

# Biotechnology and DNA vaccines for aquatic animals

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## Summary

Biotechnology has been used extensively in the development of vaccines for aquaculture. Modern molecular methods such as polymerase chain reaction (PCR), cloning and microarray analysis have facilitated antigen discovery, construction of novel candidate vaccines, and assessments of vaccine efficacy, mode of action, and host response. This review focuses on DNA vaccines for finfish to illustrate biotechnology applications in this field. Although DNA vaccines for fish rhabdoviruses continue to show the highest efficacy, DNA vaccines for several other viral and bacterial fish pathogens have now been proven to provide significant protection against pathogen challenge. Studies of the fish rhabdovirus DNA vaccines have elucidated factors that affect DNA vaccine efficacy as well as the nature of the fish innate and adaptive immune responses to DNA vaccines. As tools for managing aquatic animal disease emergencies, DNA vaccines have advantages in speed, flexibility, and safety, and one fish DNA vaccine has been licensed.

## Keywords

Aquaculture vaccine – DNA vaccine – Fish disease – Fish immune response – Marine biotechnology – Vaccine efficacy.

## Introduction

Aquatic animals are cultured for many purposes including food production, recreational fisheries, ornamental fish industries, natural resource management, and preservation of threatened and endangered species. In each case, confinement of aquatic animals typically involves conditions such as higher population densities and stress levels that can exacerbate disease processes, leading to decreased productivity through overt mortality or restrictive biosecurity measures. Control of diseases in cultured fish populations requires strict hygiene and biosecurity, but in many cases this is not sufficient to avoid the impacts of disease. Although drug treatments are effective in some cases, the use of drugs and antibiotics is increasingly limited due to concerns regarding environmental contamination and spread of antibiotic resistance. Therefore, as in human and terrestrial animal health, vaccines offer an important avenue for control of aquatic animal disease. Inactivated bacterial vaccines have been successful against a number of bacterial fish

pathogens for decades, but there are no vaccines available for many other bacterial pathogens or most fish viruses. In efforts to develop new vaccines for aquaculture, biotechnology has played a prominent role.

## Biotechnology in aquaculture vaccine research

Biotechnology has been applied at each of the stages of research toward vaccines for aquaculture, as illustrated in Figure 1:

a) antigen discovery studies: at the early stages of vaccine development cloning, protein expression systems, monoclonal antibodies and expression libraries have been used to identify protective antigen candidates that may be effective in vaccines, especially for more complex viral, bacterial and cellular pathogens;

b) construction of new candidate vaccines: polymerase chain reaction (PCR) and molecular cloning have been used to produce DNA vaccines, recombinant subunit vaccines, live attenuated bacterial and viral vaccines, modified live recombinant viruses, and viral vectors. In each case, standard techniques of molecular biology and DNA sequencing are used to verify the construct, and modifications such as different promoter elements or selection genes are incorporated by cloning;

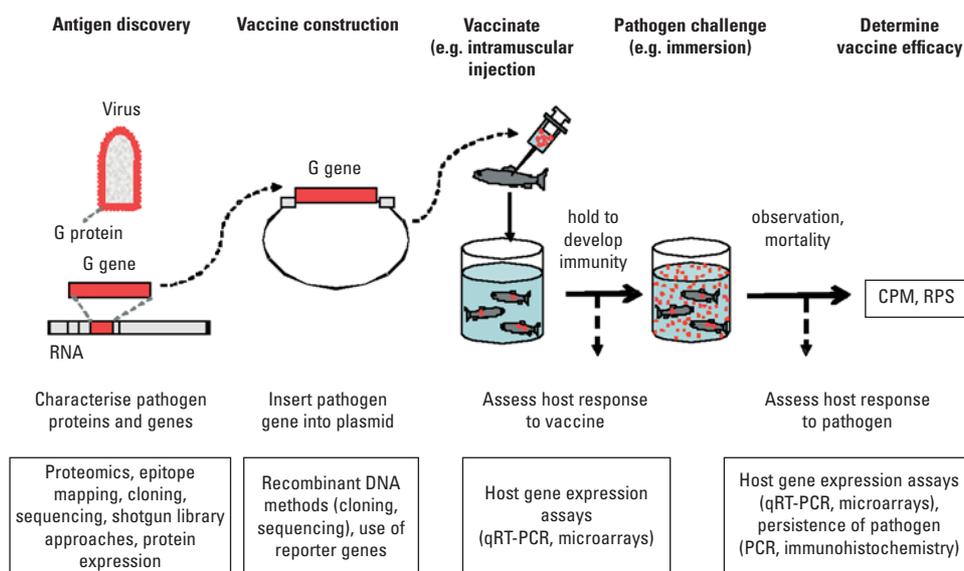
c) assessment of candidate vaccine efficacy, mode of action, and host response: reporter genes, such as luciferase or green fluorescent protein (GFP), and molecular assays such as PCR have been used to study vaccine biodistribution, kinetics of expression, and persistence in cell culture or in live animals. In recent years exciting advances have been made in investigations of the host response to vaccination or to pathogen infection. Semi-quantitative or quantitative reverse-transcriptase real time PCR (qRT-PCR) are increasingly being used to profile changes in gene expression patterns, often targeting specific host genes related to innate or adaptive immune responses. In addition, global expression profiling has been done in a small number of studies using microarrays to analyse thousands of host genes simultaneously. The goal of these efforts is to understand the protective immune response of the host, and determine if a vaccine is stimulating the response needed for protection.

Biotechnology has generated many different kinds of experimental vaccines for aquaculture such as recombinant

vaccines (20, 58, 63, 114), live attenuated recombinant viruses (13, 118), live attenuated bacterial auxotrophs (101), and DNA vaccines. This review will focus on the status of practical knowledge and basic understanding of DNA vaccines to illustrate how biotechnology has benefited fish vaccine research at all stages. There have been several reviews on fish DNA vaccines in recent years (4, 40, 41, 44, 49, 53, 64, 68). To complement the information in these earlier reviews, this paper will include an update on all aquatic animal DNA vaccines that have been tested in experimental trials, with some detail on vaccine efficacy and host response.

## Development of DNA vaccines for aquaculture

A DNA vaccine is a circular DNA plasmid that contains a gene for a protective antigenic protein from a pathogen of interest. The plasmid contains elements that allow it to be amplified to large quantities in bacterial cells, and the pathogen gene is flanked by promoter and termination elements that facilitate its expression in eukaryotic cells. Nearly all DNA vaccines use a cytomegalovirus (CMV) promoter to provide high constitutive expression levels, although several alternative promoters have been described (3, 35). When the DNA vaccine is injected into a live host the plasmid enters a small number of host cells, the pathogen gene is expressed, and the antigenic protein is synthesised within the host cell, so it is folded and processed as it would be in a natural pathogen infection. In many cases this stimulates a broad protective immune



CPM: cumulative percent mortality (as defined in the text and Figure 2)

G protein: rhabdovirus surface glycoprotein

qRT-PCR: quantitative reverse-transcriptase polymerase chain reaction

RPS: relative percent survival (as defined in the text and Figure 2)

**Fig. 1**

### Applications of biotechnology in all stages of aquaculture vaccine development

The process is illustrated using a fish rhabdovirus DNA vaccine as an example

response that includes innate immune mechanisms as well as humoral and cellular adaptive immunity. The first studies on DNA vaccines were published between 1990 and 1993, describing foreign gene expression, stimulation of immune response, and protection against influenza viral challenge in mice and chickens (83, 100, 108, 115). The first DNA vaccines for fish were reported in 1996 (5, 6), and since then DNA vaccines have been constructed and tested for many important aquatic pathogens.

The extent of DNA vaccine development for different fish pathogens depends on several factors including:

- economic importance of pathogen to aquaculture
- lack of availability of a successful vaccine produced by another strategy
- ability to culture the pathogen in the laboratory
- state of knowledge regarding protective antigen(s) of the pathogen
- availability of pathogen-free host fish of appropriate species for experimental trials
- availability of a reliable challenge model in which the disease can be re-created experimentally for testing the vaccine.

In practice, research by many investigators over the last ten years has shown that knowledge of 'the right' protective antigen and availability of a reliable challenge model are particularly critical factors in the development of a successful DNA vaccine.

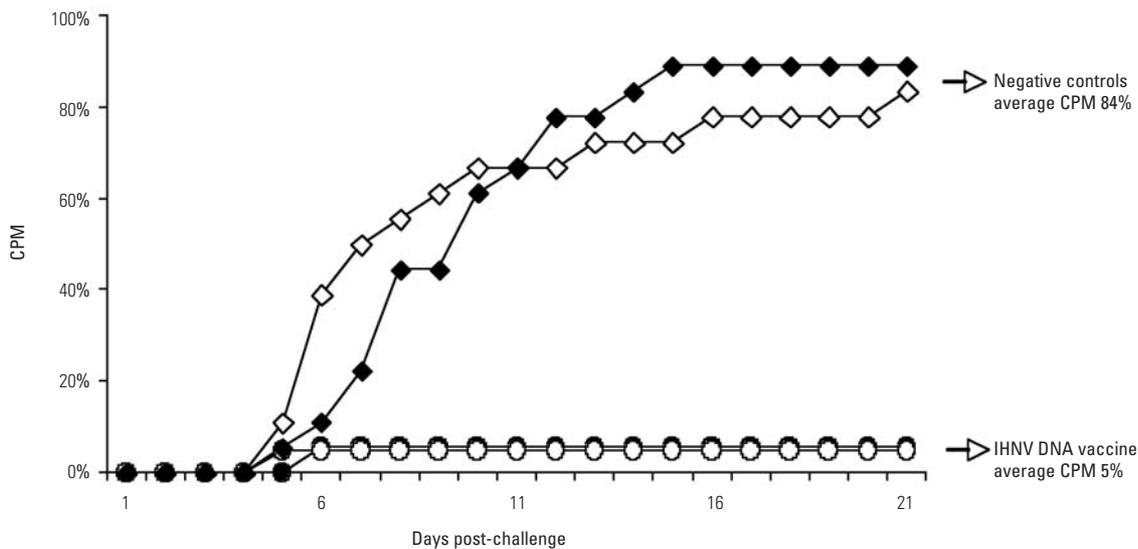
A great deal of fish DNA vaccine research has focused on viruses because of the importance of viral pathogens, the general lack of efficacious viral vaccines, the high cost of producing inactivated viral vaccines, and because viruses are relatively simple pathogens so identification of protective antigens is facilitated in many cases. DNA vaccines for the fish rhabdoviruses *Infectious haematopoietic necrosis virus* (IHNV) and *Viral haemorrhagic septicaemia virus* (VHSV) were reported to be protective in 1996 and 1998, respectively (5, 66). These are simple RNA viruses with only six genes, and the single viral surface protein (the glycoprotein, or G protein) was known to be the protective antigen (28, 29). These are also acute viral pathogens with a rapid disease course, and studies of these vaccines have been aided by reproducible challenge models in rainbow trout (*Oncorhynchus mykiss*). During the last ten years IHNV and VHSV DNA vaccines have been developed as models for investigating numerous aspects of fish DNA vaccines (16, 17, 49, 53, 64, 65, 68). They have been tested extensively by several laboratories and shown to be highly efficacious under a wide range of conditions. Other rhabdoviruses of fish share similar advantages, and *Hirame rhabdovirus* (HIRRV) in Japanese flounder (*Paralichthys olivaceus*) has now become an additional model for sophisticated studies of host responses (54, 98).

DNA vaccines for several other fish viruses initially proved more difficult to develop than the rhabdoviral vaccines. For some RNA viruses selection of protective antigen gene candidates was reasonably simple, but challenge models to test efficacy were difficult. For other RNA viruses, and large DNA viruses such as herpesviruses, identity of a protective antigen is often uncertain, although nearly all viral DNA vaccines use viral surface protein genes. Additionally, some host-pathogen relationships are more complex than others, such as the long-term chronic association of herpesviruses with their hosts, or the influence of stress in *Infectious pancreatic necrosis virus* (IPNV) infections. Despite these challenges, ongoing research has now led to demonstrations of significant efficacy for DNA vaccines against non-rhabdoviral fish viruses, as will be detailed below.

For bacterial fish pathogens there are several traditional killed vaccines, some delivered by immersion, that work extremely well. Therefore, DNA vaccine work has focused on bacteria for which killed bacterins are not effective. Identification of effective protective antigens is much more complicated for bacteria, and also for cellular parasites, so fewer DNA vaccines have been tested for these more complex pathogens. Also, disease pathogenesis is complex for some bacterial-host relationships such as infection with *Renibacterium salmoninarum* (the causative agent of bacterial kidney disease [BKD]) in salmonids. However, there are now examples of success among DNA vaccines for several fish bacteria, also detailed below.

