolates from Europe) and genotype Ia (isolates from Asia or North America) [119,120].

As with *novirhabdoviruses*, SVCV G protein is involved in cell attachment, fusion and is targeted by N-Abs (Table 3), however no epitope mapping attempts have been reported yet.

At temperatures >17 °C, SVCV naturally infected carp develop N-Abs that protect them against re-infection. It has also been shown that a protective and long lasting immunity of carp against SVCV can be obtained following i.p. or oral vaccination of carp with live virus. Furthermore, carp vaccinated by i.p. injection or oral administration with live attenuated SVCV also developed resis-

tance to re-infection [116]. Those and similar reports resulted in an inactivated preparation containing 2 strains of SVCV delivered by oil-adjuvanted i.p. injection being commercialized in Eastern Europe in 1982 [121] (Table 5). However, no reports do exist on their performance and use in the field, except for some non-peer reviewed conference abstracts that reported reduction to <1% of SVCV outbreaks in Austrian carp farms [122]. On the other hand, subunit vaccines have not proved protective so far, as exemplified by the failed attempt of commercialization by the Pharos SA company (Table 5) of a baculovirus expressed G protein [123], nor DNA vaccines have showed similar protection and reproducibility than those against *novirhabdoviruses* [122]. For instance, mixtures of 10 DNA plasmids containing partial or complete SVCV G gene fragments from the European reference strain (Fijan, Genogroup Id) tested in carp [125], have shown that the majority of groups induced little protection, except one that had a maximum of 48 RPS (64% CPM). As another example, DNA vaccines expressing the G of the North American SVCV strain from North Carolina protected carps in different trials against low, moderate and high viral dosages of challenge with the homologous virus isolate [124] (Table 4). It is not yet clear whether the difference of efficacies between *novirhabdoviruses* and SVCV DNA vaccines is due to difficulties with the challenge models in carp (for instance, previous exposure to temperature changes mimicking natural situations, might be required for highest mortalities) [116], or whether they are related to SVCV being a different genus. Further work is required to determine the best challenge conditions, dosages, delivery method, optimal vector and/or adjuvants [125], to obtain more reproducible results with DNA vaccination. With respect to future work, an infection model using zebrafish/SVCV has been described [126] that might be used not only to study interferon induction [127], but also to speed up research on possible practical SVCV DNA vaccines.

6. Salmonid alphaviruses (SAV)

Both salmon pancreas disease (PD) of Atlantic salmon in Norway, Ireland, Scotland and Canada [128] and trout sleeping disease (SD)[129] of rainbow trout in France and Italy are caused by salmonid *alphaviruses* (SAV).

SAV are enveloped spherical viruses with a positive single stranded RNA genome of ~12 kb, coding for capsid glycoproteins (E1, E2, E3 and 6K) and non-structural proteins (nsP1–4) [130] (Table 1). E1 and E2 form heterodimer glycoprotein spikes protruding from the SAV membrane. After infection, the viral particles [131] induced apoptosis most probably due to their E2 protein [132] (Table 2).

Analysis of partial nucleotide sequences of viruses isolated from PD and/or SD diseased fish showed in both cases high homologies to the genus *Alphavirus* of the family *Togaviridae* with Semliki virus forest being their type specie [9], while the study of E2 nucleotide sequences from numerous isolates from Ireland, UK, Norway, France, Italy and Spain, identified 6 different genotypes [133,134].

In non-aquatic *alphaviruses* protective epitopes inducing N-Abs have been identified on the surface glycoproteins El and E2. It was, therefore, assumed that the 2 corresponding proteins in SAV will also carry such epitopes and be useful to design recombinant subunit and/or DNA vaccines [130] (Table 3).

Field observations showed that fish surviving SAV infections were resistant to re-infection, thus suggesting the possibility to apply vaccination strategies for the control of PD and SD. Confirming those observations, live recombinant viruses [135], attenuated SAV [135] and inactivated SAV delivered by oil-adjuvanted i.p. injection [128] have been protective in rainbow trout (Table 4). As a consequence, there are 2 commercial vaccines against PD based on inactivated virus produced by Pharmaq and Intervet Norbio (Table 5). Although there are no peer-reviewed reports on their efficacies, the Intervet Norbio vaccine showed reduction in histopathological damages (equivalent to 80–90 RPS), according to previously commented conference abstracts [122] and is being used in Ireland and Norway. DNA vaccines protecting fish against SAV have not been reported yet.

7. Infectious salmon anaemia viruses (ISAV)

ISAV cause 15–100% mortalities in Atlantic salmon, thus producing severe economic losses to the greatest finfish farmed product in Europe. Furthermore, their economic impact was high because European vaccination was only allowed recently, while the first applied control strategy was to stamp out all fish diagnosed with ISA. First detected in Norway in 1984, ISAV has been isolated from Atlantic salmon in Canada, USA and Scotland and from coho salmon (*Oncorhynchus kisutch*) in Chile [136]. Because both the asymptomatic infections in marine wild finfish and the potential for emergence of new pathogenic strains, either by antigenic drift (mutations in seasonal variations), or antigenic shift (reassortment of gene segments), ISAV are a continuous thread for salmon aquaculture everywhere [137,138].

ISAV are enveloped viruses, with a 8-segmented, negative-sense, single-stranded RNA genome similar to influenza virus but with no sequence homology. RNA segments encode PB1 and 2 putative polymerases, NP nucleoprotein, PA polymerase, F surface fusion, HE surface hemaglutinin-esterase, NEP non-structural protein and M matrix proteins (Table 1) [136]. The main differences with influenza are that the hemaglutinin and esterase activities are in the same HE protein while their fusion activity is on a separated F protein [139]. Segment 7 and 8 products having type I interferon (IFN) antagonizing activity have been identified [140,141] (Table 2).

ISAV belongs to the *Orthomyxoviridae* family and to the *isavirus* genus with ISAV as the type specie [9]. The complete ISAV genome has been sequenced recently [142]. Sequence analysis of HE and F surface protein genes from numerous isolates from different worldwide geographical locations allowed to group the sequences in 3 different genotypes (I–III) [143,144]. ISAV isolates could also be further differentiated by their abundant insertion/deletions in a highly polymorphic 337–372 regions located in the HE stem [136,143]. Most of the European isolates seem to belong to genotype I [143].

The HE, one of the most variable of the ISAV genes, is responsible for receptor-binding and is the main target for N-Abs (Table 3). Furthermore, virulence, HE protein stem length and ISAV cytopathogenicity are correlated [145]. Therefore, HE was the most appropriated ISAV protein to develop subunit and/or DNA vaccines.

Results of first passive immunization experiments in salmon showed weak protection levels despite the fact that serum from survivors contained complement-dependent N-Ab activity [138]. On the other hand, oil-adjuvanted i.p. vaccines, based on inactivated ISAV from Canadian strains are the base of 3 ISAV commercial vaccines actually available and used in farmed Atlantic salmon in Canada and USA (Table 5), despite the fact that available information on those ISAV vaccines is mostly based on non-peer reviewed abstracts and internet publications since scientific reports are scarce. For instance, several non-peer reviewed conference abstracts commented by Biering et al. [122], reported oil-adjuvanted inactivated ISAV vaccines with 96 RPS (Intervet Norbio) or with 54 RPS (Microtek) but similar prototype multivalent (containing also bacterial antigens) vaccines showed 43 RPS whether or not the ISAV antigen was present. On the part of published evidence, an inactivated ISAV vaccine from AquaHealth tested by i.p. injection in salmon, resulted in 92–96 RPS when challenged by co-habitation but in 0 RPS when challenged by i.p.

injection [146]. Although, commercial vaccine development has concentrated on inactivated ISAV, some research into alternative recombinant antigens is also on going. For instance, a DNA vaccine containing the HE gene, administered as a primary dose and 2 boosters, provided some protection to salmon [147] (Table 4) and a recombinant protein vaccine produced in yeast is to date the first oral vaccine being commercialized (Centrovet in Chile with a proprietary MicroMatrix technology for encapsulation from Advanced BioNutrition Corporation) (Table 5).

8. Technological alternatives for viral fish vaccines

In the absence of any existent therapeutic methods and because farmed finfish can develop long-term acquired immunity, viral disease prevention by vaccination is considered one of the most viable strategies to control these diseases [148,149].

Ideally, a viral vaccine should mimic natural viral infections to induce the proper immune response without causing the disease. Thus compared to other alternatives, live-attenuated viral fish vaccines have many advantages, because they not only induce high protective immunity but also might disseminate from vaccinated fish, both resulting in simple delivery and low dose requirements. However, the risks of reversion to virulence and uncontrolled environmental spreading have not allowed their authorization and therefore none of them have been used in the field, despite the availability of appropriated viral strains obtained by traditional and/or recent reverse genetic technologies (i.e., VNNV, VHSV, IHNV).

