Development of novel DNA vaccines by using genetically modified antigens of fish pathogens

Doctoral Dissertation

DEVELOPMENT OF NOVEL DNA VACCINES BY USING GENETICALLY MODIFIED ANTIGENS OF FISH PATHOGENS

September 2017

Graduate School of Marine Science and Technology
Tokyo University of Marine Science and Technology
Doctoral Course of Applied Marine Bioscience

IANG SCHRONILTGEN RONDON BARRAGAN

Doctoral Dissertation

DEVELOPMENT OF NOVEL DNA VACCINES BY USING GENETICALLY MODIFIED ANTIGENS OF FISH PATHOGENS

September 2017

Graduate School of Marine Science and Technology
Tokyo University of Marine Science and Technology
Doctoral Course of Applied Marine Bioscience

IANG SCHRONILTGEN RONDON BARRAGAN

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

To Hirono sensei for the big opportunity to come to the Genome Science Lab to study at work and all the advices and recommendation during my PhD course.

Special thanks to Kondo sensei for all his time, advices, teachings, recommendations and patience with my work.

To Nozaki san for the help, for sharing her experience and skills with me and all the lab members.

To Dr. Yamashita for all his advices, support and help in the vaccination experiments at Ehime Prefecture.

To my mom Ana and Scrappy, for all the support during my life and for this time

To all my good friends (and lovely people) including Azumakawa Sayo (びわ), Maria Fernanda (Mafe), Luisa Maria Rojas (Lulu), Noel Verján, Victoria Rodriguez, Nochiri san, Rod Russell, Ann san a.k.a. Dr. Walissara :), Sherryl Hipolito, Beibei Zhao, Chung Seangmin, Totay san, Koiwai san, Tip san, Kobayashi san, Yoshikawa san, Aiko san, Samia san, Rika san, Ken Sakurai, Kaneshigue san, Miyaguchi san, Sugiyama san, Imaizumi kun, Kubo kun, Abe kun, Takase kun, for all the help, the support, the questions, the answers, the reagents, the food, the beers, the discussions, the softball, the smiles…

To the Ministry of Education, Culture, Sports, Science and Technology (MEXT) for the Monbukagakusho scholarship and the Tokyo University of Marine Science and Technology for all the support.

To the University of Tolima and the Faculty of Veterinary Medicine and Zootechny.

ABSTRACT

Vaccination is one of the prophylactic methods to protect the animals against disease. DNA vaccination have shown to induce immunity against viral and bacterial pathogens in fish, however, the induced protection showed variable results, which demands the search for new approaches to improve the vaccination efficacy. The use of sorting signals associated with an antigen in a DNA vaccine have shown promising results in animal models, taking advantage of the different sorting motifs of molecules to drive the movement of the antigens inside the cell. Among them, sorting signals from lysosomal membrane proteins can be candidates to improve the efficacy of a DNA vaccine. In the present study, lysosome-associated membrane protein-1 from Japanese flounder, *Paralichthys olivaceus*, (JfLAMP-1) was used as a carrier for the major capsid protein (MCP) from red sea bream iridovirus (RSIV) in order to evaluate its potential as DNA chimeric vaccine. First, JfLAMP-1 gene ORF was obtained by analyzing EST data from previous study in our lab and amplified by using specific primers. JfLAMP-1 amplicon was cloned in T vector, sequence was confirmed and bioinformatics analysis was done. Tissue expression analysis by RT-PCR and qPCR from gill, brain, muscle, liver, spleen, intestine, kidney, blood and Hirame natural embryo cell line was assessed in healthy animals. JfLAMP-1 gene expression in spleen was assessed under poly I:C (polyinosinic:polycytidylic acid) stimulation at 22℃ and *Edwardsiella tarda* FKC (Formalin-killed cells) injection at 15 ℃ and 22 ℃. JfLAMP-1 expression was assessed in HINAE cells by western blot and localization of the protein was evaluated by immunofluorescence assay. JfLAMP1 gene has a length of 1248 bp that encodes for 415 aa (43,8 kDa) and exhibit a signal peptide, a luminal domain, transmembrane domain and cytoplasmic domain similar with reported in higher vertebrates. JfLAMP-1 gene expresses constitutively in all the tissues with a higher expression in brain. In *E. tarda* FKC injection experiment, JfLAMP-1 mRNA level showed higher at 3 h, 12 h and 7 days post-injection at 22 ℃ and 1 day and 7 days post-injection at 15 ℃. In poly I:C stimulation, JfLAMP-1 showed no changes in the expression at mRNA level. In the protein analysis, JfLAMP-1 was detected in HINAE cells as a 56 kDa band and the immunofluorescence analysis showed it distributed in small and large granules in the cytoplasm and grouped close to the nucleus. After its characterization, JfLAMP-1 was fused with the MCP from RSIV to produce a chimeric DNA vaccine. For this, the DNA encoding the luminal domain of JfLAMP-1 was replaced with the gene for the RSIV MCP, and the construct was cloned in an expression vector (pCIneo). Japanese flounder juveniles (n=30) were distributed in the experimental groups (pCIneo, pCMCP and pCLAMP-MCP), vaccinated and the antibody titers measured 30 days

post-vaccination. Fish vaccinated with the chimeric vaccine pCLAMP-MCP showed significantly higher antibody levels than fish vaccinated with pCIneo vector harboring the MCP gene ($p<0.05$). Then a new chimeric vaccine was designed, inserting the MCP gene and keeping the luminal domain (LumD) of JfLAMP-1 gene. In this experiment, Japanese flounder juveniles (n=60) were distributed in six groups (PBS, pCIneo, pCLAMP, pCMCP, pCLAMP-MCP and pCLAMP-MCP-LumD). After 30 days of vaccination, fish vaccinated with the chimeric vaccines showed significantly higher antibody levels than those vaccinated with pCIneo vector harboring the MCP gene ($p<0.05$). The inclusion of the LumD did not induce statistically higher antibody titer than the pCLAMP-MCP. Then, a vaccination and challenge test were performed using JfLAMP-1 chimeric vaccine in a highly susceptible fish species to RSIV. For this, striped beakfish, *Oplegnathus fasciatus*, individuals were distributed in four experimental groups (PBS, pCIneo, pCMCP and pCLAMP-MCP; n=30 per group) and after 30 days of vaccination, a challenge test was done by using RSIV in low and high dose. In the vaccinated group challenged with low dose of RSIV, pCMCP and pCLAMP-MCP showed similar relative percentage of survival of 13%, however in the high dose challenge, pCLAMP-MCP vaccinated group showed relative percentage of survival of 19%, compared with 0% of the pCMCP. JfLAMP-1 chimeric vaccine induced higher protection than conventional DNA vaccine. In conclusion, chimeric DNA vaccines using sorting signals from specific molecules can be candidates to enhance the immune response against specific pathogens, for example, by modulation of the traffic of antigen.

Chapter 1.

General introduction

General introduction

Aquaculture is one the faster growing production system which reached 167.2 million tonnes in 2014, with a wide range of species and culture types, and recently there is a high demand for aquaculture products due to their nutritional value and variety (Figure 1)(FAO, 2014). In addition, the fisheries and aquaculture were included in the 2030 Agenda for Sustainable Development in order to support a sustainable development in economic, social and environmental terms for the food supply of world population (FAO, 2016b).

Among them, fish production plays an important role in aquaculture growth and its use for human consumption has outpaced population growth in the past decades, increasing at an average annual rate of 3.2% in the period 1961– 2013, double that of population growth, resulting in increasing average per capita availability (Figure 2) (FAO, 2016b) reaching above 20 kg for 2016 (FAO, 2016a).

The fish world per capita supply is almost 20 kg in average, being Asia, Europe, North America and Oceania, the continents with the high values of more than 20 kg, compared with Africa or Latin America and Caribbean. Asian countries showed the highest fish production with a high use of them for food supply.

World food fish aquaculture production in 2014 consisted of finfish (68%), molluscs (22%), crustaceans (9%) and other aquatic animal species (1%). Inland aquaculture produced 43.6 million tonnes of finfish, representing 59 % of world food fish aquaculture in 2014.

Fish production in Asia

Asia has accounted for about 89% of world aquaculture production of fish for human consumption in the past two decades with a total amount of 65`601.892 tonnes of aquaculture species, including finfish, molluscs and crustaceans (FAO, 2016b). Among Asian countries, China remains as the major producer although its participation in world fish production from aquaculture has declined slightly in the past 20 years (FAO, 2015; FAO, 2016b). Southeast Asia has shown the highest growth in aquaculture of the Asian sub-regions in recent years with a 45% increase over last decade (Ababouch & Karunasagar, 2013)

Fish production in Japan

In Japan the aquaculture and fishing industry have important roles in the diet of the population. Nevertheless, the consumption of seafood has decreased greatly due to changes in the environment surrounding food in Japan and this apparent fish consumption will remain static or decrease for 2025 (FAO, 2016b; Statistics Bureau, 2015). Japan's fishery output has been on the decline since 1989 and its 2014 fishery production totaled 4.79 million tons. Of this, marine fishery and aquaculture production amounted to 4.73 million tons (Statistics Bureau, 2015). However, among the world`s top producers and main groups of farmed species (Table 1), Japan ranked 11th at 1.1 million tons (1.2%), after Chile and Egypt (FAO, 2016b).

Japanese aquaculture production volume accounts for 22% of the total fishery and aquaculture production volume in Japan (UJNR Japan Panel, 2016). Several species are cultured in Japan, while the production of almost all species has decreased, especially carp whose production was devastated by koi herpes virus disease (UJNR Japan Panel, 2016) (Table 2).

Fish diseases in Japan

In Japanese marine fish aquaculture, diseases, such as streptococcosis and pseudotuberculosis became widespread in the past, and recently several outbreaks of viral and bacterial diseases occurred causing economic losses (Matsuyama *et al.*, 2016; Matsuyama *et al.*, 2012; Minami *et al.*, 2016; Yoshida, 2016; Yoshimizu, 2016). Beside to these, vaccines for major fish diseases have been developed with promising results (Byon *et al.*, 2005; Byon *et al.*, 2006; Kato *et al.*, 2011; Kato *et al.*, 2012; Matsuyama *et al.*, 2016; Shimmoto *et al.*, 2010; Yasuike *et al.*, 2007; Yasuike *et al.*, 2011a).

In order to ensure that fishery medicines do not remain in food, the pharmaceutical affairs act prohibits the use of medicines other than those approved by the national government (UJNR Japan Panel, 2016). To ensure proper use of medicines, including vaccines, a framework has been established whereby the prefectural fisheries experimental stations instruct methods of use and other necessary information on medicines to aquaculture operators. Import of foreign

seeds that may hold pathogens that do not exist around Japan is regulated for some fish species under the act on the protection of fishery resources (UJNR Japan Panel, 2016).

Viral diseases

As the other animal groups, fish are susceptible to several viral pathogens that affect a broad range of host (Bernoth & Crane, 1995; MacLachlan & Dubovi, 2011; Noga, 2010; Rexhepi *et al.*, 2011; Smail & Munro, 2012). The epizootiology of viral infections in fish become complex since new susceptible species or reservoirs are discovered and also because the effect of the spread of virus on wild fish population are for the most part unquantified (Crane & Hyatt, 2011; Smail & Munro, 2012) and in some cases this spread can occurred due to handling practices in fisheries (Mardones *et al.*, 2014).

Although the viruses discovered/reported in fish belong to the same families than those reported on humans or domestic livestock, there are significant differences between the ecology of viral diseases of fish and those of humans or other terrestrial vertebrates (Walker & Winton, 2010). That differences include: (1) few fish viruses are known to be vectored by arthropods, *e.g*. by parasitic crustaceans (Overstreet *et al.*, 2009); (2) wild reservoir species are often at very low densities; (3) fish are poikilotherms and temperature has an exceptionally critical role in modulating the disease process by affecting both the replication rate of the virus as well as the host immune response; (4) few fish viruses are transmitted sexually between adults. However, as occurs for avian diseases, migratory fish can serve as carriers for long-range dispersal of viral pathogens (MacLachlan & Dubovi, 2011; Walker & Winton, 2010).

For 2017, the OIE-Listed diseases from viral etiology for fish are: epizootic hematopoietic necrosis virus (EHNV), infectious hematopoietic necrosis virus (IHNV), infectious salmon anaemia virus (ISAV), koi herpesvirus (KHV), spring viremia of carp (SVC), red sea bream iridovirus (RSIV), Salmonid alphavirus (SAV) and viral haemorrhagic septicemia virus (VHSV), which are notifiable because represent a worldwide concern and can spread easily between countries with high morbidity/mortality outbreaks (OIE, 2017). Other viral disease enlisted in the OIE`s Manual Diagnostic test for Aquatic Animals include *Oncorhynchus masou* virus (OMV) and viral nervous necrosis virus (VNNV) (Bondad-Reantaso *et al.*, 2005; Crane & Hyatt, 2011; Sahoo & Goodwin, 2012; Walker & Winton, 2010).

In Asian countries, several viral diseases have been reported that caused important economic losses in fish aquaculture (Kim *et al.*, 2011; Kim *et al.*, 2005; Sahoo & Goodwin, 2012; Shinmoto *et al.*, 2009; Sohn & Park, 1998) which are demanding research in viral infection pathophysiology and prevention/control strategies, *i.e.* vaccination, genetic selection, among others (Costa & Thompson, 2016; Dong *et al.*, 2013; Li *et al.*, 2014; Lin *et al.*, 2016; Ohtani *et al.*, 2013; Robinson *et al.*, 2017). Recently, Kim *et al.* (2016) and Munang'andu (2016) described the perspective of the use of environmental samples to study the epidemiology of viral diseases in aquaculture using viral metagenomics analysis which allow to understand the biogeographic patterns of the virus spreading and also as an overture for the design of rational disease control strategies.

Fish immunity

The evolution of fish and tetrapods diverged from each other about 300 million years ago and it is natural that fishes should be the subject of investigation of the evolution of lymphoid tissues and the development of the immune system (Ellis, 1998) (Figure 3). Fish have evolved effective immune response against infections from pathogenic agents that cohabit the fish`s aquatic environment and cause disease (Thompson, 2017). Since different fish species live in diverse environments, *e.g.* cold or warm water, freshwater or seawater, and so on, these different conditions can modulate the immune response and perturbations in the environmental parameters are correlated with detriment in the immunocompetence (Bowden, 2008; Makrinos & Bowden, 2016; Vazzana *et al.*, 2017).

Similar to mammalian immunity, fish immunity comprises two groups of immune responses: Innate immunity and adaptive immunity (Tizard, 2012; Uribe *et al.*, 2011). However, the immune tissues/organs and some cells in fish differ from those described in mammals (Ellis, 1998; Secombes & Ellis, 2012; Tizard, 2012) (Table 3). For example, sharks have the Leydig organ and epigonal organ (Mattisson *et al.*, 1982; McClusky & Sulikowski, 2014; Rumfelt *et al.*, 2002) and teleost fish possess head kidney (Abdel-Aziz *et al.*, 2010; Kondera, 2014) (Figure 4) which play important roles in immune cell development and differentiation. Lymphoid organs show different roles including to provide suitable microenvironments for the development of immune effector cells, mediate negative and positive selection and regulate the efficacy of the immune response including suppression and memory (Scapigliati, 2013).

Innate immune response

Innate immune system is a collection of distinct subsytems that lack of any form of memory and work through diverse mechanisms, activated immediately when a pathogen penetrated the epithelial barriers such as mucus, skin or normal flora (Tizard, 2012). In fish, skin, gill and gut are important routes for pathogen entry because of their close contact with the aquatic environment (Secombes & Ellis, 2012; Thompson, 2017) and the sentinel cells as well as the mucosal-associated lymphoid tissue (MALT) play important roles in the surveillance and defense of possible infections (Parra *et al.*, 2016; Rombout *et al.*, 2011; Tafalla *et al.*, 2016).

Fish employ several host-derived pattern recognition receptors (PRRs) including toll-like receptors (TLRs), similar to those found in mammals to recognize molecules commonly expressed on many different microbes or pathogen-associated molecular patterns (PAMPs) and start the immune response (Fink *et al.*, 2016; Poynter *et al.*, 2015; Tizard, 2012). In fish inflammatory responses, granulocytes arrive first and their numbers peak after 12 to 24 hours in order to start the secretion of antimicrobial peptides as well as enzymes and cytokines (Tizard, 2012). In teleostean, initial inflammatory response seems to be biphasic, starting with an influx of neutrophils followed by later arrival of monocytes/macrophages (Reite & Evensen, 2006). The neutrophils move to the margin of the blood flow, getting contact with blood vessels before traversing the vessel walls originate from the head kidney, while macrophage appears in the tissues originate from blood-derived monocytes (Reite, 2005; Reite & Evensen, 2006). This is followed by a wave of macrophages and possibly lymphocytes (Tizard, 2012).

After recognition of the pathogen and chemotaxis of the immune cells to the infection/injury site, the cells start to process the antigens and to synthesize acute phase proteins including cytokines to drive the immune response based on the characteristic of the antigen (Uribe *et al.*, 2011). Several antimicrobial peptides (AMP) have been described in fish including hepcidins, β-defensins, piscidins, cathelicidins, histone-derived peptides (Katzenback, 2015). The AMP are normally present in the mucus, liver and gill tissue but also can be induced by pathogens in the tissue (Bridle *et al.*, 2011; Katzenback, 2015; Masso-Silva & Diamond, 2014). For example, in Atlantic salmon (*Salmo salar*) after bacterial challenge with *Yersinia ruckeri*, upregulation of cathelicidin was found in the gills and spleen (Bridle *et al.*, 2011). On the other hand, AMP seem to be related with several physiological processes. For example, β-defensin was dominantly expressed in pituitary and testis of orange-spotted grouper

(*Epinephelus coioides*) and its transcript level was significantly upregulated in reproductive organs from intersexual gonad to testis during the natural and artificial sex reversal (Jin *et al.*, 2010).

Intracellularly in the sentinel cells, the antigen can be processed depending of its intracellular or extracellular origin. Intracellular antigens such as virus, intracellular bacteria or intracellular protozoa are ubiquitin tagged and digested by proteasome pathway, loaded in a major histocompatibility complex (MHC) I molecule and located at cell membrane for presentation to T cells. In case of the extracellular antigens, which are engulfed and internalized in the cell, they are processed for the endosomal pathway which use lysosomes enzymes for the degradation of the antigen and its presentation by MHC II molecules for presentation to T cells. The cells are able to do this presentation of the antigen are called antigen presenting cells (APC) which are distributed throughout the organism and have some characteristics that allow the interaction with a T cell subpopulation (Iliev *et al.*, 2013; Popi *et al.*, 2016). In fish, Iliev *et al.* (2013) characterized Atlantic salmon APC based on the ability to take up soluble antigen and to migrate toward secondary lymphoid organ. This population was MHC II^+ cells able to endocytose antigen.

In the innate immune response, macrophages are the key cell to orchestrate the innate and adaptive immune response (Mills, 2015). In teleost fish, macrophages have antimicrobial mechanisms that include phagocytosis, secretion of AMP and chemotactic substances, production of reactive oxygen and nitrogen intermediates (Rieger & Barreda, 2011). Although there is not strong evidence of macrophage subpopulations M1 (inflammatory macrophage) and M2 (healing macrophage) in fish, some markers as iNOS and arginase have been proposed to discriminate the populations (Forlenza *et al.*, 2011). Wiegertjes *et al.* (2016) described the use of iNOS(B), or NOS-2(B), as marker for M1 and the use of arginase-2 as marker for M2 macrophages of teleost fish. In the same way, based on the studies on zebrafish and *Mycobacterium murinum* model, tumor necrosis factor alpha (TNF-α) was proposed as a putative although not unique marker for M1 macrophage in fish (Roca *et al.*, 2008).

Macrophages are able to act as APC, secrete cytokines and vasoactive molecules due to become an important link between innate and adaptive immune response and polarize the immune response even for bacterial, parasites or virus (Tizard, 2012; Verrier *et al.*, 2011; Wiegertjes *et al.*, 2016).

Adaptive immune response

Fish adaptive immunity elicits a specific response against a pathogen; it has a memory component that is able to quickly eliminate pathogen upon reencountering them, which is similar to higher vertebrates (Thompson, 2017). A key difference between innate and adaptive immune systems lies in their use of cell surface receptors to recognize foreign invaders. The cells of innate system use a limited number of preformed receptors that bind to PAMPs. In contrast, the cells of the adaptive immune system generate enormous number of completely new, structurally unique receptors with the possibility to recognize a broad range of foreign molecules (Covello *et al.*, 2013; Tizard, 2012) which are expressed on the surface of the cell as T-cell receptors (TCR) or B-cell receptors (BCR) or in a soluble form outside the cell (soluble BCR also called antibodies). Furthermore, MHC is a set of cell-surface molecules that the adaptive immune system uses to recognize as foreign molecules (Thompson, 2017). Although similar to mammalian MHC system, the mechanisms of peptide loading on the MHC molecules may differ in fish from those in mammals, due to the absence of some critical residues (Dijkstra *et al.*, 2013; Dijkstra *et al.*, 2007; Dijkstra *et al.*, 2003).

T- and B-cells are the main cells in the adaptive immune system and their exact timing of differentiation varies in different fish species, despite of the early appearance of T- and B-cells, the full maturation is late, and the cell-mediated immunity develops earlier than the humoral immune response (Zapata *et al.*, 2006).

