Molecular immunity in the interaction between fish and pathogen for DNA vaccine

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ABSTRACT

Cultured fish are threatened by many pathogens, especially viruses and bacteria, often with serious consequences. Vaccination is one of the most effective tools for enhancing host defense and protecting fish from pathogens. DNA vaccines are a third generation vaccine based on the administration of the gene encoding a vaccine antigen rather than the antigen itself. To date, several effective DNA vaccines that encode viral glycoproteins or other antigenic proteins have already been shown to be effective for cultured fish. This review summarizes current knowledge on fish viral pathogens and DNA vaccines against fish viral diseases, especially against hirame rhabdovirus (HIRRV), viral hemorrhagic septicemia virus (VHSV) and red seabream iridovirus (RSIV) from previous studies. Furthermore, the mechanism of interaction between the DNA vaccines and host immunity is described using mammalian evidence and data gained from using our Japanese flounder microarray chip. The efficacy of two DNA vaccines derived from pathogenic viruses such as HRV and VHSV have been evaluated through gene expression profiles. A comparison of gene expression profiles of vaccinated and unvaccinated fish suggests important evidences that DNA vaccines have a role in host immunity such as induction of MHC class I gene expression and T-cell stimulation.

Keywords: DNA vaccine, Japanese flounder (*Paralichthys olivaceus*), microarray, VHSV, HIRRV

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INTRODUCTION

Fish supplied 110 million tons of food in 2006, of which 47% (51.7 million tons) was supplied by aquaculture (FAO, 2009). The aquaculture industry grew at an amazing rate of 7.6% from 2000-2006 with an extreme growth rate of almost 40% for Vietnam (Brugère and Ridler, 2004). The industry is expected to grow more, to meet the demand of a growing global population. Japanese flounder (*Paralichthys olivaceus*, also known as olive flounder or Bastard halibut) is a major aquaculture product in Japan, Korea, and China. However, recently, the spread of an increasing array of new diseases cause epizootics that result in substantial socio-economic and environmental losses (Walker and Winton, 2010).

Fish pathogenic viruses

One of the biggest problems in aquaculture is disease caused by viruses. The OIE (World Organisation for Animal Health, originally called the Office International des Epizooties, thus its acronym) is the intergovernmental organization responsible for improving animal health worldwide (http://www.oie.int). It keeps a list of 'Fish diseases notifiable to the OIE' which means these diseases are considered to be of socio-economic and/or public health importance within countries, and significant to the international trade in aquatic animals and aquatic animal products. Of the nine fish diseases listed in 2010 by the OIE as notifiable, seven are viral. These are mostly members of the Rhabdoviridae and Iridoviridae family and they have caused tremendous socio-economic losses worldwide.

Epizootic haematopoietic necrosis (EHNV: Unassigned, *Iridoviridae, Ranavirus*) causes severe necrosis of the haematopoietic tissue of perch and rainbow trout (Whittington *et. al*., 1996; Reddacliff and Whittington, 1996).

Infectious haematopoietic necrosis (IHNV: *Mononegavirales, Rhabdoviridae, Novirhabdovirus*) causes the hemorrhage of haematopoietic tissue and nephron cells leading to death by edema. It has caused mass mortalities of salmon and trout and occurs at low water temperatures (8-15°C) (McAllister *et al*., 1974; Holway and Smith, 1973).

Spring viraemia of carp (SVCV: *Mononegavirales, Rhabdoviridae, Vesiculovirus*) is characterized by external and internal hemorrhages, peritonitis and ascites. It predominantly affects common carp (*Cyprinus carpio*) (Essbauer and Ahne, 2001).

Viral haemorrhagic septicaemia (VHSV: *Mononegavirales, Rhabdoviridae, Novirhabdovirus*) is known as one of the most serious viral pathogens of salmonids and marine fishes in the world (Mortensen *et al*., 1999). It has been known to infect eels, herring, carp, cod, perch, flatfish, salmon, and flounder (Isshik *et al.,* 2001; Dopaz *et al*., 2002). It also occurred in wild Japanese flounders during a survey on the distribution of fish viruses in wild marine fishes in Japan (Takano *et al*., 2000). The virus causes hemorrhagic lesions in internal organs and muscular tissue. The virus multiplies in endothelial cells, leucocytes, hematopoietic tissues and nephron cells.