### Laboratory trials for testing DNA vaccine efficacy

A critical factor in DNA vaccine research is efficacy trials using a laboratory challenge model. Typically, DNA vaccines are injected intramuscularly (IM) into groups of juvenile fish; negative control groups receive the same dose of vector plasmid DNA with no viral gene, vector with a reporter gene such as luciferase, buffer alone, or are left unhandled. Fish are held for a specified period of time and then challenged by intraperitoneal injection or immersion in water with a lethal dose of pathogen. Groups of vaccinated fish are also mock challenged to assess incidental mortality. Disease signs and mortality are then monitored daily for a period of time appropriate for the course of infection of the pathogen of interest. For acute pathogens such as IHNV, mortality in non-vaccinated negative control groups begins approximately one week post challenge (pc), increases exponentially for 7 to 14 days, and then levels off at a maximal value (e.g. Fig. 2). The mortality in negative control vaccine groups is then compared with mortality in vaccinated groups to assess protection afforded by the vaccine.



**Fig. 2**

**Example of determination of efficacy in a fish rhabdovirus DNA vaccine trial**

The graph shows cumulative percent mortality (CPM) in duplicate groups (filled and open symbols) of fish injected with an *Infectious haematopoietic necrosis virus* (IHNV) DNA vaccine or negative control fish injected with either buffer or a plasmid with no pathogen gene. Relative percent survival (RPS) for the vaccinated groups is calculated as  $RPS = [1 - (\text{mortality in vaccinated fish} / \text{mortality in controls})] \times 100$ , i.e.: for the data shown here  $(1 - 5/84) \times 100 = RPS\ 94$

Vaccine efficacy, or the strength of protection, is assessed in terms of:

- final cumulative percent mortality (CPM) of vaccinates and control groups at the end of the challenge period;
- relative percent survival (RPS) in vaccinated groups compared with negative control groups (43), calculated as  $RPS = [1 - (\text{CPM in vaccinates} / \text{CPM in negative controls})] \times 100$ . This metric is most valid when negative control groups reach at least 60 CPM;
- the kinetics of mortality, indicated as simple mean day to death (MDD) in different groups, or by estimating survival curves by Kaplan-Meier analysis and comparing the curves statistically;
- reduction in viral load or kinetics of viral clearance, as measured by qRT-PCR.

**Current status of viral DNA vaccines for aquaculture**

Table I provides a summary (as of 2007) of all reported trials of fish DNA vaccines containing pathogen genes. In all cases the vaccines were injected IM and expression of the pathogen gene was driven by a CMV promoter. Some vaccines included a GFP marker gene as a chimera with the pathogen gene. Most early studies were in rainbow trout, but trials have now also been conducted in Japanese

flounder, carp, turbot, Atlantic salmon, catfish, red sea bream, coho salmon, hybrid striped bass, spotted sand bass, and Asian seabass (Table I). In addition, reporter gene plasmids have demonstrated the potential for DNA vaccine expression in gilthead sea bream (*Sparus aurata*) (113). To date there have been no reports of any DNA vaccine for shellfish or crustaceans.

Among vaccines for viral fish pathogens (Table Ia), DNA vaccines containing the G gene of the fish pathogens IHNV, VHSV, and HIRRV all show reproducible, high efficacy (70 to 100 RPS) against severe viral challenges. These viral species are all within the virus taxonomic family *Rhabdoviridae*, which also contains mammalian viruses such as vesicular stomatitis virus and rabies virus (102). For each of these three fish rhabdoviruses high DNA vaccine efficacy has been shown with at least two different plasmid constructs created and tested independently in at least two different laboratories. In addition to the European freshwater VHSV DNA vaccines, there is now a DNA vaccine against a marine strain of VHSV that has shown high efficacy in flounder (16, 17). Several DNA vaccines have been constructed for another important rhabdoviral pathogen, spring viraemia of carp virus (SVCV), but it has proven more difficult to reliably demonstrate efficacy with these DNA vaccines. It is not clear whether this is due to difficulties or inconsistencies with the challenge models in carp (*Cyprinus carpio*), or whether it is an inherent feature of these vaccines, possibly related to SVCV being in a

Table Ia

**Efficacy reported in experimental challenge trials of DNA vaccines for aquatic animal pathogens: viral pathogens**

For *Infectious haematopoietic necrosis virus* and freshwater *Viral haemorrhagic septicaemia virus* only the first report is shown and subsequent reports are detailed in Tables II to V; for other pathogens all published reports are shown

Pathogen	Viral family, genome type <sup>(a)</sup>	Experimental conditions <sup>(b, c)</sup>	Gene(s) in vaccine	Vaccine dose(s), µg	Protection (RPS <sup>(d)</sup> )	Year (ref.)
<b><i>Infectious haematopoietic necrosis virus</i></b>	<i>Rhabdoviridae</i> ssRNA, minus-sense	1 g rainbow trout, challenge 6 weeks pv, 65-75% CPM in controls	G glycoprotein	10	75*	1996 (5)
<b><i>Viral haemorrhagic septicaemia virus (VHSV), freshwater</i></b>	<i>Rhabdoviridae</i>	13 g rainbow trout, challenge 52 days pv, 93% CPM in controls	G	10 50	97* 94*	1998 (66)
<b>VHSV, marine</b>	<i>Rhabdoviridae</i>	3 g Japanese flounder, challenge 1 month pv, 73-100% CPM in controls	G	10	93-100*	2005 (16)
<b>VHSV, marine</b>	<i>Rhabdoviridae</i>	10 g Japanese flounder, challenge 1 month pv, 83-100% CPM in controls	G	10	90-96*	2006 (17)
<b><i>Hirame rhabdovirus (HIRRV)</i></b>	<i>Rhabdoviridae</i>	2 g Japanese flounder, challenge 28 days pv, 100% CPM in controls	G	1 10	70* 90*	2004 (98)
<b>HIRRV</b>	<i>Rhabdoviridae</i>	3 g Japanese flounder, challenge 21 days pv, 95% CPM in controls	Partial G	5	95*	2006 (90)
<b>HIRRV</b>	<i>Rhabdoviridae</i>	10 g Japanese flounder, challenge 28 days pv, 98% CPM in controls	G	10	86*	2007 (117)
<b>Spring viraemia of carp virus (SVCV)</b>	<i>Rhabdoviridae</i>	11 g common carp, challenge 6 weeks pv, 64% CPM in controls	Mix of two different G plasmids	25 each	48*	2006 (46)
<b>SVCV</b>	<i>Rhabdoviridae</i>	1.5-4.3 g koi carp, challenge 28 days pv, 70-100% CPM in controls	G	10	50-88*	2007 (E. Emmenegger & G. Kurath, unpublished)
<b><i>Infectious pancreatic necrosis virus</i></b>	<i>Birnaviridae</i> dsRNA	20 g Atlantic salmon post-smolts, challenge 69 days pv, 34% CPM in controls	– Segment A large ORF and VP2 – VP2 alone	15 each 25	84* 29*	2004 (71)
<b><i>Infectious salmon anaemia</i></b>	<i>Orthomyxoviridae</i> ssRNA, plus-sense, segmented	20 g Atlantic salmon pre-smolts, challenge 9 weeks pv, 41% CPM in controls	Hemagglutinin- esterase HE	15 and two 15 µg boosters	40-60	2005 (71)
<b><i>Red seabream iridovirus</i></b>	<i>Iridoviridae</i> large dsDNA	5-10 g red seabream, challenge 30 days pv, 57-90% CPM in controls	Major capsid protein ORF569	25 25	43-69 48-71	2006 (18)
<b><i>Channel catfish virus (CCV)</i></b>	<i>Herpesviridae</i> large dsDNA	6-10 month-old channel catfish, challenge 4-6 weeks pv 44-66% CPM in controls	ORF6 ORF59 ORF6&59	50 50 50 each	15 38 46	2002 (76)
<b>CCV</b>	<i>Herpesviridae</i>	1 g channel catfish, challenge 5 weeks pv, 70-85% CPM in controls	ORF6 <sup>(e)</sup> ORF59 <sup>(e)</sup> ORF6&59 <sup>(e)</sup>	50 50 50 each	0 0 0	2005 (39)
<b><i>Atlantic halibut nodavirus (AHNV)</i></b>	<i>Nodaviridae</i> ssRNA, plus-sense	3.6 g turbot, challenge 35 days pv, 27% CPM in controls	C capsid protein	5	(0)	2003 (94)
<b>AHNV</b>	<i>Nodaviridae</i>	2.2 g turbot, challenge 10 weeks pv, 39-43% CPM in controls	C	20	– 7	2005 (95)

different genus from the other fish rhabdoviruses described above (102). Nevertheless, significant efficacy has recently been reported with a combination of two SVCV G plasmids in carp (46), and current work with an independently constructed SVCV DNA vaccine has shown significant efficacy in repeated trials in koi (E. Emmenegger and G. Kurath, unpublished findings).

Outside the rhabdovirus family there are now DNA vaccines that show significant protection, with moderate

levels of efficacy, against three different virus species (Table Ia): IPNV, *Infectious salmon anaemia virus* (ISAV), and *Red seabream iridovirus* (RSIV). This represents significant progress in broadening DNA vaccines for aquaculture because the viruses are from three different viral families with diverse genomes (Table Ia), including a double-stranded RNA virus, a single-stranded positive-sense RNA virus, and a large DNA virus. In each case, successful protection has been reported in a single publication. IPNV is an important pathogen of Atlantic salmon (*Salmo salar*)

**Table 1b**  
**Efficacy reported in experimental challenge trials of DNA vaccines for aquatic animal pathogens: bacterial and eukaryotic pathogens**

Pathogen	Pathogen type	Experimental conditions <sup>(b)</sup>	Gene(s) in vaccine	Vaccine dose(s), µg	Protection (RPS <sup>(d)</sup> )	Year (ref.)
<i>Aeromonas veronii</i>	Gram-negative, extracellular bacteria	10 g spotted sand bass, challenge 4 weeks pv, 41-43% CPM in controls	OMP38	20	54	2005 (112)
			OMP48	20	60	
			OMP38&48	20	62	
<i>Vibrio anguillarum</i>	Gram-negative, extracellular bacteria	10 g Asian seabass, challenge 35 days pv, 86-90% CPM in controls	OMP38	20	56*	2007 (48)
<i>Piscirickettsia salmonis</i>	Gram-negative, obligate intra-cellular bacteria	20 g coho salmon, challenge 101 days pv, 100% CPM in controls	Gene expression library from <i>P. salmonis</i>	20 and 10 µg booster	20	2003 (73)
<i>Mycobacterium marinum</i>	Acid fast/Gram-positive, facultative intra-cellular bacteria	40-50 g hybrid striped bass, challenge 90 days pv, 100% CPM in controls	Ag85A	25 50	80* 90*	2005 (77)
<i>Mycobacterium marinum</i>	Acid fast/Gram-positive, facultative intra-cellular bacteria	40-50 g hybrid striped bass, challenge 120 days pv, 100% CPM in controls	Ag85A	25 50	88-91 on day 28 pc, but 0 by day 46 pc	2006 (78)
<i>Renibacterium salmoninarum</i>	Gram-positive, facultative intra-cellular bacteria	10 g Chinook salmon, challenge 5 weeks pv, 26-32% CPM in controls	p57	10	(0)	2005 (R. Pascho, unpubl.)
<i>Cryptobia salmositica</i>	Haemoflagellate parasite	51 g rainbow trout, challenge 7 weeks pv, no mortality in controls	Metalloprotease	50	Parasitaemia reduced <sup>(f)</sup>	2007 (99)

pc: post-challenge

pv: post-vaccination

RPS: relevant percent survival

\* indicates statistically significant protection in vaccinates

(a) Viral genome type is indicated as follows:

ssRNA: single-stranded RNA

dsRNA: double-stranded RNA

dsDNA: double-stranded DNA

(b) All vaccines were delivered by intramuscular injection; where one or more booster vaccines were administered, the post-vaccination period indicated here (i.e. days/weeks pv) refers to the number of days/weeks between viral challenge and the administration of the initial vaccine dose, not subsequent booster doses

(c) Host species are rainbow trout (*Oncorhynchus mykiss*), Japanese flounder (*Paralichthys olivaceus*), common or koi carp (*Cyprinus carpio*), Atlantic salmon (*Salmo salar*), red seabream (*Pagrus major*), channel catfish (*Ictalurus punctatus*), turbot (*Scophthalmus maximus*), spotted sand bass (*Paralabrax maculatofasciatus*), Asian seabass (*Lates calcarifer*), coho salmon (*O. kisutch*), hybrid striped bass (*Morone saxatilis* × *M. chrysops*), and Chinook salmon (*O. tshawytscha*)

(d) Trials with unusually low challenge severity (less than 30 cumulative percent mortality [CPM] in controls) are noted by parentheses around RPS values

(e) These plasmid constructs, tested in 2005 by Harbottle *et al.* (39), had been tested previously by Nusbaum *et al.* in 2002 (see previous row [76]). Contrary to the 2002 results, Harbottle found that these plasmids provided no protection; Nusbaum sent the plasmids that had been used in his study to Harbottle for re-testing, and the two research groups concluded that these DNA vaccines were not protective

(f) Protection against *C. salmositica* was evident in lower parasite load, slower peak parasitaemia, and faster recovery in DNA vaccinated fish relative to negative control fish

and rainbow trout. In a complex study several different IPNV DNA vaccine constructs were tested, but protection was only observed in the treatment group that received a combination of plasmids including one that contained the entire large polyprotein open reading frame of genome segment A (72). It has been noted that the challenge model for IPNV is difficult (7), and mortality was low (32%) in negative control groups. However, the average of 5% mortality in vaccinates was consistent between triplicate groups of 50 fish, indicating significant protection. ISAV is another Atlantic salmon pathogen that emerged dramatically in European and North American salmon farms in the 1980s. A DNA vaccine containing the ISAV haemagglutinin-esterase gene, administered as a primary dose and two boosters, provided 'moderate protection' to Atlantic salmon with an RPS of 40 to 60 (71). Finally, RSIV

is a pathogen of cultured red seabream (*Pagrus major*) in Japan. In a recent study two RSIV DNA vaccines containing either the major capsid protein or a transmembrane protein domain (ORF569) were described (18). Both vaccines showed reproducible protection, providing RPS values of 43 to 71. A DNA vaccine for another large DNA iridovirus, lymphocystis disease virus has been constructed and shown to express viral antigen in Japanese flounder (119), but protection against challenge was not reported.