In contrast, most of the actual commercial fish viral vaccines are made with inactivated virus (i.e., IPNV, SAV, ISAV) (Table 5), most probably because inactivated viruses are not subject to sever regulatory constraints and might be included in multivalent bacterial injectables with a longer tradition of use in aquaculture (Table 5). However, to induce protection, inactivated virus must be adjuvanted in oil–water emulsions (which might induce growth retardation and visceral adhesions) [150] and delivered by i.p. injection (which is labour intensive, unless automatic injection machines are used). Furthermore, i.p. injection requires fish to be anaesthetised and i.p. vaccines are usually not recommended for vaccination of breeding stocks, according to the information provided by their own manufacturers. In addition, cell culture production of the large amounts of the live viruses required to be inactivated, needs extra safety containment and is a complex and costly technology. On the other hand, some inactivated vaccines might also be inconsistent in their efficacy.

With the advent of genetic engineering, the possibility of designing vaccines with recombinant viral proteins rather than with the whole virus, became a new alternative. However, the use of recombinant proteins as antigens for injectable vaccines has not fulfilled their expectations to protect fish against most viruses. For instance, although injection of recombinant G of VHSV or IHNV in rainbow trout elicited moderate levels of N-Abs, the levels of protection were low and/or irreproducible [4]. As a result only those against IPNV and a recent one to ISAV are being commercialized [46,49,122] (Tables 4 and 5).

The so called genetic immunization by using naked DNA (DNA vaccination) is the most recent technology applied to fish viral vaccines. Fish viral DNA vaccines consist of a bacterial plasmid coding for the corresponding viral protein antigen, their expression being under the control of eukaryotic elements (typically, the human cytomegalovirus promoter and the SV40 terminator) and an antibiotic resistance coding gene for plasmid preparation. Fish viral DNA vaccines are usually delivered dissolved in water by i.m. injection. For instance, i.m. injection of fish of plasmid DNA encoding novirhadoviral G genes, protected against challenge by IHNV [151] and VHSV [152] under many different laboratory controlled conditions as mentioned before. As a result of their success, the only DNA vaccine which has been approved for use in the field is the APEX-IHN DNA vaccine manufactured by Vical-Aqua Health Ltd. of Canada (Novartis) [104]. However, for several other RNA viral diseases, including VNNV, IPNV, SVCV, SAV, and ISAV, the level of protection obtained by their corresponding DNA vaccines delivered by i.m. injection, has been too low or irreproducible for commercial use (Tables 4 and 5).

DNA vaccines to fish viruses remain, therefore, an attractive alternative to traditional vaccines (attenuated, inactivated or recombinant protein subunits) because of their straight-forward design and construction, heat stability, identical production technology for any DNA vaccine which results in low production costs, easy possibility of designing multivalent vaccines, possible inclusion of molecular adjuvants, long-term storage facility, no risk of reversion to a pathogenic form and low levels of chemical impurities [4]. Inclusion of markers to differentiate infected from vaccinated fish (differentiating infected from vaccinated individuals, DIVA) is also easy to include in DNA vaccines than in other alternatives. However, DNA vaccines for most fish RNA viruses still remain to be developed at laboratory scale.

9. Alternative methods to deliver viral vaccines to the fish

Fish can be vaccinated either by injection (i.p or i.m) fish-to-fish or by mass delivery (immersion or oral administration) methods [153]. These alternatives have different advantages and disadvantages with respect to their level of protection, side-effects, practicality and cost efficiency, depending on the size of the fish to be vaccinated and the specific virus. Of all the possibilities mentioned above, only the i.p. (i.e., oil-adjuvanted VP2 IPNV recombinant protein) or i.m. (i.e., G IHNV DNA) vaccines delivered through fish-to-fish injections have been used to vaccinated fish to viral diseases (Table 5). However, oil-adjuvanted i.p. injectable vaccines have the disadvantages mentioned before while i.m. injection is an inefficient method of delivering DNA, as recognized by the authors of the IHNV DNA vaccine [104]. On the other hand, mass delivery methods such as immersion or oral deliveries, will be easiest to apply when small (fingerling) rather than large fish need to be vaccinated. For immersion vaccination the help of ultrasound [113,154] or of hyperosmotic solutions, has been reported. For oral vaccination, research has focused on protecting the antigens from digestion in the stomach/gut, for instance by using encapsulation in alginate nanoparticles [52] or matrix forming compounds (Centrovet) (Table 5). Because, mass delivery methods such as immersion or oral vaccination are not yet developed, it might be an important area for further viral fish vaccine improvement [153].

10. Comparative overview of the DNA vaccines against finfish RNA viruses

In spite of the amount of research performed, few fish viral DNA vaccines are commercialized. No live attenuated vaccines are currently licensed, and only one is a DNA vaccine. Thus, most of fish viral vaccines for sale (Table 5) and/or their corresponding patents (Table 6) are based upon inactivated virus or viral recombinant proteins delivered by i.p. injection in oil-adjuvants. For instance, inactivated vaccines against IPNV are being used in salmon culture, despite the fact that not all their performances have been reported in peer-reviewed journals and most of them would probably benefit from improvements. Different is the case for the fish *novirhabdoviruses* such as VHSV and IHNV, since DNA vaccines offer excellent well-demonstrated protection. However, despite DNA vaccines against IHNV being commercial in Canada, they have not

Table 6
Finfish vaccine related-patents in the world intellectual property organization (WIPO).

	Concept	Organization	Country	Number
Viruses				
VNNV	VNNV	Schweitzer Che. Co.	USA	WO2004/050142
	Inactivated VNNV	Novartis A.G.	Swiss	WO2004/074467
	Empty capsids VLPs	AFSSA	France	WO2005/112994
IPNV	Synthetic peptides	Proteus Mol. Design	UK	WO1994/004565
	Empty capsids VLPs	Maryland U.	USA	WO1999/050419
	Yeast VP2, VP3	Aberdeen Univ.	UK	WO2002/038770
	VP2	Maryland Univ.	USA	WO2003/013597
	IPNV	Schweitzer Che. Co.	USA	WO2004/050142
	Plant VP2, VP3	Novartis A.G.	Swiss	WO2004/055190
	Yeast VP2	Advanced Bionutrition	USA	WO2008/140610
VHSV	Attenuated VHSV	Kolbl, O.	Germany	WO1989/005154
	Nv defective novirhabdoviruses	INRA	France	WO2003/097090
	Fusion mutant G genes	INIA	Spain	WO2006/035082
	Recombinant novirhabdoviruses	INRA	France	WO2007/144773
IHNV	G protein	RNA Inc.	Korea	WO2002/036618
	G protein	Novartis A.G.	Swiss	WO2004/026338
SVCV	Attenuated SVCV	Kolbl, O.	Germany	WO1989/005154
	G gene	US Geological Survey	USA	WO2009/002376
SAV	C fragment epitope	Intervet	USA	WO2007/031572
ISAV	Immunogenic proteins	Genomar A.S.	Norway	WO2000/072878
	Surface antigens/genes	Novartis A.G.	Swiss	WO2001/010469
	Viral proteins/genes	Akzo Nobel N.V.	Netherlands	WO2001/049712
	Antigenic polypeptides	Univ. Aberdeeen	Scotland	WO2001/066569
	DNA	Microtek Int.	Canada	WO2002/079231
	M1, M2 proteins	Azco Nobel N.V.	Netherlands	WO2002/026784
	48 kDa fragment	Akzo Nobel N.V.	Netherlands	WO2003/035680
Methods				
Vector	Transposons	INIA	Spain	WO2007/080203
Delivery	Ultrasound	INIA	Spain	WO2006/035084
	Oral	Wageningen Univ.	Netherlands	WO2006/080842
	Oral	Riemser Arzneimittel A.G.	Germany	WO2006/092168
	Recombinant microalgae	New Mexico Univ.	USA	WO2008/027235
	DNA in food	Sol. Biotecnologicas	Chile	WO2008/077413
	Protein nanocarriers	Kapsid Link SL	Spain	WO2009/103752
	Bacteriophages	Big DNA Ltd.	UK	WO2009/138752
Adjuvants	IHNV G	Novartis AG	USA	WO2004/026338
	siRNA	Novartis AG	USA	WO2004/085645
	Novirhaddoviral G	Forinnova A.S.	UK	WO2005/123121
	RNA interference	Advanced Bionutrition	USA	WO2005/079236
	dsRNA	Hokkaido U.	Japan	WO2010/024284

According to data in WIPO (http://www.wipo.int/pctdb/en/index.jsp)

been approved in Europe. On the other hand, while VNNV, SVCV, SAV and ISAV inactivated vaccines have been tested at the laboratory scale, and commercial SAV or lSAV vaccines are currently available, there is none commercial for VNNV or SVCV (Table 5). Alternative DNA vaccines are also not available since protection with the corresponding N-Ab targeted viral protein genes of IPNV, SVCV or ISAV, was only moderate while no protection data has been reported for VNNV or SAV (Table 4).