Infectious salmon anaemia (ISAV: Unassigned, *Isavirus*) is often associated with an haemorrhagic kidney disease (Lovely *et al*., 1999). ISAV has caused severe economic losses to Atlantic salmon cultured in Europe, North America, and Chile (Rodger and Richards, 1998; Falk *et al*., 1997; Mjaaland *et al*., 1997).

Iridoviruses, including Red seabream iridoviral disease (RSIV: *Iridoviridae*) are large icosahedral cytoplasmic DNA viruses that have been isolated from a diverse number of invertebrate and vertebrate hosts (Williams *et al*., 2000). RSIV has caused significant economic losses in aquaculture. This disease was first observed in red seabream (*Pagrus major*) cultured in Shikoku Island, Japan in 1990 (Matsuoka et. al., 1996). A survey on the extent of RSIV infection showed that this virus infects 31 marine fish species including 28 Perciformes, two Pleuronectiformes and one Tetraodontiformes (Kawakami and Nakajima, 2002). Gross pathologies can include severe anemia, petechia of the gills and enlargement of the spleen.

Koi herpesvirus disease (KHV: *Herpesvirales, Alloherpesviridae, Cyprinivirus*) is characterized by papillomas on the caudal fin region. KHV has caused enormous losses around the world (Bretzinger *et al.*, 1999; Ariav *et al*., 1999).

Aside from these major viral diseases, several other viruses have caused much damage to fish aquaculture species around the world (Table 1). Some of the most notable are:

Viral nervous necrosis virus (VNNV: Unassigned, *Nodaviridae, Betanodavirus*) can infect several fish species (Tanaka *et al.,* 2003). It induces abnormal swimming behavior, encephalopathy and retinopathy. Since it was first discovered, several similar cases have been reported around the world. One of the most prominent species is the striped jack viral nervous necrosis virus (SJNNV).

Hirame rhabdovirus (HIRRV: *Mononegavirales, Rhabdoviridae, Novirhabdovirus*) is mostly associated with epizootics and heavy losses in aquaculture (Kimura *et al*., 1986) and is primarily a pathogen of Japanese flounder. It is composed of five structural proteins with similarity to those of IHNV and VHSV (Nishizawa *et al*., 1991). Transmission occurs mainly by shedding from infected fish, and the viruses are spread by waterborne contact (Wolf, 1988). The early targets for the viruses are the gills, the esophagus, cardiac stomach region, and mucus-secreting glands.

Lymphocystis disease virus (LCDV: Unassigned, *Iridoviridae, Lymphocystivirus*) causes transformation and enlargement of cells of the skin and in the connective tissue of internal organs. Infected cells undergo massive hypertrophy and encapsidation by an extracellular hyaline matrix. LCD is characterized as a chronic benign disease with rare mortality. Infection rates are increased by stress factors (Wolf, 1988).

Marine birnavirus (MABV: Unassigned, *Birnaviridae, Aquabirnavirus*) has been isolated in flounders (Jung *et al.*, 2008). It causes accumulation of ascitic fluid in the abdominal

Table 1.
Viral Families causing major diseases in fish

Group[a]	Order[b]	Family	Representative Species
A. DNA Viruses			
I: dsDNA	Caudovirales/	Iridoviridae	Epizootic haematopoietic necrosis virus (EHNV)*
	Herpesvirales		Red seabream iridovirus (RSIV)*
			Lymphocystis disease viruses (LCDV)†
		Herpesviridae	Koi herpesvirus (KHV, or CyHV-3)*[c]
			Channel catfish herpesvirus (CCHV)
B. RNA Viruses			
III: dsRNA		Reoviridae	Golden shiner reovirus (GSRV)
			Grass carp reovirus (GCRV)
		Birnaviridae	Infectious pancreatic necrosis virus (IPNV)
			Marine fish birnaviruses (MABV)†
IV: ss(+)RNA	Nidovirales /	Caliciviridae	San Miguel sea lion virus (SMSV)
	Picornavirales	Togaviridae	Salmon pancreatic disease virus (SPDV)
			Sleeping disease virus of rainbow trout (SDV)
		Nodaviridae	Viral nervous necrosis virus (VNNV)†
V: ss(-)RNA	Mononegavirales	Orthomyxoviridae	Eel viruses (A1B, EV1 and EV2)
			Infectious salmon anaemia virus (ISAV)*
		Rhabdoviridae	Infectious haematopoietic necrosis virus (IHNV)*
			Viral haemorrhagic septicaemia virus (VHSV)*†
			Spring viraemia of carp virus (SVCV)*
			Hirame rhabdovirus (HIRRV)†
VI: RT-Transcribing		Retroviridae	Walleye dermal sarcoma virus (WDSV)