In addition to DNA vaccines that provide significant protection against experimental challenge, it is also important to understand which DNA vaccines conclusively do not elicit protection. DNA vaccines containing internal viral nucleocapsid protein (N) genes are clearly not protective for IHNV, HIRRV, or ISAV (5, 22, 71, 90, 117).

DNA vaccines containing two different genes from a fish herpesvirus, *Channel catfish virus* (CCV), were initially reported to be protective in catfish (76), but later work by another investigator was unable to confirm the protection despite using the same viral genes (39) (Table Ia). In a fine example of cooperative science, the analogous vaccines constructed independently in the two research laboratories were tested side-by-side, providing a conclusive demonstration that these DNA vaccines did not protect catfish against CCV under the conditions tested (39). As another example, a DNA vaccine containing the capsid protein (C) of *Atlantic halibut nodavirus* (AHNV) clearly did not provide protection in two different challenge studies with juvenile turbot (*Scophthalmus maximus*) (94, 95). This is notable because expression of the C protein was confirmed in DNA vaccinated fish, and treatment groups vaccinated with a recombinant C protein vaccine in the same vaccine trial did show protection. Thus, the C protein was indeed a protective antigen, but it was not protective when expressed from a DNA vaccine. This serves as an important reminder that DNA vaccines will not be the best vaccine option for all pathogens.

### Status of bacterial and eukaryotic pathogen DNA vaccines for aquaculture

As mentioned earlier, research on fish bacterial DNA vaccines has focused on species for which traditional bacterins are not efficacious. There are now reports of successful DNA vaccines for two extracellular Gram-negative bacterial fish pathogens (Table Ib). For both *Aeromonas veronii* and *Vibrio anguillarum*, DNA vaccines encoding major outer membrane proteins provided moderate protection with RPS values of 54 to 62 (48, 112). The success of these vaccines builds on antigen characterisation studies identifying OMP48 as a good candidate antigen in *A. veronii* (111). Similar candidate antigen work has been done for *Aeromonas hydrophila*, leading to cloning of beta-haemolysin (37) and an extracellular serine-protease (19), but vaccine trials have not been reported.

DNA vaccines have also been constructed and tested for three important intracellular bacterial fish pathogens (Table Ib). For *Piscirickettsia salmonis* a complex mixture of DNA vaccine plasmids containing an expression library of bacterial genomic DNA provided partial protection against a severe challenge in coho salmon (73), but further studies to identify the specific protective antigen(s) have not been reported. As an alternative approach there has also been work on identifying immunoreactive proteins of *P. salmonis* for use as candidate antigens in DNA vaccines (14). For *Mycobacterium marinum* in hybrid striped bass there have been two reports describing a DNA vaccine containing a gene encoding one of the major secreted fibronectin-binding proteins, Ag85A (77, 78). This vaccine provides

excellent protection against a severe experimental challenge, but the protection appears to be of short duration, with a high level of delayed mortality occurring in vaccinates after 28 days post-challenge. Finally, there have been some studies investigating DNA vaccines against *Renibacterium salmoninarum*, the agent of BKD in salmon. Grayson *et al.* used DNA vaccine plasmids containing five individual *R. salmoninarum* proteins to explore the host response, but they were not tested for efficacy as vaccines (38). In a thorough test of several BKD vaccines in Chinook salmon (2) an unreported treatment group that received a DNA vaccine encoding the highly expressed *Msa* (p57) virulence factor showed no protection (Table Ib) (S.W. Alcorn and R. Pascho, personal communication).

DNA vaccines against eukaryotic parasites of fish are generally at an early stage of development. Due to the higher complexity of these organisms much of the recent effort is directed toward identification of appropriate candidate antigen genes that might be protective as DNA vaccines. One of the best characterised systems is for the protozoan ciliate *Ichthyophthirius multifiliis* (Ich), where the host protective immune response has been described and protective immobilisation antigens (i-antigens) of the parasite are known (15, 59). Expression of these antigens from synthetic genes has been accomplished and DNA vaccines containing these genes stimulate specific antibodies in catfish, but to date they have not provided significant protection against challenge (59). In another parasite system DNA vaccines expressing protease genes from the haemoflagellate parasite *Cryptobia salmositica* have recently been tested in vaccine trials in salmonid fish (99). The experimental challenge was not lethal for this pathogen but rainbow trout that received a DNA vaccine expressing the metalloprotease virulence factor had lower parasite loads, a slower peak of parasitaemia, and faster recovery relative to control fish. This is the first published demonstration of protective effects of a fish parasite DNA vaccine in fish.

### Factors that affect DNA vaccine efficacy

The majority of our understanding of factors that affect fish DNA vaccine efficacy comes from numerous publications on fish rhabdovirus DNA vaccines. As expected for any well-studied vaccines, the specific level of protection observed with the IHNV and VHSV DNA vaccines varies with several factors, including vaccine dose, delivery route, host species, host size/age, length of time between vaccination and viral challenge, severity of challenge, and relatedness of the challenge virus strain to the strain represented by the vaccine. In addition, the interplay of these factors can influence efficacy. For example, vaccine trials with high viral challenge severity may result in lower protection (RPS) than trials with a more moderate challenge pressure, or a higher vaccine dose may

compensate for reduced efficacy seen in challenges with heterologous viral strains. Despite these complexities DNA vaccines for IHNV and VHSV reliably demonstrate significant protection under varying conditions.

### Severity of challenge

In experimental challenge studies the severity of the pathogen challenge is generally controlled by the pathogen dose, duration, and route of exposure. However, even in well-known systems factors such as variation in fish condition, water quality or seasonality can lead to variation in final mortality produced. Therefore, it is essential to consider vaccine efficacy reported in terms of RPS in light of the level of mortality observed in control fish that did not receive the candidate vaccine being tested. If control fish groups experienced only low mortality, then calculated RPS can imply high efficacy that may not be effective at higher pathogen challenge levels. Conversely, if negative control fish groups have 100% mortality this severe challenge could overwhelm protection that might be evident at less severe challenge pressure. In many published DNA vaccine trials negative control groups reach 75 to 100% mortality, so the vaccines are typically tested for ability to provide protection against severe viral challenge (Table I). The effect of challenge severity on vaccine performance is not only important for interpreting results of experimental trials, but it is also relevant in field applications, where pathogen pressure may vary from low levels in endemic areas to high levels during epidemics.

### Vaccine dose

The majority of fish DNA vaccines have been tested at single doses ranging from 5 µg to 50 µg (Table I). However, for fish rhabdovirus DNA vaccines against IHNV, VHSV and HIRRV, a single IM injection of a 1.0 µg dose, with no adjuvant or boosters, is sufficient to provide a high level of protection in juvenile fish. The minimal effective dose has been defined for IHNV and VHSV DNA vaccines in different sizes of rainbow trout (Table II). In juvenile animals (approximately 1 g to 10 g) vaccine doses of 0.1 µg to 1.0 µg reproducibly provide 70 to 100% RPS, and doses as low as 1 ng provide reduced but significant levels of protection. In sub-adult trout of 100 g to 120 g higher vaccine doses are required: 0.1 µg is not protective but 0.5 µg to 1.0 µg provides strong protection (Table II). Levels of protection are comparable in the rhabdoviral systems and protection appears to be dose dependent in several studies (23, 61, 70).

### Fish life stage

Most tests of fish DNA vaccines have been conducted in juvenile fish, where epidemics frequently occur in aquaculture. As detailed in Tables I and II, many DNA vaccines perform well at this early life stage. Trials in sub-adult fish have been successful for IHNV and VHSV DNA

vaccines in rainbow trout (55, 70), and for an IHNV DNA vaccine in Atlantic salmon smolts (104). The *Mycobacterium marinum* vaccine showed strong but transient efficacy in 50 g hybrid striped bass (Table I) (77, 78). Interestingly, high doses of the IHNV DNA vaccine do not appear to provide protection to sexually mature rainbow trout (S.E. LaPatra, personal communication) or returning mature sockeye salmon (*O. nerka*) (Garver, Varney and Kurath, unpublished findings).

### Protection against heterologous virus strains

An important factor for vaccine efficacy is the ability to provide broad protection against genetically diverse strains of the same viral species. Extensive genetic typing and phylogenetic analyses of VHSV field isolates have defined four major genotypes (I to IV) (27, 93). European genotype I virus isolates differ by up to 6% in nucleotide sequence and several subtypes (Ia-Ie) have been defined. The G gene in the well-studied VHSV DNA vaccine pcDNA3vhsG (61) is from a genotype Ia virus. The majority of publications using this vaccine describe homologous challenges, but it has also been tested for efficacy against two heterologous VHSV strains from genotype Ia (67) and genotype Ic (62). In both cases, pcDNA3vhsG provided significant protection, but with reduced efficacy (54 to 78 RPS) compared to simultaneous challenges with the homologous virus strain (77 to 100 RPS). Cross-genotype challenges assessing efficacy against VHSV strains from European genotypes II and III or the North American genotype IV have not been reported. The marine VHSV DNA vaccine that is efficacious in flounder (16, 17) carries a genotype IV strain G gene but it has not been tested in heterologous challenges.

Phylogenetic analyses of IHNV field isolates have defined three major genogroups in North America, designated U, M, and L, as well as several subgroups within genogroup M (M-A through M-F) (51, 105, 106). The average pairwise nucleotide diversity between virus isolates in different IHNV genogroups is 4% to 6%. The two IHNV DNA vaccines used in most research studies, pCMV4-G and pIHNVw-G, contain G genes from IHNV strains in the U and M-A genogroups, respectively (5, 22). The viral strains used to construct these vaccines have low virulence in rainbow trout so they are typically tested using heterologous challenges with more virulent strains from the M genogroup. A high dose of the U genogroup IHNV vaccine provided significant protection (75 to 87 RPS) to rainbow trout challenged with two M genogroup viral strains (5, 47). A low dose (0.1 µg) of the M genogroup vaccine provided significant protection (56 to 100 RPS) to rainbow trout challenged with six heterologous viral strains from the M and U genogroups (23). The IHNV M genogroup DNA vaccine also provided significant protection against heterologous U and L genogroup viral strains in other salmonid host species where the U and

Table II

**Efficacy of fish rhabdovirus glycoprotein gene DNA vaccines in experimental trials testing various doses of vaccine in different sizes of rainbow trout or Japanese flounder**