T cells can be divided in two groups depending on the TCR expressed on the surface: αβ and γδ (Buonocore *et al.*, 2012; Nam *et al.*, 2003). The majority of T-cells expressed αβ TCR and they can recognize peptides on the surface of APC on secondary lymphoid organs. On the other hand, T-cells expressing γδ TCR reside in epithelial layers of mucosal tissues where they work as effector cells showing cytotoxic activity but they do not recognize peptides processes and presented by APC (Buonocore *et al.*, 2012; Koizumi *et al.*, 1991). Previously, Nam *et al.* (2003) reported the four genes (α, β, γ, δ) in Japanese flounder and Lee *et al.* (2013) found their expression at mRNA level commonly expressed in the immune-related organ such as spleen, kidney and gill, however weak expressed in fin and eye. In adult zebrafish was estimated that the 2×10^5 T cells contain at least unique 1.5×10^4 TCRαβ pairs, present in low frequency in the zebrafish TCRαβ repertoire with bias for just some V-J combinations (Covacu *et al.*, 2016). This can be related with and impaired T cell immunity as it was demonstrated in

murine models against virus (Yager *et al.*, 2008). In juveniles of *Dicentrarchus labrax*, Buonocore *et al.,* (2012) showed the upregulated expression of γTCR in the head kidney and down-regulated in intestine after in vivo infection with betanodavirus.

In addition, two big subpopulations of T-cells have been described based on the expression of a cluster of differentiation (CD) on the cell surface, $CD8^+$ and $CD4^+$ (Tizard, 2012). $CD8^+$ T cells as well as NK cells, another effector cells from the same lineage, are effector cells than can be cytotoxic and lyse target cells by two mechanisms, namely granule exocytosis, in which pore-forming substance and granzymes are released, and FasL/Fas interaction, both of which require membrane contact with the target cell (Fischer *et al.*, 2013). These pore-forming molecules as well as granzymes have been already reported in teleost (Athanasopoulou *et al.*, 2009; Hwang *et al.*, 2004; Praveen *et al.*, 2006)

$CD4^+$ T cells are also called T helper (Th) cells and play a pivotal role in the polarization of the immune response to cell-mediated immunity (Th1-type response) or antibody-mediated immunity (Th2-type response) based on the MHC class in which the peptide antigen is loaded in the APC (Kono & Korenaga, 2013; Yamaguchi *et al.*, 2015). The Th17-type of response, which is related with mucosal immunity and the induction of antimicrobial peptides, was also reported in fish (Zhang *et al.*, 2013; Zhang *et al.*, 2014). After antigen presentation, a specific types of cytokine are released which stimulate the immune response, mainly interferon (IFN)-γ for Th1 responses, interleukin-4 for Th2 responses and interleukin-6 for Th17 responses (Kaiko *et al.*, 2008; Kono & Korenaga, 2013; Korenaga *et al.*, 2013; Tizard, 2012) (Figure 5).

B-cells are able to differentiate to plasma cells which can produce antibodies (Tizard, 2012). In mice and humans, 3 principal classes of B lymphocytes have been described on the basis of their ontogeny and anatomic localization: B1 and B2 B-cells, this last one which are divided in marginal zone (MZ) and follicular (FO) B-cells (Hoffman *et al.*, 2016). B1 lymphocytes express the surface markers CD5, $B220^{lo}$, IgM^{hi}, and IgD^{lo} while B2 lymphocytes are $CD23^-$ and when they are located in celomic cavities, which are the major site of their homing and proliferation (Oliveira *et al.*, 2005).

In teleost, these cells reside in the anterior and posterior kidney, spleen, gut lamina propia, and blood (Abelli *et al.*, 1997; Rombout *et al.*, 1993) and, similar to mammals, the B cells use the same tissue for their development as plasma cells for their residence (Fillatreau *et al.*, 2013). B cells of fish retain many innate characteristics and functions and consequently are one of the early responders to inflammation (Castro *et al.*, 2017). In general, in the ontogeny of fish B cells, Ig-producing cells appear earlier in freshwater species than marine species and they appear first in head kidney, followed by the spleen, and finally in the MALT and the surface expression of Ig occurs earlier than cytoplasmic expression (Salinas, 2015; Salinas *et al.*, 2011; Zapata *et al.*, 2006). The difference in the development of B cell in freshwater fish vs marine fish could be due to very distinct ecological strategies, greater egg size and earlier larvae development in freshwater fish (Salinas *et al.*, 2011).

In bony fish, B cell subsets can be distinguished according to their expression of distinct immunoglobulin (Ig) class combinations, IgM and D, or IgT only (Fillatreau *et al.*, 2013). IgM constitutes the main systemic Ig and IgT plays the prevalent role in mucosal surfaces (Magadan *et al.*, 2015; Mashoof & Criscitiello, 2016). However, the existence of different B cell subsets is still controversial, mainly because of the scarce availability of specific antibodies against differentially expressed markers (Castro *et al.*, 2017), however IgM+ B cells expressing CD9 were detected in rainbow trout which is related with "innate-like" B lymphocytes or B1 B cells from higher vertebrates (Castro *et al.*, 2015). In addition, IgM+ B cells have shown phagocytic activity for soluble and particulate antigen, inducible expression of MHC II, and the ability to present antigens to T CD4+ cells (Li *et al.*, 2006; Zhu *et al.*, 2014) which resemble B1 B cells from mammals (Abos *et al.*, 2016; Popi *et al.*, 2016). In the same way, fish can produce natural antibodies at a level that is regulated in the absence of antigenic stimulation (Uribe *et al.*, 2011) which support fish B cells as equivalent to B1 cells from mammals (Boes, 2000). IgM-secreting cells have been proved to be up-regulated in fish injected with either *E. coli* or VHSV when compared to fish injected with saline (Castro *et al.*, 2017).

Immunity to virus

Once virus spreads beyond a few host cells a variety of non-specific and specific host defensive responses will be elicited (Smail & Munro, 2012). Similar to mammals, specific

receptors and cells are responsible to recognize and to induce the immune response against virus in fish (Scapigliati, 2013; Scapigliati *et al.*, 2010; Somamoto *et al.*, 2002). In case of innate immunity, TLR3 and TLR7/TLR8 are the main intracellular sensors of the foreign nucleic acids from virus, double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA), respectively (Pietretti & Wiegertjes, 2014) and TLR22 which recognize dsRNA can be a cell surface TLR3 analog sensing the dsRNA outside the cell (Matsuo *et al.*, 2008; Pietretti & Wiegertjes, 2014; Su *et al.*, 2012). Another intracellular sensor for viral nucleic acid described in fish includes the proteins: nucleotide-binding oligomerization domain - NOD (NOD-like) (Thanasaksiri *et al.*, 2017), melanoma differentiation-associated 5 - MDA5 (Ohtani *et al.*, 2011) and the laboratory of genetics and physiology 2 - LGP2 (Chang *et al.*, 2011; Han *et al.*, 2016).

After recognition and during the antigen processing, several cytokines are secreted in order to orchestrate the chemotaxis of immune cells as well as to induce a Th response depending on the type of antigen and the MHC restriction (Tizard, 2012). The first innate immune antiviral defenses are the interferon (IFN) and IFN-induced genes (ISGs) (Verrier *et al.*, 2011). In fish, two subfamilies of IFN have been described: type I (α and β) and II (γ) IFN. However, the inflammatory functions of teleost type II IFNs have not been fully characterized, especially in the case of those species possessing two genes (Pereiro *et al.*, 2016). Both types of IFN act as antiviral molecules and causes susceptible cells to express potent antiviral mechanisms to limit further viral growth and spread (Haller *et al.*, 2006).

Type I IFNs are polypeptides secreted by infected cells and induce cell-intrinsic antimicrobial states in infected and close cells that limit the dissemination of pathogens, particularly virus (Ivashkiv & Donlin, 2014). Type II IFN (known as IFN-γ) potentiates inflammation during viral infection, favor the expression of those genes directly related with the activity of macrophages and had anti-inflammatory effects during bacterial disease (Pereiro *et al.*, 2016) mainly by inhibiting the production of pro-inflammatory IL-1 and IL-8, as well as by inducing expression of suppressors of cytokine signaling (SOCS) (Mühl & Pfeilschifter, 2003). The IFN pathways are coordinated by intracellular signaling molecules. Most of these signaling molecules, including IFN regulatory factors (IRFs), Janus kinases (JAKs), signal transducer and activator of transcription (STAT) proteins, protein inhibitors of activated STAT (PIAS), and SOCS, are present in cartilaginous fish, as well as in Osteichthyes (Secombes & Zou, 2017).

After stimulation several ISGs are induced and coded for antiviral proteins such as double-stranded RNA- activated protein kinase (PKR), myxovirus resistance protein (Mx), the 2`–5` oligoadenylate synthetases (2`,5`-OAS) (Qu *et al.*, 2013). Tetrapods and fish share a number of ISGs that are remarkably conserved, indicating that the interferon system is an ancient and fundamental part of the immune system of gnathostomes (Verrier *et al.*, 2011). In fish, some ISGs have been characterized which are upregulated under viral stimulation or poly I:C injection (Røkenes *et al.*, 2007; Seppola *et al.*, 2007; Yasuike *et al.*, 2011b; Zhang *et al.*, 2007) and inhibit viral replication in the host (Secombes & Zou, 2017).

The presentation of the antigen to the effective immune cells occurs, in order to start a strong and specific immune response. B cells and T cells are components of adaptive immunity, being responsible for antibody production and cell-mediated cytotoxicity, respectively (Fischer *et al.*, 2006; Tizard, 2012). Castro *et al.* (2017) showed that upon intraperitoneal antigen stimulation (bacterial or viral), peritoneal B cell population increase which can occur due to increasing in the traffic from the lymphoid organs or local proliferation. Similar behavior was reported in the muscle after intramuscular injection of a DNA vaccine (Castro *et al.*, 2014). On the other side, cytotoxic T-lymphocytes (CTL) have been described as antiviral effector cells in fish playing a role in the control of early viral infection (Somamoto *et al.*, 2002) and the recognition of virally infected cells is MHC class I restricted (Fischer *et al.*, 2006). The diversity of B cell and T cell repertoire receptor diversity is driven by the viral antigens, towards a virus specific response and higher clonotypic diversity, which have been shown in the DNA vaccination (Castro *et al.*, 2011).

Vaccination

Vaccines are one of the prophylactic strategies used in animal production, including companion animals (Day *et al.*, 2016; Day *et al.*, 2015), to induce protective immunity against a corresponding infectious agent or multiple infectious agents as in the case of multivalent vaccines (Brun, 2016; Lee *et al.*, 2012; Walz *et al.*, 2015; Walz *et al.*, 2017; Wang *et al.*, 2012). However, their use and regulation are dependent on the policies of each country and based on the specific epidemiology of the area or zone. Previously, the world health organization (WHO) defined some general regulatory requirements (Shin *et al.*, 2011). Similarly, the Pan American Health Organization (PANDRH, 2010) and Japan have their own regulation policies for human

and veterinary products, including vaccines (MAFF & NVAL, 2013; Nakayama & Aruga, 2015).

Many types of vaccines have been developed starting from live vaccines, modified-live vaccines, attenuated vaccines by formalin or heat, protein-based vaccines, DNA vaccines, among others in order to induce protection, using mainly immunodominant antigens (Nuñez-Ortiz *et al.*, 2016; Saul & O'Brien, 2017; Sequeira *et al.*, 2017; Walz *et al.*, 2017; Zheng *et al.*, 2016). DNA vaccines became an alternative, due to some advantages including that they are relatively simple to produce and safe to administer and because they are not associated with a viral coat, since naked nucleic acids are not generally subject to neutralizing antibody reactions that can hamper the clinical efficacy of vaccines based on recombinant viruses (Pereira *et al.*, 2014; Restifo *et al.*, 2000; Starodubova *et al.*, 2010). DNA vaccine 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 (Kurath, 2008). However, some concerns exist about the use of "naked" plasmid DNA and the possibility of integration on the host genome, also known as insertional mutagenesis (Alonso & Leong, 2013; Starodubova *et al.*, 2010; Tonheim *et al.*, 2008) and unfortunately the induced immune response is not as strong as virus vectors or bacteria (Restifo *et al.*, 2000). The mechanism of immune stimulation by nucleic acid vaccines start with the use of the host's transcriptional and translational machinery to produce the antigen coded in the plasmid, *i.e.* protein, with conformation and posttranslational modification patterns identical, in most cases, to those which occur during normal infection (Whitton *et al.*, 1999). This polypeptide product can be recognized by the immune system and its early uptake is made by myocytes which can present antigen to immune cells (Restifo *et al.*, 2000) and the subsequent production of cytokines promotes the immune response (Løvås *et al.*, 2014). DNA immunization induces both humoral and cellular immunity including both CD4+ and CD8+ T-cells, which often are protective against microbial challenge (Whitton *et al.*, 1999)(Figure 6).

The administration of the vaccine includes the mucosal/oral route, immersion or injection through the intraperitoneal (i.p.) or intramuscular (i.m.) route (Embregts & Forlenza, 2016). In the same way, some adjuvants as well as new delivery vehicles have been reported (Behera & Swain, 2011; Gvili *et al.*, 2007; Tafalla *et al.*, 2013; Vimal *et al.*, 2014; Zheng *et al.*, 2016). In addition, Kanellos *et al.* (1999) showed that the mode and place of injection can influence the distribution of the vaccine in fish tissues. Besides the i.p., i.m., immersion and

oral vaccination, LaPatra *et al.* (2015) and Salinas *et al.* (2015) showed the viability to use nasal delivery of vaccines for IHNV and enteric red mouth virus vaccines in rainbow trout, eliciting innate immunity and conferring early protection against experimental infection. Previously was demonstrated that the mucosa surfaces are important in the antigen uptake (Moore *et al.*, 1998). However, this type of delivery method has not been proved in DNA vaccines.

In addition, the effective concentration of plasmid for protection depends on the size of the fish. Previously, Corbeil *et al.* (2000) described that the minimal dose of DNA vaccine against IHNV can be as little as 1-10 ng of DNA vaccine per fish, which is enough to induce partial to complete protection in fry against IHNV challenge doses of 10^3-10^4 plaque forming units/ml. However, in large fish 10 ng of DNA vaccine per gram body weight by i.m. administration is required to induce protection against IHNV or VHSV in rainbow trout (Lorenzen *et al.*, 2002a).

DNA vaccines in fish

Several vaccines are available in fish aquaculture, most of them targeting bacterial pathogens and only a few are raised against viruses (Alonso & Leong, 2013; Embregts & Forlenza, 2016; Park *et al.*, 2012). For bacterial fish pathogens, killed vaccines have been shown good results, and the development of DNA vaccines is focused on bacteria which bacterins are not effective (Kurath, 2008).

In fish, several studies have demonstrated the potential use of DNA vaccines, nevertheless, just few have been approved for commercial use (Alonso & Leong, 2013; EMA, 2016). The fate of DNA vaccine in fish have been reviewed previously (Gillund *et al.*, 2008; Seternes *et al.*, 2007; Tonheim *et al.*, 2008; Tonheim *et al.*, 2008; Tonheim *et al.*, 2007) however still exist the concern about consumption of DNA-vaccinated fish meat and the spills or waste of DNA vaccine to the environment from the production process (Tonheim *et al.*, 2008). In the same way, some uncertainties exist regard to whether DNA vaccines persist degradation in tissues, or in the environment and if the fish should be label as a genetic-modified organism (GMO) (Myhr, 2017). If a DNA vaccinated animal is considered to be a GMO the environmental legislation on the deliberate release of GMOs by producers should be accomplished (Hølvold *et al.*, 2014).

After injection of DNA vaccine, injected plasmid can be taken up by migrating cells passing through the muscle or may diffuse to transfect cells at distant sites (Heppell *et al.*, 1998). Thus, DNA from vaccine has been found in different cell populations as scavenger endothelial cells in Atlantic cod heart (Seternes *et al.*, 2007), myocytes in rainbow trout and zebrafish (Boudinot *et al.*, 1998; Castro *et al.*, 2014; Einer-Jensen *et al.*, 2009; Heppell *et al.*, 1998), gills tissue in rainbow trout and zebrafish (Heppell *et al.*, 1998) and Atlantic salmon kidney cells (Tonheim *et al.*, 2008). On oral and i.m. vaccination experiments, DNA vaccine transcripts were found in gills, kidney, spleen and intestinal tissues showing the distribution of the vaccine through internal and external organs of vaccinated fish (Ballesteros *et al.*, 2015; Vimal *et al.*, 2016). This shows that the spread of the vaccine can be wide and it can go to different tissues where the degradation rate can be higher (Gillund *et al.*, 2008; Gillund *et al.*, 2008) and it appeared that head kidney preferentially acts as a scavenger tissue, clearing the plasmid from blood circulation (Ballesteros *et al.*, 2012). Nevertheless, the persistence time on tissues showed higher variability between studies (Anderson *et al.*, 1996; Heppell *et al.*, 1998; Salonius *et al.*, 2007; Vimal *et al.*, 2016). In addition, Heppell *et al.* (1998) described that the expression of a DNA vaccine in cells other than myocytes, especially if it includes professional APC, could potentially contribute to improved immune responses, and could also lead to a more rapid decrease of the expression level of the foreign gene due to the higher turnover rate of these cells.

Immune response of fish in antiviral DNA vaccination

Same as described for higher vertebrates, DNA vaccine in fish can induce cell-mediated and antibody-mediated immunity (Kurath, 2008). In case of viral vaccination, the protection against virus-infection is non-specific during the first 18 days after injection whilst the later protection is virus specific and long lasting (Lorenzen *et al.*, 2002b). Several antiviral genes have been showed upregulated after DNA vaccination and they can be responsible to orchestrate the early immune response against vaccine antigen and the subsequent antiviral state (Byon *et al.*, 2005; Caipang *et al.*, 2003; Kim *et al.*, 2000; Robertsen, 2008) (Table 4). The route of administration of the vaccine showed similar gene upregulation, which was dose-dependent (Ballesteros *et al.*, 2015).

Utke *et al.* (2008) showed that vaccination by using VHSV G and N protein in a DNA vaccine (plasmid) provoked the activation of antiviral cytotoxic cells (CTLs and NK cells).

Also, after DNA vaccination, T cell repertoire of anti-VHSV TCRs is large enough to allow the selection of diverse private responses using different VβJβ combinations in different individuals (Boudinot *et al.*, 2001) which can enhance the immune response against specific pathogen challenge. At transcription level, DNA vaccination showed to be able to induce $CD4^+$ and $CD8^+$ gene expression upregulation which are necessary to start Th1- or Th2-type responses. In IHNV DNA vaccination by oral route, CD4 and CD8 gene expression was significantly higher than i.m. injection, and CD8 expression levels were lower than those of CD4, nevertheless, the i.m. injection of the vaccine did not induce significant levels of CD8 gene expression in kidney and spleen of vaccinated fish (Ballesteros *et al.*, 2015). This demonstrated that the route of vaccine administration may influence the immune response.

On the other hand, Castro *et al.* (2014) showed that DNA vaccination by intramuscular injection induced a large infiltration of both IgM+ and IgT+ cells B cells which was accompanied with an up-regulation of chemokines CXCL11_L1, CK5B, CK6 and CXCR3B genes, that possible contribute to the observed leukocyte recruitment to the muscle. Oral and i.m. vaccination, induced IgM and IgT gene expression as well as antibody production (Ballesteros *et al.*, 2015; Vimal *et al.*, 2016).

DNA vaccination have shown to induce cross-protection in bacterial and viral experiments. Previously, Xu *et al.* (2017) showed that a DNA vaccine against a Chinese strain of IHNV not only provided significant protection against challenge with the parental IHNV strain SD-12 (genogroup J), but provided almost the same protection against intragenogroup challenge with other Chinese IHNV field strains. In the same way, Lorenzen *et al.* (2002b) showed that VHSV and IHNV DNA vaccines induced significant protection in rainbow trout against VHSV in challenge experiments performed 4 and 7 days post vaccination. In the same way, these two DNA vaccines induced lower protection against *Y. ruckeri* and *A. salmonicida*. The immune response and protection was attributed to the induction of non-specific anti-viral defense mechanisms which are gradually replaced by a more specific immune response.

Recently, the improvement of the DNA vaccines against bacteria and virus by using new immunodominant antigens, adjuvants as well as delivery methods have been reported in fish (Chang *et al.*, 2015; Fu *et al.*, 2015; He *et al.*, 2014; Liu *et al.*, 2017). Thus, Zheng *et al.* (2016) showed that an oral DNA vaccine against turbot reddish body iridovirus (TRBIV) based on chitosan nanoparticles induced higher survival after challenge and higher upregulation of

immune-related genes, mainly TNF-α in the hindgut tissue of turbot, than the conventional DNA vaccine. In the same way, in Asian sea bass, Vimal *et al.* (2014) using chitosan–tripolyphosphate (CS/TPP) nanoparticles as vehicles for a DNA vaccine against nodavirus (virus nervous necrosis virus), evidenced a higher survival rate after challenge and higher antibody titer at 3 weeks after vaccination compared with the conventional DNA vaccine.

Research objective

Vaccination is a prophylactic method that can be useful to increase the immunity against specific pathogens and to reduce the mortality and economic losses due to diseased animals. DNA vaccines have shown promising results to induce immunity against fish pathogens. Chimeric antigens coded in a DNA vaccine can improve the immunity against the antigens and the use of sorting signals in the chimeric molecule allows to drive the immune response. In this research, lysosome-associated membrane protein-1 (LAMP-1) from Japanese flounder was used as an antigen carrier in a chimeric DNA vaccine.

References

Ababouch, L., & Karunasagar, I. (2013, September 29-October 3). *Global fisheries and aquaculture: Opportunities and challenges.* Paper presented at the 10th World Seafood Congress, Saint John`s, Canada.