As commented above, most vaccination formulations using whole viruses whether those are delivered as infection, attenuated and/or inactivated, seem to have some effectiveness with different RNA viruses, while recombinant viral proteins (except those for aquabirnaviruses) or DNA vaccines (except those of *novirhabdoviruses*) are usually not so effective. The reason for these differences remains obscure, since we are only beginning to understand the interactions between finfish and their pathogenic viruses [155]. At least one of the differences is that while whole virus includes all the viral proteins and genomic RNA, most recombinant protein or DNA vaccines only contain the N-Ab targeted component (subunit vaccines). In addition, replication of viral RNA from live vaccines produces double stranded RNA intermediaries which activate Toll-like receptor 3 (TLR3), while oil-adjuvants also activate several other TLRs [156,157]. Perhaps not only N-Ab targeted proteins/genes but also viral RNA or dsRNA, would need to be simultaneously delivered with N-Ab targeted proteins to induce higher protection responses, but this possibility has not been tested. On the other hand, other viral proteins that interfere with host defences, such as those inducing apoptosis (VP5 of IPNV, VHSV, M of IHNV) or interfering with IFN signalling (NS of ISAV) (Table 3), should also be included in the subunit vaccines for maximal efficacy. For instance, many of the identified viral interferences to the cell they infect refer to apoptosis. Viral interference with apoptosis might be a way to increase their pathogenicity, since apoptosis is a natural way for cells to die without releasing danger signals such as *hmgb1*, a pleitropic cytokine [158]. Therefore, apoptosis might be promoted by viral infection to avoid necrotic cellular death which will induce *hmgb1* secretion and therefore alert the fish host of the infection. Because there are no reports, we might only speculate about the possible inclusion in new DNA vaccine formulations of these viral RNA, dsRNA and/or interfering viral proteins to activate TLRs and/or

to reduce viral interferences, respectively, and thus potentiate subunit vaccine efficacy to obtain similar protection levels than those using whole viruses.

11. Safety/regulatory aspects of fish DNA vaccines

Since fish DNA vaccines have not been licensed in Europe, their safety requirements will need to be regulated based on the previous general rules for DNA vaccines of the European Agency for Evaluation of Medical Products. However, some issues are specific of fish, for instance, the differentiation between a DNA-vaccinated fish and a genetically modified organism (GMO) needs to be further clarified, since different national regulatory organizations maintain different criteria. Thus, the British Agriculture and Environment Biotechnology Commission and the Danish Medical Authorities consider that a DNA-vaccinated fish is a GMO only if the foreign DNA is integrated into the fish genome, while the Norwegian Directorate for Nature Management considers any fish containing foreign DNA as a GMO and the Norwegian Biotechnology Advisory Board recommends that a DNA vaccinated fish should only be considered a GMO if the foreign DNA is integrated and cause either negative effects or is inherited by the offspring [159]. Detailed discussions on these aspects of DNA vaccines have been published by the Norwegian Biotechnology Advisory Board (www.bion.no/publikasjoner/regulation_of_DNA_vaccines.pdf), the Danish Institute for Food and Veterinary Research (www.dfvf.dk/Files/Filer/Publikationer/DNAvaccines_report_-_Final1.doc) [159] and the American FDA (www.fda.gov/cber/gdlns/plasdnavac.pdf). Because of present uncertainties as to what extent DNA vaccines persist into the fish and/or are sheded to the environment [160], European authorities still debate whether to label DNA vaccinated fish as GMOs or not [161]. The debate is not trivial because labelling DNA vaccinated fish as GMOs may negatively affect the willingness to consume DNA vaccinated fish.

The knowledge of the effects and pathways that the DNA follows after being delivered to the fish are, therefore, central to the above mentioned safety concerns. Thus, after delivery to the fish, the DNA might: (i) induce fish immune responses and protection, distribute through fish tissues, insert into the fish genome and/or to be degraded and (ii) be released to the environment or to consumers either from the vaccinated fish and/or from accidental spilling [162]. Those different pathways might affect the vaccinated fish welfare, the environment impact uncertainties and the consumer acceptance, generating their corresponding safety concerns [159,161]. Safety concerns on fish welfare are raised because the DNA induced immune responses might not only be the intended (induction of protection), but also some unintended (autoimmunity, tolerance, cross-protection to other pathogens, etc.), which might decrease fish welfare and aquaculture production. Nevertheless, concerns on fish welfare are low because, as mentioned before, most actual DNA vaccines carry only a single viral gene and therefore they are non-infectious and there is no risk of transferring and disseminating the disease with the vaccine. As a consequence, DNA vaccines are safer to the fish health than attenuated live virus or even inactivated virus. Furthermore, fish DNA vaccines do not require oil adjuvants, and therefore do not cause the post-vaccination oil side effects which affect fish welfare. The other safety concerns are mostly due to the distribution of the DNA vaccine after vaccination which depend on the delivery route. For instance, after i.m. injection most of the DNA plasmid remains at the injection site and the rest distributes to various fish tissues disappearing shortly after vaccination but leaving small amounts of long-term persisting DNA plasmid [104,163]. Although theoretical calculations suggest that the probability of integration of those persisting amounts of DNA into the fish genome are smaller than the chances of natural mutation, no detailed experimental estimations are yet available. Nevertheless, controlling the fate of the DNA (perhaps by including some special sequences to control DNA stability out of the fish or with time), could have an important influence on whether Europeans perceive the risk as acceptable or not. Although, even in small amounts, persisting DNA, might be susceptible for environmental release to the aquatic ecosystem or to consumers eating the vaccinated fish, even smaller amounts are likely to remain months or years later at the time of consumption. Also, compared with the total amount of DNA in the food, the persistent DNA will be a negligible amount. On the other hand, no effects have been detected in human volunteers receiving mg doses of plasmid DNA in previous safety tests [164].

Still other safety concerns might be originated by the presence of the human cytomegalovirus (CMV) promoter to control expression of the viral protein in most of the actual plasmids used for fish DNA vaccines, including the one approved against IHNV [104]. In this regard, it might be expected that the use of control elements (promoters and terminators) from fish origin (all-fish-plasmids) could contribute to a more acceptable consumer alternative. As one example of this alternative, the carp β-actin promoter has been used to raise immunity against VHSV [165]. However, concerns are also raised as to whether the use of fish sequences in DNA vaccines would increase the levels of homologous recombination and thus their possible insertion into the fish genomes. To address this former concern, enhancers and cores from several fish promoters [166] are being combined to design new hybrid promoters (data not published) in an attempt to reduce their possible genome insertion probabilities while conserving the all-fish sequences. Alternatives to replace terminators of mammalian or viral origin for those of fish origin, are also being searched (data not published).

Finally, the commercialization of the IHNV DNA vaccine in Canada is arguing for a similar use of a VHSV DNA vaccine in Europe. Thus, after ~5 years of use of the DNA vaccine for IHNV virus approved for commercialization by the Canadian Food Inspection Agency in July of 2005 [104], there have been no IHN epizootic or negative safety issues to the fish or to the consumer. Because local regulatory authorities were concerned with the potential impact to the environment and to human consumers due to the novelty of the technology [104], the persistence of the DNA plasmids in the i.m. injected fish was studied before approval was granted. Among other considerations, because laboratory-scale testing demonstrated 99% reduction of plasmid copy numbers at the site of injection after ~2 months, faster reduction rate in other tissues, lack of measurable DNA vaccine in gonadal tissue (suggesting a low probability of germ-line transmission), and no adverse effects to salmon, the DNA vaccine was approved. While the evidences mentioned above convinced Canada authorities, those results have not been accepted in Europe. Field-testing is still been performed for IHNV in Atlantic salmon in Canada and for VHSV in Denmark during the latest years, but no detailed reports have been yet published [159]. Those ongoing studies will compare many different physiological and immunological data in vaccinated and unvaccinated fish under field conditions to provide further information on the safety of fish DNA vaccines.

12. Conclusions

Intensive aquaculture is growing more rapidly than all other food animal-producing sectors. To achieve higher levels of production, their viral disease problems must be addressed, since viral outbreaks cause high mortality, severe economical losses and important ecological impacts. An intensive aquaculture, without prevention of the spreading of the viruses they generate, will be unsustainable [167]. However, there are not yet any effective treat-

ments other than destroying all fish in infected farms, avoiding fish movements to and from infected areas and, in some cases, vaccination.