Virus	Experimental conditions <sup>(a)</sup>	Vaccine dose ( $\mu$ g)	Relative percent survival <sup>(b)</sup>	Ref.
<b>Minimum effective vaccine dose in juvenile fish (1 to 10 g)</b>				
IHNV	2.0 g rbt, challenge 4 to 6 weeks pv, 36-58% CPM in controls	10	100*	22
		5	97*	
		1.0	97*	
	1.8 g rbt, challenge 6 weeks pv, 30-48% CPM in controls	5	100*	23
		1	100*	
		0.1	97*	
		0.01	100*	
		0.01	90*	
	0.8 g rbt, challenge 6 weeks pv, 2-60% CPM in controls	0.01	83*	23
0.001		61*		
VHSV	3.5 g rbt, challenge 60 days pv, 96-97% CPM controls	10	78*	67
		1	90*	
		0.1	77*	
	3 to 4 g rbt, challenge 51 days pv, 92-100% CPM in controls	1.0	100*	61
		0.1	97*	
		0.01	78*	
		0.001	14*	
	10 g rbt, challenge 4 weeks pv, 80% CPM in controls	0.5	73*	70
		0.1	53*	
0.01		43*		
HIRRV	2 g flounder, challenge 4 weeks pv, 100% CPM in controls	10	90*	98
		1.0	70*	
<b>Minimum effective vaccine dose in sub-adult fish</b>				
IHNV	120 g rbt, challenge 6 weeks pv, 90% CPM controls	25	100*	55
		10	100*	
		1	100*	
	110 g rbt, challenge 6 weeks pv, 40-50% CPM in controls	10	100*	55
		1	100*	
VHSV	100 g rbt, challenge 4 weeks pv, 34% CPM in controls	0.5	59*	70
		0.1	0	
		0.01	12	

rbt: rainbow trout  
 flounder: Japanese flounder  
 IHNV: *Infectious haematopoietic necrosis virus*  
 VHSV: *Viral haemorrhagic septicaemia virus*  
 HIRRV: *Hirame rhabdovirus*

(a) Vaccines were injected intramuscularly and viral challenge was 4 to 10 weeks post-vaccination (pv). Specific DNA vaccines in these trials are pIHNw-G for IHNV, pcDNA3vhsG for VHSV, and pCMV-HIRg for HIRRV. Challenge severity is indicated by cumulative percent mortality (CPM) in control groups of unhandled fish or fish injected with vector DNA, vector with a non-viral gene (e.g. luciferase), or buffer

(b) Efficacy is shown as relative percent survival (RPS, see text) of fish in vaccinated groups compared with control groups

\* indicates statistically significant protection in vaccinates

L strains are more virulent (34). In a thorough summary of efficacy trials with the M genogroup IHNV DNA vaccine (pIHNw-G), Garver *et al.* observed that when the vaccine and viral challenge strains were derived from different genogroups (cross-genogroup), the protection was significant, but consistently lower (52 to 68 RPS) than protection with intra-genogroup challenges (89 to 97 RPS) (34). Combined with the VHSV heterologous challenge studies, these results suggest that fish rhabdovirus DNA vaccines are capable of providing broad, significant protection, but efficacy varies with the relatedness of the challenge strain to the strain used to create the vaccine.

## Distribution and persistence of DNA vaccines in fish

The fate of DNA vaccine plasmids in fish has been investigated using both reporter gene constructs and rhabdovirus DNA vaccines. Studies with reporter genes (e.g. CAT,  $\beta$ gal, luciferase, or GFP) in several different host species have shown that the great majority of expression is focused in muscle tissue at the site of delivery, although some expression in other tissues, at lower levels and for shorter duration, has been occasionally observed (6, 36, 41, 87, 96, 107, 113). The same studies often report

relatively long-term persistence of plasmid DNA or reporter gene expression in muscle, including luciferase activity two years after DNA vaccination in living glass catfish (*Kryptopterus bicirrhus*) (26).

Similar studies have been conducted with IHNV and VHSV DNA vaccines, in which plasmid DNA is detected by PCR and expression of the G protein is detected by immunohistochemistry using G-specific monoclonal antibodies. Upon injection plasmid DNA is rapidly distributed throughout the fish, but it subsequently clears from systemic tissues and persists at detectable levels only in muscle tissue at times longer than 7 to 14 days post-injection (33, 88). Expression of G protein is consistently most prevalent in injection site muscle tissue (9, 33, 62, 66, 88), although trace amounts of G protein have been detected in kidney and thymus (33). G protein expression peaks at 2 to 3 weeks post-injection and has not been detected beyond a month, possibly due to the lower sensitivity of immunohistochemistry compared with reporter gene assays (33, 62). IHNV vaccine plasmid DNA quantities in muscle decline by over 99% from day 1 to 35 post-vaccination (88) but then persist in amounts detectable by PCR for at least 90 days or longer (33, 88). With regard to the mechanism of plasmid DNA elimination from fish, a recent study using reporter plasmids and radioactively labelled plasmid DNA has shown a scavenger mechanism involving specific endocytosis and degradation of plasmid DNA in endocardial cells of cod (*Gadhus morhua* L.) (91).

### Host responses to DNA vaccines in fish

The goal of exploring host response is to define features of the 'protective immune response' that may have more general application in fish health research and vaccine development. Again, the fish rhabdovirus systems have generated the most advanced knowledge available. The first insight into the mechanisms of fish host response to DNA vaccination came from observations on the onset and duration of protection provided by the IHNV and VHSV DNA vaccines. These temporal features have been defined in multiple efficacy trials with challenges conducted at various times from one day to two years post-vaccination (Table III). For both vaccines the onset of strong significant protection is as early as 4 to 8 days post-vaccination (56, 61, 69). For the VHSV vaccine high efficacy has been demonstrated up to 9 months post-vaccination. The IHNV vaccine showed high efficacy for 3 months and then slightly reduced but significant efficacy (65 to 69 RPS) for 6 to 24 months post-vaccination. This detailed knowledge of the timing and magnitude of protective efficacy has been used to define three phases of the host response to DNA vaccination (50, 53, 64, 68).

### Three-phase model of host response to fish rhabdovirus DNA vaccines

The first phase is the early anti-viral response (EAVR), which confers strong protection with high RPS values as early as 4 to 8 days after vaccination. During this phase protection is non-specific, so that fish vaccinated with a rhabdovirus DNA vaccine are cross-protected against challenge with other fish rhabdoviral species, and also against the unrelated Atlantic halibut nodavirus, but not against bacterial pathogens (47, 56, 69, 94). The cross-protective capability is transient, so that protection becomes specific in a matter of several weeks, marking the end of the EAVR. The subsequent specific antiviral response (SAVR) phase is characterised by continuing high efficacy protection that is specific for the species represented in the DNA vaccine. The SAVR lasts for several months in rainbow trout and correlates with appearance of specific neutralising antibodies stimulated by the vaccines. The last phase is the long-term anti-viral response (LAVR), defined by reduced but significant protective efficacy and decline or absence of detectable neutralising antibodies (50). There have been many studies using biotechnology to elucidate the host innate or adaptive immune response mechanism(s) that are active during these phases of response to DNA vaccines. These have been recently reviewed in detail (53) so they will be treated only briefly here.

### Gene expression studies by quantitative RT-PCR

Host gene expression changes in response to DNA vaccination have been particularly well studied early after vaccination, during the EAVR, to define the host immune response that provides rapid non-specific protection against viral pathogens. The most commonly assayed gene is the interferon-inducible Mx gene, which functions as a reliable indicator of activation of the anti-viral type 1 interferon pathway. Several studies have used semi-quantitative or quantitative RT-PCR to show increased expression of the Mx gene in response to fish rhabdovirus DNA vaccines (1, 9, 70, 80, 81) and induction of the Mx protein has been shown by immunoblot (47). Kinetics of Mx induction have been shown to vary between rainbow trout and Atlantic salmon (1), and differential regulation of three trout Mx isoforms in response to a VHSV DNA vaccine has been described (97).

In addition to Mx, VHSV virally-induced genes designated VIG-1, VIG-2, and VIG-8, which are also induced by interferon, have been shown to be strongly induced by DNA vaccination (11, 12, 80). Using a suite of qRT-PCR assays for eight trout cytokine and cytokine-related genes, Purcell *et al.* reported that the only response in spleen tissue that was specific to DNA vaccination was up-regulation of the interferon inducible Mx and Vig-8 genes (80). Takano *et al.* reported up-regulation of seven immune

**Table III****Onset and duration of protection provided by *Infectious haematopoietic necrosis virus (IHNV)* and *Viral haemorrhagic septicaemia virus (VHSV)* DNA vaccines in rainbow trout**

Entries are as described in Table II

Experimental conditions	Challenge time post-vaccination <sup>(a)</sup>	Relative percent survival in different trials	Ref.
<b>IHNV DNA vaccine and IHNV challenge</b>			
2.0 g rbt, 1.0 µg vaccine, 27-98% CPM in controls	1 d	12, 0, 8	56
	2 d	0, 75*	
	4 d	93*, 53*, 88*	
	7 d	96*, 76*, 96*, 98*	
	14 d	74*, 93*	
	21 d	96*	
	28 d	99*, 81*	
	28 d	97*	
58 d	68*		
80 d	48*		
2.5 g rbt, 0.1 µg vaccine, 76-96% CPM in controls	3 mo.	100*	50
	6 mo.	69*	
	13 mo.	65*	
	25 mo.	66*	
226 g rbt, 10 µg vaccine, 60% CPM in controls	12 mo.	83*	50
<b>VHSV DNA vaccine and VHSV challenge</b>			
4 g rbt, 1.0 µg vaccine, 94-98% CPM in controls	8 d	66*	61
	19 d	88*	
	28 d	97*	
	61 d	97*	
	112 d	79*	
	168 d	64*	
0.6 g rbt, 1.0 µg vaccine, 92% CPM in controls	9 d	98*	65
2 to 4 g rbt, 1.0 µg vaccine, 86-99% CPM in controls	4 d	78*	69
	7 d	80*	
	60 d	99*	
	84 d	95*	
10 g rbt, (0.5, 5, 10) µg vaccine, 90% CPM in controls	7 d	80 to 90*	70
4.5 g rbt, 0.5 µg vaccine, 80-86% CPM in controls	4 wk	76*	70
	8 wk	85*	
100 g rbt, (0.5, 5, 10) µg vaccine, 48% CPM in controls	7 d	92 to 96*	70
100 g rbt, 0.5 µg vaccine, 85% CPM in controls	9 mo.	88*	70

rbt: rainbow trout

(a) Timing of challenge is given in days (d), weeks (wk), or months (mo.) post-vaccination

\* Indicates statistically significant protection in vaccinates

related genes including MHC and T-cell receptor genes 7 days after HIRRV DNA vaccination of flounder (98). As qRT-PCR assays become available for more immune genes from more host species, common features of protective host responses will emerge. At present the main consistent observation is induction of interferon-inducible genes such as Mx and VIG genes 7 to 10 days post-vaccination with fish rhabdovirus DNA vaccines. The magnitude of induction is greatest in muscle at the injection site, but

there is also significant up-regulation of Mx and VIG gene expression in systemic tissues such as spleen, liver, and kidney, indicating systemic activation of the innate interferon 1 anti-viral pathway (53, 81).

### Gene expression studies by microarray analysis

Comprehensive surveys using microarray technology to assess global host transcriptional gene expression changes in response to DNA vaccination have been reported in

three publications. The first study used a 779 feature microarray to investigate gene expression changes in kidney tissue collected from Japanese flounder 1, 3, 7 and 21 days after IM injection with a marine VHSV G gene DNA vaccine (16). The maximum response was at 3 days post-vaccination, when genes associated with both non-specific and specific immunity were up-regulated. Interferon inducible genes including the Mx and interferon regulatory factor genes were the most strongly induced genes in this systemic tissue. In the second publication a salmonid microarray with 16,008 features was used to analyse gene expression changes in rainbow trout muscle tissue 7 days after vaccination with an IHNV DNA vaccine (81). At this primary expression site there was up-regulation of genes associated with antigen presenting cells, lymphocytes, leucocytes, inflammation, antigen presentation, and interferon pathways. Confirmatory qRT-PCR analysis of selected genes at systemic sites (gill, spleen, kidney) of the same fish showed induction of genes associated with the type 1 interferon pathway to be the main systemic response. Finally, a 796 feature microarray was used to assess gene expression changes in kidney of Japanese flounder vaccinated with a highly protective HIRRV-G DNA vaccine, in comparison with a non-protective HIRRV-N gene DNA vaccine (117). Results identified five genes specifically up-regulated 7 days after vaccination with the protective HIRRV-G vaccine, including three known interferon inducible genes (Mx, ISG15, and ISG56) and two unidentified genes.

The combined results of these microarray studies and qRT-PCR assays of selected genes provide a consistent conclusion in which the high efficacy of fish rhabdovirus DNA vaccines is correlated with rapid up-regulation of genes associated with the type 1 interferon pathway (e.g. Mx and VIG genes) during the EAVR, at both primary and systemic tissue sites. Although up-regulation of genes associated with an inflammatory response was observed at the vaccine injection site, this was not observed at systemic sites where virus replication occurs (81). To date, few gene expression studies at later time points have been conducted, so changes associated with specific protection during the SAVR or LAVR remain to be characterised.