Abdel-Aziz, E.-S. H., Abdu, S. B. S., Ali, T. E.-S., & Fouad, H. F. (2010). Haemopoiesis in the head kidney of tilapia, *Oreochromis niloticus* (Teleostei: Cichlidae): a morphological (optical and ultrastructural) study. *Fish Physiology and Biochemistry, 36*(3), 323-336.

Abelli, L., Picchietti, S., Romano, N., Mastrolia, L., & Scapigliati, G. (1997). Immunohistochemistry of gut-associated lymphoid tissue of the sea bass, *Dicentrarchus labrax* (L.). *Fish & Shellfish Immunology, 7*(4), 235-245.

Abos, B., Wang, T., Castro, R., Granja, A. G., Leal, E., Havixbeck, J., Luque, A., Barreda, D. R., Secombes, C. J., & Tafalla, C. (2016). Distinct differentiation programs triggered by IL-6 and LPS in teleost IgM(+) B cells in the absence of germinal centers. *Scientific Reports, 6*, 30004.

Acosta, F., Collet, B., Lorenzen, N., & Ellis, A. E. (2006). Expression of the glycoprotein of viral haemorrhagic septicaemia virus (VHSV) on the surface of the fish cell line RTG-P1 induces type 1 interferon expression in neighbouring cells. *Fish & Shellfish Immunology, 21*(3), 272-278.

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 Immunology, 18*(1), 81-89.

Alonso, M., & Leong, J. A. (2013). Licensed DNA vaccines against infectious hematopoietic necrosis virus (IHNV). *Recent Patents on DNA Gene Sequences, 7*(1), 62-65.

Anderson, E. D., Mourich, D. V., & Leong, J. A. (1996). Gene expression in rainbow trout (*Oncorhynchus mykiss*) following intramuscular injection of DNA. *Molecular Marine Biology and Biotechnology, 5*(2), 105-113.

Athanasopoulou, S., Marioli, D., Mikrou, A., Papanastasiou, A. D., & Zarkadis, I. K. (2009). Cloning and characterization of the trout perforin. *Fish & Shellfish Immunology, 26*(6), 908-912.

Ballesteros, N. A., Alonso, M., Saint-Jean, S. R., & Perez-Prieto, S. I. (2015). An oral DNA vaccine against infectious haematopoietic necrosis virus (IHNV) encapsulated in alginate microspheres induces dose-dependent immune responses and significant

protection in rainbow trout (*Oncorrhynchus mykiss*). *Fish & Shellfish Immunology, 45*(2), 877-888.

Ballesteros, N. A., Saint-Jean, S. S., Perez-Prieto, S. I., & Coll, J. M. (2012). Trout oral VP2 DNA vaccination mimics transcriptional responses occurring after infection with infectious pancreatic necrosis virus (IPNV). *Fish & Shellfish Immunology, 33*(6), 1249-1257.

Behera, T., & Swain, P. (2011). Antigen adsorbed calcium phosphate nanoparticles stimulate both innate and adaptive immune response in fish, *Labeo rohita* H. *Cellular Immunology, 271*.

Bernoth, E.-M., & Crane, M. S. J. (1995). Viral diseases of aquarium fish. *Seminars in Avian and Exotic Pet Medicine, 4*(2), 103-110.

Boes, M. (2000). Role of natural and immune IgM antibodies in immune responses. *Molecular Immunology, 37*(18), 1141-1149.

Bondad-Reantaso, M. G., Subasinghe, R. P., Arthur, J. R., Ogawa, K., Chinabut, S., Adlard, R., Tan, Z., & Shariff, M. (2005). Disease and health management in Asian aquaculture. *Veterinary Parasitology, 132*(3–4), 249-272.

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 protective immunity and nonspecific response in rainbow trout. *Virology, 249*(2), 297-306.

Boudinot, P., Boubekeur, S., & Benmansour, A. (2001). Rhabdovirus infection induces public and private T cell responses in teleost fish. *Journal of Immunology, 167*(11), 6202-6209.

Bowden, T. J. (2008). Modulation of the immune system of fish by their environment. *Fish & Shellfish Immunology, 25*(4), 373-383.

Bridle, A., Nosworthy, E., Polinski, M., & Nowak, B. (2011). Evidence of an antimicrobial-Immunomodulatory role of atlantic salmon cathelicidins during infection with *Yersinia ruckeri*. *PLoS ONE, 6*(8), e23417.

Brun, A. (2016). Vaccines and vaccination for veterinary viral diseases: A general overview. *Methods in Molecular Biology, 1349*, 1-24.

Buchmann, K. (2014). Evolution of innate immunity: Clues from invertebrates via fish to mammals. *Frontiers in Immunology, 5*, 459.

Buonocore, F., Castro, R., Randelli, E., Lefranc, M.-P., Six, A., Kuhl, H., Reinhardt, R., Facchiano, A., Boudinot, P., & Scapigliati, G. (2012). Diversity, molecular

characterization and expression of T cell receptor γ in a teleost fish, the Sea Bass (*Dicentrarchus labrax*, L). *PLoS ONE, 7*(10), e47957.

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 Immunology, 18*(2), 135-147.

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*(7), 921-930.

Caipang, C. M. A., Hirono, I., & Aoki, T. (2003). In vitro inhibition of fish rhabdoviruses by Japanese flounder, *Paralichthys olivaceus* Mx. *Virology, 317*(2), 373-382.

Carmona, S. J., Teichmann, S. A., Ferreira, L., Macaulay, I. C., Stubbington, M. J., Cvejic, A., & Gfeller, D. (2017). Single-cell transcriptome analysis of fish immune cells provides insight into the evolution of vertebrate immune cell types. *Genome Research, 27*(3), 451-461.

Castro, R., Abós, B., González, L., Aquilino, C., Pignatelli, J., & Tafalla, C. (2015). Molecular characterization of CD9 and CD63, two tetraspanin family members expressed in trout B lymphocytes. *Developmental & Comparative Immunology, 51*(1), 116-125.

Castro, R., Abós, B., González, L., Granja, A. G., & Tafalla, C. (2017). Expansion and differentiation of IgM+ B cells in the rainbow trout peritoneal cavity in response to different antigens. *Developmental & Comparative Immunology, 70*, 119-127.

Castro, R., Bernard, D., Lefranc, M. P., Six, A., Benmansour, A., & Boudinot, P. (2011). T cell diversity and TcR repertoires in teleost fish. *Fish & Shellfish Immunology, 31*(5), 644-654.

Castro, R., Martínez-Alonso, S., Fischer, U., Haro, N. Á. d., Soto-Lampe, V., Wang, T., Secombes, C. J., Lorenzen, N., Lorenzen, E., & Tafalla, C. (2014). DNA vaccination against a fish rhabdovirus promotes an early chemokine-related recruitment of B cells to the muscle. *Vaccine, 32*(10), 1160-1168.

Chang, C.-J., Sun, B., & Robertsen, B. (2015). Adjuvant activity of fish type I interferon shown in a virus DNA vaccination model. *Vaccine, 33*(21), 2442-2448.

Chang, M., Collet, B., Nie, P., Lester, K., Campbell, S., Secombes, C. J., & Zou, J. (2011). Expression and functional characterization of the RIG-I-like receptors MDA5 and

LGP2 in rainbow trout (*Oncorhynchus mykiss*). *Journal of Virology, 85*(16), 8403-8412.

Chico, V., Martinez-Lopez, A., Ortega-Villaizan, M., Falco, A., Perez, L., Coll, J. M., & Estepa, A. (2010). Pepscan mapping of viral hemorrhagic septicemia virus glycoprotein G major lineal determinants implicated in triggering host cell antiviral responses mediated by type I interferon. *Journal of Virology, 84*(14), 7140-7150.

Corbeil, S., LaPatra, S. E., Anderson, E. D., & Kurath, G. (2000). Nanogram quantities of a DNA vaccine protect rainbow trout fry against heterologous strains of infectious hematopoietic necrosis virus. *Vaccine, 18*(25), 2817-2824.

Costa, J. Z., & Thompson, K. D. (2016). Understanding the interaction between Betanodavirus and its host for the development of prophylactic measures for viral encephalopathy and retinopathy. *Fish & Shellfish Immunology, 53*, 35-49.

Covacu, R., Philip, H., Jaronen, M., Almeida, J., Kenison, J., Darko, S., Chao, C.-C., Yaari, G., Louzoun, Y., Carmel, L., Douek, D. C., Efroni, S., & Quintana, F. J. (2016). System-wide analysis of the T-cell response. *Cell Reports, 14*(11), 2733-2744.

Covello, J. M., Bird, S., Morrison, R. N., Bridle, A. R., Battaglene, S. C., Secombes, C. J., & Nowak, B. F. (2013). Isolation of RAG-1 and IgM transcripts from the striped trumpeter (*Latris lineata*), and their expression as markers for development of the adaptive immune response. *Fish & Shellfish Immunology, 34*(3), 778-788.

Crane, M., & Hyatt, A. (2011). Viruses of fish: An overview of significant pathogens. *Viruses, 3*(11), 2025-2046.

Day, M. J., Horzinek, M. C., Schultz, R. D., & Squires, R. A. (2016). WSAVA Guidelines for the vaccination of dogs and cats. *Journal of Small Animal Practice, 57*(1), E1-E45.

Day, M. J., Karkare, U., Schultz, R. D., Squires, R., & Tsujimoto, H. (2015). Recommendations on vaccination for Asian small animal practitioners: a report of the WSAVA Vaccination Guidelines Group. *Journal of Small Animal Practice, 56*(2), 77-95.

Dijkstra, J. M., Grimholt, U., Leong, J., Koop, B. F., & Hashimoto, K. (2013). Comprehensive analysis of MHC class II genes in teleost fish genomes reveals dispensability of the peptide-loading DM system in a large part of vertebrates. *BMC Evolutionary Biology, 13*(1), 260.

Dijkstra, J. M., Katagiri, T., Hosomichi, K., Yanagiya, K., Inoko, H., Ototake, M., Aoki, T., Hashimoto, K., & Shiina, T. (2007). A third broad lineage of major histocompatibility

complex (MHC) class I in teleost fish; MHC class II linkage and processed genes. *Immunogenetics, 59*.

Dijkstra, J. M., Kiryu, I., Kollner, B., Yoshiura, Y., & Ototake, M. (2003). MHC class II invariant chain homologues in rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology, 15*.

Dong, Y., Weng, S., He, J., & Dong, C. (2013). Field trial tests of FKC vaccines against RSIV genotype Megalocytivirus in cage-cultured mandarin fish (*Siniperca chuatsi*) in an inland reservoir. *Fish & Shellfish Immunology, 35*(5), 1598-1603.

Einer-Jensen, K., Delgado, L., Lorenzen, E., Bovo, G., Evensen, O., Lapatra, S., & Lorenzen, N. (2009). Dual DNA vaccination of rainbow trout (*Oncorhynchus mykiss*) against two different rhabdoviruses, VHSV and IHNV, induces specific divalent protection. *Vaccine, 27*(8), 1248-1253.

Ellis, A. E. (1998). Immunology of fishes. In P. Pastoret, P. Griebel, H. Bazin, & A. Govaerts (Eds.), *Handbook of Vertebrate Immunology* (1st ed., pp. 3-62). San Diego: Academic Press.

EMA. (2016). First DNA vaccine in the EU recommended for use in salmon. Clynav to protect Atlantic salmon from serious infectious disease [Press release]. Retrieved from http://www.ema.europa.eu/docs/en_GB/document_library/Press_release/2016/04/WC500205214.pdf

Embregts, C. W. E., & Forlenza, M. (2016). Oral vaccination of fish: Lessons from humans and veterinary species. *Developmental & Comparative Immunology, 64*, 118-137.

FAO. (2014). *The state of world fisheries and aquaculture: Opportunities and challenges* (F. a. A. O. o. t. U. Nations Ed.). Rome: Food and Agriculture Organization of the United Nations.

FAO. (2015). *Fisheries and aquaculture topics. Fisheries statistics and information.* Retrieved from Rome: http://www.fao.org/fishery/information/en

FAO. (2016a, July 7th, 2016). Global per capita fish consumption rises above 20 kilograms a year. Retrieved from http://www.fao.org/news/story/en/item/421871/icode/

FAO. (2016b). *The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all* (FAO Ed.). Rome: FAO.

Fillatreau, S., Six, A., Magadan, S., Castro, R., Sunyer, J. O., & Boudinot, P. (2013). The astonishing diversity of Ig classes and B cell repertoires in teleost fish. *Frontiers in Immunology, 4*, 28.

Fink, I. R., Pietretti, D., Voogdt, C. G. P., Westphal, A. H., Savelkoul, H. F. J., Forlenza, M., & Wiegertjes, G. F. (2016). Molecular and functional characterization of Toll-like receptor (Tlr)1 and Tlr2 in common carp (*Cyprinus carpio*). *Fish & Shellfish Immunology, 56*, 70-83.

Fischer, U., Koppang, E. O., & Nakanishi, T. (2013). Teleost T and NK cell immunity. *Fish & Shellfish Immunology, 35*(2), 197-206.

Fischer, U., Utke, K., Somamoto, T., Köllner, B., Ototake, M., & Nakanishi, T. (2006). Cytotoxic activities of fish leucocytes. *Fish & Shellfish Immunology, 20*(2), 209-226.

Forlenza, M., Fink, I. R., Raes, G., & Wiegertjes, G. F. (2011). Heterogeneity of macrophage activation in fish. *Developmental & Comparative Immunology, 35*(12), 1246-1255.

Fu, X., Li, N., Lin, Q., Guo, H., Liu, L., Huang, Z., & Wu, S. (2015). Early protein ORF086 is an effective vaccine candidate for infectious spleen and kidney necrosis virus in mandarin fish *Siniperca chuatsi*. *Fish & Shellfish Immunology, 46*(2), 200-205.

Gillund, F., Dalmo, R., Tonheim, T. C., Seternes, T., & Myhr, A. I. (2008). DNA vaccination in aquaculture — Expert judgments of impacts on environment and fish health. *Aquaculture, 284*(1–4), 25-34.

Gillund, F., Kjolberg, K. A., von Krauss, M. K., & Myhr, A. I. (2008). Do uncertainty analyses reveal uncertainties? Using the introduction of DNA vaccines to aquaculture as a case. *Science of The Total Environment, 407*(1), 185-196.

Gillund, F., Tonheim, T., Seternes, T., Dalmo, R. A., & Myhr, A. I. (2008). DNA vaccination in aquaculture –Expert judgements of impact on environment and fish health. *Aquaculture, 284,* 25-34.

Gvili, K., Benny, O., Danino, D., & Machluf, M. (2007). Poly(D,L-lactide-co-glycolide acid) nanoparticles for DNA delivery: waiving preparation complexity and increasing efficiency. *Biopolymers, 85*, 379-391.

Haller, O., Kochs, G., & Weber, F. (2006). The interferon response circuit: Induction and suppression by pathogenic viruses. *Virology, 344*(1), 119-130.

Han, J., Wang, Y., Chu, Q., & Xu, T. (2016). The evolution and functional characterization of miiuy croaker cytosolic gene LGP2 involved in immune response. *Fish & Shellfish Immunology, 58*, 193-202.

He, Y., Wang, K.-y., Xiao, D., Chen, D.-f., Huang, L., Liu, T., Wang, J., Geng, Y., Wang, E.-l., & Yang, Q. (2014). A recombinant truncated surface immunogenic protein (tSip) plus adjuvant FIA confers active protection against Group B streptococcus infection in tilapia. *Vaccine, 32*(51), 7025-7032.

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 Immunology, 8*(4), 271-286.

Hoffman, W., Lakkis, F. G., & Chalasani, G. (2016). B Cells, antibodies, and more. *Clinical Journal of the American Society of Nephrology : CJASN, 11*(1), 137-154.

Hølvold, L. B., Myhr, A. I., & Dalmo, R. A. (2014). Strategies and hurdles using DNA vaccines to fish. *Veterinary Research, 45*(1), 21-21.

Hwang, J. Y., Ohira, T., Hirono, I., & Aoki, T. (2004). A pore-forming protein, perforin, from a non-mammalian organism, Japanese flounder, *Paralichthys olivaceus*. *Immunogenetics, 56*(5), 360-367.

Iliev, D. B., Thim, H., Lagos, L., Olsen, R., & Jørgensen, J. B. (2013). Homing of antigen-presenting cells in head kidney and spleen – Salmon head kidney hosts diverse APC types. *Frontiers in Immunology, 4*, 137, 1-13.

Ivashkiv, L. B., & Donlin, L. T. (2014). Regulation of type I interferon responses. *Nature reviews. Immunology, 14*(1), 36-49.

Jin, J.-Y., Zhou, L., Wang, Y., Li, Z., Zhao, J.-G., Zhang, Q.-Y., & Gui, J.-F. (2010). Antibacterial and antiviral roles of a fish β-defensin expressed both in pituitary and testis. *PLoS ONE, 5*(12), e12883.

Kaiko, G. E., Horvat, J. C., Beagley, K. W., & Hansbro, P. M. (2008). Immunological decision-making: how does the immune system decide to mount a helper T-cell response? *Immunology, 123*(3), 326-338.

Kanellos, T., Sylvester, I. D., Howard, C. R., & Russell, P. H. (1999). DNA is as effective as protein at inducing antibody in fish. *Vaccine, 17*(7–8), 965-972.

Kato, G., Kato, K., Saito, K., Pe, Y., Kondo, H., Aoki, T., & Hirono, I. (2011). Vaccine efficacy of Mycobacterium bovis BCG against *Mycobacterium* sp. infection in amberjack *Seriola dumerili*. *Fish & Shellfish Immunology, 30*(2), 467-472.

Kato, G., Kondo, H., Aoki, T., & Hirono, I. (2012). Mycobacterium bovis BCG vaccine induces non-specific immune responses in Japanese flounder against *Nocardia seriolae*. *Fish & Shellfish Immunology, 33*(2), 243-250.

Katzenback, B. A. (2015). Antimicrobial peptides as mediators of innate immunity in teleosts. *Biology, 4*(4), 607-639.

Kim, C. H., Johnson, M. C., Drennan, J. D., Simon, B. E., Thomann, E., & Leong, J. C. (2000). DNA vaccines encoding viral glycoproteins induce nonspecific immunity and Mx protein synthesis in fish. *Journal of Virology, 74*(15), 7048-7054.

Kim, W. S., Nishizawa, T., Kim, J., Suebsing, R., Jung, S., & Oh, M. (2011). Korean and Japanese isolates of viral hemorrhagic septicemia virus from olive flounder are pathogenic to rainbow trout fry. *Fish Pathology, 46*(4), 112-115.

Kim, W. S., Oh, M. J., Jung, S. J., Kim, Y. J., & Kitamura, S. (2005). Characterization of an iridovirus detected from cultured turbot *Scophthalmus maximus* in Korea. *Diseases of Aquatic Organisms, 64*(2), 175-180.

Kim, Y., Aw, T. G., & Rose, J. B. (2016). Transporting ocean viromes: Invasion of the aquatic biosphere. *PLoS ONE, 11*(4), e0152671.

Koizumi, H., Liu, C. C., Zheng, L. M., Joag, S. V., Bayne, N. K., Holoshitz, J., & Young, J. D. (1991). Expression of perforin and serine esterases by human gamma/delta T cells. *Journal of Experimental Medicine, 173*(2), 499-502.

Kondera, E. (2014). Cell composition of the head kidney of European chub (*Squalius cephalus* L.). *Archives of Polish Fisheries, 22*(4), 271-280.

Kono, T., & Korenaga, H. (2013). Cytokine gene expression in CD4 positive cells of the Japanese Pufferfish, *Takifugu rubripes*. *PLoS ONE, 8*(6), e66364.

Korenaga, H., Nagamine, R., Sakai, M., & Kono, T. (2013). Expression profile of cytokine genes in Fugu monocytes stimulated with TLR agonists. *International Immunopharmacology, 17*(2), 390-399.

Kurath, G. (2008). Biotechnology and DNA vaccines for aquatic animals. *Revue Scientifique et Technique, 27*, 175 - 196.

LaPatra, S., Kao, S., Erhardt, E. B., & Salinas, I. (2015). Evaluation of dual nasal delivery of infectious hematopoietic necrosis virus and enteric red mouth vaccines in rainbow trout (*Oncorhynchus mykiss*). *Vaccine, 33*(6), 771-776.

Lee, N.-H., Lee, J.-A., Park, S.-Y., Song, C.-S., Choi, I.-S., & Lee, J.-B. (2012). A review of vaccine development and research for industry animals in Korea. *Clinical and Experimental Vaccine Research, 1*(1), 18-34.

Lee, Y. M., Lee, J.-H., Noh, J. K., Kim, H. C., Park, C.-J., Park, J.-W., Hwang, I. J., & Kim, S. Y. (2013). Stage and tissue specific expression of four TCR subunits in olive flounder (*Paralichthys olivaceus*). *Development & Reproduction, 17*(4), 329-335.

Li, J., Barreda, D. R., Zhang, Y. A., Boshra, H., Gelman, A. E., Lapatra, S., Tort, L., & Sunyer, J. O. (2006). B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. *Nature Immunology, 7*(10), 1116-1124.

Li, P., Yan, Y., Wei, S., Wei, J., Gao, R., Huang, X., Huang, Y., Jiang, G., & Qin, Q. (2014). Isolation and characterization of a new class of DNA aptamers specific binding to Singapore grouper iridovirus (SGIV) with antiviral activities. *Virus Research, 188*, 146-154.