With the exception of DNA vaccination to *novirhabdoviruses*, only viral infections, live attenuated and/or some oil-adjuvanted whole virus vaccines, produced acceptable levels of reproducible protection. The promising results obtained by DNA vaccination to *novirhabdoviruses* indicate that this technology could also be applied to other fish vaccines in the future, but at present, DNA vaccines have not been yet developed for other fish RNA viral diseases, even at the laboratory scale. To achieve progress in this field, further co-operation between more basic and applied science should be encouraged (i.e., immunology and vaccinology). Thus, although progress in the understanding of the fish immune systems, specially on their cellular and mucosal immunities, has been addressed in recent years by European community founded research, more studies and further connection with practical needs are still required.

To obtain similar protection levels than those stimulated by whole viruses (live, attenuated or oil-adjuvanted), other specific viral components (dsRNA or viral proteins interfering with fish defences) and/or molecular adjuvants, might have to be included in new DNA vaccine formulations. On the other hand, more suitable delivery methods need to be developed in order to make vaccination of small fish economically feasible. Alternatively, immunization of brood stocks to protect small fish might need to be developed (for instance for VNNV or IPNV). Also, more studies of crossprotection among different genotypes are needed to estimate the needs for multivalent vaccines to protect to different local variants in each viral specie since very few reports do exist on this issue.

Ideal DNA vaccine formulations must not only avoid finfish death and viral persistence in asymptomatic carriers, but also be safe for the environment and human consumption [168]. Since, the first focus of DNA vaccine researchers is to prove their efficacy, aspects such as those mentioned above have been seldom included perhaps due to the lack of negative impacts on environment or consumers of the present oil-adjuvanted viral vaccines. In this respect, although there are field tests going on by using present DNA vaccines driven by the CMV promoter, the use of alternative fish control elements should also be considered. Finally, authorities and scientists need to achieve better transparency of regulatory and safety issues to inform consumers about the positive effects the use of safer viral DNA vaccines might have in aquaculture.

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References

[1] EFSA. Aquatic species susceptible to diseases listed in Directive 2006/88/EC; Scientific Opinion of the Panel on Animal Health and Welfare (AHAW). Eur Food Safe Authority J 2006;88:5–144, http://www.efsa.europa.eu/fr/scdocs/doc/808.pdf.
[2] Stone DM, Way K, Dixon PF. Nucleotide sequence of the glycoprotein gene of viral haemorrhagic septicemia (VHS) viruses from different geographical areas: a link between VHS in farmed fish species and viruses isolated from North Sea cod (*Gadus morhua* L.). J Gen Virol 1997;78:1319–26.
[3] Brudeseth BE, Evensen O. Occurrence of viral haemorrhagic septicaemia virus (VHSV) in wild marine fish species in the coastal regions of Norway. Dis Aquat Organ 2002;52(1):21–8.
[4] Kurath G. Biotechnology and DNA vaccines for aquatic animals. Rev Sci Technol 2008;27(1):175–96.
[5] Patel S, Korsnes K, Bergh O, Vik-Mo F, Pedersen J, Nerland AH. Nodavirus in farmed Atlantic cod *Gadus morhua* in Norway. Dis Aquat Organ 2007;77:169–73.
[6] Gagne N, Johnson SC, Cook-Versloot M, MacKinnon AM, Olivier G. Molecular detection and characterization of nodavirus in several marine fish species from the northeastern Atlantic. Dis Aquat Organ 2004;62:181–9.
[7] Munday BL, Kwang J, Moody N. Betanodavirus infections of'teleost fish: a review. J Fish Dis 2002;25:127–42.
[8] Su YC, Wu JL, Hong JR. Betanodavirus non-structural protein B2: a novel necrotic death factor that induces mitochondria-mediated cell death in fish cells. Virology 2009;385:143–54.
[9] ICTV. International Committee on Taxonomy of Viruses (ICTV index of viruses); 2009, http://www.ncbi.nlm.nih.govIICTVdbIIclvlindex.htm.
[10] Skliris GP, Krondiris JV, Sideris DC, Shinn AP, Starkey WG, Richards RH. Phylogenetic and antigenic characterization of new fish nodavirus isolates from Europe and Asia. Virus Res 2001;75:59–67.
[11] Nishizawa T, Furuhashi M, Nagai T, Nakai T, Muroga K. Genomic classification of fish nodaviruses by molecular phylogenetic analysis of the coat protein gene. Appl Environ Microbiol 1997;63(4):1633–6.
[12] Johansen R, Sommerset I, Torud B, Korsnes K, Hjortaas MJ, Nilsen F, et al. Characterization of nodavirus and viral encephalopathy and retinopathy in farmed turbot, *Scophthalmus maximus* (L.). J Fish Dis 2004;27:591–601.
[13] Costa JZ, Adams A, Bron JE, Thompson KD, Starkey WG, Richards RH. Identification of B-cell epitopes on the betanodavirus capsid protein. J Fish Dis 2007;30(7):419–26.
[14] Baudin-Laurencin F, Richards R. Nodavirus workshop. Bull Eur Ass Fish Pathol 1999;19:284–5.
[15] Yamashita H, Fujita Y, Kawakami H, Nakai T. The efficacy of inactivated virus vaccine against viral nervous necrosis (NNV). Fish Pathol 2005;40:15–21.
[16] Kai YH, Chi SC. Efficacies of inactivated vaccines against betanodavirus in grouper larvae (*Epinephelus coioides*) by bath immunization. Vaccine 2008;26(11):1450–7.
[17] Lin CS, Lu MW, Tang L, Liu WT, Chao CB, Lin CJ, et al. Characterization of virus-like particles assembled in a recombinant baculovirus system expressing the capsid protein of a fish nodavirus. Virology 2001;290:50–8.
[18] Thiery R, Cozien J, Cabon J, Lamour F, Baud M, Schneemann A. Induction of a protective immune response against viral nervous necrosis in the European sea bass *Dicentrarchus labrax* by using betanodavirus virus-like particles. J Virol 2006;80(20):10201–7.
[19] Liu W, Hsu CH, Chang CY, Chen HH, Lin CS. Immune response against grouper nervous necrosis virus by vaccination of virus-like particles. Vaccine 2006;24(37–39):6282–7.
[20] Husgard S, Grotmol S, Hjeltnes BK, Rodseth OM, Biering E. Irnmune response to a recombinant capsid protein of striped jack nervous necrosis virus (SJNNV) in turbot *Scophthalmus maximus* and Atlantic halibut Hippoglossus hippoglossus, and evaluation of a vaccine against SJNNY. Dis Aquat Organ 2001;45:33–44.
[21] Yuasa K, Koesharyani, Roza D, Mori K, Katata M, Nakai T. Immune response of humpback grouper, *Cromileptes altivelis* (Valenciennes) injected with the recombinant coat protein of betanodavirus. J Fish Dis 2002;25:53–6.
[22] Coeurdacier JL, Laporte F, Pepin JF. Preliminary approach to find synthetic peptides from nodavirus capsid potentially protective against sea bass viral encephalopathy and retinopathy. Fish Shellfish Immunol 2003;14(5):435–47.
[23] Tanaka S, Morí K, Arimoto M, Iwamoto T, Nakai T. Protective irnrnunity of sevenband grouper, *Epinephelus septemfasciatus* (Thunberg), against experimental viral nervous necrosis. J Fish Dis 2001;24:15–22.
[24] Yamashita H, Mori K, Kuroda A, Nakai T. Neutralizing antibody levels for protection against betanodavirus infection in sevenband grouper, *Epinephelus septemfasciatus* (Thunberg), immunized with an inactivated virus vaccine. J Fish Dis 2009;32(9):767–75.
[25] Oshima S, Hata J, Segawa C, Yamashita S. Mother to fry, successful transfer of immunity against infectious haematopoietic necrosis virus infection in rainbow trout. J Gen Virol 1996;77:2441–5.
[26] Lillehaug A, Sevatdal S, Endal T. Passive transfer of specific maternal immunity does not protect Atlantic salmon (*Salmo salar* L) fry against yersiniosis. Fish Shellfish Immunol 1996;6:521–35.
[27] Sommerset I, Skern R, Biering E, Bleie H, Fiksdal IU, Grove S, et al. Protection against Atlantic halibut nodavirus in turbot is induced by recombinant capsid protein vaccination but not following DNA vaccination. Fish Shellfish Immunol 2005;18:13–29.
[28] Takizawa N, Adachi K, Kobayashi N. Establishment of reverse genetics system of betanodavirus for the efficient recovery of infectious particles. J Virol Methods 2008;151(2):271–6.
[29] Urquhart K, Murray AG, Gregory A, O'Dea M, Munro LA, Smail DA, et al. Estimation of infectious dose and viral shedding rates for infectious pancreatic necrosis virus in Atlantic salmon, *Salmo salar* L., post-smolts. J Fish Dis 2008;31:879–87.
[30] Saint-Jean SR, Borrego JJ, Perez-Prieto SI. Infectious pancreatic necrosis virus: biology, pathogenesis, and diagnostic methods. Adv Virus Res 2003;62:113–65.
[31] Collet B, Munro ES, Gahlawat S, Acosta F, Garcia J, Roemelt C, et al. Infectious pancreatic necrosis virus suppresses type I interferon signalling in rainbow trout gonad cell line but not in Atlantic salmon macrophages. Fish Shellfish Immunol 2007;22:44–56.
[32] Hong JR, Lin TL, Hsu YL, Wu JL. Apoptosis precedes necrosis of fish cell line with infectious pancreatic necrosis virus infection. Virology 1998;250:76–84.
[33] Lin JG, Zhang CX, Suzuki S. An anti-apoptosis gene of the Bcl-2 family from Marine Birnavirus inhibiting apoptosis of insect cells infected with baculovirus. Virus Genes 2005;31(2):185–93.