### Antibody responses

The generation of specific humoral antibody has been detected in response to many proteins expressed from DNA vaccines in fish. In general, development of detectable titres of neutralising antibodies (NAb) correlates with protection against pathogen challenge. The magnitude and kinetics of NAb responses to fish rhabdovirus DNA vaccines have been recently reviewed (53, 64, 68) and are summarised in Table IV. High levels of seroconversion have been reported in sub-adult rainbow trout (~ 100 g average weight) 4 to 5 weeks post-vaccination with rhabdovirus DNA vaccines and in

juvenile fish at 6 to 12 weeks post-vaccination. As mentioned earlier, the timing of these antibody responses correlates with the specific SAVR phase of protection by fish rhabdovirus vaccines. Many studies report higher levels of protection (RPS) than seroconversion, indicating that protection in some fish occurs in the absence of detectable neutralising antibodies (see bold RPS values in Table IV). This may be due to biological activity of antibody at titres below detection, or it may indicate contributions of other mechanisms such as non-neutralising antibodies or cellular immune responses.

### Cellular responses

Cell-mediated immune responses involving cytotoxic T lymphocytes (CTLs) and the TH1 subset of T helper cells are induced by DNA vaccines in mammals, contributing in many cases to protection elicited by these vaccines. In the past, a lack of reagents to assess T cell activity in fish has limited explorations of cellular responses to fish DNA vaccines, but recently, advances have been made in our understanding of cytotoxic cells in fish. Sequences of several genes encoding major T-cell related molecules from fish are now available (32), and clonal expansion of T cells has been described in response to both VHSV infection and DNA vaccination (8, 10). In addition, an important experimental system based on an MHC-matched homozygous rainbow trout and cell line model has been used to demonstrate specific cell-mediated cytotoxicity against VHSV-infected cells (25). This system has recently been used to investigate cell-mediated immune responses to DNA vaccines carrying either the G or N genes of VHSV (109). Peripheral blood leukocytes (PBL) from trout immunised with the VHSV-G DNA vaccine specifically killed both MHC-matched and xenogeneic target cells that had been infected with VHSV, indicating both innate (NK cells) and adaptive (CTL) cellular responses to the DNA vaccine. In contrast, PBL from trout injected with the VHSV-N gene DNA vaccine, which is not protective, only killed VHSV-infected MHC matched target cells, indicating only the adaptive CTL response. Additional interesting results in the same paper include evidence of seasonality in the CTL response to the VHSV-N DNA vaccine and specific homing of leukocytes transferred from DNA vaccinated fish into naïve fish. This comprises the first demonstration of specific and non-specific cell-mediated cytotoxicity in response to a fish DNA vaccine, confirming similarities with mammalian DNA vaccines.

### Delivery of DNA vaccines in fish

As for mammalian DNA vaccines, intramuscular injection is the most commonly used delivery method in experimental studies with fish DNA vaccines. However, due to the practical need to vaccinate extremely high numbers of animals in aquaculture (often hundreds of

**Table IV**  
**Detection of specific neutralising antibody elicited by rhabdovirus glycoprotein gene DNA vaccines in fish, and correlation with protection against viral challenge**

Vaccine G gene	Experimental conditions <sup>(a)</sup>	Percentage of neutralising antibody positive (time post-vaccination) <sup>(b)</sup>	Protection (RPS) <sup>(c)</sup>	Ref.
<b>Infectious haematopoietic necrosis virus</b>	2 g rbt, 0.1 µg vaccine	0 (27 d)	<b>100</b>	21
	2 g rbt, 0.01 to 5 µg vaccine	100 (6 wk)	97 to 100	23
	2 g rbt, 1 µg vaccine	0 (80 d)	<b>48</b>	22
	57 to 73 g At. sal., 25 µg vaccine	33 (8 wk)	<b>90 to 100</b>	104
	3 g sockeye, 0.1 µg vaccine	100 (62 d)	61 <sup>(d)</sup>	34
	3 g sockeye, 1.0 µg vaccine	100 (62 d)	86 <sup>(d)</sup>	34
	3 g chinook, 0.1 µg vaccine	80 (76 d)	71 <sup>(d)</sup>	34
	3 g chinook, 1.0 µg vaccine	100 (76 d)	78 <sup>(d)</sup>	34
	110 g rbt, 0.1 µg vaccine	0 (10 wk)	0 <sup>(d)</sup>	55
	110 to 120 g rbt, 1 µg vaccine	40 to 60 (10 wk)	<b>100</b> <sup>(d)</sup>	55
	110 to 120 g rbt, 10 µg vaccine	80 to 100 (10 wk)	100 <sup>(d)</sup>	55
	120 g rbt, 25 µg vaccine	100 (10 wk)	100 <sup>(d)</sup>	55
	2.5 g rbt, 0.1 µg vaccine	100 (3 mo.)	100	50
	"	20 (6 mo.)	<b>69</b>	50
	"	40 (12 mo.)	<b>65</b>	50
	"	0 (25 mo.)	<b>66</b>	50
<b>Viral haemorrhagic septicaemia virus</b>	13 g rbt, 5 to 10 µg vaccine	72 (67 d)	<b>94</b> <sup>(d)</sup>	66
	13 g rbt, 5 µg vaccine	60 (67 d)	<b>94</b> <sup>(d)</sup>	66
	4.5 g rbt, 0.5 µg vaccine	0 (4 wk)	<b>76</b>	70
	"	50 (8 wk)	<b>85</b>	70
	10 g rbt, 0.01 µg vaccine	0 (4 wk)	<b>43</b>	70
	10 g rbt, 0.1 µg vaccine	0 (4 wk)	<b>53</b>	70
	10 g rbt, 0.5 µg vaccine	25 (4 wk)	<b>73</b>	70
	100 g rbt, 0.01 µg vaccine	0 (4 wk)	12	70
	100 g rbt, 0.1 µg vaccine	0 (4 wk)	0	70
	100 g rbt, 0.5 µg vaccine	0 (4 wk)	<b>59</b>	70
	100 g rbt, 0.5 µg vaccine	0 (9 mo.)	<b>85</b>	70

NAb: neutralising antibody

(a) Hosts are as follows:

rbt: rainbow trout (*Oncorhynchus mykiss*)

At. sal.: Atlantic salmon (*Salmo salar*)

Sockeye: sockeye salmon (*O. nerka*)

Chinook: Chinook salmon (*O. tshawytscha*)

(b) Sera from individual fish or from pools of 2 to 5 fish were assayed by plaque neutralisation titre assays and % NAb positive indicates seroprevalence as the number of positive serum samples/number of samples tested. Each row represents data from 3 to 50 serum samples, with most experiments in the range of 5 to 10 samples. Sera were collected from DNA vaccinated, non-challenged fish at times given in days (d), weeks (wk), or months (mo.) post-vaccination

(c) Protection in terms of relative percent survival (RPS) is as described in the text. Entries in **bold** indicate experiments where the percent of fish protected was greater than the percent seropositive, indicating protection without detectable NAb titres in some proportion of fish

(d) In these entries viral challenges that generated the RPS data were initiated 15 to 30 days before sera were collected to determine the % NAb positive in non-challenged control groups. In all other studies sera were collected at the same time as the initiation of viral challenge

CPM: cumulative percent mortality (as defined in the text and Figure 2)

G protein: rhabdovirus surface glycoprotein

qRT-PCR: quantitative reverse-transcriptase polymerase chain reaction

RPS: relative percent survival (as defined in the text and Figure 2)

thousands), alternative delivery methods aimed at cost-effective mass delivery are being actively explored. Gene gun delivery to epidermal tissues has been shown to be highly effective for expression of reporter genes in trout and Japanese flounder (36, 57, 96, 107), and it elicited strong protection with an IHNV DNA vaccine (21).

Unfortunately, to date, intraperitoneal injection of IHNV DNA vaccines provides reduced or no protection (0 to 50 RPS) (21, 70). Delivery methods that have proven ineffective for the IHNV DNA vaccine include intrabuccal administration, skin scarification, and immersion in water containing DNA-coated beads (21). In addition, an attempt to deliver the IHNV DNA vaccine to eggs by water-hardening them in the presence of the DNA vaccine did not result in either protection or immune tolerance in the progeny (52). Mass delivery methods under investigation include automated injection, needle-less injection devices, oral delivery in feed, encapsulation in polymers, liposomes or chitosan for oral or immersion delivery (82, 84, 85, 86), ultrasound-enhanced immersion delivery (31, 74, 75), DNA-coated microspheres, and delivery by heterologous viral vectors (79) or attenuated bacteria transfected with DNA vaccine plasmids (24, 92). In most studies luciferase or GFP reporter gene plasmids have been used to assess success of novel delivery methods.

### Licensing and field trials of fish rhabdovirus DNA vaccines

DNA vaccines represent a relatively new technology in the field of vaccine research. Although there are human DNA vaccines in clinical trials, none are currently licensed. However, two DNA vaccines have been licensed for use in animals, both approved in the summer of 2005. One was a DNA vaccine against IHNV in Atlantic salmon, licensed in Canada, and the other was a DNA vaccine for West Nile virus in horses, licensed in the United States. In support of the licensing of the IHNV DNA vaccine an extensive field vaccination programme including 1.6 million sea-reared Atlantic salmon was conducted in British Columbia, Canada, in 2004/2005, using the approved IHNV DNA vaccine (88). Sub-groups of vaccinated and control fish were subjected to controlled experimental laboratory IHNV challenges that confirmed protective efficacy with RPS levels of 71 and 64 at 4 and 17 months post-vaccination, respectively (88). However, due to the absence of natural viral challenge at the testing sites, the field efficacy of the vaccine remains to be confirmed (D. Robertson, personal communication). During the same period, a small-scale field-testing of a VHS DNA vaccine was performed in Denmark with rainbow trout. The experimental fish were not approved for human consumption and therefore had to be kept in enclosed net-cages positioned in ponds with VHS outbreaks. Although a high cage-to-cage variability was seen, the overall result

indicated that the vaccine was effective. However, more extensive testing, including vaccination of whole farm populations, is needed for confirmation (N. Lorenzen, personal communication).

## DNA vaccines as tools for responding to aquatic animal disease emergencies

Although emergencies inherently cannot be predicted, in general terms aquatic animal disease emergencies will be of three different types:

- known pathogens in new geographic locales or new host species, e.g. current VHSV emergence in many new hosts in the Great Lakes region of North America
- previously unknown pathogens in common aquaculture species, e.g. ISAV in Atlantic salmon farms in the 1980s
- previously unknown pathogens in new aquaculture species, e.g. white sturgeon iridovirus in cultured sturgeon.

For emergencies involving known pathogens a vaccine may be available, and if so, it is likely to be a valuable tool in preventing the spread of disease to nearby stocks and facilities. If it is a DNA vaccine, research so far suggests it is likely to be successful in the new situation: the IHNV vaccine developed and tested in trout has been shown to work well in Atlantic salmon (104), and sockeye and Chinook salmon (34).

If the emergency involves a previously unknown pathogen the first requirement is to identify the causative agent, and then to identify candidate antigenic protein(s) and gene(s) that may be effective in vaccine development. Biotechnology can greatly accelerate these early steps in the process of response, as shown in Figure 1. A limitation for previously unknown pathogens will be the need for a reliable laboratory challenge model to test vaccine efficacy, but this would apply for any type of new vaccine.

In response to emergencies with either known or novel pathogens, DNA vaccines have important advantages in speed, flexibility, and safety, as follows:

- *speed*: the simple construction of DNA vaccines and modern-day ease of cloning facilitate rapid construction and scale-up of novel vaccines, providing a relatively fast response once relevant pathogen gene(s) have been identified;
- *flexibility*: again due to the ease of cloning, one can relatively easily construct several vaccines with different candidate genes, combinations of genes, or genes from new

or different strains of a pathogen. If efficacy trials suggest that a DNA vaccine needs to be modified, that can also be accommodated relatively easily;

– *safety*: DNA vaccines are noted for having low inherent risk because they are not infectious agents like attenuated or modified live vaccines, and there is no risk of incomplete inactivation as for killed viral vaccines or bacterins. In addition, risk assessment studies have found no detectable adverse effects by histopathology (50) and no evidence of integration of DNA vaccines into the host DNA (6, 45, 88, 103). Thus, as DNA vaccines become better understood and accepted in the future, there may be less need for extensive safety testing as required with attenuated viral or bacterial pathogens.

With regard to the probability of success of a new DNA vaccine, experience suggests that this will depend on the type of pathogen involved. If the new pathogen is identified as a rhabdovirus then high efficacy is very likely. If it is a new strain of a known rhabdovirus, existing DNA vaccines or new vaccines specific to the new strain should be effective. This has proven true for the marine strain of VHSV in flounder (16), and in the near future this may be tested again with vaccines to the Great Lakes strain of VHSV. If the pathogen is a new species of virus or bacteria similar to a species for which an efficacious DNA vaccine has been developed then use of the analogous gene(s) and vaccine construction should enhance chances of success.