*OIE listed disease

†Infectious to *P. olivaceus*

[a] Viral Groups are arranged according to Baltimore classification (I, III-VI)

[b] Viruses are classified using ICTV nomenclature

[c] KHV is classified as belonging to the Alloherpesviridae family using ICTV classification

cavity, congestion in the liver and absence of food in the intestine with hemorrhages. External signs can be hemorrhages on the body surface and white nodules in the kidney and spleen.

Advantages of vaccine use

Several strategies have been implemented to alleviate the damage caused by these diseases. One of the most commonly used strategy against disease, in general, is chemotherapy by using antibiotics. However, there is cause for serious concern regarding the overutilization of antibiotics, which can lead to serious environmental damage by upsetting the natural microbial population and can hasten the emergence of antibiotic resistant pathogens (Huovinen, 1999a; Huovinen, 1999b; Park *et al*., 2009). Moreover, antibiotics are not effective against viruses. Therefore, an effective strategy against viral pathogens must be considered. One of the most promising techniques is vaccination (Gudding *et al*., 1999; Hastein *et al*., 2005).

The principal difference between antibiotics and vaccines is that antibiotics are a therapeutic strategy, to be administered upon the onset of a disease. Vaccines, on the other hand, are a preventive strategy, and depend on stimulating the immune system of the target animal. Also, antibiotics are active mostly against bacterial pathogens, and will be effective only if an appropriate concentration is maintained. Vaccines, upon stimulation of the host immune system, need not be maintained, and the duration of protection surpasses that of antibiotics (Grisez and Tan, 2005).

Administration

Vaccines can be delivered orally, by immersion, or by injection. Vaccines to be delivered orally are integrated into the diet (by mixing, top-dressing, or bioencapsulation). Oral administration is the most straightforward and easiest method, but problems with getting the vaccine intact through the digestive system are prevalent. Attempts to improve this method have mostly focused on protecting the vaccine from digestive enzymes (Ellis, 1998). Immersion relies on stimulating the immune response of the skin and gills of the fish to protect it from future infection. It can be done by dipping (higher concentration of the vaccine, shorter exposure time), or bathing (lower concentration, longer exposure) (Nakanishi and Ototake, 1997). Though it is theoretically practical for large amounts of small fish, it can suffer the same problems with oral vaccines. Vaccination by injection is more direct, and the antigenic effect may be stimulated by an adjuvant (Grisez and Tan, 2005). Protection from vaccines delivered by injection can last for 6 months to a year. The vaccine is injected into the muscle (IM) or into the body cavity [intracoelomic (ICe), or intraperitoneal (IP)]. The injection also allows for multiple antigens to be combined into a single vaccine (Evelyn, 2002). However, since all fish must be handled individually, this method requires more time, labor, and skilled personnel.

Vaccine development

Development of vaccines is a time-consuming and laborious process. As with any drug-development process, it initially involves the screening of potential candidates, which will lead to the identification and isolation of the therapeutic agent/vaccine (discovery).

Characterization of the epidemiology and pathogenesis of the etiological agent, in order to identify targets for immune response or key virulence factors, would help in focusing the screening effort, in the enhancement of the potential antigen by genetic engineering, or in the development of DNA constructs for 3rd generation vaccines (DNA vaccines). A challenge model, to facilitate testing, which must show consistently reproducible and significant data, must be developed (Gudding *et al*., 1999). The candidate vaccines must undergo several in vivo and ultimately in vitro trials to test for positive and/or undesirable effects. With the use of genetic engineering techniques, a vaccine candidate can be streamlined to attenuate any undesired traits and/or to increase desired effects. The candidate can then go to 'production process development', wherein the method for large scale production and eventual delivery must be optimized. In all this, potential expenditure for production must be realistically assessed with the potential cost of the final product. This candidate must then undergo stringent tests for quality and safety to pass registration with relevant government agencies. Finally, the product must be marketed. Ideally, the final vaccine should: a) be safe for the fish, the caretaker, and the end-user; b) have a broad and effective protective spectrum; c) provide lasting protection; d) be easy to administer; e) be easy and cost-effective to manufacture; and f) be easily licensed or registered.