Lin, K., Zhu, Z., Ge, H., Zheng, L., Huang, Z., & Wu, S. (2016). Immunity to nervous necrosis virus infections of orange-spotted grouper (*Epinephelus coioides*) by vaccination with virus-like particles. *Fish & Shellfish Immunology, 56*, 136-143.

Liu, X., Zhang, H., Jiao, C., Liu, Q., Zhang, Y., & Xiao, J. (2017). Flagellin enhances the immunoprotection of formalin-inactivated *Edwardsiella tarda* vaccine in turbot. *Vaccine, 35*(2), 369-374.

Lorenzen, N., Lorenzen, E., Einer-Jensen, K., & Lapatra, S. E. (2002a). DNA vaccines as a tool for analysing the protective immune response against rhabdoviruses in rainbow trout. *Fish & Shellfish Immunology, 12*(5), 439-453.

Lorenzen, N., Lorenzen, E., Einer-Jensen, K., & LaPatra, S. E. (2002b). Immunity induced shortly after DNA vaccination of rainbow trout against rhabdoviruses protects against heterologous virus but not against bacterial pathogens. *Developmental & Comparative Immunology, 26*.

Løvås, T.-O., Bruusgaard, J. C., Øynebråten, I., Gundersen, K., & Bogen, B. (2014). DNA vaccines: MHC II-targeted vaccine protein produced by transfected muscle fibres induces a local inflammatory cell infiltrate in mice. *PLoS ONE, 9*(10), e108069.

MacLachlan, J., & Dubovi, E. (2011). *Fenner`s veterinary virology* (J. MacLachlan & E. Dubovi Eds. 4th ed.). San Diego, USA: Elsevier.

MAFF, & NVAL. (2013). *Outline of Regulation System of Veterinary Medicinal Products (VMPs) in Japan.* . Tokyo, Japan: Ministry of Agriculture, Forestry and Fisheries (MAFF) and National Veterinary Assay Laboratory (NVAL) Retrieved from http://www.maff.go.jp/nval/english/pdf/outline130325.pdf.

Magadan, S., Sunyer, O. J., & Boudinot, P. (2015). Unique features of fish immune repertoires: Particularities of adaptive immunity within the largest group of vertebrates. *Results and Problems in Cell Differentiation, 57*, 235-264.

Makrinos, D. L., & Bowden, T. J. (2016). Natural environmental impacts on teleost immune function. *Fish & Shellfish Immunology, 53*, 50-57.

Mardones, F. O., Martinez-Lopez, B., Valdes-Donoso, P., Carpenter, T. E., & Perez, A. M. (2014). The role of fish movements and the spread of infectious salmon anemia virus (ISAV) in Chile, 2007–2009. *Preventive Veterinary Medicine, 114*(1), 37-46.

Mashoof, S., & Criscitiello, M. F. (2016). Fish Immunoglobulins. *Biology (Basel), 5*(4).

Masso-Silva, J. A., & Diamond, G. (2014). Antimicrobial peptides from fish. *Pharmaceuticals, 7*(3), 265-310.

Matsuo, A., Oshiumi, H., Tsujita, T., Mitani, H., Kasai, H., Yoshimizu, M., Matsumoto, M., & Seya, T. (2008). Teleost TLR22 recognizes RNA duplex to induce IFN and protect cells from birnaviruses. *Journal of Immunology, 181*(5), 3474-3485.

Matsuyama, T., Minami, T., Fukuda, Y., Sano, N., Sakai, T., Takano, T., & Nakayasu, C. (2016). Passive immunization against red sea bream iridoviral disease in five marine fish species. *Fish Pathology, 51*(1), 32-35.

Matsuyama, T., Nakayasu, C., Fujiwara, A., Kurita, J., Takano, T., Ito, T., & Sano, M. (2012). Ontogeny of anti-viral hemorrhagic septicemia virus (VHSV) immunity in developing Japanese flounder. *Developmental & Comparative Immunology, 37*, 313-322.

Mattisson, A., xe, & nge, R. (1982). The cellular structure of the Leydig organ in the shark, *Etmopterus spinax* (L.). *Biological Bulletin, 162*(2), 182-194.

Mazon, A. F., Huising, M. O., Taverne-Thiele, A. J., Bastiaans, J., & Verburg-van Kemenade, B. M. L. (2007). The first appearance of Rodlet cells in carp (*Cyprinus carpio* L.) ontogeny and their possible roles during stress and parasite infection. *Fish & Shellfish Immunology, 22*(1–2), 27-37.

McClusky, L. M., & Sulikowski, J. (2014). The epigonal organ and mature pole of the testis in the recreationally fished blue shark (*Prionace glauca*): histochemico-functional correlates. *Journal of Anatomy, 225*(6), 614-624.

Mills, C. D. (2015). Anatomy of a Discovery: M1 and M2 Macrophages. *Frontiers in Immunology, 6*, 212.

Minami, T., Iwata, K., Shimahara, Y., & Yuasa, K. (2016). *Vibrio harveyi* infection in farmed greater Amberjack, *Seriola dumerili*. *Fish Pathology, 51*(1), 1-7.

Moore, F. E., Garcia, E. G., Lobbardi, R., Jain, E., Tang, Q., Moore, J. C., Cortes, M., Molodtsov, A., Kasheta, M., Luo, C. C., Garcia, A. J., Mylvaganam, R., Yoder, J. A., Blackburn, J. S., Sadreyev, R. I., Ceol, C. J., North, T. E., & Langenau, D. M. (2016). Single-cell transcriptional analysis of normal, aberrant, and malignant hematopoiesis in zebrafish. *Journal of Experimental Medicine, 213*(6), 979-992.

Moore, J. D., Ototake, M., & Nakanishi, T. (1998). Particulate antigen uptake during immersion immunisation of fish: The effectiveness of prolonged exposure and the roles of skin and gill. *Fish & Shellfish Immunology, 8*(6), 393-408.

Mühl, H., & Pfeilschifter, J. (2003). Anti-inflammatory properties of pro-inflammatory interferon-γ. *International Immunopharmacology, 3*(9), 1247-1255.

Munang'andu, H. M. (2016). Environmental viral metagenomics analyses in aquaculture: Applications in epidemiology and disease control. *Frontiers in Microbiology, 7*, 1986.

Myhr, A. I. (2017). DNA vaccines: Regulatory considerations and safety aspects. *Current Issues in Molecular Biology, 22*, 79-88.

Nakayama, Y., & Aruga, A. (2015). Comparison of current regulatory status for gene-based vaccines in the U.S., Europe and Japan. *Vaccines, 3*(1), 186-202.

Nam, B. H., Hirono, I., & Aoki, T. (2003). The four TCR genes of teleost fish: the cDNA and genomic DNA analysis of Japanese flounder (*Paralichthys olivaceus*) TCR alpha-, beta-, gamma-, and delta-chains. *Journal of Immunology, 170*(6), 3081-3090.

Noga, E. J. (2010). Problems 77 Through 88. In E. J. Noga (Ed.), *Fish Disease: Diagnosis and treatment* (2nd ed., pp. 269-303). Iowa: Blackwell Publishing, Inc.

Nuñez-Ortiz, N., Pascoli, F., Picchietti, S., Buonocore, F., Bernini, C., Toson, M., Scapigliati, G., & Toffan, A. (2016). A formalin-inactivated immunogen against viral encephalopathy and retinopathy (VER) disease in European sea bass (*Dicentrarchus labrax*): immunological and protection effects. *Veterinary Research, 47*(1), 89.

Ohtani, M., Hikima, J.-i., Jung, T.-S., Kondo, H., Hirono, I., Takeyama, H., & Aoki, T. (2013). Variable domain antibodies specific for viral hemorrhagic septicemia virus (VHSV) selected from a randomized IgNAR phage display library. *Fish & Shellfish Immunology, 34*(2), 724-728.

Ohtani, M., Hikima, J.-i., Kondo, H., Hirono, I., Jung, T.-S., & Aoki, T. (2011). Characterization and antiviral function of a cytosolic sensor gene, MDA5, in Japanese flounder, *Paralichthys olivaceus*. *Developmental & Comparative Immunology, 35*(5), 554-562.

OIE. (2017). OIE-Listed diseases, infections and infestations in force in 2017. Retrieved from http://www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2017/

Oliveira, F. L., Aguiar, A. M., Borojevic, R., & El-Cheikh, M. C. (2005). IgE expression on the surface of B1 and B2 lymphocytes in experimental murine schistosomiasis. *Brazilian Journal of Medical and Biological Research, 38*(7), 1033-1042.

Ou-yang, Z., Wang, P., Huang, X., Cai, J., Huang, Y., Wei, S., Ji, H., Wei, J., Zhou, Y., & Qin, Q. (2012). Immunogenicity and protective effects of inactivated Singapore grouper iridovirus (SGIV) vaccines in orange-spotted grouper, *Epinephelus coioides*. *Developmental & Comparative Immunology, 38*(2), 254-261.

Overstreet, R. M., Jovonovich, J., & Ma, H. (2009). Parasitic crustaceans as vectors of viruses, with an emphasis on three penaeid viruses. *Integrative and Comparative Biology, 49*(2), 127-141.

PANDRH. Harmonized requirements for the licensing of vaccines in the americas and guidelines for preparation of application, (2010). Pan American Network on Drug Regulatory Harmonization. Pan American Health Organization. Washington D.C. 1-40.

Park, S. B., Aoki, T., & Jung, T. S. (2012). Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish. *Veterinary Research, 43*(1), 1-11.

Parra, D., Korytář, T., Takizawa, F., & Sunyer, J. O. (2016). B cells and their role in the teleost gut. *Developmental & Comparative Immunology, 64*, 150-166.

Pereira, V., Zurita-Turk, M., Saraiva, T., De Castro, C., Souza, B., Mancha Agresti, P., Lima, F., Pfeiffer, V., Azevedo, M., Rocha, C., Pontes, D., Azevedo, V., & Miyoshi, A. (2014). DNA vaccines approach: From concepts to applications. *World Journal of Vaccines, 4*, 50-71.

Pereiro, P., Forn-Cuni, G., Figueras, A., & Novoa, B. (2016). Pathogen-dependent role of turbot (*Scophthalmus maximus*) interferon-gamma. *Fish & Shellfish Immunology, 59*, 25-35.

Pietretti, D., & Wiegertjes, G. F. (2014). Ligand specificities of Toll-like receptors in fish: Indications from infection studies. *Developmental & Comparative Immunology, 43*(2), 205-222.

Popi, A. F., Longo-Maugéri, I. M., & Mariano, M. (2016). An overview of B-1 cells as antigen-presenting cells. *Frontiers in Immunology, 7*(138).

Poynter, S., Lisser, G., Monjo, A., & DeWitte-Orr, S. (2015). Sensors of infection: Viral nucleic acid PRRs in fish. *Biology, 4*, 460-493.

Praveen, K., Leary Iii, J. H., Evans, D. L., & Jaso-Friedmann, L. (2006). Nonspecific cytotoxic cells of teleosts are armed with multiple granzymes and other components of the granule exocytosis pathway. *Molecular Immunology, 43*(8), 1152-1162.

Qu, H., Yang, L., Meng, S., Xu, L., Bi, Y., Jia, X., Li, J., Sun, L., & Liu, W. (2013). The differential antiviral activities of chicken interferon α (ChIFN-α) and ChIFN-β are related to distinct interferon-stimulated gene expression. *PLoS ONE, 8*(3), e59307.

Reite, O. B. (2005). The rodlet cells of teleostean fish: their potential role in host defence in relation to the role of mast cells/eosinophilic granule cells. *Fish & Shellfish Immunology, 19*(3), 253-267.

Reite, O. B., & Evensen, Ø. (2006). Inflammatory cells of teleostean fish: A review focusing on mast cells/eosinophilic granule cells and rodlet cells. *Fish & Shellfish Immunology, 20*(2), 192-208.

Restifo, N. P., Ying, H., Hwang, L., & Leitner, W. W. (2000). The promise of nucleic acid vaccines. *Gene Therapy, 7*(2), 89-92.

Rexhepi, A., Bërxholi, K., Scheinert, P., Hamidi, A., & Sherifi, K. (2011). Study of viral diseases in some freshwater fish in the Republic of Kosovo. *Veterinarski Arhiv, 8*(3), 405-413.

Rieger, A. M., & Barreda, D. R. (2011). Antimicrobial mechanisms of fish leukocytes. *Developmental & Comparative Immunology, 35*(12), 1238-1245.

Robertsen, B. (2008). Expression of interferon and interferon-induced genes in salmonids in response to virus infection, interferon-inducing compounds and vaccination. *Fish & Shellfish Immunology, 25*(4), 351-357.

Robinson, N. A., Gjedrem, T., & Quillet, E. (2017). Improvement fo disease resistance by genetic methods. In G. Jeney (Ed.), *Fish Diseases: Prevention and Control Strategies* (1st ed., pp. 21-45). London, UK: Elsevier.

Roca, F. J., Mulero, I., López-Muñoz, A., Sepulcre, M. P., Renshaw, S. A., Meseguer, J., & Mulero, V. (2008). Evolution of the inflammatory response in vertebrates: Fish TNF-α is a powerful activator of endothelial cells but hardly activates phagocytes. *Journal of Immunology, 181*(7), 5071-5081.

Røkenes, T. P., Larsen, R., & Robertsen, B. (2007). Atlantic salmon ISG15: Expression and conjugation to cellular proteins in response to interferon, double-stranded RNA and virus infections. *Molecular Immunology, 44*(5), 950-959.

Rombout, J. H., Taverne-Thiele, A. J., & Villena, M. I. (1993). The gut-associated lymphoid tissue (GALT) of carp (*Cyprinus carpio* L.): an immunocytochemical analysis. *Developmental & Comparative Immunology, 17*(1), 55-66.

Rombout, J. H. W. M., Abelli, L., Picchietti, S., Scapigliati, G., & Kiron, V. (2011). Teleost intestinal immunology. *Fish & Shellfish Immunology, 31*(5), 616-626.

Rumfelt, L. L., McKinney, E. C., Taylor, E., & Flajnik, M. F. (2002). The development of primary and secondary lymphoid tissues in the nurse shark *Ginglymostoma cirratum*: B-cell zones precede dendritic cell immigration and T-cell zone formation during ontogeny of the spleen. *Scandinavian Journal of Immunology, 56*(2), 130-148.

Sahoo, P. K., & Goodwin, A. E. (2012). Viruses of freshwater finfish in the asian-pacific region. *Indian Journal of Virology, 23*(2), 99-105.

Salinas, I. (2015). The mucosal immune system of teleost fish. *Biology (Basel), 4*(3), 525-539.

Salinas, I., LaPatra, S. E., & Erhardt, E. B. (2015). Nasal vaccination of young rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis and enteric red mouth disease. *Developmental & Comparative Immunology, 53*(1), 105-111.

Salinas, I., Zhang, Y. A., & Sunyer, J. O. (2011). Mucosal immunoglobulins and B cells of teleost fish. *Developmental & Comparative Immunology, 35*(12), 1346-1365.

Salonius, K., Simard, N., Harland, R., & Ulmer, J. B. (2007). The road to licensure of a DNA vaccine. *Current Opinion in Investigational Drugs, 8*(8), 635-641.

Saul, A., & O'Brien, K. L. (2017). Prioritizing vaccines for developing world diseases. *Vaccine, 35, Supplement 1*, A16-A19.

Scapigliati, G. (2013). Functional aspects of fish lymphocytes. *Developmental & Comparative Immunology, 41*(2), 200-208.

Scapigliati, G., Buonocore, F., Randelli, E., Casani, D., Meloni, S., Zarletti, G., Tiberi, M., Pietretti, D., Boschi, I., Manchado, M., Martin-Antonio, B., Jimenez-Cantizano, R., Bovo, G., Borghesan, F., Lorenzen, N., Einer-Jensen, K., Adams, S., Thompson, K., Alonso, C., Bejar, J., Cano, I., Borrego, J. J., & Alvarez, M. C. (2010). Cellular and molecular immune responses of the sea bass (*Dicentrarchus labrax*) experimentally infected with Betanodavirus. *Fish & Shellfish Immunology, 28*.

Secombes, C. J., & Ellis, A. E. (2012). The immunology of teleosts. In R. Roberts (Ed.), *Fish Pathology* (4th ed., pp. 144-166). Oxford,UK: Wiley-Blackwell.

Secombes, C. J., & Zou, J. (2017). Evolution of interferons and interferon receptors. *Frontiers in Immunology, 8,* 209.

Seppola, M., Stenvik, J., Steiro, K., Solstad, T., Robertsen, B., & Jensen, I. (2007). Sequence and expression analysis of an interferon stimulated gene (ISG15) from Atlantic cod (*Gadus morhua* L.). *Developmental & Comparative Immunology, 31*(2), 156-171.

Sequeira, D. P., Correia, R., Carrondo, M. J., Roldao, A., Teixeira, A. P., & Alves, P. M. (2017). Combining stable insect cell lines with baculovirus-mediated expression for multi-HA influenza VLP production. *Vaccine*. doi: 10.1016/j.vaccine.2017.02.043.

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.). *Journal of Experimental Biology, 210*(Pt 12), 2091-2103.

Seternes, T., Tonheim, T. C., Løvoll, M., Bøgwald, J., & Dalmo, R. A. (2007). Specific endocytosis and degradation of naked DNA in the endocardial cells of cod (*Gadus morhua* L.). *J Exp Biol, 210*.

Shimmoto, H., Kawai, K., Ikawa, T., & Oshima, S. (2010). Protection of red sea bream *Pagrus major* against red sea bream iridovirus infection by vaccination with a recombinant viral protein. *Microbiology and Immunology, 54*(3), 135-142.

Shin, J., Lei, D., Conrad, C., Knezevic, I., & Wood, D. (2011). International regulatory requirements for vaccine safety and potency testing: a WHO perspective. *Procedia in Vaccinology, 5*, 164-170.

Shinmoto, H., Taniguchi, K., Ikawa, T., Kawai, K., & Oshima, S.-i. (2009). Phenotypic diversity of infectious red sea bream iridovirus isolates from cultured fish in Japan. *Applied Environmental Microbiology, 75*(11), 3535-3541.

Smail, D. A., & Munro, E. S. (2012). The virology of teleosts. In R. Roberts (Ed.), *Fish Pathology* (4th ed., pp. 186-291). Oxford,UK: Wiley-Blackwell.

Sohn, S.-G., & Park, M.-A. (1998). Viral diseases of cultured marine fish and shrimp in Korea. *Fish Pathology, 33*(4), 189-192.

Somamoto, T., Nakanishi, T., & Okamoto, N. (2002). Role of specific cell-mediated cytotoxicity in protecting fish from viral infections. *Virology, 297*(1), 120-127.

Starodubova, E. S., Isaguliants, M. G., & Karpov, V. L. (2010). Regulation of immunogen processing: signal sequences and their application for the new generation of DNA-vaccines. *Acta Naturae, 2*(1), 53-60.

Statistics Bureau. (2015). *Statistical handbook of Japan*. 19-1 Wakamatsu-cho, Shinjuku-ku, Tokyo 162-8668 Japan: Statistics Bureau Ministry of Internal Affairs and Communications Japan Retrieved from http://www.stat.go.jp/english/data/handbook/pdf/2015all.pdf.

Su, J., Heng, J., Huang, T., Peng, L., Yang, C., & Li, Q. (2012). Identification, mRNA expression and genomic structure of TLR22 and its association with GCRV susceptibility/resistance in grass carp (*Ctenopharyngodon idella*). *Developmental & Comparative Immunology, 36*(2), 450-462.

Tafalla, C., Bøgwald, J., & Dalmo, R. A. (2013). Adjuvants and immunostimulants in fish vaccines: Current knowledge and future perspectives. *Fish & Shellfish Immunology, 35*(6), 1740-1750.

Tafalla, C., Leal, E., Yamaguchi, T., & Fischer, U. (2016). T cell immunity in the teleost digestive tract. *Developmental & Comparative Immunology, 64*, 167-177.

Thanasaksiri, K., Hirono, I., & Kondo, H. (2017). Molecular cloning and expression analysis of NOD-like receptor 5 in Japanese flounder (*Paralichthys olivaceus*) after injection with two different formalin-killed pathogenic bacteria and poly (I:C). *Developmental & Comparative Immunology, 67*, 481-484.

Thompson, K. (2017). Immunology: Improvement of innate and adaptive immunity. In G. Jeney (Ed.), *Fish diseases: prevention and control strategies* (1st ed., pp. 3-14). London, UK: Elsevier.

Tizard, I. (2012). *Veterinary Immunology* (9th ed.). London, UK: WB Saunders Company.

Tonheim, T., Dalmo, R. A., Bøgwald, J., & Seternes, T. (2008). Specific uptake of plasmid DNA without reporter gene expression in Atlantic salmon (*Salmo salar* L.) kidney after intramuscular administration. *Fish & Shellfish Immunology, 24*, 90-101.

Tonheim, T. C., Bøgwald, J., & Dalmo, R. A. (2008). What happens to the DNA vaccine in fish? A review of current knowledge. *Fish & Shellfish Immunology, 25*(1–2), 1-18.

Tonheim, T. C., Leirvik, J., Løvoll, M., Myhr, A. I., Bøgwald, J., & Dalmo, R. A. (2007). Detection of supercoiled plasmid DNA and luciferase expression in Atlantic salmon (*Salmo salar* L.) 535 days after injection. *Fish & Shellfish Immunology, 23*.

Tort, L., Balasch, J., & MacKenzie, S. (2003). Fish immune system. A crossroads between innate and adaptive responses. *Inmunologia, 22*(3), 277-286.