## Concluding remarks

DNA vaccines constitute a promising future approach to managing aquatic animal diseases, including 'regular' disease impacts and emergencies. After ten years of research on aquatic animal DNA vaccines the current state of knowledge is as follows:

- they are highly efficacious for a small number of important pathogens, specifically for rhabdoviruses
- they also appear to work for some other viruses and some bacteria, but with more moderate efficacy; reproducibility needs to be proven
- they are likely to work, at least moderately, for other pathogens not yet tested
- it is probable that they will not work for all pathogens.

In the history of the field it is fortuitous that the first aquatic DNA vaccine tested was for a rhabdovirus, because the high efficacy observed in that vaccine stimulated active research efforts with many other pathogens. This is oddly similar to the history of fish bacterial vaccines, where the first bacterins tested in the 1970s worked very well and stimulated intense study of other bacterial vaccines, but

eventually the original *Vibrio* bacterins proved to be exceptional in their high efficacy (30). Thus, still today there are many important fish bacteria for which there is no effective vaccine available. It is too early to know whether this will be similar for DNA vaccines.

It may be that fish rhabdoviruses, as a class of pathogens, are particularly amenable to DNA vaccines because the rhabdoviral G protein may function as a pathogen-associated molecular pattern (PAMP) to stimulate strong innate immunity (53). Even if this proves to be true, DNA vaccines will still be a major benefit to aquaculture because rhabdoviruses comprise an important class of economically significant fish pathogens, represented prominently among the aquatic animal pathogens listed as reportable in the *Aquatic Animal Health Code* of the World Organisation for Animal Health (OIE) (116). The fish rhabdovirus DNA vaccines also serve as important research models for exploring the basis of high efficacy, in hopes of revealing mechanisms that can be extended to other important fish pathogens including bacteria and parasites. Where DNA vaccines show partial efficacy it may also be useful to test some of the numerous strategies currently under investigation for enhancing mammalian DNA vaccines, such as prime-boost combinations or co-expression of factors that stimulate enhanced or modified immune responses (60, 110). Recent studies provide encouraging results showing that co-expression of the chemokine IL-8 with a VHSV DNA vaccine modifies the cytokine responses in rainbow trout (42, 89).

In summary, the increase in culture of current and new aquaculture species requires the ability to rapidly develop novel vaccines in response to disease emergencies. Already DNA vaccines provide protection against fish pathogens that are economically important worldwide, and for which traditional vaccines have thus far not proven successful. At present the major challenge continues to be the need for mass delivery methods appropriate to large-scale fish culture. Considering the critical importance of this practical barrier, exploration of novel delivery strategies must be encouraged strongly within the field. As an aside, biotechnology must also continue to be applied in development of other novel vaccine types, since DNA vaccines are not likely to be optimal for all pathogens. Due to the early stage of DNA vaccine acceptance in general, regulatory issues still present some challenges, but the licensing of the IHNV DNA vaccine in Canada is encouraging. Assuming these challenges can be met in the future, the extent of use in the field will ultimately depend on economic factors based on the cost of the vaccines per delivered dose. If these vaccines are to realise their potential in contributing to health of cultured fish worldwide they must be made available at a cost that is commensurate with the relatively low value of individual animals in most aquaculture settings. Finally, we look forward to future demonstrations of DNA vaccine efficacy

in the field, against real-world ambient pathogen challenge. These vaccines may then begin to contribute to understanding DNA vaccine field efficacy, which is currently unexplored for any DNA vaccine.

Garth Traxler, Scott LaPatra, Ron Pascho, Diane Morrison, Linda Rhodes, Marta Gomez-Chiarri, Maureen Purcell, and Jim Winton.

## Acknowledgements

The author is grateful to several colleagues who provided unpublished information or comments on the manuscript: thanks to Niels Lorenzen, Kira Salenius, Kyle Garver,



## La biotechnologie et les vaccins à ADN utilisés chez les animaux aquatiques

G. Kurath

### Résumé

Le développement des vaccins utilisés en aquaculture est largement redevable à la biotechnologie. Les méthodes moléculaires modernes telles que l'amplification en chaîne par la polymérase (PCR), le clonage et l'analyse par microdamier ont permis de découvrir des antigènes, d'élaborer de nouveaux candidats vaccins et d'évaluer l'efficacité et le mode d'action de ces vaccins, ainsi que la réponse induite chez l'hôte. Pour illustrer les applications de la biotechnologie sur le terrain, l'auteur décrit les vaccins à ADN utilisés chez les poissons. Si les vaccins à ADN les plus efficaces à ce jour sont ceux qui ont été développés contre les rhabdovirus chez les poissons, d'autres vaccins à ADN dirigés contre d'autres virus et bactéries affectant les poissons ont fait leurs preuves et confèrent une protection avérée contre ces agents pathogènes. Des études sur les vaccins à ADN dirigés contre les rhabdovirus chez les poissons ont mis en lumière les facteurs susceptibles de peser sur l'efficacité des vaccins à ADN, ainsi que la nature de l'immunité innée ou acquise sollicitée par ces vaccins. Utilisés dans le cadre de la gestion des urgences sanitaires affectant les animaux aquatiques, les vaccins à ADN présentent de nombreux avantages en termes de rapidité, de souplesse et de sécurité ; un vaccin à ADN destiné aux poissons a déjà reçu l'autorisation de mise sur le marché.

### Mots-clés

Biotechnologie marine – Efficacité des vaccins – Maladie des poissons – Réponse immune chez les poissons – Vaccin à ADN – Vaccin en aquaculture.



## Biotecnología y vacunas de ADN para animales acuáticos

G. Kurath

### Resumen

La biotecnología ha sido utilizada con profusión para elaborar vacunas destinadas a la acuicultura. Los métodos moleculares modernos como la reacción en cadena de la polimerasa (PCR), la clonación o el análisis de micromatrices han facilitado el descubrimiento de antígenos, la obtención de nuevas protovacunas y la evaluación de la eficacia y el modo de acción de las vacunas, y también de la respuesta en el animal receptor. El autor se centra en las vacunas de ADN destinadas a los peces como exponente de las aplicaciones de la biotecnología en este terreno. Aunque las vacunas de ADN contra las rhabdovirus de los peces siguen siendo las más eficaces, ahora está demostrado que estas vacunas también ofrecen una protección importante contra la infección por otros varios patógenos de los peces, tanto víricos como bacterianos. El estudio de las vacunas de ADN contra las rhabdovirus de los peces ha servido para dilucidar los factores que influyen en su eficacia y desentrañar la naturaleza de la respuesta inmunitaria (innata y adaptativa) del animal a esas vacunas. Gracias a su velocidad de acción, flexibilidad e inocuidad, las vacunas de ADN son muy útiles como instrumento de lucha en caso de emergencia sanitaria en animales acuáticos. Ya existe una vacuna de ADN para peces que ha obtenido licencia de comercialización.

### Palabras clave

Biotecnología marina – Eficacia de las vacunas – Enfermedad de los peces – Respuesta inmunitaria en los peces – Vacuna de ADN – Vacuna para la acuicultura.



## References

1. Acosta F, Petrie A., Lockhart K., Lorenzen N. & Ellis A.E. (2005). – Kinetics of Mx expression in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar* L.) parr in response to VHS-DNA vaccination. *Fish Shellfish Immunol.*, **18**, 81-89.
2. Alcorn S.W., Murray A.L., Pascho R.J. & Varney J. (2005). – A cohabitation challenge to compare the efficacies of vaccines for bacterial kidney disease (BKD) in chinook salmon *Oncorhynchus tshawytscha*. *Dis. aquat. Organisms*, **63**, 151-160.
3. Alonso M., Johnson M., Simon B. & Leong J.A. (2003). – A fish specific expression vector containing the interferon regulatory factor 1A (IRF1A) promoter for genetic immunization of fish. *Vaccine*, **21**, 1591-1600.
4. Anderson E.D. & Leong J.-A.C. (2000). – Development of DNA vaccines for salmonid fish. In *Methods in molecular medicine*, Vol. 29, DNA vaccines: methods and protocols (D.B. Lowrie & R.G. Whalen, eds). Humana Press, Totawa, New Jersey, 105-121.
5. Anderson E.D., Mourich D.V., Fahrenkrug S.C., La Patra S.E., Shepard J. & Leong J.C. (1996). – Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. *Molec. mar. Biol. Biotechnol.*, **5**, 114-122.
6. Anderson E.D., Mourich D.V. & Leong J.C. (1996). – Gene expression in rainbow trout (*Oncorhynchus mykiss*) following intramuscular injection of DNA. *Molec. mar. Biol. Biotechnol.*, **5**, 105-113.
7. Biering E., Villoing S., Sommerset I. & Christie K.E. (2005). – Update on viral vaccines for fish. *Dev. Biol. Standard.*, **121**, 97-113.
8. Boudinot P., Bernard D., Boubekeur S., Thoulouze M.-I., Bremont M. & Benmansour A. (2004). – The glycoprotein of a fish rhabdovirus profiles the virus-specific T-cell repertoire in rainbow trout. *J. gen. Virol.*, **85**, 3099-3108.

9. Boudinot P, Blanco M., de Kinkelin P. & Benmansour A. (1998). – Combined DNA immunization with the glycoprotein gene of viral hemorrhagic septicemia virus and infectious hematopoietic necrosis virus induces double-specific immunity and nonspecific responses in rainbow trout. *Virology*, **249**, 297-306.
10. Boudinot P, Boubekeur S. & Benmansour A. (2001). – Rhabdovirus infection induces public and private T cell responses in teleost fish. *J. Immunol.*, **167**, 6202-6209.
11. Boudinot P, Massin P, Blanco M., Riffault S. & Benmansour A. (1999). – Vig-1, a new fish gene induced by the rhabdovirus glycoprotein, has a virus-induced homologue in humans and shares conserved motifs with the MoaA family. *J. Virol.*, **73**, 1846-1852.
12. Boudinot P, Salhi S., Blanco M. & Benmansour A. (2001). – Viral haemorrhagic septicaemia virus induces vig-2, a new interferon-responsive gene in rainbow trout. *Fish Shellfish Immunol.*, **11**, 383-397.
13. Bremont M. (2005). – Reverse genetics on fish rhabdoviruses: tools to study the pathogenesis of fish rhabdoviruses. *Curr. Top. Microbiol. Immunol.*, **292**, 119-141.
14. Brouwers H.J.M. (2004). – Identification of immunoreactive protein encoding genes of the fish pathogen *Piscirickettsia salmonis* and evaluation of their use in genetic vaccination. PhD thesis, University of Prince Edward Island, Canada.
15. Buchmann K., Sigh J., Nielsen C.V. & Dalgaard M. (2001). – Host responses against the fish parasitizing ciliate *Ichthyophthirius multifiliis*. *Vet. Parasitol.*, **100**, 105-116.
16. Byon J.Y., Ohira T., Hirono I. & Aoki T. (2005). – Use of a cDNA microarray to study immunity against viral hemorrhagic septicemia (VHS) in Japanese flounder (*Paralichthys olivaceus*) following DNA vaccination. *Fish Shellfish Immunol.*, **18**, 135-147.
17. Byon J.Y., Ohira T., Hirono I. & Aoki T. (2006). – Comparative immune responses in Japanese flounder, *Paralichthys olivaceus* after vaccination with viral hemorrhagic septicemia virus (VHSV) recombinant glycoprotein and DNA vaccine using a microarray analysis. *Vaccine*, **24**, 921-930.
18. Caipang C.M., Takano T., Hirono I. & Aoki T. (2006). – Genetic vaccines protect red seabream, *Pagrus major*, upon challenge with red seabream iridovirus (RSIV). *Fish Shellfish Immunol.*, **21**, 130-138.
19. Chu W. & Lu C. (2004). – Cloning and sequence analysis of an extracellular serine-protease gene of *Aeromonas hydrophila* J-1. *J. Fish. China*, **28**, 84-88.
20. Clark T.G. & Cassidy-Hanley D. (2005). – Recombinant subunit vaccines: potentials and constraints. *Dev. Biol. Standard.*, **121**, 153-163.
21. Corbeil S., Kurath G. & LaPatra S.E. (2000). – Fish DNA vaccine against infectious hematopoietic necrosis virus: efficacy of various routes of immunization. *Fish Shellfish Immunol.*, **10**, 711-723.
22. Corbeil S., LaPatra S.E., Anderson E.D., Jones J., Vincent B., Hsu Y.L. & Kurath G. (1999). – Evaluation of the protective immunogenicity of the N, P, M, NV and G proteins of infectious hematopoietic necrosis virus in rainbow trout *Oncorhynchus mykiss* using DNA vaccines. *Dis. aquat. Organisms*, **39**, 29-36.
23. Corbeil S., LaPatra S.E., Anderson E.D. & Kurath G. (2000). – Nanogram quantities of a DNA vaccine protect rainbow trout fry against heterologous virus strains of infectious hematopoietic necrosis virus. *Vaccine*, **18**, 2817-2824.
24. Dietrich G., Kolb-Maurer A., Spreng S., Scharlt M., Goebel W. & Gentschev I. (2001). – Gram-positive and Gram-negative bacteria as carrier systems for DNA vaccines. *Vaccine*, **19**, 2506-2512.
25. Dijkstra J.M., Kollner B., Aoyagi K., Sawamoto Y., Kuroda A., Ototake M., Nakanishi T. & Fischer U. (2003). – The rainbow trout classical MHC class I molecule Onmy-UBA\*501 is expressed in similar cell types as mammalian classical MHC class I molecules. *Fish Shellfish Immunol.*, **14**, 1-23.
26. Dijkstra J.M., Okamoto H., Ototake M. & Nakanishi T. (2001). – Luciferase expression 2 years after DNA injection in glass catfish (*Kryptopterus bicirrhus*). *Fish Shellfish Immunol.*, **11**, 199-202.
27. Einer-Jensen K., Ahrens P., Forsberg R. & Lorenzen N. (2004). – Evolution of the fish rhabdovirus viral hemorrhagic septicaemia virus. *J. gen. Virol.*, **85**, 1167-1179.
28. Engelking H.M. & Leong J.C. (1989). – Glycoprotein from infectious hematopoietic necrosis virus (IHNV) induces protective immunity against five IHNV types. *J. aquat. Anim. Hlth*, **1**, 291-300.
29. Engelking H.M. & Leong J.C. (1989). – The glycoprotein of infectious hematopoietic necrosis virus elicits neutralizing antibody and protective responses. *Virus Res.*, **13**, 213-230.
30. Evelyn T.P. (1997). – A historical review of fish vaccinology. *Dev. Biol. Standard.*, **90**, 3-12.
31. Fernandez-Alonso M., Rocha A. & Coll J.M. (2001). – DNA vaccination by immersion and ultrasound to trout viral hemorrhagic septicaemia virus. *Vaccine*, **19**, 3067-3075.
32. Fischer U., Utke K., Somamoto T., Kollner B., Ototake M. & Nakanishi T. (2006). – Cytotoxic activities of fish leucocytes. *Fish Shellfish Immunol.*, **20**, 209-226.
33. Garver K.A., Conway C.M., Elliott D.G. & Kurath G. (2005). – Analysis of DNA vaccinated fish reveals antigen in muscle, kidney and thymus, and transient histopathological changes. *Mar. Biotechnol.*, **7**, 540-553.
34. Garver K.A., LaPatra S.E. & Kurath G. (2005). – Efficacy of an infectious hematopoietic necrosis (IHN) virus DNA vaccine in Chinook *Oncorhynchus tshawytscha* and sockeye *O. nerka* salmon. *Dis. aquat. Organisms*, **64**, 13-22.