Types of vaccines

First generation vaccines utilize the whole pathogen – live, weakened, or killed. These include bacterins, which are composed of killed pathogenic cells that stimulate the humoral (antibody) immune response; and live, attenuated vaccines, which can stimulate both cell-mediated (killer T-cells T_K, helper T-cells, T_H) and antibodies. This is currently the most commonly used type in aquaculture (Hastein *et al*., 2005). The biggest danger is that live pathogens can revert to a dangerous form, while bacterins are ineffective against several pathogens. Second generation vaccines utilizes antigenic subunits of the whole pathogen, such as recombinant protein antigens (toxoids), or other components. These can elicit T_H and antibody responses but not a T_K response. Third generation vaccines or DNA vaccines are directly inoculated DNA constructs that encode a specific antigen under the control of a eukaryotic promoter to stimulate *in vivo* synthesis of immunogenic protein and immune responses. After the vaccinated protein is expressed, it generates an immune response against the DNA-encoded immunogen. This mechanism has been revealed in mammals, when mice were inoculated with plasmids expressing human growth, but developed antibodies instead (Tang *et al*., 1992). The advantage of DNA vaccines is that they have been shown to elicit antibody, T_H, and cytotoxic T lymphocyte (CTL) response. DNA vaccine techniques have been investigated in cultured fish (Lorenzen *et al*., 1998, 1999, 2009; Corbeil *et al*., 1999; 2000) and there is currently tremendous interest in the development of DNA vaccines for fish (Biering *et al*., 2005; Kurath, 2005; Lorenzen and LaPatra, 2005).

DNA vaccines against viral diseases in fish

To date, several DNA vaccines have been examined for use in fish viral diseases (Table 2), especially against viral haemorrhagic septicaemia virus (VHSV), hirame rhabdovirus (HIRRV) and red seabream iridovirus (RSIV).

Table 2.
Summary of DNA vaccines against fish viral diseases

Disease	Antigens	Species	Reference
A. DNA Viruses			
Channel catfish herpesvirus (CCHV)	Several open reading frame (ORF)59, ORF6	Channel catfish	Nusbaum *et al*., 2002
Red seabream iridovirus (RSIV)	Major capsid protein (MCP) and an ORF569	Red seabream	Caipang *et al*., 2006
Lymphocystis disease virus (LCDV)	MCP	Japanese flounder	Tian and Yu, 2010
B. RNA Viruses			
Infectious hematopoietic necrosis virus (IHNV)	Glycoprotein	Rainbow trout, Atlantic salmon	Anderson *et al*., 1996 Leong *et al*., 1997 Winton, 1997 Corbeil *et al*., 2000
Viral haemorrhagic septicaemia virus (VHSV)	Glycoprotein	Rainbow trout	Boudinot *et al*., 1998 Heppell *et al*., 1998 Lorenzen *et al*., 1998 McLauchlan *et al*., 2003
		Japanese flounder	Byon *et al*., 2005 Byon *et al*., 2006
Infectious pancreatic necrosis virus (IPNV)	VP2	Brown trout Rainbow trout	de las Heras *et al*., 2009 de las Heras *et al*., 2010
Atlantic halibut nodavirus (AHNV)	Coat protein (weakly effective)	Turbot	Sommerset *et al*., 2005
	Glycoprotein of VHSV		Sommerset *et al*., 2003
Hirame rhabdovirus	Glycoprotein	Japanese flounder	Takano *et al*., 2004
Spring viraemia of carp virus (SVCV)	Glycoprotein	Common carp Koi carp	Kanellos *et al*., 2006 Emmenegger and Kurath, 2008
Infectious salmon anaemia virus (ISAV)	Hemagglutinin-esterase (HE) (weakly effective)	Atlantic salmon	Mikalsen *et al*., 2005
VHSV and IHNV (Mixed DNA vaccine)	Glycoproteins of VHSV and IHNV	Rainbow trout	Einer-Jensen *et al*., 2009