[34] Cutrin JM, Olveira JG, Barja JL, Dopazo CP. Diversity of infectious pancreatic necrosis virus strains isolated from fish, shellfish, and other reservoirs in Northwestern Spain. Appl Environ Microbiol 2000;66(2): 839–43.
[35] Nishizawa T, Kinoshita S, Yoshimizu M. An approach for genogrouping of Japanese isolates of aquabirnaviruses in a new genogroup. VII, based on the VP2/NS junction region. J Gen Virol 2005;86(Pt 7):1973–8.
[36] Heppell J, Tarrab E, Lecomte J, Berthiaume L, Arella M. Strain variability and localization of important epitopes on the major structural protein (VP2) of infectious pancreatic necrosis virus. Virology 1995;214:40–9.
[37] Liao L, Dobos P. Mapping of a serotype specific epitope of the major capsid protein VP2 of infectious pancreatic necrosis virus. Virology 1995;209: 684–7.
[38] Santi N, Vakharia VN, Evensen O. Identification of putative motifs involved in the virulence of infectious pancreatic necrosis virus. Virology 2004;322:31–40.
[39] Bain N, Gregory A, Raynard RS. Genetic analysis of infectious pancreatic necrosis virus from Scotland. J Fish Dis 2008;31:37–47.
[40] Bowden TJ, Smail DA, Ellis AE. Development of a reproducible infectious pancreatic necrosis virus challenge model for Atlantic salmon, *Salmo salar* L. J Fish Dis 2002;25:555–63.
[41] Bowden TJ, Lockhart K, Smail DA, Ellis AE. Experimental challenge of post-smolts with IPNV: mortalities do not depend on population density. J Fish Dis 2003;26:309–12.
[42] Midtlyng P. Vaccination. In: Evensen O, Rimstad E, Stagg R, Brun E, Midtlyng P, Skjelstad B, Johansen LH, Jensen I, editors. IPN in salmonids: a review. Trondheim: FHL and YESO; 2003. p. 85–95.
[43] Allnutt FCT, Bowers RM, Rowe CG, Vakharia VN, LaPatra SE, Dhar AK. Antigenicity of infectious pancreatic necrosis virus VP2 subviral particles expressed in yeast. Vaccine 2007;25:4880–8.
[44] Shivappa RB, McAllister PE, Edwards GH, Santi N, Evensen O, Vakharia VN. Development of a subunit vaccine for infectious pancreatic necrosis virus using a baculovirus insect/larvae system. Dev Biol 2005;121:165–74.
[45] McKenna BM, Fitzpatrick RM, Phenix KV, Todd D, Vaughan LM, Atkins GJ. Formation of infectious pancreatic necrosis virus-like particles following expression of segment A by recombinant Semliki Forest virus. Mar Biotechnol 2001;3(2):103–10.
[46] Christie KE. Immunization with viral antigens: infectious pancreatic necrosis. In: Gudding R, Lillehaug A, Midtlying, Journal P, Brown F, editors. Fish vaccinology. Developments Biological Standarization, vol. 90. Basel Karger; 1997. p. 191–9.
[47] Fridholm H, Eliasson L, Everitt E. Immunogenicity properties of authentic and heterologously synthesized structural protein VP2 of infectious pancreatic necrosis virus. Viral Immunol 2007;20:635–48.
[48] Mikalsen AB, Torgersen J, Alestrom P, Hellemann AL, Koppang EO, Rimstad E. Protection of atlantic salmon *Salmo salar* against infectious pancreatic necrosis after DNA vaccination. Dis Aquat Organ 2004;60:11–20.
[49] Frost P, Ness A. Vaccination of Atlantic salmon with recombinant VP2 of infectious pancreatic necrosis virus (IPNV), added to a multivalent vaccine, suppresses viral replication following IPNV challenge. Fish Shellfish Immunol 1997;7:609–19.
[50] Frost P, Borsheim K, Endresen C. Analysis of the antibody response in Atlantic salmon against recombinant VP2 ofinfectious pancreatic necrosis virus (IPNV). Fish Shellfish Immunol 1998;8:447–56.
[51] Colquhoun DJ, Skjerve E, Poppe TT. Pseudomonas fluorescens, infectious pancreatic necrosis virus and environmental stress as potential factors in the development of vaccine related adhesions in Atlantic salmon, *Salmo salar* L. J Fish Dis 1998;21:355–63.
[52] delasHeras AI, Saint-Jean SR, Pérez-Prieto SI. Immunogenic and protective effects of an oral DNA vaccine against infectious pancreatic necrosis virus in fish. Fish Shellfish Immunol 2010;28:562–70.
[53] Skall HF, Olesen NJ, Mellergaard S. Viral haemorrhagic septicaemia virus in marine fish and its implications for fish farming—a review. J Fish Dis 2005;28(9):509–29.
[54] Schutze H, Mundt E, Mettenleiter TC. Complete genomic sequence of viral hemorrhagic septicemia virus, a fish rhabdovirus. Virus Genes 1999;19(1):59–65.
[55] Hill BJ. Physico-chemical and serological characterization of five rhabdoviruses infecting fish. J Gen Virol 1975;27(3):369–78.
[56] Nishizawa T, Iida H, Takano R, Isshiki T, Nakajima K, Muroga K. Genetic relatedness among Japanese, American and European isolates of viral hemorrhagic septicemia virus (VHSV) based on partial G and P genes. Dis Aquat Organ 2002;48(2):143–8.
[57] Schutze H, Enzmann PJ, Kuchling R, Mundt E, Niemann H, Mettenleiter TC. complete genomic sequence of the fish rhabdovirus infectious hematopoietic necrosis virus. J Gen Virol 1995;76:2519–27.
[58] Thoulouze MI, Bouguyon E, Carpentier C, Bremont M. Essential role of the NV protein of Novirhabdovirus for pathogenicity in rainbow trout. J Virol 2004;78(8):4098–107.
[59] Biacchesi S, Lamoureux A, Merour E, Bernard J, Bremont M. Limited interference at the early stage of infection between two recombinant novirhabdoviruses: viral hemorrhagic septicemia virus and infectious hematopoietic necrosis virus. J Virol 2010;84(19):10038–50.
[60] Du C, Zhang Q, Li C, Miao D, Gui J. Induction of apoptosis in a carp leucocyte cell line infected with turbot (*Scophthalmus maximus* L.) rhabdovirus. Virus Res 2004;101(2):119–26.
[61] Bjorklund HV, Johansson TR, Rinne A. Rhabdovirus-induced apoptosis in a fish cell line is inhibited by a human endogenous acid cysteine proteinase inhibitor. J Virol 1997;71(7):5658–62.
[62] Chiou PP, Kim CH, Ormonde P, Leong JAC. Infectious hematopoietic necrosis virus matrix protein inhibits host-directed gene expression and induces morphological changes of apoptosis in cell cultures. J Virol 2000;74(16):7619–27.
[63] Einer-Jensen K, Krogh TN, Roepstorff P, Lorenzen N. Characterization of intramolecular disulphide bonds and secondary modifications of the glycoprotein from viral haemorrhagic septicaemia virus (VHSV), a fish rhabdovirus. J Virol 1998;72:10189–96.
[64] Walker PJ, Kongsuwan K. Deduced structural model for animal rhabdovirus glycoproteins. J Gen Virol 1999;80:1211–20.
[65] Nishizawa T, Yoshimizu M, Winton J, Ahne W, Kimura T. Characterization of structural proteins of Hirame Rhabdovirus, Hrv. Dis Aquat Organ 1991;10(3):167–72.
[66] Nishizawa T, Yoshimizu M, Winton JR, Kimura T. Comparison of genome size and synthesis of structural proteins of Hirame Rhabdovirus. Infectious hematopoietic necrosis virus, and viral hemorrhagic septicemia virus. Fish Pathol 1991;26(2):77–81.
[67] Einer-Jensen K, Ahrens P, Lorenzen N. Parallel phylogenetic analyses using the N,G or Nv gene from a fixed group of VHSV isolates reveal the same overall genetic typing. Dis Aquat Organ 2005;67(1–2):39–45.
[68] Winton JR. Immunization with viral antigens: infectious haematopoietic necrosis. In: Gudding R, Lillehaug A, Midtlying PJ, Brown F, editors. Fish vaccinology. Developments biological standardization, vol. 90. Basel Karger; 1997. p. 211–20.
[69] Johansson T, Einer-Jensen K, Batts W, Ahrens P, Bjorkblom C, Kurath G, et al. Genetic and serological typing of European infectious haematopoietic necrosis virus (IHNV) isolates. Dis Aquat Organ 2009;86(3):213–21.
[70] Enzmann PJ, Castric J, Bovo G, Thiery R, Fichtner D, Schutze H, et al. Evolution of infectious hematopoietic necrosis virus (IHNV), a fish rhabdovirus, in Europe over 20 years: implications for control. Dis Aquat Organ 2010;89(1):9–15.
[71] Jonstrup SP, Gray T, Kahns S, Skall HF, Snow M, Olesen NJ. Fish-Pathogens.eu/vhsv: a user-friendly viral haemorrhagic septicaemia virus isolate and sequence database. J Fish Dis 2009;32:925–9.
[72] Jonstrup SP, Schuetze H, Kurath G, Gray T, Jensen BB, Olesen NJ. An isolate and sequence database of infectious haematopoietic necrosis virus (IHNV). J Fish Dis 2010;33(6):469–71.
[73] Lorenzen N, LaPatra SE. Immunity to rhabdoviruses in rainbow trout: the antibody response. Fish Shellfish Immunol 1999;9:345–60.
[74] Estepa A, Coll JM. Pepscan mapping and fusion related properties of the major phosphatidylserine-binding domain of the glycoprotein of viral hemorrhagic septicemia virus, a salmonid rhabdovirus. Virology 1996;216:60–70.
[75] Nuñez E, Fernandez AM, Estepa A, Gonzalez-Ros JM, Gavilanes F, Coll JM. Phospholipid interactions of a peptide from the fusion-related domain of the glycoprotein of VHSV, a fish rhabdovirus. Virology 1998;243:322–30.
[76] Rocha A, Ruiz S, Tafalla C, Coll JM. Conformation and fusion defective mutants in the hypothetical phospholipid-binding and fusion peptides of the protein G of viral haemorrhagic septicemia salmonid rhabdovirus. J Virol 2004;78:9115–22.
[77] Roche S, Bressanelli S, Rey FA, Gaudin Y. Crystal structure of the low-pH form of the vesicular stomatitis virus glycoprotein G. Science 2006;313(5784):187–91.
[78] Roche S, Rey FA, Gaudin Y, Bressanelli S. Structure of the prefusion form of the vesicular stomatitis virus glycoprotein G. Science 2007;315(5813):843–8.
[79] Bearzotti M, Monnier AF, Vende P, Grosclaude J, DeKinkelin P, Benmansour A. The glycoprotein of viral hemorrhagic septicemia virus (VHSV): antigenicity and role in virulence. Vet Res 1995;26:413–22.
[80] Roberti KA, Rohovec JS, Winton JR. Vaccination of rainbow trout against infectious hematopoietic necrosis (IHN) by using attenuated mutants selected by neutralizing monoclonal antibodies. J Aquat Anim Health 1998;10(4):328–37.
[81] Winton JR. Molecular approaches to fish vaccines. J Appl Ichthyol 1998;14(3–4):153–8.
[82] de Kinkelin P, Bearzotti-Le Berre M, Bernard J. Viral hemorrhagic septicemia of rainbow trout: selection of a thermoresistant virus variant and comparison of polypeptide synthesis with the wild-type virus strain. J Virol 1980;36(3):652–8.
[83] Ristow SS, LaPatra SE, Dixon R, Pedrow CR, Shewmaker WD, Park JW, et al. Responses of cloned rainbow trout *Oncorhynchus mykiss* to an attenuated strain of infectious hematopoietic necrosis virus. Dis Aquat Organ 2000;42(3):163–72.
[84] DeKinkelin P, Bearzotti M. Immunization of rainbow trout against viral hemorrhagic septicaemia (VHS) with a thermoresistant variant of the virus. Dev Biol Stand 1981;49:431–9.
[85] Enzmann PJ, Fichtner D, Schutze H, Walliser G. Development of vaccines against VHS and IHN: oral application, molecular marker and discrimination of vaccinated fish from infected populations. J Appl Ichthyol 1998;14(3–4):179–83.
[86] Biacchesi S, Thoulouze M -I, Bearzotti M, Yu Y -X, Bremont M. Recovery of NV knockout infectious hematopoietic necrosis virus expressing foreign genes. J Virol 2000;74(23):11247–53.
[87] Biacchesi S, Bearzotti M, Bouguyon E, Bremont M. Heterologous exchanges of the glycoprotein and the matrix protein in a Novirhabdovirus. J Virol 2002;76:2881–9.
[88] Biacchesi S, LeBerre M, Lamoureux A, Louise Y, Lauret E, Boudinot P, et al. Mitochondrial antiviral signaling protein plays a major role in induction