35. Gomez-Chiarri M. & Chiaverini L.A. (1999). – Evaluation of eukaryotic promoters for the construction of DNA vaccines for aquaculture. *Gene Analysis Tech.*, **15**, 121-124.
36. Gomez-Chiarri M., Livingston S.K., Muro-Cacho C., Sanders S. & Levin R.P. (1996). – Introduction of foreign genes into the tissue of live fish by direct injection and particle bombardment. *Dis. aquat. Organisms*, **27**, 5-12.
37. Gong H., Lin T., Yu F., Dong C., Chen R., Yang J. & Chen Z. (2003). – Cloning and expression of the beta-hemolysin gene of *Aeromonas hydrophila* strain isolated from *Anguilla anguilla*. *J. Fish. China*, **27**, 124-130.
38. Grayson T.H., Cooper L.F., Wrathmell A.B., Roper J., Evenden A.J. & Gilpin M.L. (2002). – Host responses to *Renibacterium salmoninarum* and specific components of the pathogen reveal the mechanisms of immune suppression and activation. *Immunology*, **106**, 273-283.
39. Harbottle H., Plant K.P. & Thune R.L. (2005). – DNA vaccination against channel catfish virus results in minimal immune response and is not efficacious against challenge. *J. aquat. Anim. Hlth*, **17**, 251-262.
40. Heppell J. & Davis H.L. (2000). – Application of DNA vaccine technology to aquaculture. *Adv. Drug Deliv. Rev.*, **43**, 29-43.
41. Heppell J., Lorenzen N., Armstrong N.K., Wu T., Lorenzen E., Einer-Jensen K., Schorr J. & Davis H.L. (1998). – Development of DNA vaccines for fish: vector design, intramuscular injection and antigen expression using viral haemorrhagic septicaemia virus genes as model. *Fish Shellfish Immunol.*, **8**, 271-286.
42. Jimenez N., Coll J., Salguero F.J. & Tafalla C. (2006). – Co-injection of interleukin 8 with the glycoprotein gene from viral haemorrhagic septicaemia virus (VHSV) modulates the cytokine response in rainbow trout (*Oncorhynchus mykiss*). *Vaccine*, **24**, 5615-5626.
43. Johnson K.A., Flynn J.K. & Amend D.F. (1982). – Onset of immunity in salmonid fry vaccinated by direct immersion in *Vibrio anguillarum* and *Yersinia ruckeri* bacterins. *J. Fish Dis.*, **5**, 197-205.
44. Jones S.R.M. (2001). – Plasmids in DNA vaccination. In *Plasmids for therapy and vaccination* (M. Schleef, ed.). Wiley-VCH, Weinheim, 169-191.
45. Kanellos T., Sylvester I.D., Ambali A.G., Howard C.R. & Russell P.H. (1999). – The safety and longevity of DNA vaccines for fish. *Immunology*, **96**, 307-313.
46. Kanellos T., Sylvester I.D., D'Mello F., Howard C.R., Mackie A., Dixon P.F., Chang K.C., Ramstad A., Midtlyng P.J. & Russell P.H. (2006). – DNA vaccination can protect *Cyprinus Carpio* against spring viraemia of carp virus. *Vaccine*, **24**, 4927-4933.
47. Kim C.H., Johnson M.C., Drennan J.D., Simon B.E., Thomann E. & Leong J.A. (2000). – DNA vaccines encoding viral glycoproteins induce nonspecific immunity and Mx protein synthesis in fish. *J. Virol.*, **74**, 7048-7054.
48. Kumar S.R., Parameswaran V., Ahmed V.P., Musthaq S.S. & Hameed A.S. (2007). – Protective efficiency of DNA vaccination in Asian seabass (*Lates calcarifer*) against *Vibrio anguillarum*. *Fish Shellfish Immunol.*, **23**, 316-326.
49. Kurath G. (2005). – Overview of recent DNA vaccine development for fish. In *Progress in fish vaccinology* (P.J. Midtlyng, ed.). *Dev. Biol.*, **121**, 201-213.
50. Kurath G., Garver K.A., Corbeil S., Elliott D.G., Anderson E.D. & LaPatra S.E. (2006). – Protective immunity and lack of histopathological damage two years after DNA vaccination against infectious hematopoietic necrosis virus in trout. *Vaccine*, **16**, 345-354.
51. Kurath G., Garver K.A., Troyer R.M., Emmenegger E.J., Einer-Jensen K. & Anderson E.D. (2003). – Phylogeography of infectious haematopoietic necrosis virus in North America. *J. gen. Virol.*, **84**, 803-814.
52. Kurath G. & Pascho R.J. (2005). – Presence of an IHN DNA vaccine during water hardening of rainbow trout eggs does not result in either immune tolerance or protective immunity. *Am. Fish. Soc. Fish Hlth Section Newsl.*, **33**, 21-25.
53. Kurath G., Purcell M.K. & Garver K.A. (2007). – Fish rhabdovirus models for understanding host response to DNA vaccines. *CAB Reviews*, **2**, 1-12.
54. Kurobe T., Yasuike M., Kimura T., Hirono K. & Aoki T. (2005). – Expression profiling of immune-related genes from Japanese flounder *Paralichthys olivaceus* kidney cells using cDNA microarrays. *Dev. comp. Immunol.*, **29**, 515-523.
55. LaPatra S.E., Corbeil S., Jones G.R., Shewmaker W.D. & Kurath G. (2000). – The dose-dependent effect on protection and humoral response to a DNA vaccine against IHN virus in subyearling rainbow trout. *J. aquat. Anim. Hlth*, **12**, 181-188.
56. LaPatra S.E., Corbeil S., Jones G.R., Shewmaker W.D., Lorenzen N., Anderson E.D. & Kurath G. (2001). – Protection of rainbow trout against infectious hematopoietic necrosis virus four days after specific or semi-specific DNA vaccination. *Vaccine*, **19**, 4011-4019.
57. Lee J.Y., Hirono I.I. & Aoki T. (2000). – Stable expression of a foreign gene, delivered by gene gun, in the muscle of rainbow trout *Oncorhynchus mykiss*. *Mar. Biotechnol.*, **2**, 254-258.
58. Leong J.C., Anderson E., Bootland L.M., Chiou P.W., Johnson M., Kim C., Mourich D. & Trobridge G. (1997). – Fish vaccine antigens produced or delivered by recombinant DNA technologies. *Dev. Biol. Standard.*, **90**, 267-277.

59. Lin Y., Cheng G., Wang X. & Clark T.G. (2002). – The use of synthetic genes for the expression of ciliate proteins in heterologous systems. *Gene*, **288**, 85-94.
60. Liu M.A., Wahren B. & Karlsson-Hedestam G.B. (2006). – DNA vaccines: recent developments and future possibilities. *Hum. Gene Ther.*, **17** (11), 1051-1061.
61. Lorenzen E., Einer-Jensen K., Martinussen T., LaPatra S.E. & Lorenzen N. (2000). – DNA vaccination of rainbow trout against viral hemorrhagic septicemia virus: a dose-response and time-course study. *J. aquat. Anim. Hlth*, **12**, 167-180.
62. Lorenzen E., Lorenzen N., Einer-Jensen K., Brudeseth B. & Evensen O. (2005). – Time course study of an in situ expression of antigens following DNA-vaccination against VHS in rainbow trout (*Oncorhynchus mykiss* Walbaum) fry. *Fish Shellfish Immunol.*, **19**, 27-41.
63. Lorenzen N. (1999). – Recombinant vaccines: experimental and applied aspects. *Fish Shellfish Immunol.*, **9**, 361-365.
64. Lorenzen N. & LaPatra S.E. (2005). – DNA vaccines for aquacultured fish. In *Biotechnology applications in animal health*. *Rev. sci. tech. Off. int. Epiz.*, **24** (1), 201-213.
65. Lorenzen N., Lorenzen E. & Einer-Jensen K. (2001). – Immunity to viral haemorrhagic septicaemia (VHS) following DNA vaccination of rainbow trout at an early life-stage. *Fish Shellfish Immunol.*, **11**, 585-591.
66. Lorenzen N., Lorenzen E., Einer-Jensen K., Heppell J., Wu T. & Davis H.L. (1998). – Protective immunity to VHS in rainbow trout (*Oncorhynchus mykiss* Walbaum) following DNA vaccination. *Fish Shellfish Immunol.*, **8**, 261-270.
67. Lorenzen N., Lorenzen E., Einer-Jensen K., Heppell J., Wu T. & Davis H.L. (1999). – Genetic vaccination of rainbow trout against viral haemorrhagic septicaemia virus: small amounts of plasmid DNA protect against a heterologous serotype. *Virus Res.*, **63**, 19-25.
68. Lorenzen N., Lorenzen E., Einer-Jensen K. & LaPatra S.E. (2002). – DNA vaccines as a tool for analysing the protective immune response against rhabdoviruses in rainbow trout. *Fish Shellfish Immunol.*, **12**, 439-453.
69. Lorenzen N., Lorenzen E., Einer-Jensen K. & La Patra S.E. (2002). – Immunity induced shortly after DNA vaccination of rainbow trout against rhabdoviruses protects against heterologous virus but not against bacterial pathogens. *Dev. comp. Immunol.*, **26**, 173-179.
70. McLauchlan P.E., Collet B., Ingerslev E., Secombes C.J., Lorenzen N. & Ellis A.E. (2003). – DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout: size, dose, route of injections and duration of protection-early protection correlates with Mx expression. *Fish Shellfish Immunol.*, **15**, 39-50.
71. Mikalsen A.B., Sindre H., Torgersen J. & Rimstad E. (2005). – Protective effects of a DNA vaccine expressing the infectious salmon anemia virus hemagglutinin-esterase in Atlantic salmon. *Vaccine*, **23**, 4895-4905.
72. Mikalsen A.B., Torgersen J., Alestrom P., Hellemann A.L., Koppang E.O. & Rimstad E. (2004). – Protection of Atlantic salmon *Salmo salar* against infectious pancreatic necrosis after DNA vaccination. *Dis. aquat. Organisms*, **60**, 11-20.
73. Miquel A., Muller I., Ferrer P., Valenzuela P.D.T. & Burzio L.O. (2003). – Immunoresponse of coho salmon immunized with a gene expression library from *Piscirickettsia salmonis*. *Biol. Res.*, **36**, 313-323.
74. Navot N., Kimmel E. & Avtalion R.R. (2004). – Enhancement of antigen uptake and antibody production in goldfish (*Carassius auratus*) following bath immunization and ultrasound treatment. *Vaccine*, **22**, 2660-2666.
75. Navot N., Kimmel E. & Avtalion R.R. (2005). – Immunisation of fish by bath immersion using ultrasound. *Dev. Biol. Standard.*, **121**, 135-142.
76. Nusbaum K.E., Smith B.F., DeInnocentes P. & Bird R.C. (2002). – Protective immunity induced by DNA vaccination of channel catfish with early and late transcripts of the channel catfish herpesvirus (IHV-1). *Vet. Immunol. Immunopathol.*, **84**, 151-168.
77. Pasnik D.J. & Smith S.A. (2005). – Immunogenic and protective effects of a DNA vaccine for *Mycobacterium marinum* in fish. *Vet. Immunol. Immunopathol.*, **103**, 195-206.
78. Pasnik D.J. & Smith S.A. (2006). – Immune and histopathological responses of DNA-vaccinated hybrid striped bass *Morone saxatilis* × *M. chrysops* after acute *Mycobacterium marinum* infection. *Dis. aquat. Organisms*, **73**, 33-41.
79. Phenix K.V., McKenna B., Fitzpatrick R., Vaughan L., Atkins G., Liljestrom P. & Todd D. (2000). – Cell culture evaluation of the Semliki Forest virus expression system as a novel approach for antigen delivery and expression in fish. *Mar. Biotechnol.*, **2**, 27-37.
80. Purcell M.K., Kurath G., Garver K.A., Herwig R.P. & Winton J.R. (2004). – Quantitative expression profiling of immune response genes in rainbow trout following infectious haematopoietic necrosis virus (IHNV) infection of DNA vaccination. *Fish Shellfish Immunol.*, **17**, 447-462.
81. Purcell M.K., Nichols K.M., Winton J.R., Kurath G., Thorgaard G.H., Wheeler P., Hansen J.D., Herwig R.P. & Park L.K. (2006). – Comprehensive gene expression profiling following DNA vaccination of rainbow trout against *Infectious hematopoietic necrosis virus*. *Molec. Immunol.*, **43**, 2089-2106.
82. Ramos E.A., Relucio J.L. & Torres-Villanueva C.A. (2005). – Gene expression in tilapia following oral delivery of chitosan-encapsulated plasmid DNA incorporated into fish feeds. *Mar. Biotechnol.*, **7**, 89-94.
83. Robinson H.L., Hunt L.A. & Webster R.G. (1993). – Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA. *Vaccine*, **11**, 957-960.