The glycoprotein (G-protein) gene of VHSV is a highly immunogenic viral protein when used as a DNA vaccine although DNA vaccination with the nucleocapsid (N) protein, phosphoprotein, non-virion, and matrix protein genes of IHNV were shown to be inefficient in rainbow trout (Lorenzen *et al*., 1999; Corbeil *et al*., 1999). Immunization using the VHSV G-protein gene in Japanese flounder, however, showed a high protective efficiency with 93% relative percentage survival (RPS) (Byon *et al*., 2005).

A DNA vaccine encoding HIRRV G-protein gene provided strong protection against HIRRV (Takano *et al*., 2004). Fourteen days post-HIRRV-challenge, the RPSs of fish infected with 1 and 10 μg plasmid DNA vaccine were 70.5 and 90.1%, respectively. However, a DNA vaccine encoding the N-protein gene was inefficient against HIRRV

(Yasuike *et al*., 2010). Interestingly, the gene expression patterns during HIRRV infection between fish vaccinated with the G- and N-protein were substantially different, as shown in the next section.

Vaccination protocols, using formalin-killed virus, have been found to be highly efficient in protecting fish against RSIV (Nakajima *et al*., 1997; Kawakami and Nakajima, 2002). However, the use of whole-killed antigen vaccines has its own limitations, which include poor induction of cell-mediated immunity and poor immunogenicity (Davis and McCluskie, 1999). DNA vaccines encoding the viral major capsid protein (MCP) and an open reading frame (ORF) containing a transmembrane domain have been successfully used against RSIV in red seabream (Caipang et. al., 2006). The RPS of fish treated with the DNA vaccines and their combination ranged from 42.8 to 71.4%. These vaccines significantly induced the expression of MHC class I transcript in the vaccinated fish 15 to 30 days post immunization (Caipang *et al*., 2006).

Immune-related genes in Japanese flounder immunized with DNA vaccines

To understand the immunological response to DNA vaccination, it is necessary for an effective technology to comprehensively analyze the transcripts expressed by the vaccines. Microarrays are specially treated glass slides robotically spotted with thousands of genes (Schena *et al*., 1995). We have previously analyzed the expressions of about 2000 Japanese flounder ESTs with microarray chips (Kurobe *et al*., 2005; Byon *et al*., 2005, 2006; Yasuike *et al*., 2007; Dumrongphol *et al*., 2009). These chips have been used to evaluate the effectiveness of DNA vaccines in Japanese flounder infected with three viral pathogens (such as VHSV and HIRRV).

A microarray analysis of Japanese flounder immunized with DNA vaccine encoding VHSV G-protein was conducted to understand the gene expression patterns of the non-specific and specific immune responses to the vaccination (Byon et. al., 2005, 2006). Non-specific immune response genes such as NK Kupffer cells receptor, MIP1-a and Mx1 protein gene were observed to be up-regulated at 1 and 3 days post-immunization, while the specific immune response genes containing the CD20, CD8 alpha chain, CD40 and B lymphocyte cell adhesion molecule were also up-regulated during that time. These results suggested that the VHSV G-protein gene elicits strong humoral and cellular immune responses, which may play an important role in protecting the fish during viral infections.

Microarray analyses showed differential gene expressions in Japanese flounder in response to DNA vaccination by HIRRV G- and N-proteins (Yasuike *et al*., 2007, 2010). Five genes, including the interferon-stimulated gene 15kDa (ISG15), ISG56, Mx and two unknown genes, were strongly induced after the injection by the HRV G-protein, but not N-protein. The three genes are known as type I interferon (IFN)-inducible genes, which inhibit viral replication or protein synthesis (Caipang *et al*., 2003; Haller and Kochs, 2000; Samuel, 2001; Guo *et al*., 2000), suggesting that stimulation of the type I IFN system protects against HRV infection. Furthermore, a microarray analysis of pHRV-G-vaccinated flounder infected with HIRRV showed up-regulation of several genes within 3 days post-infection

(Yasuike *et al*., 2010). These included genes with homology to mammalian T cell activation-related (such as Cytohesin-1, CXCR3, CARD11/CARMA1, gp96, CaMKII, DAP10, DC-SIGN, PA28α and α2m) and complement system (such as CD59, MASP-2 and complement factor H).