UJNR Japan Panel. (2016). *2015 Annual report on aquaculture in Japan*. Retrieved from Tokyo, Japan: http://nria.fra.affrc.go.jp/ujnr/PDF/2015AnnualReport_j.pdf

Uribe, C., Folch, H., Enriquez, R., & Moran, G. (2011). Innate and adaptive immunity in teleost fish: a review. *Veterinarni Medicina, 56*(10), 486-503.

Utke, K., Kock, H., Schuetze, H., Bergmann, S. M., Lorenzen, N., Einer-Jensen, K., Köllner, B., Dalmo, R. A., Vesely, T., Ototake, M., & Fischer, U. (2008). Cell-mediated immune responses in rainbow trout after DNA immunization against the viral hemorrhagic septicemia virus. *Developmental & Comparative Immunology, 32*(3), 239-252.

Vazzana, M., Celi, M., Arizza, V., Calandra, G., Buscaino, G., Ferrantelli, V., Bracciali, C., & Sarà, G. (2017). Noise elicits hematological stress parameters in Mediterranean

damselfish (*Chromis chromis*, perciformes): A mesocosm study. *Fish & Shellfish Immunology, 62*, 147-152.

Verjan, N., Ooi, E. L., Nochi, T., Kondo, H., Hirono, I., Aoki, T., Kiyono, H., & Yuki, Y. (2008). A soluble nonglycosylated recombinant infectious hematopoietic necrosis virus (IHNV) G-protein induces IFNs in rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology, 25*(1–2), 170-180.

Verrier, E. R., Langevin, C., Benmansour, A., & Boudinot, P. (2011). Early antiviral response and virus-induced genes in fish. *Developmental & Comparative Immunology, 35*(12), 1204-1214.

Vimal, S., Farook, M. A., Madan, N., Abdul Majeed, S., Nambi, K. S. N., Taju, G., Sundar raj, N., Venu, S., Subburaj, R., Thirunavukkarasu, A. R., & Sahul Hameed, A. S. (2016). Development, distribution and expression of a DNA vaccine against nodavirus in Asian Seabass, *Lates calcarifier* (Bloch, 1790). *Aquaculture Research, 47*(4), 1209-1220.

Vimal, S., Majeed, S. A., Nambi, K. S. N., Madan, N., Farook, M. A., Venkatesan, C., Taju, G., Venu, S., Subburai, R., Thirunavukkarasu, A. R., & Hameed, A. S. (2014). Delivery of DNA vaccine using chitosan–tripolyphosphate (CS/TPP) nanoparticles in Asian sea bass, *Lates calcarifer* (Bloch, 1790) for protection against nodavirus infection. *Aquaculture, 420*.

Walker, P. J., & Winton, J. R. (2010). Emerging viral diseases of fish and shrimp. *Veterinary Research, 41*(6), 51.

Walz, P. H., Edmondson, M. A., Riddell, K. P., Braden, T. D., Gard, J. A., Bayne, J., Joiner, K. S., Galik, P. K., Zuidhof, S., & Givens, M. D. (2015). Effect of vaccination with a multivalent modified-live viral vaccine on reproductive performance in synchronized beef heifers. *Theriogenology, 83*(5), 822-831.

Walz, P. H., Givens, M. D., Rodning, S. P., Riddell, K. P., Brodersen, B. W., Scruggs, D., Short, T., & Grotelueschen, D. (2017). Evaluation of reproductive protection against bovine viral diarrhea virus and bovine herpesvirus-1 afforded by annual revaccination with modified-live viral or combination modified-live/killed viral vaccines after primary vaccination with modified-live viral vaccine. *Vaccine, 35*(7), 1046-1054.

Wang, X., Feng, N., Ge, J., Shuai, L., Peng, L., Gao, Y., Yang, S., Xia, X., & Bu, Z. (2012). Recombinant canine distemper virus serves as bivalent live vaccine against rabies and canine distemper. *Vaccine, 30*(34), 5067-5072.

Whitton, J. L., Rodriguez, F., Zhang, J., & Hassett, D. E. (1999). DNA immunization: mechanistic studies. *Vaccine, 17*(13-14), 1612-1619.

Wiegertjes, G. F., Wentzel, A. S., Spaink, H. P., Elks, P. M., & Fink, I. R. (2016). Polarization of immune responses in fish: The 'macrophages first' point of view. *Molecular Immunology, 69*, 146-156.

Xu, L., Zhao, J., Liu, M., Kurath, G., Ren, G., Lapatra, S. E., Yin, J., Liu, H., Feng, J., & Lu, T. (2017). A effective DNA vaccine against diverse genotype J infectious hematopoietic necrosis virus strains prevalent in China. *Vaccine, 35*(18), 2420-2426.

Yager, E. J., Ahmed, M., Lanzer, K., Randall, T. D., Woodland, D. L., & Blackman, M. A. (2008). Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus. *Journal of Experimental Medicine, 205*(3), 711-723.

Yamaguchi, T., Takizawa, F., Fischer, U., & Dijkstra, J. (2015). Along the axis between type 1 and type 2 Immunity; Principles conserved in evolution from fish to mammals. *Biology, 4*(4), 814.

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 Immunology, 23*(3), 531-541.

Yasuike, M., Kondo, H., Hirono, I., & Aoki, T. (2011a). Gene expression profile of HIRRV G and N protein gene vaccinated Japanese flounder, *Paralichthys olivaceus* during HIRRV infection. *Comparative Immunology, Microbiology and Infectious Diseases, 34*(2), 103-110.

Yasuike, M., Kondo, H., Hirono, I., & Aoki, T. (2011b). Identification and characterization of Japanese flounder, *Paralichthys olivaceus* interferon-stimulated gene 15 (Jf-ISG15). *Comparative Immunology, Microbiology and Infectious Diseases, 34*(1), 83-91.

Yoshida, T. (2016). Streptococcosis in aquaculture. *Fish Pathology, 51*(2), 44-48.

Yoshimizu, M. (2016). Bacterial kidney disease of salmonids. *Fish Pathology, 51*(2), 49-53.

Zapata, A., Diez, B., Cejalvo, T., Gutierrez-de Frias, C., & Cortes, A. (2006). Ontogeny of the immune system of fish. *Fish & Shellfish Immunology, 20*(2), 126-136.

Zhang, H., Fei, C., Wu, H., Yang, M., Liu, Q., Wang, Q., & Zhang, Y. (2013). Transcriptome profiling reveals Th17-like immune responses induced in zebrafish bath-vaccinated with a live attenuated *Vibrio anguillarum*. *PLoS ONE, 8*(9), e73871.

Zhang, H., Shen, B., Wu, H., Gao, L., Liu, Q., Wang, Q., Xiao, J., & Zhang, Y. (2014). Th17-like immune response in fish mucosal tissues after administration of live attenuated *Vibrio anguillarum* via different vaccination routes. *Fish & Shellfish Immunology, 37*(2), 229-238.

Zhang, M., Hu, Y. H., Xiao, Z. Z., Sun, Y., & Sun, L. (2012). Construction and analysis of experimental DNA vaccines against megalocytivirus. *Fish & Shellfish Immunology, 33*, 1192-1198.

Zhang, Y.-B., Wang, Y.-L., & Gui, J.-F. (2007). Identification and characterization of two homologues of interferon-stimulated gene ISG15 in crucian carp. *Fish & Shellfish Immunology, 23*(1), 52-61.

Zheng, F., Liu, H., Sun, X., Zhang, Y., Zhang, B., Teng, Z., Hou, Y., & Wang, B. (2016). Development of oral DNA vaccine based on chitosan nanoparticles for the immunization against reddish body iridovirus in turbots (*Scophthalmus maximus*). *Aquaculture, 452*, 263-271.

Zhu, L. Y., Lin, A. F., Shao, T., Nie, L., Dong, W. R., Xiang, L. X., & Shao, J. Z. (2014). B cells in teleost fish act as pivotal initiating APCs in priming adaptive immunity: an evolutionary perspective on the origin of the B-1 cell subset and B7 molecules. *Journal of Immunology, 192*(6), 2699-2714.

Table 1. World top producers and main groups of finfish species*

Major producer	Finfish		Total aquatic animals	Total aquaculture production
	Inland aquaculture	Marine/coastal aquaculture		
China	260297.7	1189.7	45469	58795.3
Indonesia	2857.6	782.3	4253.9	14330.9
India	4391.1	90	4881	4884
Vietnam	2478.5	208.5	3397.1	3411.4
Philippines	299.3	373	788	2337.6
Bangladesh	1733.1	93.7	1956.9	1956.9
Rep. of Korea	17.2	83.4	480.4	1567.4
Norway	0.1	1330.4	1332.5	1332.5
Chile	68.7	899.4	1214.5	1227.4
Egypt	1129.9	…	1137.1	1137.1
Japan	33.8	238.7	657	1020.4
Myanmar	901.9	1.8	962.2	964.3
Thailand	401	19.6	934.8	934.8
Brazil	474.3	…	561.8	562.5
Malaysia	106.3	64.3	275.7	521
World	**43559.3**	**6302.6**	**73783.7**	**101090.7**

*Based on the data published by FAO (2016b)

Table 2. Production (thousands tons) by fishery type and species in Japan (2000-2014)*

Fishery type and species	2000	2005	2010	2013	2014
Marine fisheries	5022	4457	4122	3734	3739
Tunas	286	239	208	189	187
Bonito	341	370	303	284	258
Sardine	150	28	70	218	202
Mackerels	346	620	492	386	502
Alaska Pollack	300	194	251	230	198
Crabs	42	34	32	30	30
Squids	624	330	267	228	206
Marine aquaculture	1231	1212	1111	997	987
Yellowtails	137	160	139	150	136
Oysters	221	219	200	164	184
Laver	392	387	329	316	267
Wakame seaweed	67	63	52	51	44
Pearl (tons)	30	29	21	20	20
Inland water fisheries	71	#54	#40	31	#31
Salmons and trouts	17	#19	#14	13	#11
Sweetfish	11	#7	#3	2	#2
Shellfishes	20	#14	#14	11	#12
Inland water aquaculture	61	#42	39	30	34
Eel	24	19	21	14	18
Trouts	15	12	9	8	8
Common carp	11	4	4	3	3
Total	**6384**	**5765**	**5313**	**4792**	**4789**

*Based on the data published by Statistics Bureau (2015), # Marked break in series

Table 3. Main differences between components of immune system from fish and mammals*

Feature	Fish	Mammals
Hematopoietic tissue	Head kidney (bony fish) Epigonal and Leydig organs (cartilaginous fish)	Bone marrow
Lymph nodes	Absent	Present
Thymus involution	Species-dependent, influence by seasonal and hormonal cycles	Influence by age
Gut-associated lymphoid tissue	Lymphoid aggregates (not organized)	Peyer patches
Natural killer cells	Putative NK-Like cells (based on single cells transcriptome analysis)	Present
Rodlet cells	Present	Absent
Eosinophilic granule cells	Present	Putative homologue (Mast cells or eosinophils)
Germinal centers	Absent	Present
Ig heavy chain isotypes	IgM, IgD, IgT/Z (bony fish) IgM, IgX/IgR, IgW, NAR(C) (cartilaginous fish) IgM redox forms	IgM, IgA, IgD, IgE, IgG
Ig gene rearragements	Multicluster (mainly in cartilaginous fish)	Translocon
Non-specific diversity	Several C3 isoforms	No C3 isoforms
Antibody affinity	Low	High
Antibody response	Slow	Fast
Memory response	Weak	Strong
Affinity maturation	Low or absent	High

*Based on Buchmann (2014); Carmona *et al.* (2017); Mashoof and Criscitiello (2016); Mazon *et al.* (2007); Moore *et al.* (2016); Reite and Evensen (2006); Tort *et al.* (2003)

Table 4. Genes upregulated after DNA vaccination against virus in fish

Gene	Vaccine or antigen*	Reference
Mx	VHSV	Acosta *et al.* (2005)
	HIRV	Yasuike *et al.* (2007)
	IHNV	Ballesteros *et al.* (2015); Verjan *et al.* (2008); Xu *et al.* (2017)
	TRBIV	Zheng *et al.* (2016)
	SGIV	Ou-yang *et al.* (2012)
	RBIV-C1	Zhang *et al.* (2012)
	SHRV	Kim *et al.* (2000)
	SVCV	
	IPNV	Ballesteros *et al.* (2012)
vig-1	IHNV	Ballesteros *et al.* (2015)
vig-2		
ISG15	HIRV	Yasuike *et al.* (2007)
	SGIV	Ou-yang *et al.* (2012)
ISG56	HIRV	Yasuike *et al.* (2007)
LB3		
Type I-IFN	VHSV	Acosta *et al.* (2006); Acosta *et al.* (2005); Chico *et al.* (2010)
	IHNV	Verjan *et al.* (2008)
	TRBIV	Zheng *et al.* (2016)
	RBIV-C1	Zhang *et al.* (2012)
	IPNV	Ballesteros *et al.* (2012)
IL-1β	SGIV	Ou-yang *et al.* (2012)
	RBIV-C1	Zhang *et al.* (2012)
IL-8	SGIV	Ou-yang *et al.* (2012)
	RBIV-C1	Zhang *et al.* (2012)
	IPNV	Ballesteros *et al.* (2012)
TNF-α	TRBIV	Zheng *et al.* (2016)
	SGIV	Ou-yang *et al.* (2012)
	RBIV-C1	Zhang *et al.* (2012)
	IPNV	Ballesteros *et al.* (2012)
MHC (I-II)	TRBIV	Zheng *et al.* (2016)
	SGIV	Ou-yang *et al.* (2012)

	RBIV-C1	Zhang *et al.* (2012)
	IPNV	Ballesteros *et al.* (2012)
TLR	IHNV	Ballesteros *et al.* (2015)
CD4		
CD8		
IgM, IgT	IHNV	Ballesteros *et al.* (2015)
	IPNV	Ballesteros *et al.* (2012)

*Viral hemorrhagic septicemia virus (VHSV), Hirame rhabdovirus (HIRV), Turbot reddish body iridovirus (TRBIV), Singapore grouper iridovirus (SGIV), infectious pancreatic necrosis virus (IPNV), Infectious hematopoietic necrosis virus (IHNV), rock bream iridovirus isolate 1 from China (RBIV-C1), snakehead rhabdovirus (SHRV), or spring viremia of carp virus (SVCV).

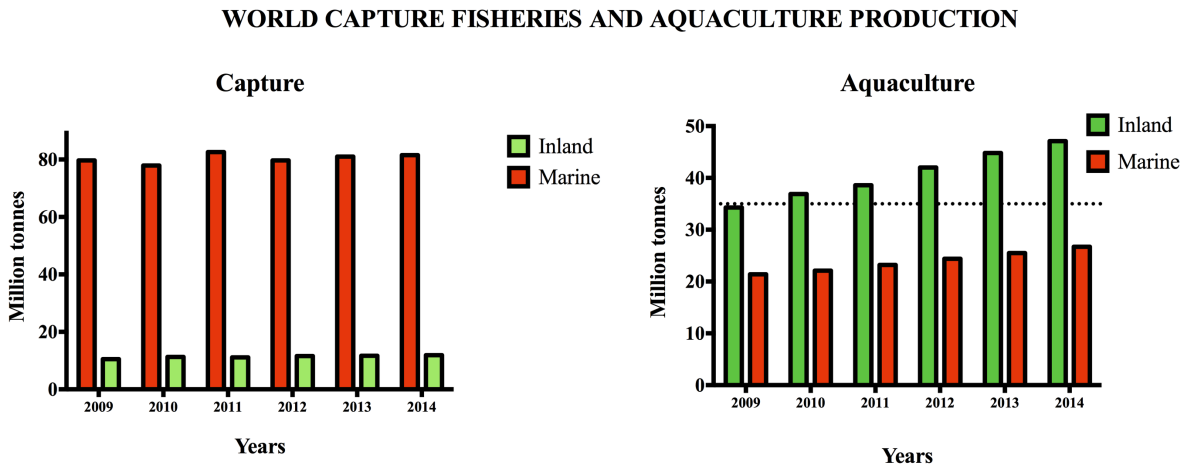

Figure 1. World capture fisheries and aquaculture production, 2009-2014. Based on FAO (2016b)

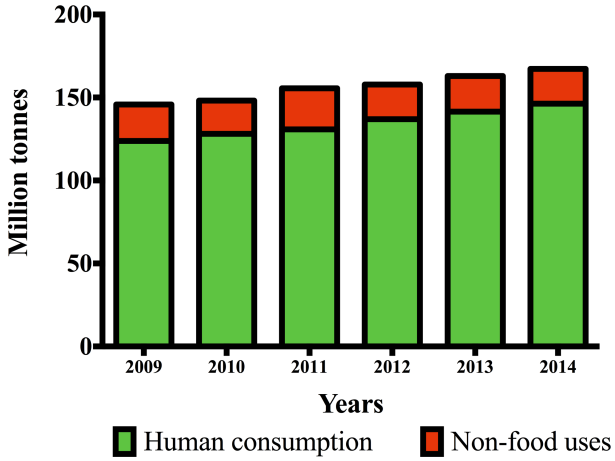

Figure 2. World fish utilization, 2009-2014. Based on FAO (2016b)

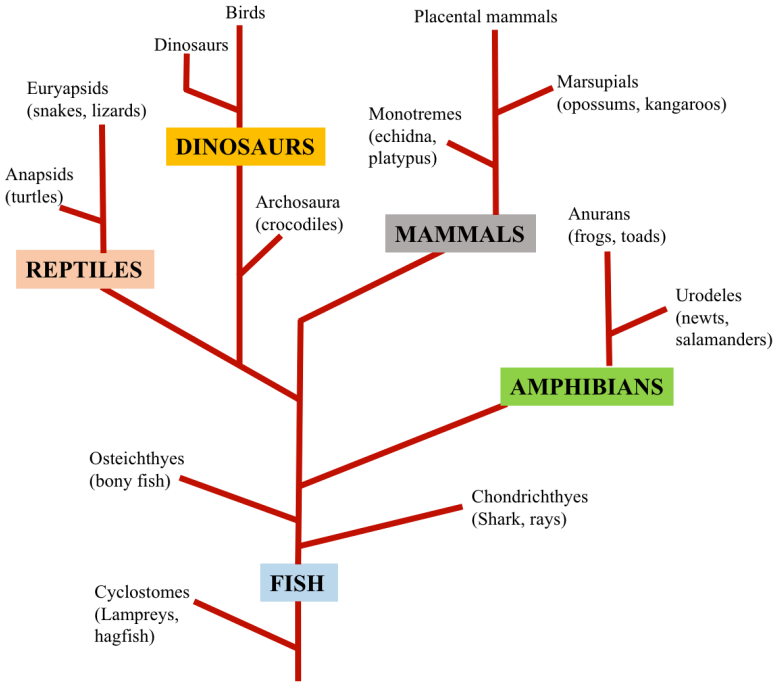

Figure 3. Simplified phylogenetic tree showing the major relationships among the vertebrates (based on Tizard (2012))

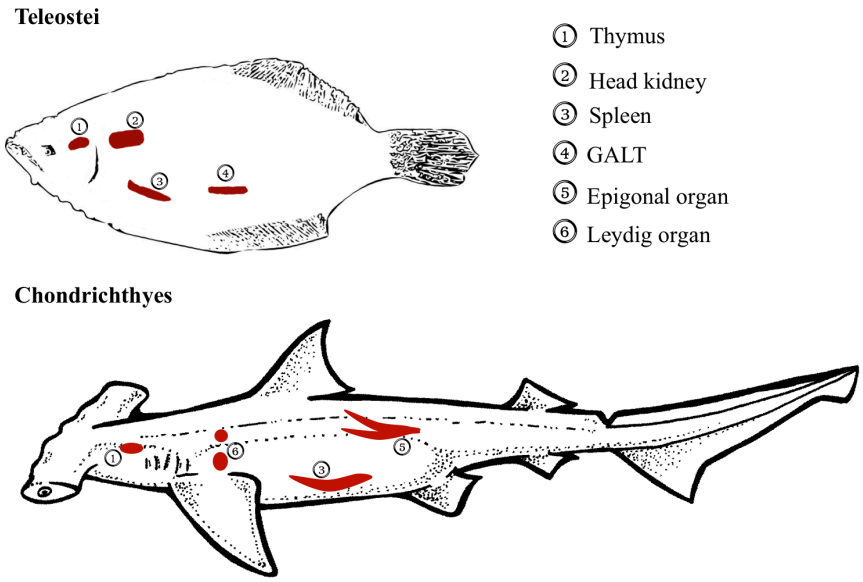

Figure 4. Lymphoid organs in bony fish (Teleostei) and cartilaginous fish (Chondrichtyes).