84. Romøren K., Thu B.J. & Evensen Ø. (2002). – Immersion delivery of plasmid DNA. II. A study of the potentials of a chitosan based delivery system in rainbow trout (*Oncorhynchus mykiss*) fry. *J. controlled Release*, **85**, 215-225.
85. Romøren K., Thu B.J. & Evensen Ø. (2004). – Expression of luciferase in selected organs following delivery of naked and formulated DNA to rainbow trout (*Oncorhynchus mykiss*) by different routes of administration. *Fish Shellfish Immunol.*, **16**, 251-264.
86. Romøren K., Thu B.J., Smistad G. & Evensen Ø. (2002). – Immersion delivery of plasmid DNA. I. A study of the potentials of a liposomal delivery system in rainbow trout (*Oncorhynchus mykiss*) fry. *J. controlled Release*, **85**, 203-213.
87. Russell P.H., Kanellos T., Sylvester I.D., Chang K.C. & Howard C.R. (1998). – Nucleic acid immunisation with a reporter gene results in antibody production in goldfish (*Carassius auratus* L.). *Fish Shellfish Immunol.*, **8**, 121-128.
88. Salenius K., Simard N., Harland R. & Ulmer J.B. (2007). – The road to licensure of a DNA vaccine. *Curr. Opin. investig. Drugs*, **8**, 635-641.
89. Sanchez E., Coll J. & Tafalla C. (2007). – Expression of inducible CC chemokines in rainbow trout (*Oncorhynchus mykiss*) in response to a viral haemorrhagic septicemia virus (VHSV) DNA vaccine and interleukin 8. *Dev. comp. Immunol.*, **31**, 916-926.
90. Seo J.I., Kim K.H., Kim S.G., Oh M.J., Nam S.W., Kim Y.T. & Choi T.J. (2006). – Protection of flounder against hirame rhabdovirus (HIRRV) with a DNA vaccine containing the glycoprotein gene. *Vaccine*, **24**, 1009-1015.
91. Seternes T., Tonheim T.C., Lovoll M., Bogwald J. & Dalmo R.A. (2007). – Specific endocytosis and degradation of naked DNA in the endocardial cells of cod (*Gadus morhua* L.). *J. experim. Biol.*, **210**, 2091-2103.
92. Simon B., Nomellini J., Chiou P., Bingle W., Thornton J., Smit J. & Leong J.A. (2001). – Recombinant vaccines against infectious hematopoietic necrosis virus: production by the *Caulobacter crescentus* S-layer protein secretion system and evaluation in laboratory trials. *Dis. aquat. Organisms*, **44**, 17-27.
93. Snow M., Bain N., Black J., Taupin V., Cunningham C.O., King J.A., Skall H.F. & Raynard R.S. (2004). – Genetic population structure of marine viral haemorrhagic septicemia virus (VHSV). *Dis. aquat. Organisms*, **61**, 11-21.
94. Sommerset I., Lorenzen E., Lorenzen N., Bleie H. & Nerland A.H. (2003). – A DNA vaccine directed against a rainbow trout rhabdovirus induces early protection against a nodavirus challenge in turbot. *Vaccine*, **21**, 4661-4667.
95. Sommerset I., Skern R., Biering E., Bleie H., Fiksdal I.U., Grove S. & Nerland A.H. (2005). – Protection against Atlantic halibut nodavirus in turbot is induced by recombinant capsid protein vaccination but not following DNA vaccination. *Fish Shellfish Immunol.*, **18**, 13-29.
96. Sudha P.M., Low S., Kwang J. & Gong Z. (2001). – Multiple tissue transformation in adult zebrafish by gene gun bombardment and muscular injection of naked DNA. *Mar. Biotechnol.*, **3**, 119-125.
97. Tafalla C., Chico V., Perez L., Coll J.M. & Estepa A. (2007). – *In vitro* and *in vivo* differential expression of rainbow trout (*Oncorhynchus mykiss*) Mx isoforms in response to viral haemorrhagic septicemia virus (VHSV) G gene, poly I:C and VHSV. *Fish Shellfish Immunol.*, **23**, 210-221.
98. Takano T., Iwahori A., Hirono I. & Aoki T. (2004). – Development of a DNA vaccine against hirame rhabdovirus and analysis of the expression of immune-related genes after DNA vaccination. *Fish Shellfish Immunol.*, **17**, 367-374.
99. Tan C.W., Jesudhasan P. & Woo P.T. (2007). – Towards a metalloprotease-DNA vaccine against piscine cryptobiosis caused by *Cryptobia salmositica*. *Parasitol. Res.*, **102**, 265-275.
100. Tang D.C., DeVit M. & Johnston S.A. (1992). – Genetic immunization is a simple method for eliciting an immune response. *Nature*, **356**, 152-154.
101. Thune R.L., Fernandez D.H., Hawke J.P. & Miller R. (2003). – Construction of a safe, stable, efficacious vaccine against *Photobacterium damsela* ssp. *piscicida*. *Dis. aquat. Organisms*, **57**, 51-58.
102. Tordo N., Benmansour A., Calisher C., Dietzgen R.G., Fang R.-X., Jackson A.O., Kurath G., Nadin-Davis S., Tesh R.B. & Walker P.J. (2005). – Family *Rhabdoviridae*. In *Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses* (C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger & L.A. Ball, eds), Academic Press, 1162 pp.
103. Torgersen J., Collas P. & Aleström P. (2000). – Gene-gun-mediated transfer of reporter genes to somatic zebrafish (*Danio rerio*) tissues. *Mar. Biotechnol.*, **2**, 293-300.
104. Traxler G.S., Anderson E., LaPatra S.E., Richard J., Shewmaker B. & Kurath G. (1999). – Naked DNA vaccination of Atlantic salmon *Salmo salar* against IHNV. *Dis. aquat. Organisms*, **38**, 183-190.
105. Troyer R.M. & Kurath G. (2003). – Molecular epidemiology of infectious hematopoietic necrosis virus reveals complex virus traffic and evolution within southern Idaho aquaculture. *Dis. aquat. Organisms*, **55**, 175-185.
106. Troyer R.M., LaPatra S.E. & Kurath G. (2000). – Genetic analyses reveal unusually high diversity of infectious hematopoietic necrosis virus in rainbow trout aquaculture. *J. gen. Virol.*, **81**, 2823-2832.
107. Tucker C., Endo M., Hirono I. & Aoki T. (2000). – Assessment of DNA vaccine potential for juvenile Japanese flounder *Paralichthys olivaceous*, through the introduction of reporter genes by particle bombardment and histopathology. *Vaccine*, **19**, 801-809.

108. Ulmer J.B., Donnelly J.J., Parker S.E., Rhodes G.H., Felgner P.L., Dwarki V.J., Gromkowski S.H., Deck R.R., DeWitt C.M., Friedman A. *et al.* (1993). – Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science*, **259**, 1745-1749.
109. Utke K., Kock H., Schuetze H., Bergmann S.M., Lorenzen N., Einer-Jensen K., Kollner B., Dalmo R.A., Vesely T., Ototake M. & Fischer U. (2007). – Cell-mediated immune responses in rainbow trout after DNA immunization against the viral hemorrhagic septicemia virus. *Dev. comp. Immunol.*, **32** (3), 239-252.
110. Van Drunen Littel-van den Hurk S., Babiuk S.L. & Babiuk L.A. (2004). – Strategies for improved formulation and delivery of DNA vaccines to veterinary target species. *Immunol. Rev.*, **199**, 113-125.
111. Vazquez-Juarez R., Barrera-Saldana H., Hernandez-Saavedra N., Gomez-Chiarri M. & Ascencio F. (2003). – Molecular cloning, sequencing, and characterization of omp48, the gene encoding for an antigenic outer membrane protein from *Aeromonas veronii*. *J. appl. Microbiol.*, **94**, 908-918.
112. Vazquez-Juarez R.C., Gomez-Chiarri M., Barrera-Saldana H., Hernandez-Saavedra N., Dumas S. & Ascencio F. (2005). – Evaluation of DNA vaccination of spotted sand bass (*Paralabrax maculatofasciatus*) with two major outer-membrane protein-encoding genes from *Aeromonas veronii*. *Fish Shellfish Immunol.*, **19**, 153-163.
113. Verri T., Ingrosso L., Chiloiro R., Danieli A., Zonno V., Alifano P., Romano N., Scapigliati G., Vilella S. & Storelli C. (2003). – Assessment of DNA vaccine potential for gilthead sea bream (*Sparus aurata*) by intramuscular injection of a reporter gene. *Fish Shellfish Immunol.*, **15**, 283-295.
114. Winton J. (1998). – Molecular approaches to fish vaccines. *J. appl. Ichthyol.*, **14**, 153-158.
115. Wolff J.A., Malone R.W., Williams P., Chong W., Acsadi G., Jani A. & Felgner P.L. (1990). – Direct gene transfer into mouse muscle *in vivo*. *Science*, **247**, 1465-1468.
116. World Organisation for Animal Health (OIE) (2007). – Chapter 1.2.3. Diseases listed by the OIE. *In Aquatic Animal Health Code*, 10th Ed. OIE, Paris, 23-24. Also available at: [http://www.oie.int/eng/normes/fcode/en\\_chapitre\\_1.2.3.htm](http://www.oie.int/eng/normes/fcode/en_chapitre_1.2.3.htm) (accessed on 28 February 2008).
117. Yasuike M., Kondo H., Hirono I. & Aoki T. (2007). – Difference in Japanese flounder, *Paralichthys olivaceus* gene expression profile following hirame rhabdovirus (HIRRV) G and N protein DNA vaccination. *Fish Shellfish Immunol.*, **23**, 531-541.
118. Zhang H.G. & Hanson L.A. (1995). – Deletion of thymidine kinase gene attenuates channel catfish herpesvirus while maintaining infectivity. *Virology*, **209**, 658-663.
119. Zheng F.R., Sun X.Q., Liu H.Z. & Zhang J.X. (2006). – Study on the distribution and expression of a DNA vaccine against lymphocystis disease virus in Japanese flounder (*Paralichthys olivaceus*). *Aquaculture*, **261**, 1128-1134.