Molecular and cellular interactions between DNA vaccines and host immunity

The schematic pathway of the host immune mechanisms that DNA vaccines effectively activate in mammals is shown in Fig. 1. A DNA vaccine administered to the host body can activate the host immunity in two pathways. In one pathway, the encoded antigenic protein is expressed in non-immune cells (including muscle cells and skin cells) or antigen-presenting cells (APC), such as dendritic cells (DC). The antigen peptide is presented by MHC class I or II to enhance T cell differentiation to CD4+ T cells and CD8+ CTL (Rice *et al.*, 2008). The CD4+ T cells also enhance B cell differentiation to specific antibody producing cells (Coban *et al*., 2008; Stevenson *et al*., 2010). In the other pathway, DNA of the vaccine is directly recognized by some receptors including CpG DNA sensor [*i.e.* Toll-like receptor (TLR)-9], B-form DNA sensor [*i.e.* Z-DNA binding protein-1 (ZBP, also known as DAI)] and inflammasome [*i.e.* NACHT-, LRR- and pyrin domain (PYD)-containing proteins (NALP3)]. Recognition by these receptors stimulates type-I interferon (IFN) and pro-inflammatory cytokine gene expressions as an adjuvant (Coban *et al*., 2008;

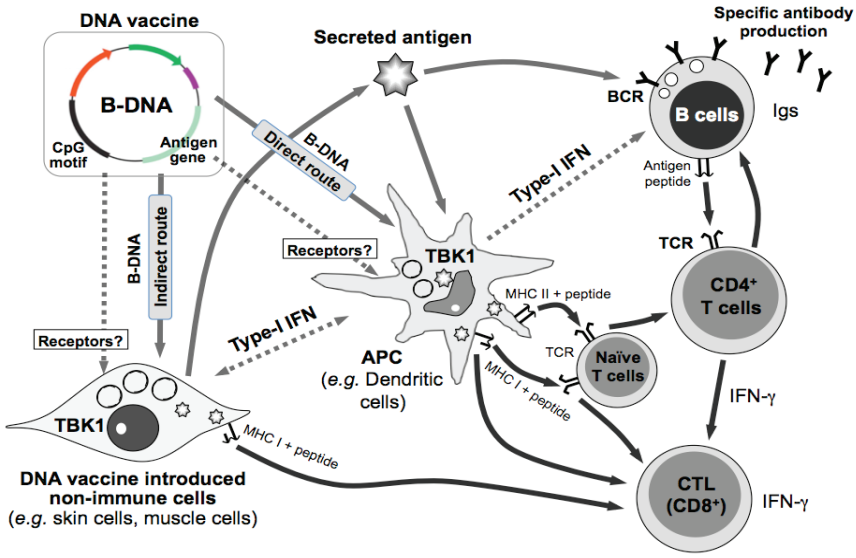

Figure 1. Schematic pathway of immune response mediated by DNA vaccines (solid lines), and the adjuvanticity reaction (broken lines). Abbreviations: B-DNA, B-form DNA; TBK1, TANK-binding kinase-1; IFN, interferon; APC, antigen-presenting cell; BCR, B-cell receptor; TCR, T-cell receptor; MHC I (or II), major histocompatibility complex class I (or II); CTL, cytotoxic T-cell.

Stevenson *et al*., 2010). In these recognition cascades, TANK-binding kinase-1 (TBK1) mediates the adjuvant effect of DNA vaccines as a key molecule, which is necessary for DNA-vaccine-induced immunogenicity (Ishii *et al*., 2008).

The microarray results showed that DNA vaccination significantly induced the expression of MHC class I transcripts (Caipang *et al*., 2006), and increased the expression of T cell activation-related genes (Yasuike *et al*., 2010). These results suggest that teleosts have an interaction pathway similar to that in mammals. Therefore, development of DNA vaccines in teleost fish will be greatly aided by understanding the cellular responses and receptors to DNA vaccines.

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