of the fish innate immune response against RNA and DNA viruses. J Virol 2009;83(16):7815–27.
[89] Ammayappan A, Lapatra SE, Vakharia VN. A vaccinia-virus-free reverse genetics system for infectious hematopoietic necrosis virus. J Virol Methods 2010;167(2):132–9.
[90] Benmansour A, de Kinkelin P. Live fish vaccines: history and perspectives. Dev Biol Stand 1997;90:279–89.
[91] Lorenzen N, Olesen NJ. Immunization with viral antigens: viral haemorrhagic septicemia. Dev Biol Stand 1997;90:201–9.
[92] DeKinkelin P. Vaccination against viral haemorrhagic septicaemia. In: Ellis AE, editor. Fish vaccination. Academic Press; 1988. p. 172–92.
[93] Estepa A, Thiry M, Coll JM. Recombinant protein fragments from haemorrhagic septicaemia rhabdovirus stimulate trout leucocyte anamnestic in vitro responses. J Gen Virol 1994;75:1329–38.
[94] Lorenzen N, Olesen NJ, Jorgensen PEV, Etzerodt M, Holtet TL, Thogersen HC. Molecular-cloning and expression in *Escherichia coli* of the glycoprotein gene of Vhs virus, and immunization of rainbow-trout with the recombinant protein. J Gen Virol 1993;74:623–30.
[95] Simon B, Nomellini J, Chiou P, Bingle W, Thornton J, Smit J, et al. Recombinant vaccines against infectious hematopoietic necrosis virus: production by the *Caulobacter crescentus* S-layer protein secretion system and evaluation in laboratory trials. Dis Aquat Organ 2001;44(1):17–27.
[96] Noonan B, Enzmann PJ, Trust TJ. Recombinant infectious necrosis virus and viral hemorrhagic septicemia virus glycoprotein epitopes expressed in *Aeromonas salmonicida* induce protective immunity in rainbow trout (*Onchorynchus mykiss*). Appl Environ Microbiol 1995;61:3586–91.
[97] Koener JF, Leong JC. Expresion of the glycoprotein gene from a fish rhabdovirus by using baculovirus vectors. J Virol 1990;64:428–30.
[98] Cain KD, Byrne KM, Brassfield AL, Lapatra SE, Ristow SS. Temperature dependent characteristics of a recombinant infectious hematopoietic necrosis virus glycoprotein produced in insect cells. Dis Aquat Organ 1999;36:1–10.
[99] Lorenzen N, Olesen NJ, Jorgensen PEV. Antibody response in rainbow trout to VHS virus proteins. Fish Shellfish Immunol 1993;3:461–73.
[100] Xu L, Mourich DV, Engelking HM, Ristow S, Arnzen J, Leong JC. Epitope mapping and characterization of the infectious hematopoietic necrosis virus glycoprotein, using fusion proteins synthesized in *Escherichia coli*. J Virol 1991;65:1611–5.
[101] Emmenegger E, Landolt M, LaPatra S, Winton J. Immunogenicity of synthetic peptides representing antigenic determinants on the infectious hematopoietic necrosis virus glycoprotein. Dis Aquat Organ 1997;28:175–84.
[102] Anderson ED, Mourich DV, Fahrenkrug SC, LaPatra SC, Shepherd J, Leong JC. Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. Mol Mar Biol Biotechnol 1996;5:114–22.
[103] Kim CH, Johnson MC, Drennan JD, Simon BE, Thomann E, Leong JA. DNA vaccines encoding viral glycoproteins induce nonspecific immunity and Mx protein synthesis in fish. J Virol 2000;74:7048–54.
[104] Salonius K, Simard N, Harland R, Ulmer JB. The road to licensure of a DNA vaccine. Curr Opin Investig Drugs 2007;8:635–41.
[105] Einer-Jensen K, Delgado L, Lorenzen E, Bovo G, Evensen O, Lapatra S, et al. Dual DNA vaccination of rainbow trout (*Oncorhynchus mykiss*) against two different rhabdoviruses, VHSV and IHNV, induces specific divalent protection. Vaccine 2009;27(8):1248–53.
[106] Lorenzen E, Einer-Jensen K, Martinussen T, LaPatra SE, Lorenzen N. DNA vaccination of rainbow trout against viral hemorrhagic septicemia virus: a dose–response and time-course study. J Aquat Anim Health 2000;12(3):167–80.
[107] Lorenzen E, Einer-Jensen K, Rasmussen JS, Kjaer TE, Collet B, Secombes CJ, et al. The protective mechanisms induced by a fish rhabdovirus DNA vaccine depend on temperature. Vaccine 2009;27:3870–80.
[108] Kurath G, Garver KA, Corbeil S, Elliott DG, Anderson ED, LaPatra SE. Protective immunity and lack of histopathological damage two years after DNA vaccination against infectious hematopoietic necrosis virus in trout. Vaccine 2006;24(3):345–54.
[109] Lorenzen N, Lorenzen E, Einer-Jensen K, LaPatra SE. Immunity induced shortly after DNA vaccination of rainbow trout against rhabdoviruses protects against heterologous virus but not against bacterial pathogens. Dev Comp Immunol 2002;26(2):173–9.
[110] Garver KA, LaPatra SE, Kurath G. Efficacy of an infectious hematopoietic necrosis (IHN) virus DNA vaccine in Chinook Oncorhynchus tshawytscha and sockeye O-nerka salmon. Dis Aquat Organ 2005;64(1):13–22.
[111] Kurath G, Garver KA, LaPatra SE, Purcell MK. Resistance and protective immunity in Redfish Lake sockeye salmon exposed to M type infectious hematopoietic necrosis virus (IHNV). J Aquat Anim Health 2010;22(2):129–39.
[112] Adelmann M, Kollner B, Bergmann SM, Fischer U, Lange B, Weitschies W, et al. Development of an oral vaccine for immunisation of rainbow trout (*Oncorhynchus mykiss*) against viral haemorrhagic septicaemia. Vaccine 2008;26(6):837–44.
[113] Fernandez-Alonso M, Rocha A, Coll JM. DNA vaccination by immersion and ultrasound to trout viral haemorrhagic septicaemia virus. Vaccine 2001;19:3067–75.
[114] Novoa B, Romero A, Mulero V, Rodriguez I, Fernandez I, Figueras A. Zebrafish (*Danio rerio*) as a model for the study of vaccination against viral haemorrhagic septicemia virus (VHSV). Vaccine 2006;24(31–32):5806–16.