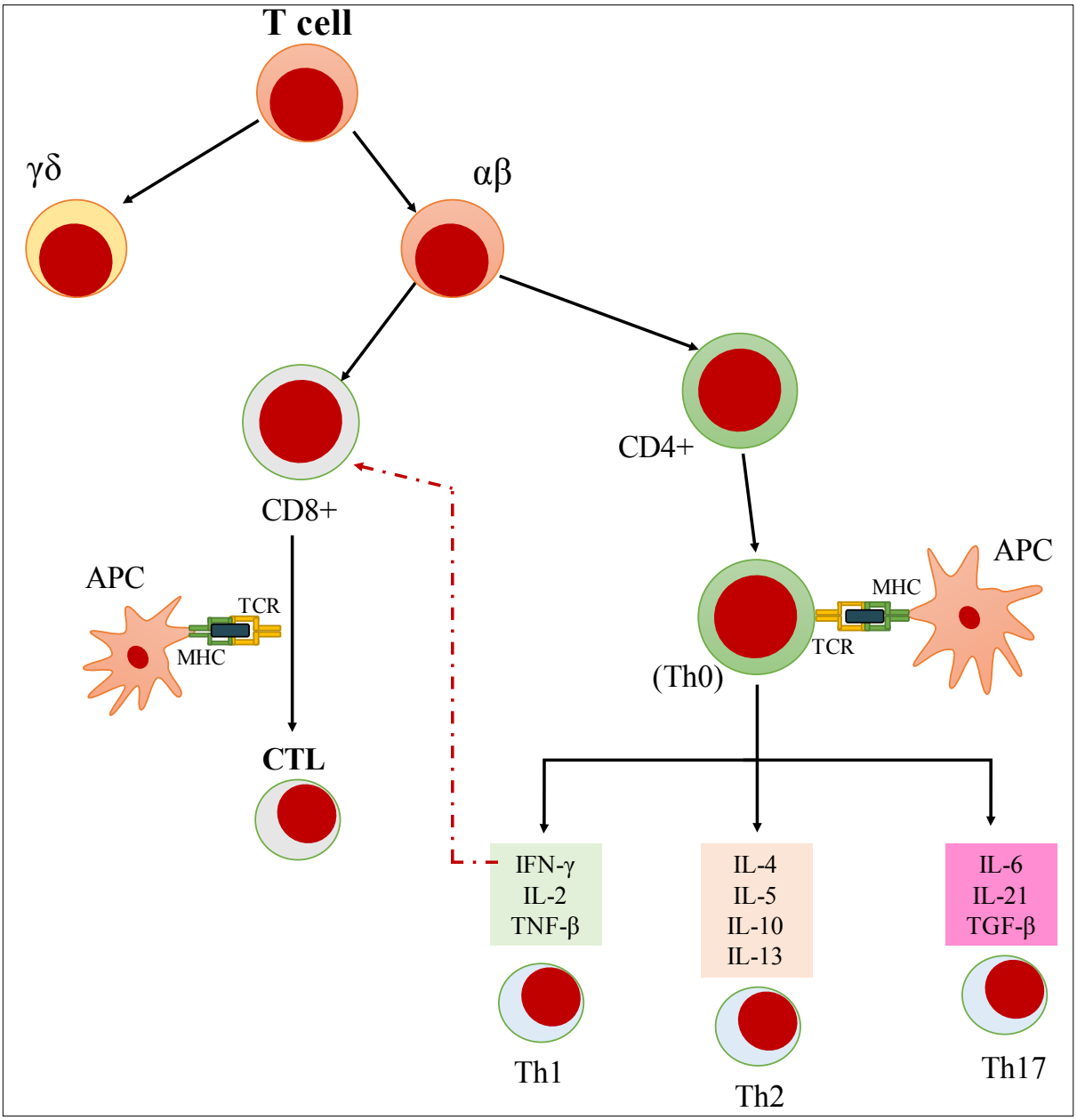

Figure 5. T-cell populations and Th responses. T-cells belong to two subpopulations: αβ and γδ. αβ T-cells can be subdivided in CD8+ and CD4+. CD8+ T cells can become cytotoxic lymphocytes (CTL) after contact with professional APC or after stimulation with IFN-γ. CD4+ T cells can be stimulated by APC via MHC-TCR interaction which induces a specific profile of cytokines that starts Th immune responses (Th1, Th2, Th17) based on the origin of the antigen intracellular or intracellular. The Th response depends on the cytokine secreted, and this induced inhibitory signal on the other, *e.g.* IFN-γ in the Th1 response inhibits the secretion of IL-4 which decrease the Th2 response and viceversa.

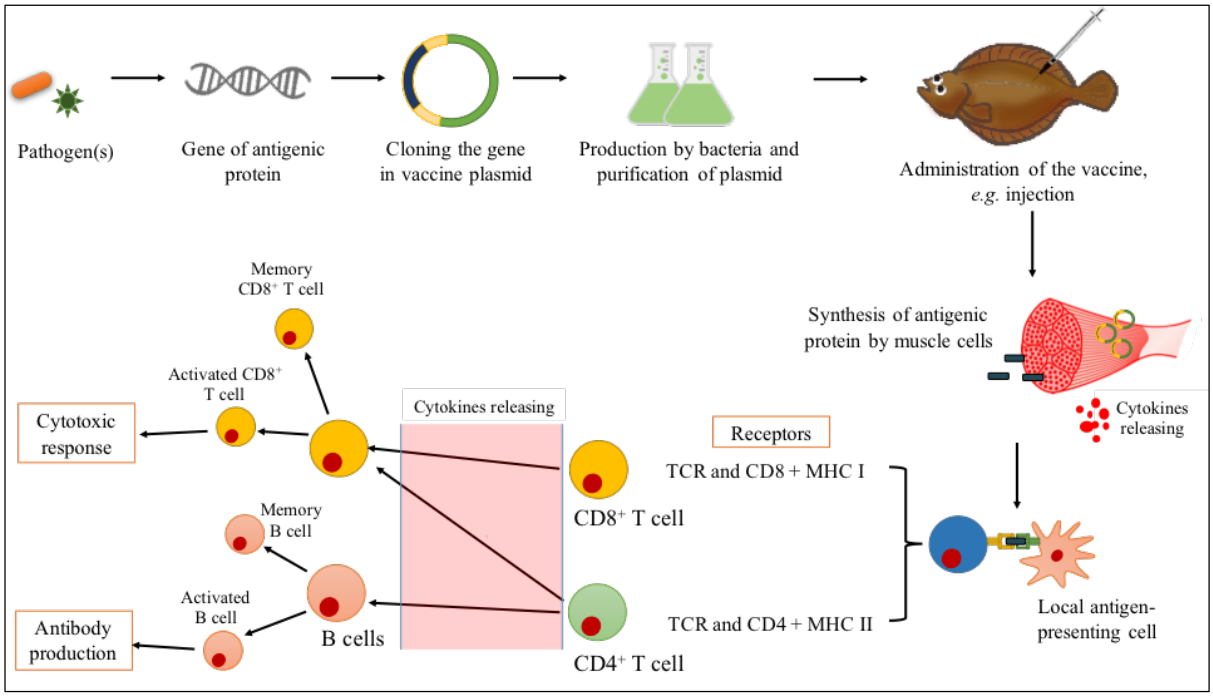

Figure 6. Schematic explanation of DNA vaccination. A gene coding for a specific antigenic protein from a pathogen is isolated and cloned in the vaccine plasmid, which is transformed in a competent cell (*e.g.* bacteria, yeast), produced at large scale and purified. After purification, the vaccine is administered and the muscle cells endocytose the plasmid and synthesize the antigenic protein. The antigenic protein is released into the extracellular space as well as presented on the cell surface, where local antigen-presenting cells initiate the immune response. The secretion of cytokines helps in the recruitment of immune cells and the early local inflammatory response. Antigenic peptides can be presented to T cells by MHCI or MHCII which can stimulate Th1 or Th2 immune responses, respectively, and specific cytokines in each response are secreted. Th1 responses are characterized for the stimulation of $CD8^+$ T cells while Th2 response is for $CD4^+$ T cells. Th1 immune response generates a cytotoxic response by $CD8^+$ activated T cells and Th2 immune response generates and antibody production by activated B cells (plasma cells). In both responses, memory cells are generated.

Chapter 2.

Characterization of lysosome-associated membrane protein-1 (LAMP-1) in Japanese flounder (*Paralichthys olivaceus*)

Abstract

Lysosomes play an important role in cellular metabolism and several lysosomal proteins are pivotal for physiological processes including cell-to-cell interactions, embryonic development, antigen presentation and autophagy. Lysosome associated membrane protein-1 (LAMP-1) is one the main lysosomal membrane proteins that interact in immune responses and mediates the antigen processing inside the cell. In the Japanese flounder, *Paralichthys olivaceus*, the lysosomal protein has not bet yet characterized. The present study aimed to characterize the LAMP-1 gene in Japanese flounder through tissue distribution and expression analysis. Japanese flounder LAMP-1 (JfLAMP-1) gene ORF was obtained by analyzing EST data from previous study in our lab, amplified and cloned by using specific primers. Tissue expression of JfLAMP-1 gene was assessed from several tissues in healthy animals and in spleen under *Edwardsiella tarda* FKC (Formalin-killed cells) and poly I:C injection. JfLAMP-1 expression was assessed in HINAE cells by western blot and localization of the protein was evaluated by immunofluorescence. JfLAMP-1 gene has a length of 1248 bp that encodes for 415 aa (43,8 kDa) and exhibit a signal peptide and 3 conserved domains (luminal domain, transmembrane domain and cytoplasmic domain) similar with reported in higher vertebrates. JfLAMP-1 gene expresses constitutively in all the tissues examined. At *E. tarda* FKC injection JfLAMP-1 expression can be upregulated at 3 h, 12 h and 3 days for 22 ℃ and 1 day and 7 days for 15 ℃. No changes in mRNA level of JfLAMP-1 were detected on poly I:C injected animals. JfLAMP-1 was detected in HINAE cells as a 56 kDa band and the immunofluorescence analysis showed it distributed in small and large granules in the cytoplasm and grouped close to the nucleus. The characterization of lysosomal proteins in fish increase the basic biology knowledge to understand the immune response as well as open possibilities for new cell marker in cell development or the use of lysosomal proteins in immunization strategies.

Keywords: Lysosome, Japanese flounder, gene expression, tissue distribution

1. Introduction

Lysosomes are found in all eukaryotic cell types and participate with the endocytic pathway (Johnson *et al.*, 2016; Luzio *et al.*, 2014). They are characterized by a highly acidic lumen, rich in hydrolytic enzymes and responsible for degradation of macromolecules from the extracellular environment (Sun-Wada *et al.*, 2003) as well as interaction with major histocompatibility complex (MHC) class II molecules (Villadangos, 2001; Watts, 2012). Lysosomes generate and maintain an acidic lumen by means of the vacuolar H^+-ATPase and their position in the cells as well as their pH can be related with heterogeneous function in the cell (Johnson *et al.*, 2016; Lübke *et al.*, 2009).

In general, lysosomes constitute up to 5% of the intracellular volume of animal cells (Luzio *et al.*, 2014) and their morphology and level differ between tissues and cell types which allows to have several roles in physiology (Klumperman & Raposo, 2014; Sun-Wada *et al.*, 2003). For example, in cytotoxic T lymphocytes (CTL) and a small number of other cell types, lysosomes are also secretory organelles called as lytic granules that contain specialized secretory proteins in addition to lysosomal hydrolases (Page *et al.*, 1998). Approximately 13 lysosome-related organelles (LRO) have been described in different cell types and include melanosomes, platelet dense granules, Weibel-Palade bodies and neutrophil azurophil granules (Bonifacino, 2004; Jani *et al.*, 2016; Luzio *et al.*, 2014) and the congenital deficiency of lysosomes or LRO or some lysosomal proteins have been associated with several diseases and syndromes (Callahan *et al.*, 2009; Jani *et al.*, 2016)

Lysosomal membrane proteins are highly glycosylated (Fukuda, 1991; Hatakeyama *et al.*, 2014) and it was estimated that lysosome associated membrane proteins (LAMPs) and lysosome integrated membrane proteins (LIMP) constitute about 50% of all proteins in lysosomal membrane (Hunziker *et al.*, 1996; Marsh *et al.*, 1987) and some of them can be expressed on the cell surface (Furuta *et al.*, 1999; Hatakeyama *et al.*, 2014; Tompkins *et al.*, 2006). LAMP-1 and LAMP-2 are physiologically essentials and the deficiency of them in mice are embryonic lethal, with the accumulation of autophagic bodies in several tissues (Eskelinen *et al.*, 2004; Terasawa *et al.*, 2016). Furthermore, late endosomes/lysosomes as well as phagosomes lacking LAMP-1 and LAMP-2 had reduced ability to move toward the

microtubule-organizing center, likely precluding their interaction with each other (Huynh *et al.*, 2007).

LAMPs are type I transmembrane proteins with a large luminal domain, one transmembrane domain and a C-terminal cytoplasmic tail (Eskelinen, 2006). Luminal domain is glycosylated with some O-glycans and a large number of N-glycans, most of which are of the complex poly-N- acetyllactosamine type, thus explaining the low p*I* between 2 – 4 exhibited by the proteins (Eskelinen *et al.*, 2003). In addition, the short terminal cytoplasmic tail, corresponding to 10-11 amino acids, determines the sorting of LAMPs (Dahlgren *et al.*, 1995) and their tissue distribution (Furuta *et al.*, 1999).

Besides of its function as interacting molecules, LAMP-1 showed to protect natural killer cells from the degranulation-associated damage (Cohnen *et al.*, 2013) and it is highly expressed in some tumors with a possible role in cell-to-cell adhesion and migration (Jensen *et al.*, 2013). Recently, it was used to the design of vaccines against some diseases (Arruda *et al.*, 2006; Dhalia *et al.*, 2009; Nawaratna *et al.*, 2015). In Japanese flounder (*Paralichthys olivaceus*), LAMP molecules are not yet characterized. The aim of this study is to characterize the Japanese flounder LAMP-1 (JfLAMP-1) gene through tissue distribution and expression analysis.

2. Material and methods

2.1. JfLAMP-1 Cloning

Based on EST analyses of Japanese flounder transcripts (Kondo *et al.*, 2014), primers were designed to amplify the open reading frame (ORF) of Japanese flounder LAMP-1 (JfLAMP-1) (Table 1), including restriction enzymes sites for *Nde*I and *Eco*RI and cDNA from spleen was used as a template. PCR products were ligated and cloned into pGEM T-easy vector (Promega, USA) and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with a 3130xl genetic analyzer (Applied Biosystems). Putative signal peptide was predicted using SignalP (http://www.cbs.dtu.dk./services/SignalP/) and InterproScan was used to predict the domains (luminal, transmembrane and cytoplasmic) (http://www.ebi.ac.uk/interpro/). NetNGlyc and NetOGlyc programs were used for prediction of N-linked and O-linked glycosylation sites (http://www.cbs.dtu.dk./services/NetNGlyc/, http://www.cbs.dtu.dk./services/NetOGlyc/). Multiple sequence alignments were generated using GENETYX-MAC 16.0.7 software (Software Development Co., Ltd., Tokyo, Japan) and Geneious® 8.1.7 (Biomatters Limited) (Kearse *et al.*, 2012), and identity values were calculated using SIAS tool (http://imed.med.ucm.es/Tools/sias.html).

2.2. Tissue expression

For JfLAMP-1 mRNA expression analysis, total RNA was extracted from healthy Japanese flounder (n=3) using RNAiso plus reagent and RNAiso blood reagent (Takara Bio. Inc., Otsu, Japan) from several tissues including gill, brain, muscle, liver, spleen, intestine, kidney and blood. In the same way, total RNA was extracted from HINAE (hirame natural embryo) cell line (Kasai & Yoshimizu, 2001), which was maintained in Leibovitz's L-15 (Life Technologies, Carlsbad, CA, USA) medium containing 10% FBS (Life Technologies), 100 units/ml of penicillin, 100 μg/ml of streptomycin 25 °C. cDNA was synthesized using SuperScript™ III First Strand synthesis system M-MLV (Life Technologies).

2.2.1. RT-PCR and qPCR (quantitative real-time PCR)

RT-PCR was performed with cDNA templates (5 times diluted) from each tissue using specific primer sets for JfLAMP-1 (Table 1) under the following PCR conditions: initial

denaturation at 95 °C for 4 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final extension of 72 °C for 7 min. EF-1α gene was used as an internal control. The PCR products were electrophoresed on 1% agarose gels.

For qPCR, specific primer sets (Table 1) were designed and the reaction was carried out using SYBR green PCR master mix (Applied Biosystems) on a StepOnePlus Real-time PCR system (Applied Biosystems) according to the manufacturer's protocol. EF-1α was used as a housekeeping gene for internal control. The expression levels of JfLAMP-1 gene were normalized to the expression level of the housekeeping gene, calculated using the $2^{\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), and were expressed as fold change relative to blood (set as 1).

2.2.2. JfLAMP-1 expression in *Edwardsiella tarda* FKC-injection model

Juveniles of Japanese flounder (average weight of 4.2 g) were distributed 200-l tanks supplying filtered seawater at 15°C and 22°C. Fish were injected i.p. with a dose of 2.3 x 10^8 cfu/fish of FKC *Edwardsiella tarda*. Spleen was sampled at different sampling times (5 fish per group/sampling time): 0 h (before injection), 3 h, 6 h, 12 h, day 1, day 2, day 7 and day 14 post injection. RNA extraction as well as cDNA synthesis were done as describe above. Primers sets used for the analysis are enlisted in Table 1.

2.2.3. JfLAMP-1 expression in polyinosinic-polycytidylic acid (poly I:C) stimulation

Japanese flounder (average weight of 15 g) individuals were distributed 200-l tanks supplying filtered seawater at 22°C. Fish were injected intraperitoneally with a dose of 100 µg of poly I:C/fish. Spleen was sampled from experimental animals at different sampling times (5 fish/sampling time): 0 h (before injection), 3 h, 6 h, 12 h, day 1, day 2, day 7 and day 14 post injection. RNA extraction and cDNA synthesis was made as describe above. JfLAMP-1 expression was assessed by qPCR analysis and EF-1α was used as a housekeeping gene for the normalization of the data. Primers used for the analysis are enlisted in Table 1.

2.2.4. Expression of JfLAMP-1 in HINAE cells

Expression of JfLAMP-1 in HINAE cells was assessed by western blot and immunofluorescence using rabbit anti-JfLAMP -1 as a primary antibody (1:1000). Antiserum

was developed by immunizing rabbit with a JfLAMP-1 synthetic peptide sequence, NH2-C+**GRKRSHAGYQTI-**COOH (Eurofinsgenomics Co Ltd, Japan) and its specificity was confirmed by using the recombinant JfLAMP-1 (rJfLAMP-1) in a western blot analysis. rJfLAMP-1 was produced using pET-32a expression vector and transformed in *Escherichia coli* BL21(D3) strain.

For western blotting, cultured HINAE cells after confluent stage were removed from culture flask by trypsin digestion and washed three times with phosphate buffer saline (PBS). Cell pellet was suspended in PBS, mixed with 2x SDS sample buffer (ratio 1:1) and analyzed by sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) (12%) and western blot. Anti-Rabbit IgG (Fc) AP conjugate (Promega, USA) was used as secondary antibody (1:3000). For immunofluorescence detection, HINAE cells were cultured in 6-well plates with a coverslip in the bottom. Cells were washed in PBS by triplicate and fixed in the coverslip. Primary antibody diluted in PBS-T (PBS + Tween 20) was added to each well and incubated for 1 h. Then, the cells were washed thrice with PBS-T and the secondary antibody (Goat Anti-Rabbit IgG FITC; Cayman chemical, USA) was added and incubated in dark condition for 1 h. Cells were washed three times and then their nucleus were stained using Hoechst (Invitrogen, USA) and observed under fluorescence microscope (Nikon Eclipse C*i*, camera DS-Ri1; Nikon, Japan). Images were analyzed using Fiji (ImageJ) software (Schindelin *et al.*, 2012).

2.4. Statistical analysis

Differences in gene expression were evaluated with a *t*-test. Statistical analyses were done with GraphPad Prism v 6.0 (La Jolla, CA, USA). Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. cDNA cloning and characterization of JfLAMP-1

The ORF for JfLAMP-1 (accession number: LC127058) has a length of 1248 bp that encodes for 415 aa (43,8 kDa). The 3'UTR had a polyadenylation signal (AATAA) 854 bp downstream of the translation termination codon (TGA)(Figure 1). JfLAMP-1 contains the signal peptide and 3 domains: luminal domain, transmembrane domain and cytoplasmic domain. In the same way, 9 predicted N-glycosylation sites and 14 predicted O-glycosylation sites were detected (Figure 1). Eight conserved cysteine residues are present in the predicted protein, which have been reported to form 4 disulphide bridges (Carlsson & Fukuda, 1989). Luminal domain showed a high O-glycosylation region (hinge region) which is rich in threonine and proline and divide the luminal in two fragments or loops (Figure 2). The HAG**Y**QT**I** motif, which is responsible for the sorting of molecule inside the cell (Braulke & Bonifacino, 2009), was in the position 409-415 and it is the most conserved region between LAMP-1s. Transmembrane domain also showed high identity between LAMP-1 molecules (Figure 3). The LAMP-1 amino acid sequences showed a pairwise identity of 51.8% among the species analyzed.

Phylogenetic analysis based on the amino acid sequences of LAMP from vertebrates showed that several fish species were grouped in the same cluster and LAMP-1 from Fugu, Asian sea bass, Bicolor damselfish and Black rockcod were the closest to JfLAMP-1 (Figure 4). On the other hand, identity index showed values in a range from 39% to 92% among the species (Table 2)

3.2. Tissue expression of JfLAMP-1

JfLAMP-1 mRNA was detected in blood, gills, brain, muscle, liver, spleen, intestine, kidney and HINAE cells as a constitutive gene (Figure 5a & 9a). However, in liver the mRNA levels were slightly lower compared with the others. Relative gene expression of JfLAMP-1 by qPCR showed the brain with high values (Figure 5b).

In *E. tarda* FKC-injected fish, the JfLAMP-1 gene expression in spleen was significantly upregulated at 3h, 12h ($p<0.05$) and 3 days ($p<0.01$) after injection at 22℃ (Figure 6) and at 1 day and 7 days post-injection at 15℃ (Figure 7). However, in poly I:C stimulation, there were no changes in the JfLAMP-1 gene expression at any time assessed (Figure 8).