[115] Encinas P, Rodriguez-Milla MA, Novoa B, Estepa A, Figueras A, Coll JM. Zebrafish fin immune responses during high mortality infections with viral haemorrhagic septicemia rhabdovirus. A proteomic and transcriptomic approach. BMC Genomics 2010;11:518–34.
[116] Ahne W, Bjorklund HV, Essbauer S, Fijan N, Kurath G, Winton JR. Spring viremia of carp (SVC). Dis Aquat Organ 2002;52:261–72.
[117] Goodwin AE. Spring Viremia of Carp Virus (SVCV): global status of outbreaks, diagnosis, surveillance, and research. Isr J Aquacul-Bamidgeh 2009;61:180–7.
[118] Hoffmann B, Schutze H, Mettenleiter TC. Determination of the complete genomic sequence and analysis of the gene products of the virus of Spring Viremia of Carp, a fish rhabdovirus. Virus Res 2002;84(1–2):89–100.
[119] Stone DM, Ahne W, Denham KL, Dixon PF, Liu CT, Sheppard AM, et al. Nucleotide sequence analysis of the glycoprotein gene of putative spring viraemia of carp virus and pike fry rhabdovirus isolates reveals four genogroups. Dis Aquat Organ 2003;53(3):203–10.
[120] Warg JV, Dikkeboom AL, Goodwin AE, Snekvik K, Whitney J. Comparison of multiple genes of spring viremia of carp viruses isolated in the United States. Virus Genes 2007;35:87–95.
[121] Sommerset I, Krossoy B, Biering E, Frost P. Vaccines for fish in aquaculture. Expert Rev Vaccines 2005;4:89–101.
[122] Biering E, Villoing S, Sommerset I, Christie KE. Update on viral vaccines for fish. Dev Biol 2005;121:97–113.
[123] Dixon P. Immunization with viral antigens: viral diseases of carp and catfish. Dev Biol Stand 1997;90:221–32.
[124] Emmenegger EJ, Kurath G. DNA vaccine protects ornamental koi (*Cyprinus carpio* koi) against North American spring viremia of carp virus. Vaccine 2008;26:6415–21.
[125] Kanellos T, Sylvester ID, D'Mello F, Howard CR, Mackie A, Dixon PF, et al. DNA vaccination can protect *Cyprinus carpio* against spring viraemia of carp virus. Vaccine 2006;24(23):4927–33.
[126] Sanders GE, Batts WN, Winton JR. Susceptibility of zebrafish (*Danio rerio*) to a model pathogen, spring viremia of carp virus. Comp Med 2003;53:514–21.
[127] Levraud JP, Boudinot P, Colin I, Benmansour A, Peyrieras N, Herbomel P, et al. Identification of the zebrafish IFN receptor: implications for the origin of the vertebrate IFN system. J Immunol 2007;178(7):4385–94.
[128] McLoughlin MF, Graham DA. Alphavirus infections in salmonids—a review. J Fish Dis 2007;30:511–31.
[129] Weston J, Villoing S, Bremont M, Castric J, Pfeffer M, Jewhurst V, et al. Comparison of two aquatic alphaviruses, salmon pancreas disease virus and sleeping disease virus, by using genome sequence analysis, monoclonal reactivity, and cross-infection. J Virol 2002;76:6155–63.
[130] Villoing S, Bearzotti M, Chilmonczyk S, Castric J, Bremont M. Rainbow trout sleeping disease virus is an atypical alphavirus. J Virol 2000;74:173–83.
[131] Hu J, Cai XF, Yan G. Alphavirus M1 induces apoptosis of malignant glioma cells via downregulation and nucleolar translocation of p21WAF1/CIP1 protein. Cell Cycle 2009;8(20):3328–39.
[132] Ubol S, Tucker PC, Griffin DE, Hardwick JM. Neurovirulent strains of Alphavirus induce apoptosis in bcl-2-expressing cells: role of a single amino acid change in the E2 glycoprotein. Proc Natl Acad Sci USA 1994;91(11):5202–6.
[133] Fringuelli E, Rowley HM, Wilson JC, Hunter R, Rodger H, Graham DA. Phylogenetic analyses and molecular epidemiology of European salmonid alphaviruses (SAV) based on partial E2 and nsP3 gene nucleotide sequences. J Fish Dis 2008;31(11):811–23.
[134] Weston JH, Graham DA, Branson E, Rowley HM, Walker IW, Jewhurst VA, et al. Nucleotide sequence variation in salmonid alphaviruses from outbreaks of salmon pancreas disease and sleeping disease. Dis Aquat Organ 2005;66:105–11.
[135] Moriette C, Leberre M, Lamoureux A, Lai TL, Bremont M. Recovery of a recombinant salmonid alphavirus fully attenuated and protective for rainbow trout. J Virol 2006;80:4088–98.
[136] Cottet L, Rivas A, Cortez M, Sandino AM, Spencer E. Infectious salmon anemia virus-genetics and pathogenesis. Virus Res 2010.
[137] Gregory A, Munro LA, Snow M, Urquhart KL, Murray AG, Raynard RS. An experimental investigation on aspects of infectious salmon anaemia virus (ISAV) infection dynamics in seawater Atlantic salmon, *Salmo salar* L. J Fish Dis 2009;32:481–9.
[138] Kibenge FS, Munir K, Kibenge MJ, Joseph T, Moneke E. Infectious salmon anemia virus: causative agent, pathogenesis and immunity. Anim Health Res Rev 2004;5(1):65–78.
[139] Clouthier SC, Rector T, Brown NE, Anderson ED. Genomic organization of infectious salmon anaemia virus. J Gen Virol 2002;83(Pt 2):421–8.
[140] Garcia-Rosado E, Markussen T, Kileng O, Baekkevold ES, Robertsen B, Mjaaland S, et al. Molecular and functional characterization of two infectious salmon anaemia virus (ISAV) proteins with type I interferon antagonizing activity. Virus Res 2008;133:228–38.
[141] McBeath AJ, Collet B, Paley R, Duraffour S, Aspehaug V, Biering E, et al. Identification of an interferon antagonist protein encoded by segment 7 of infectious salmon anaemia virus. Virus Res 2006;115(2):176–84.
[142] Merour E, Leberre M, Lamoureux A, Bernard J, Bremont M, Biacchesi S. Completion of the full-length genome sequence of the infectious salmon anaemia virus (ISAV), an aquatic orthomyxovirus-like, and characterization of monoclonal antibodies. J Gen Virol; in press, doi:vir.0.027417-0.
[143] Kibenge FSB, Godoy MG, Wang YW, Kibenge MJT, Gherardelli V, Mansilla S, et al. Infectious salmon anaemia virus (ISAV) isolated from the ISA disease outbreaks in Chile diverged from ISAV isolates from Norway around