Protein expression analysis of JfLAMP-1 by western blot showed a band of ~56 kDa (Figure 9c). The specificity of the primary antiserum used for the protein analysis was confirmed in the rJfLAMP-1 which appeared as a band of ~44 kDa (Figure 9b). In the immunofluorescence analysis in HINAE cells, the expression of JfLAMP-1 showed with a throughout distribution in the small and large granules in the cytoplasm and grouped close to the nucleus (Figure 10).

4. Discussion

4.1. JfLAMP-1 characterization

This is the first characterization of LAMP-1 in fish, however, LAMP-3 was reported previously (Johansson *et al.*, 2012), which showed similarities in the sorting motif. Human LAMP genes are present in different chromosomes suggesting that they diverged early in evolution of vertebrates and have distinct functions (Furuta *et al.*, 1999). In the present study, JfLAMP-1 showed molecular mass and conserved domains similar to LAMP-1 in higher vertebrates (Carlsson *et al.*, 1988; Chen *et al.*, 1988; Fambrough *et al.*, 1988; Fukuda *et al.*, 1988; Mane *et al.*, 1989). Transmembrane and cytoplasmic domains of JfLAMP-1 showed high identity and the YXXØ- sorting signal specifically the motif –HAG**Y**QT**I** was highly conserved. YXXØ motifs are essential for the intracellular targeting after biosynthesis including rapid internalization of LAMP-1 from the plasma membrane, interaction with transmembrane proteins as well as with *trans*-Golgi network and endosomes (Bonifacino & Traub, 2003; Fukuda, 1991; Hunziker & Geuze, 1996; Hunziker *et al.*, 1996).

JfLAMP-1 showed many potential N- and O- glycosylation sites as reported previously in humans (Carlsson *et al.*, 1988; Mane *et al.*, 1989), which can increase the size of the molecule and this glycosyl moiety can constitutes about 60% of the total mass (Eskelinen *et al.*, 2003). N-glycosylation seems to be important for the stability of the proteins in the lysosomal membrane (Barriocanal *et al.*, 1986; Kundra & Kornfeld, 1999) and as candidate to receptor site for binding microbial lectins (Carlsson & Fukuda, 1989; Dahlgren *et al.*, 1995). Opposite to human LAMP-1 (Fukuda *et al.*, 1988), the N-glycosylation sites in JfLAMP-1 are far from the transmembrane domain. The proximity of N-glycosylation sites to the transmembrane domain has been described as pivotal for formation of polylactosaminoglycans (Fukuda*, et al.*, 1988) and the reduction in these polylactosaminoglycans are correlated with the cell differentiation of colonic human adenocarcinoma (Brockhausen *et al.*, 1991; Youakim *et al.*, 1989). On the other hand, JfLAMP-1 O-linked glycosylations are distributed in clusters at the hinge region which is rich in proline and threonine, similar to described in another species (Carlsson *et al.*, 1988; Chen *et al.*, 1988; Fambrough *et al.*, 1988; Mane *et al.*, 1989) and divide the luminal domain in two segments and together with the cysteine residues and disulphide bonds can induce an immunoglobulin-like loop (Carlsson & Fukuda, 1989). These O-linked

glycosylations likely protect this region from intraluminal lysosomal proteases (Carlsson *et al.*, 1993).

4.2. Expression of JfLAMP-1 in tissues

The expression of JfLAMP-1 mRNA was detected in all the examined tissues as a constitutive gene, similar to the expression of another genes of the LAMP family (Johansson *et al.*, 2012). In human, Furuta *et al.*, (1999) assessed the expression of LAMP-1 in different tissues (cerebral cortex, colonic mucosa, kidney cortex, liver, lung, pancreas, prostate, spleen, and uterine myometrium) by immunohistochemistry and Northern analysis showing constitutive expression in all of them. However, the expression of LAMP-1 in brain neurons was virtually absent. In case of JfLAMP-1, quantitative expression analysis showed higher relative values in brain tissue. On the other hand, JfLAMP-1 was highly expressed in HINAE cells which is similar to F9 embryonal carcinoma cells where the LAMP-1 is high and can be modulated by retinoic acid in a time- and dose-dependent manner (Amos & Lotan, 1990). This high expression of LAMP-1 as well as its glycosylation state in embryonic stages is associated with early stages of cell differentiation in which LAMP-1 can modulate the process together with another molecule (Amos & Lotan, 1990; Brockhausen *et al.*, 1991; Romero *et al.*, 1993; Youakim *et al.*, 1989). Expression of LAMP-1 can differ between cell populations or cellular compartments (*e.g.* granules) (Dahlgren *et al.*, 1995) and the variation in the expression of LAMP-1 in fish cells can be related with cell differentiation and investigated in the future as a cell development marker.

The western blot analysis of JfLAMP-1 in HINAE cells showed a band of approximately 56 kDa, which was also present in other tissues examined previously. Although the predicted size of JfLAMP-1 is 43.8 kDa, N- and O-glycosylations of the molecule may increase its weight as demonstrated in other species, where LAMP-1 can also appear as a "smear" band of broad range size (*e.g.* 90-120 kDa) (Amos & Lotan, 1990; Carlsson *et al.*, 1988; Eskelinen *et al.*, 2003; Mane *et al.*, 1989; Zhou *et al.*, 1993). The size of the rLAMP-1 was detected as a band of 44 kDa which support the assumption of change of the protein mass due to the glycosyl moiety, due to the lack of post-translational modifications in recombinant protein produced in prokaryotic host (Sahdev *et al.*, 2008).

By immunofluorescence analysis, JfLAMP-1 appeared distributed in cytoplasmic granules with different intensity and frequently grouped in the perinuclear area. These findings of the distribution are similar to the results reported in embryonic (Fehrenbacher *et al.*, 2008) and differentiated cells (Dahlgren *et al.*, 1995; Jensen *et al.*, 2013; Parkinson-Lawrence *et al.*, 2005; Sadaka *et al.*, 2009; Sarafian *et al.*, 2006; Zhou *et al.*, 1993).

Previous studies have reported that FKC-injected Japanese flounder showed upregulation of immune-related genes (Dumrongphol *et al.*, 2009; Kondo *et al.*, 2014; Lyu *et al.*, 2016). Kondo *et al.* (2014) previously reported that *E. tarda* FKC-immunization differentially upregulates clusters of immune-related genes mainly those related with cellular and metabolic process. In this study, JfLAMP-1 expression in *E. tarda* FKC-injected Japanese flounder was assessed by real-time PCR showing an upregulation at 3h, 12h and 3 days post-injection. However, there is not any reports of the evaluation of gene expression of LAMP-1 in fish or neither in FKC nor bacterial challenge model. Nevertheless, in case of rainbow trout LAMP-3 gene *in vivo* and *in vitro* studies were done (Johansson *et al.*, 2012). *In vivo* challenge with *Yersinia ruckeri* increased the expression of LAMP-3 in gills and head kidney 24 h after i.p. challenge and after 48 h the level expression return to basal. On the other hand, *in vitro* study showed that following Poly I:C and *E. coli* lipopolysaccharide stimulation of mononuclear cell line an upregulation of its expression was observed at 8 h post-stimulation which return to basal levels at 24 h. In our study the upregulation was observed at 3 h post-injection which is agreement with the observation of Huynh *et al.* (2007) who demonstrated that the displacement for phagolysosome fusion was evidenced at 3 h in fibroblast and the role of LAMP-1 in the late endosome interaction (mediated by Rab7).

Poly I:C is a synthetic dsRNA compound that mimic the effect of viral dsRNA antigen and have been used to detect antiviral response in fish (Røkenes *et al.*, 2007; Seppola *et al.*, 2007; Yasuike *et al.*, 2011). In the present study, the stimulation with poly I:C did not induce changes in the gene expression of JfLAMP-1 which is supported in studies by Watanabe *et al.* (2011) who showed that poly I:C was uptaked by receptor on the plasma membrane and moved to the TLR3-positive early endosomes, but did not colocalize with LAMP-1, demonstrating that LAMP-1 may not participate in the processing pathway. In contrast, Zou *et al.* (2013) demonstrated that poly I:C was colocalized with LAMP-1 in dendritic cells at 2 h after

stimulation but this colocalization disappear at 12h.

In conclusion, JfLAMP-1 gene was characterized and its tissue expression under normal condition as well as *E. tarda* FKC and poly I:C stimulation, which showed that JfLAMP-1 is a constitutive gene and can participate in the immune response against bacteria.

References

Amos, B., & Lotan, R. (1990). Modulation of lysosomal-associated membrane glycoproteins during retinoic acid-induced embryonal carcinoma cell differentiation. *Journal of Biological Chemistry, 265*(31), 19192-19198.

Arruda, L. B., Sim, D., Chikhlikar, P. R., Maciel, M., Jr., Akasaki, K., August, J. T., & Marques, E. T. (2006). Dendritic cell-lysosomal-associated membrane protein (LAMP) and LAMP-1-HIV-1 gag chimeras have distinct cellular trafficking pathways and prime T and B cell responses to a diverse repertoire of epitopes. *Journal of Immunology, 177*(4), 2265-2275.

Barriocanal, J. G., Bonifacino, J. S., Yuan, L., & Sandoval, I. V. (1986). Biosynthesis, glycosylation, movement through the Golgi system, and transport to lysosomes by an N-linked carbohydrate-independent mechanism of three lysosomal integral membrane proteins. *Journal of Biological Chemistry, 261*(35), 16755-16763.

Bonifacino, J. S. (2004). Insights into the biogenesis of lysosome-related organelles from the study of the Hermansky-Pudlak syndrome. *Annals of the New York Academy of Sciences, 1038*(1), 103-114.

Bonifacino, J. S., & Traub, L. M. (2003). Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annual Review of Biochemistry, 72*, 395-447.

Braulke, T., & Bonifacino, J. S. (2009). Sorting of lysosomal proteins. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1793*(4), 605-614.

Brockhausen, I., Romero, P. A., & Herscovics, A. (1991). Glycosyltransferase changes upon differentiation of CaCo-2 human colonic adenocarcinoma cells. *Cancer Research, 51*(12), 3136-3142.

Callahan, J. W., Bagshaw, R. D., & Mahuran, D. J. (2009). The integral membrane of lysosomes: Its proteins and their roles in disease. *Journal of Proteomics, 72*(1), 23-33.

Carlsson, S. R., & Fukuda, M. (1989). Structure of human lysosomal membrane glycoprotein 1. Assignment of disulfide bonds and visualization of its domain arrangement. *Journal of Biological Chemistry, 264*.

Carlsson, S. R., Lycksell, P. O., & Fukuda, M. (1993). Assignment of O-glycan attachment sites to the hinge-like regions of human lysosomal membrane glycoproteins lamp-1 and lamp-2. *Archives of Biochemistry Biophysics, 304*(1), 65-73.

Carlsson, S. R., Roth, J., Piller, F., & Fukuda, M. (1988). Isolation and characterization of human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. Major

sialoglycoproteins carrying polylactosaminoglycan. *Journal of Biological Chemistry, 263*(35), 18911-18919.

Chen, J. W., Cha, Y., Yuksel, K. U., Gracy, R. W., & August, J. T. (1988). Isolation and sequencing of a cDNA clone encoding lysosomal membrane glycoprotein mouse LAMP-1. Sequence similarity to proteins bearing onco-differentiation antigens. *Journal of Biological Chemistry, 263*(18), 8754-8758.

Cohnen, A., Chiang, S. C., Stojanovic, A., Schmidt, H., Claus, M., Saftig, P., Janssen, O., Cerwenka, A., Bryceson, Y. T., & Watzl, C. (2013). Surface CD107a/LAMP-1 protects natural killer cells from degranulation-associated damage. *Blood, 122*(8), 1411-1418.

Dahlgren, C., Carlsson, S. R., Karlsson, A., Lundqvist, H., & Sjolin, C. (1995). The lysosomal membrane glycoproteins Lamp-1 and Lamp-2 are present in mobilizable organelles, but are absent from the azurophil granules of human neutrophils. *Biochemical Journal, 311 (Pt 2)*, 667-674.

Dhalia, R., Maciel Jr., M., Cruz, F. S. P., Viana, I. F. T., Palma, M. L., August, T., & Marques Jr., E. T. A. (2009). Membrane and envelope virus proteins co-expressed as lysosome associated membrane protein (LAMP) fused antigens: a potential tool to develop DNA vaccines against flaviviruses. *Anais da Academia Brasileira de Ciências, 81*, 663-669.

Dumrongphol, Y., Hirota, T., Kondo, H., Aoki, T., & Hirono, I. (2009). Identification of novel genes in Japanese flounder (*Paralichthys olivaceus*) head kidney up-regulated after vaccination with *Streptococcus iniae* formalin-killed cells. *Fish & Shellfish Immunology, 26*(1), 197-200.

Eskelinen, E. L. (2006). Roles of LAMP-1 and LAMP-2 in lysosome biogenesis and autophagy. *Molecular Aspects of Medicine, 27*(5-6), 495-502.

Eskelinen, E. L., Schmidt, C. K., Neu, S., Willenborg, M., Fuertes, G., Salvador, N., Tanaka, Y., Lullmann-Rauch, R., Hartmann, D., Heeren, J., von Figura, K., Knecht, E., & Saftig, P. (2004). Disturbed cholesterol traffic but normal proteolytic function in LAMP-1/LAMP-2 double-deficient fibroblasts. *Molecular Biology of the Cell, 15*.

Eskelinen, E. L., Tanaka, Y., & Saftig, P. (2003). At the acidic edge: emerging functions for lysosomal membrane proteins. *Trends in Cell Biology, 13*(3), 137-145.

Fambrough, D. M., Takeyasu, K., Lippincott-Schwarz, J., & Siegel, N. R. (1988). Structure of LEP100, a glycoprotein that shuttles between lysosomes and the plasma membrane, deduced from the nucleotide sequence of the encoding cDNA. *Journal of Cell Biology, 106*(1), 61-67.

Fehrenbacher, N., Bastholm, L., Kirkegaard-Sorensen, T., Rafn, B., Bottzauw, T., Nielsen, C., Weber, E., Shirasawa, S., Kallunki, T., & Jaattela, M. (2008). Sensitization to the lysosomal cell death pathway by oncogene-induced down-regulation of lysosome-associated membrane proteins 1 and 2. *Cancer Research, 68*(16), 6623-6633.

Fukuda, M. (1991). Lysosomal membrane glycoproteins. Structure, biosynthesis, and intracellular trafficking. *Journal of Biological Chemistry, 266*(32), 21327-21330.

Fukuda, M., Guan, J. L., & Rose, J. K. (1988). A membrane-anchored form but not the secretory form of human chorionic gonadotropin-alpha chain acquires polylactosaminoglycan. *Journal of Biological Chemistry, 263*(11), 5314-5318.

Fukuda, M., Viitala, J., Matteson, J., & Carlsson, S. R. (1988). Cloning of cDNAs encoding human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. Comparison of their deduced amino acid sequences. *Journal of Biological Chemistry, 263*(35), 18920-18928.

Furuta, K., Yang, X. L., Chen, J. S., Hamilton, S. R., & August, J. T. (1999). Differential expression of the lysosome-associated membrane proteins in normal human tissues. *Archives of Biochemistry and Biophysics, 365*(1), 75-82.

Hatakeyama, Y., Hatakeyama, J., Oka, K., Tsuruga, E., Inai, T., Anan, H., & Sawa, Y. (2014). Immunohistochemical study of amelogenin and lysosome-associate membrane proteins (LAMPs) in cartilage. *International Journal of Morphology, 32*, 618-626.

Hunziker, W., & Geuze, H. J. (1996). Intracellular trafficking of lysosomal membrane proteins. *Bioessays, 18*(5), 379-389.

Hunziker, W., Simmen, T., & Honing, S. (1996). Trafficking of lysosomal membrane proteins in polarized kidney cells. *Nephrologie, 17*(7), 347-350.

Huynh, K. K., Eskelinen, E. L., Scott, C. C., Malevanets, A., Saftig, P., & Grinstein, S. (2007). LAMP proteins are required for fusion of lysosomes with phagosomes. *EMBO Journal, 26*(2), 313-324.

Jani, R. A., Mahanty, S., & Setty, S. R. G. (2016). SNAREs in the maturation and function of LROs. *Bioarchitecture, 6*(1), 1-11.

Jensen, S. S., Aaberg-Jessen, C., Christensen, K. G., & Kristensen, B. (2013). Expression of the lysosomal-associated membrane protein-1 (LAMP-1) in astrocytomas. *International Journal of Clinical and Experimental Pathology, 6*(7), 1294-1305.

Johansson, P., Corripio-Miyar, Y., Wang, T., Collet, B., Secombes, C. J., & Zou, J. (2012). Characterisation and expression analysis of the rainbow trout (*Oncorhynchus mykiss*)

homologue of the human dendritic cell marker CD208/lysosomal associated membrane protein 3. *Developmental & Comparative Immunology, 37*(3–4), 402-413.

Johnson, D. E., Ostrowski, P., Jaumouillé, V., & Grinstein, S. (2016). The position of lysosomes within the cell determines their luminal pH. *Journal of Cell Biology, 212*(6), 677-692.

Kasai, H., & Yoshimizu, M. (2001). Establishment of two Japanese flounder embryo cell lines. *Bulletin of Fisheries Sciences Hokkaido University, 52*, 67-70.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., & Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics, 28*(12), 1647-1649.

Klumperman, J., & Raposo, G. (2014). The Complex Ultrastructure of the Endolysosomal System. *Cold Spring Harbor Perspectives in Biology, 6*(10).

Kondo, H., Kawana, Y., Suzuki, Y., & Hirono, I. (2014). Comprehensive gene expression profiling in Japanese flounder kidney after injection with two different formalin-killed pathogenic bacteria. *Fish & Shellfish Immunology, 41*(2), 437-440.

Kundra, R., & Kornfeld, S. (1999). Asparagine-linked oligosaccharides protect Lamp-1 and Lamp-2 from intracellular proteolysis. *Journal of Biological Chemistry, 274*(43), 31039-31046.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods, 25*(4), 402-408.

Lübke, T., Lobel, P., & Sleat, D. E. (2009). Proteomics of the lysosome. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1793*(4), 625-635.

Luzio, J. P., Hackmann, Y., Dieckmann, N. M. G., & Griffiths, G. M. (2014). The biogenesis of lysosomes and lysosome-related organelles. *Cold Spring Harbor Perspectives in Biology, 6*(9), a016840.

Lyu, Z.-Z., Zhao, B.-B., Koiwai, K., Hirono, I., & Kondo, H. (2016). Identification of endonuclease domain-containing 1 gene in Japanese flounder *Paralichthys olivaceus*. *Fish & Shellfish Immunology, 50*, 43-49.

Mane, S. M., Marzella, L., Bainton, D. F., Holt, V. K., Cha, Y., Hildreth, J. E., & August, J. T. (1989). Purification and characterization of human lysosomal membrane glycoproteins. *Archives of Biochemistry and Biophysics, 268*(1), 360-378.

Marsh, M., Schmid, S., Kern, H., Harms, E., Male, P., Mellman, I., & Helenius, A. (1987). Rapid analytical and preparative isolation of functional endosomes by free flow electrophoresis. *Journal of Cell Biology, 104*(4), 875-886.

Nawaratna, S. S., Gobert, G. N., Willis, C., Mulvenna, J., Hofmann, A., McManus, D. P., & Jones, M. K. (2015). Lysosome-associated membrane glycoprotein (LAMP)--preliminary study on a hidden antigen target for vaccination against schistosomiasis. *Scientific Reports, 5*, 15069.

Page, L. J., Darmon, A. J., Uellner, R., & Griffiths, G. M. (1998). L is for lytic granules: lysosomes that kill. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1401*(2), 146-156.

Parkinson-Lawrence, E. J., Dean, C. J., Chang, M., Hopwood, J. J., Meikle, P. J., & Brooks, D. A. (2005). Immunochemical analysis of CD107a (LAMP-1). *Cellular Immunology, 236*(1–2), 161-166.

Røkenes, T. P., Larsen, R., & Robertsen, B. (2007). Atlantic salmon ISG15: Expression and conjugation to cellular proteins in response to interferon, double-stranded RNA and virus infections. *Molecular Immunology, 44*(5), 950-959.

Romero, P. A., Way, T., & Herscovics, A. (1993). Lamp-1 does not acquire the large polylactosaminoglycans characteristic of F9 cells. *Biochemical Journal, 296*(Pt 1), 253-257.

Sadaka, C., Marloie-Provost, M. A., Soumelis, V., & Benaroch, P. (2009). Developmental regulation of MHC II expression and transport in human plasmacytoid-derived dendritic cells. *Blood, 113*(10), 2127-2135.

Sahdev, S., Khattar, S. K., & Saini, K. S. (2008). Production of active eukaryotic proteins through bacterial expression systems: a review of the existing biotechnology strategies. *Molecular and Cellular Biochemistry, 307*(1-2), 249-264.

Sarafian, V., Jans, R., & Poumay, Y. (2006). Expression of lysosome-associated membrane protein 1 (Lamp-1) and galectins in human keratinocytes is regulated by differentiation. *Archives of Dermatology Research, 298*(2), 73-81.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods, 9*(7), 676-682.

Seppola, M., Stenvik, J., Steiro, K., Solstad, T., Robertsen, B., & Jensen, I. (2007). Sequence and expression analysis of an interferon stimulated gene (ISG15) from Atlantic cod (*Gadus morhua* L.). *Developmental & Comparative Immunology, 31*(2), 156-171.

Sun-Wada, G. H., Wada, Y., & Futai, M. (2003). Lysosome and lysosome-related organelles responsible for specialized functions in higher organisms, with special emphasis on vacuolar-type proton ATPase. *Cell Structure and Function, 28*(5), 455-463.