1996 and was disseminated around 2005, based on surface glycoprotein gene sequences. Virol J 2009;6:1–16.
[144] Krossoy B, Nilsen F, Falk K, Endresen C, Nylund A. Phylogenetic analysis of infectious salmon anaemia virus isolates from Norway, Canada and Scotland. Dis Aquat Organ 2001;44(1):1–6.
[145] Kibenge FS, Kibenge MJ, Wang Y, Qian B, Hariharan S, McGeachy S. Mapping of putative virulence motifs on infectious salmon anemia virus surface glycoprotein genes. J Gen Virol 2007;88(Pt 11):3100–11.
[146] Jones RM, MacKinnon AM, Salonius K. Vaccination of freshwater-reared Atlantic salmon reduces mortality associated with infectious salmion anemia virus. Bull Eur Ass Fish Pathol 1999;19:98–100.
[147] Mikalsen AB, Sindre H, Torgersen J, Rimstad E. Protective effects of a DNA vaccine expressing the infectious salmon anemia virus hemagglutinin-esterase in Atlantic salmon. Vaccine 2005;23:4895–905.
[148] COM539. Animal Health Strategy for the European Union (2007–2013): "Prevention is better than cure"; 2007, 26 pp. http://ec.europa.eu/food/animal/diseases/strategy/docs/animal_health_strategy_en.pdf.
[149] COM545. Action Plan for the implementation of the EU Animal Health Strategy; 2008, 8 pp. http://ec.europa.eu/food/animal/diseases/strategy/docs/COMM_PDF_COM_2008_0545_F_EN_AUTRE_PROC_LEG_NOUVELLE.pdf.
[150] Lund RA, Midtlyng PJ, Hansen LP. Post-vaccination intra-abdominal adhesions as a marker to identify Atlantic salmon, *Salmo salar* L., escaped from commercial fish farms. Aquaculture 1997;154:27–37.
[151] Anderson ED, Mourich DV, Leong JC. Gene expression in rainbow trout (*Onchorynchus mykiss*) following intramuscular injection of DNA. Mol Mar Biol Biotechnol 1996;5:105–13.
[152] Lorenzen N, Lorenzen E, Einer-Jensen K, Heppell J, Wu T, Davis H. Protective immunity to VHS in rainbow trout (*Oncorhynchus mykiss*, Walbaum) following DNA vaccination. Fish Shellfish Immunol 1998;8(4):261–70.
[153] Horne MT. Technical aspects of the administration of vaccines. Fish Vaccinol 1997;90:79–89.
[154] Navot N, Kimmel E, Avtalion RR. Immunization of fish by bath immersion using ultrasound. Dev Biol 2005;121:135–42.
[155] Workenhe ST, Rise ML, Kibenge MJ, Kibenge FS. The fight between the teleost fish immune response and aquatic viruses. Mol Immunol 2010;47(16):2525–36.
[156] Akira S. Toll-like receptor signaling. J Biol Chem 2003;278(40):38105–8.
[157] Kawai T, Akira S. TLR signaling. Cell Death Differ 2006;13(5):816–25.
[158] Bianchi ME, Manfredi AA. High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity. Immunol Rev 2007;220:35–46.
[159] Lorenzen N, LaPatra SE. DNA vaccines for aquacultured fish. Rev Sci Technol Off Int Epizoot 2005;24:201–13.
[160] Gillund F, Kjolberg KA, von Krauss MK, Myhr AI. Do uncertainty analyses reveal uncertainties? Using the introduction of DNA vaccines to aquaculture as a case. Sci Total Environ 2008;407(1):185–96.
[161] Myhr AI, Dalmo RA. Introduction of genetic engineering in aquaculture; ecological and ethical implications for science and governance. Aquaculture 2005;250:542–54.
[162] Tonheim TC, Bogwald J, Dalmo RA. What happens to the DNA vaccine in fish? A review of current knowledge. Fish Shellfish Immunol 2008;25(1–2):1–18.
[163] Garver KA, Conway CM, Elliot DG, Kurath G. Analysis of DNA-vaccinated fish reveals viral antigen in muscle, kidney and thymus and transient histopathological changes. Mar Biotechnol 2005;7:540–53.
[164] Donnelly J, Berry K, Ulmer JB. Technical and regulatory hurdles for DNA vaccines. Int J Parasitol 2003;33(5–6):457–67.
[165] Chico V, Ortega-Villaizan M, Falco A, Tafalla C, Perez L, Coll JM, et al. The immunogenicity of viral haemorragic septicaemia rhabdovirus (VHSV) DNA vaccines can depend on plasmid regulatory sequences. Vaccine 2009;27(13):1938–48.
[166] Ruiz S, Tafalla C, Cuesta A, Estepa A, Coll JM. In vitro search for alternative promoters to the human immediate early-cytomegalovirus (IE-CMV) to express the G gene of viral haemorrhagic septicemia virus (VHSV) in fish epithelial cells. Vaccine 2008;26:6620–9.
[167] COM162. Building a sustainable future for aquaculture. A new impetus for the Strategy for the Sustainable Development of European Aquaculture; 2009, 12 pp. http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2009:0162:FIN:EN:PDF.
[168] COUNCIL. Directive 2006/88/EC on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals; 2006. L328, p. 14–56 http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:328:0014:0056:EN:PDF.
[169] Espinoza JC, Cortes-Gutierrez M, Kuznar J. Necrosis of infectious pancreatic necrosis virus (IPNV) infected cells rarely is preceded by apoptosis. Virus Res 2005;109:133–8.
[170] Imajoh M, Hirayama T, Oshima S. Frequent occurrence of apoptosis is not associated with pathogenic infectious pancreatic necrosis virus (IPNV) during persistent infection. Fish Shellfish Immunol 2005;18:163–77.
[171] Griffin DE, Hardwick JM. Regulators of apoptosis on the road to persistent alphavirus infection. Annu Rev Microbiol 1997;51:565–92.
[172] Byon JY, Ohira T, Hirono I, Aoki T. Use of a cDNA microarray to study immunity against viral hemorrhagic septicemia (VHS) in Japanese flounder (*Paralichthys olivaceus*) following DNA vaccination. Fish Shellfish Immunol 2005;18(2):135–47.
[173] Lorenzen N, Lorenzen E, Eoner-jensen K, Heppell J, Wu T, Davis H. Protective immunity to VHS in rainbow trout (*Oncorhynchus mykiss*, Walbaum) following DNA vaccination. Fish Shellfish Immunol 1998;8:261–70.
[174] Traxler GS, Anderson E, LaPatra SE, Richard J, Shewmaker B, Kurath G. Naked DNA vaccination of Atlantic salmon *Salmo salar* against IHNV. Dis Aquat Organ 1999;38:183–90.
[175] Lopez-Doriga MV, Smail DA, Smith RJ, Domenech A, Castric J, Smith PD, et al. Isolation of salmon pancreas disease virus (SPDV) in cell culture and its ability to protect against infection by the 'wild-type' agent. Fish Shellfish Immunol 2001;11:505–22.