Terasawa, K., Tomabechi, Y., Ikeda, M., Ehara, H., Kukimoto-Niino, M., Wakiyama, M., Podyma-Inoue, K. A., Rajapakshe, A. R., Watabe, T., Shirouzu, M., & Hara-Yokoyama, M. (2016). Lysosome-associated membrane proteins-1 and -2 (LAMP-1 and LAMP-2) assemble via distinct modes. *Biochemical and Biophysical Research Communications, 479*(3), 489-495.

Tompkins, K., George, A., & Veis, A. (2006). Characterization of a mouse amelogenin [A−4]/M59 cell surface receptor. *Bone, 38*(2), 172-180.

Villadangos, J. A. (2001). Presentation of antigens by MHC class II molecules: getting the most out of them. *Molecular Immunology, 38*(5), 329-346.

Watanabe, A., Tatematsu, M., Saeki, K., Shibata, S., Shime, H., Yoshimura, A., Obuse, C., Seya, T., & Matsumoto, M. (2011). Raftlin is involved in the nucleocapture complex to induce poly(I:C)-mediated TLR3 activation. *Journal of Biological Chemistry, 286*(12), 10702-10711.

Watts, C. (2012). The endosome–lysosome pathway and information generation in the immune system. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1824*(1), 14-21.

Yasuike, M., Kondo, H., Hirono, I., & Aoki, T. (2011). Identification and characterization of Japanese flounder, *Paralichthys olivaceus* interferon-stimulated gene 15 (Jf-ISG15). *Comparative Immunology, Microbiology and Infectious Diseases, 34*(1), 83-91.

Youakim, A., Romero, P. A., Yee, K., Carlsson, S. R., Fukuda, M., & Herscovics, A. (1989). Decrease in polylactosaminoglycans associated with lysosomal membrane glycoproteins during differentiation of CaCo-2 human colonic adenocarcinoma cells. *Cancer Research, 49*(24 Part 1), 6889-6895.

Zhou, B. K., Boissy, R. E., Pifko-Hirst, S., Moran, D. J., & Orlow, S. J. (1993). Lysosome-associated membrane protein-1 (LAMP-1) is the melanocyte vesicular membrane glycoprotein band II. *Journal of Investigative Dermatology, 100*(2), 110-114.

Zou, J., Kawai, T., Tsuchida, T., Kozaki, T., Tanaka, H., Shin, K.-S., Kumar, H., & Akira, S. (2013). Poly IC triggers a cathepsin D- and IPS-1-dependent pathway to enhance cytokine production and mediate dendritic cell necroptosis. *Immunity, 38*(4), 717-728.

Table 1. PCR primers used in this study.

Target	**Usage**	**Primer sequence (5′–3′)***
JfLAMP ORF	**Cloning F**	AACATATGGAACTCTCTCACACGGT
	Cloning R	GAATTCGATGGTCTGGTATCC
JfLAMP-1	**RT PCR**	GCGGCCGCATGGAACTCTCTC
	RT PCR	GGCTTCACATAATCCAGACACATTT
	qPCR F	GTTCAACATCTCCTACGTCTCAAAA
	qPCR R	GGCTTCACATAATCCAGACACATTT
EF1α	**RT PCR/qPCR F**	CTCGGGCATAGACTCGTGGT
	RT PCR/qPCR R	CATGGTCGTGACCTTCGCTC

*Restriction enzyme sites (*Nde*I and *Eco*RI) are underlined at the start of the sequence.

Table 2. Amino acid identity index (%) in LAMP-1 from different species compared with the JfLAMP-1*.

		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	Japanese flounder	**83.61**	**77.59**	**65.54**	**75.9**	**71.32**	**63.65**	**68.68**	**70.84**	**65.06**	**65.3**	**53.64**	**48.66**	**42.4**	**39.8**	**39.03**
2	Asian seabass		81.68	70.02	78.41	74.58	67.07	73.3	75.53	68.58	66.66	55.58	51.09	42.92	40.29	40.76
3	Bicolor damselfish			66.5	77.83	72.53	67.07	69.41	71.8	66.74	63.61	52.66	49.14	42.65	39.06	40
4	Three-spined stickleback				69.04	63.3	56.58	61.16	62.67	57.41	57.99	46.84	47.93	37.61	36.85	36.69
5	Black rockcod					72.42	66.58	67.71	69.85	63.15	62.05	54.61	49.39	42.38	39.55	40.28
6	Fugu						64.63	68.93	68.82	63.54	61.87	52.18	47.93	44.6	37.59	38.12
7	Nile tilapia							59.75	61.95	61.46	57.07	48.04	45.6	42.92	39.06	38.04
8	Amazon molly								92.71	66.99	62.13	52.18	49.87	40.29	37.1	37.62
9	Southern platyfish									67.7	61.48	52.42	50.12	41.14	38.32	38.36
10	Japanese medaka										59.8	49.75	46.71	44.01	38.82	38.84
11	Salmon											52.18	50.85	41.28	38.08	37.41
12	Channel catfish												49.63	39.56	39.06	37.86
13	Zebrafish													40.38	37.34	37.71
14	Australian ghostshark														41.27	41.24
15	Mouse															66.09
16	Human															

*Genbank accession numbers or Ensembl protein ID: Japanese flounder LAMP-1 (LC127058), Fugu uncharacterized protein (H2V4H1), Zebrafish LAMP-1 (Q6P299), Japanese medaka uncharacterized protein (H2LIM4), Nile tilapia (I3J577), Amazon molly LAMP-1 (XP_007554035.1), Three-spined stickleback uncharacterized protein (G3PRL1), Channel catfish LAMP-1 (W5UCE1), Salmon LAMP-1 (C0H980), Asian sea bass predicted LAMP-1 (XP_018539319.1), Bicolor damselfish predicted LAMP-1 (XP_008273729.1), Black rockcod predicted LAMP-1 (XP_010784816.1), Southern platyfish uncharacterized protein (M4AXY6), Australian ghostshark LAMP-1 (V9KCK3), Mouse LAMP-1 (Q9DC13), Human LAMP-1 (NP_005552.3).

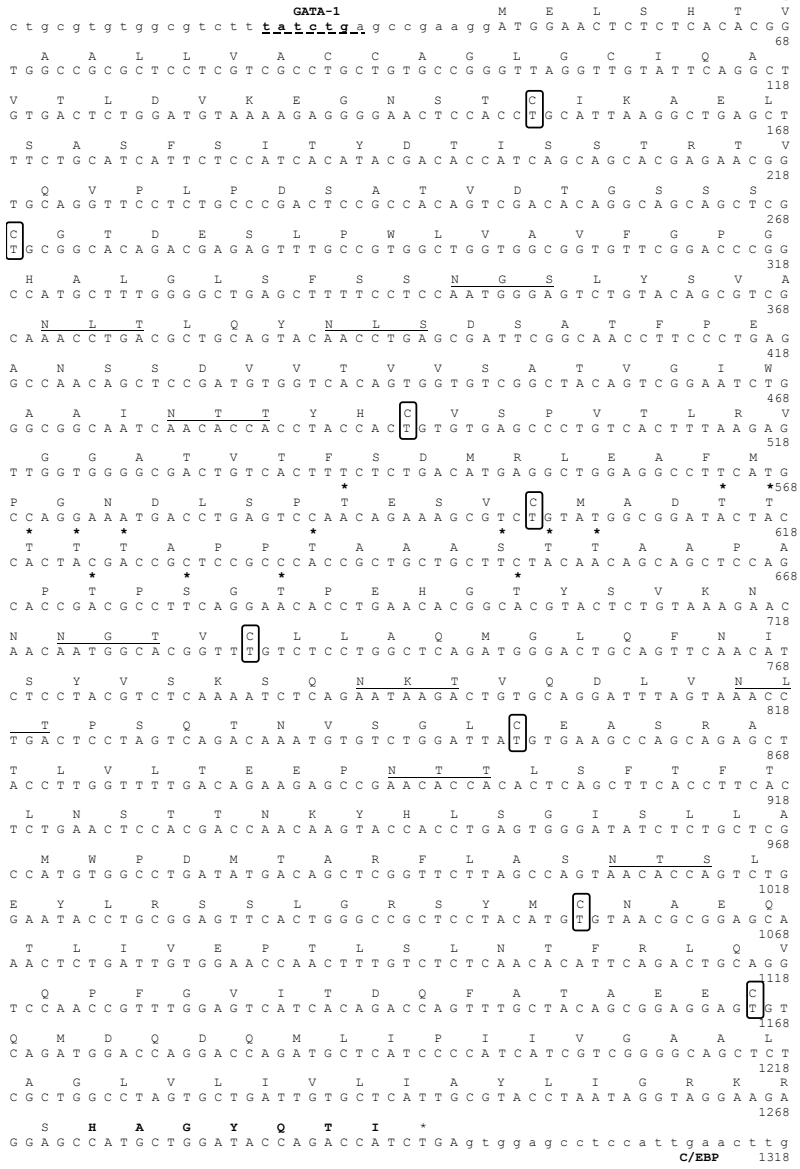

Figure 1. Japanese flounder LAMP-1 nucleotide/amino acid sequence analysis. N-linked glycosylation sites are continuous underlined and O-linked glycosylation sites are marked by asterisks. Conserved cysteine residues are boxed.

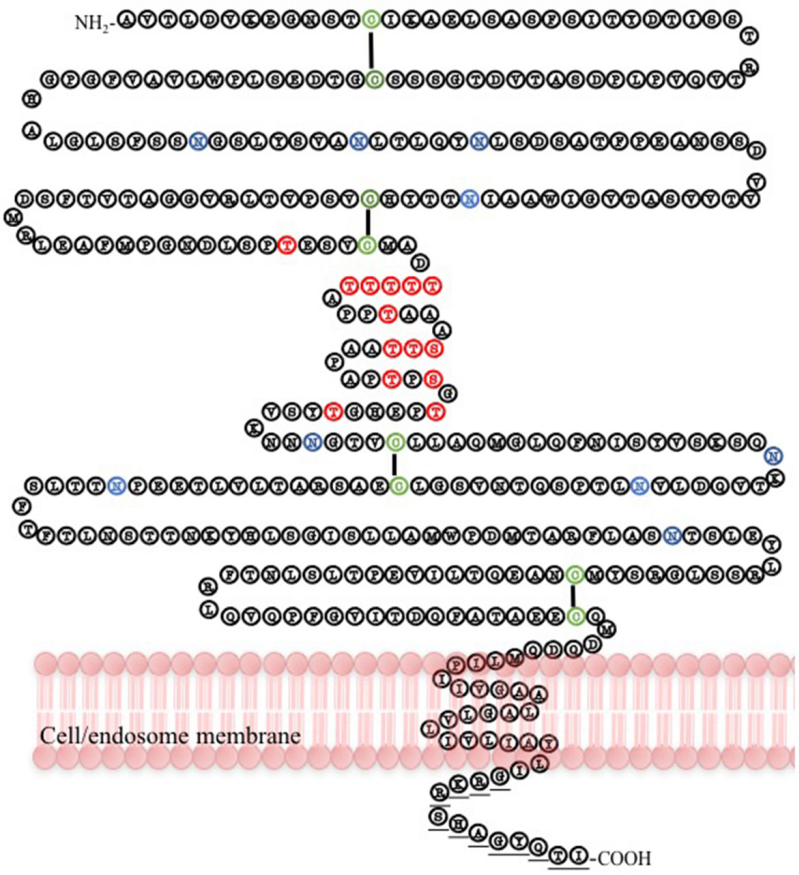

Figure 2. Schematic model of Japanese flounder LAMP-1 peptide (without the signal peptide). Conserved Cysteine residues (green), N-glycosylation (blue), O-glycosylation sites (red), Cytoplasmic domain (underlined). Disulphide bonds appear between conserved cysteine residues. Model was build based on the human LAMP-1 structure described in Carlsson and Fukuda (1989).

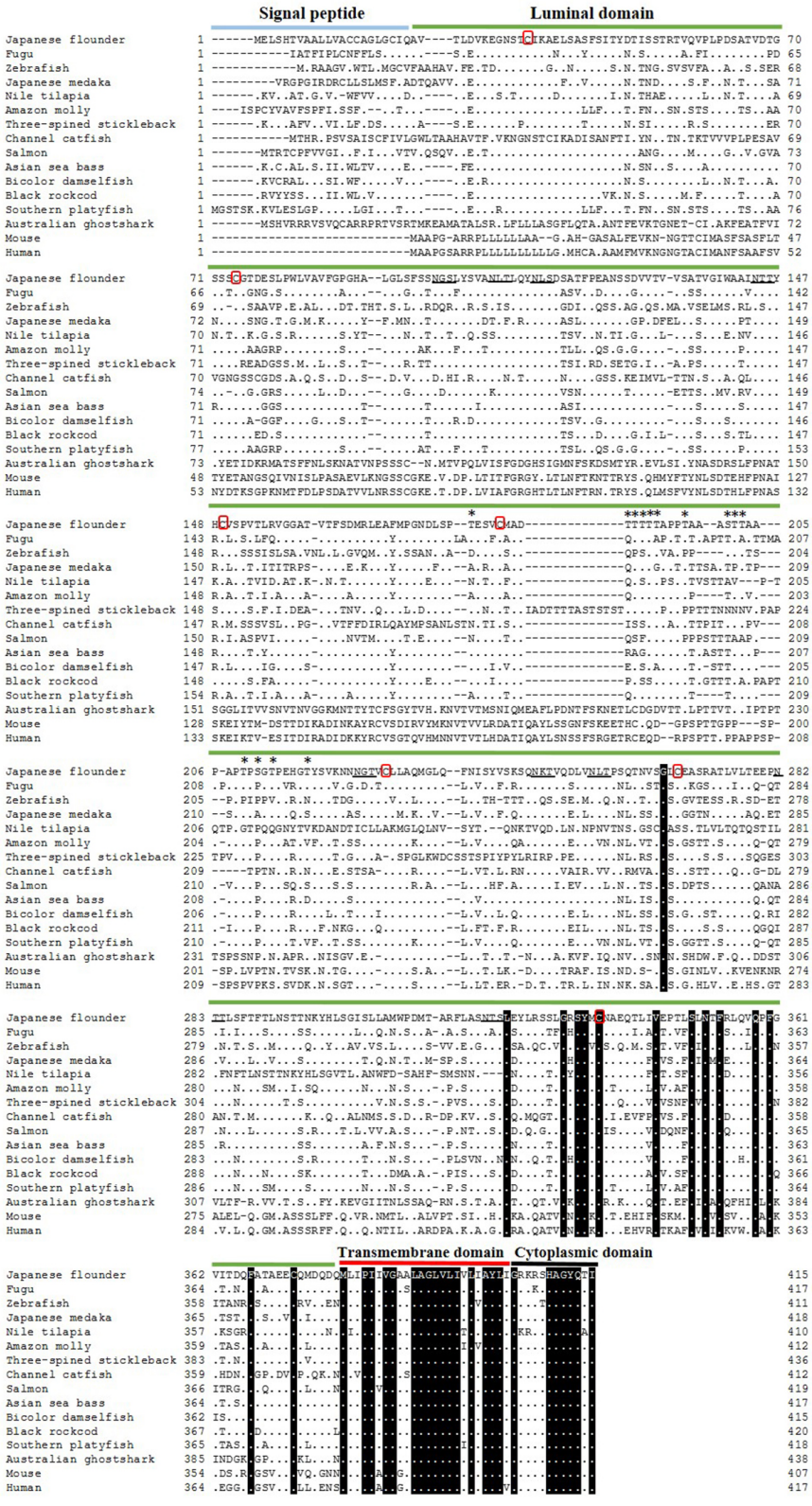

Figure 3. Multiple sequence alignment of LAMP-1 amino acid sequence. The amino acid sequences were obtained from Genbank accession numbers or Ensembl protein ID: Japanese flounder LAMP-1 (LC127058), Fugu uncharacterized protein (H2V4H1), Zebrafish LAMP-1 (Q6P299), Japanese medaka uncharacterized protein (H2LIM4), Nile tilapia (I3J577), Amazon molly LAMP-1 (XP_007554035.1), Three-spined stickleback uncharacterized protein

(G3PRL1), Channel catfish LAMP-1 (W5UCE1), Salmon LAMP-1 (C0H980), Asian sea bass predicted LAMP-1 (XP_018539319.1), Bicolor damselfish predicted LAMP-1 (XP_008273729.1), Black rockcod predicted LAMP-1 (XP_010784816.1), Southern platyfish uncharacterized protein (M4AXY6), Australian ghostshark LAMP-1 (V9KCK3), Mouse LAMP-1 (Q9DC13), Human LAMP-1 (NP_005552.3). N-linked glycosylation sites are continuous underlined and O-linked glycosylation sites are marked by asterisks. Conserved cysteine residues are boxed.

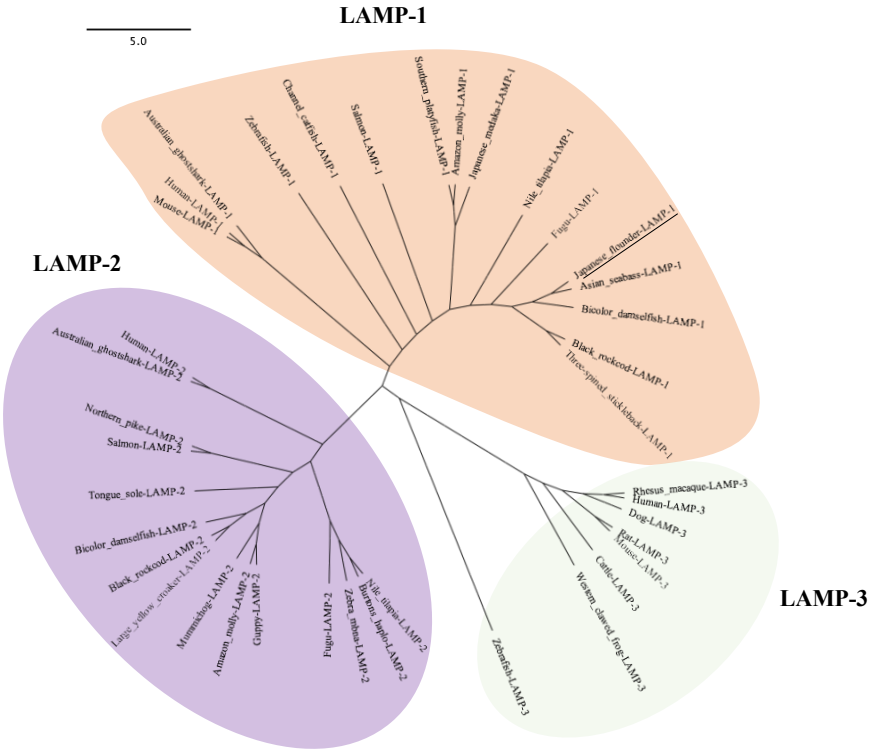

Figure 4. The Neighbor-joining phylogenetic tree of LAMP family members. The bootstrap analysis used 1000 replications. The amino acid sequences were obtained from Genbank accession numbers or Ensembl protein ID: Japanese flounder LAMP-1 (LC127058), Fugu uncharacterized protein (H2V4H1), Zebrafish LAMP-1 (Q6P299), Japanese medaka uncharacterized protein (H2LIM4), Nile tilapia (I3J577), Amazon molly LAMP-1 (XP_007554035.1), Three-spined stickleback uncharacterized protein (G3PRL1), Channel catfish LAMP-1 (W5UCE1), Salmon LAMP-1 (C0H980), Southern platyfish uncharacterized protein (M4AXY6), Australian ghostshark LAMP-1 (V9KCK3), Mouse LAMP-1 (Q9DC13), Human LAMP-1 (NP_005552.3). Green spotted pufferfish chromosome 5 SCAF14581 (Q4SHX6), Zebrafish LAMP2 (NP_001013551.1), Guppy predicted LAMP2 X1 (XP_008418857.1), Fugu predicted LAMP2 X3 (XP_011609168), Japanese medaka predicted LAMP2 X3 (XP_011478225.1), Salmon LAMP2 (NP_001133282.1), Asian sea bass predicted LAMP-1 (XP_018539319.1), Bicolor damselfish predicted LAMP-1 (XP_008273729.1), Black rockcod predicted LAMP-1 (XP_010784816.1), Nile tilapia predicted LAMP2 X1 (XP_005467465.1), Large yellow croaker predicted LAMP2 X2 (XP_010730273.1), Amazon molly predicted LAMP2 X2 (XP_007569760.1), Mummichog predicted LAMP2 (XP_012717205.1), Tongue sole predicted LAMP2 (XP_008324674.1), Atlantic herring predicted LAMP2 X1 (XP_012697575), Australian ghostshark LAMP2 (NP_001279230.1), Bicolor damselfish predicted LAMP2 (XP_008274116), Northern pike predicted LAMP2 (XP_010894624.1), Black rockcod predicted LAMP2 X2 (XP_010764723), Burton's haplo

predicted LAMP2 (XP_005913220), West Indian ocean coelacanth predicted LAMP2 (XP_006003901.1), Zebra mbna predicted LAMP2 X3 (XP_004545812.1), Human LAMP2 (AAB35426), Cattle – Bull LAMP3 (NP_001095605), Dog predicted LAMP3 X1 (XP_848889.2), Zebrafish predicted LAMP3 (XP_001342688.2), Western clawed frog predicted LAMP3 (XP_002936919.2), Rat LAMP3 (NP_001012015.1), Mouse LAMP3 (NP_796330.2), Rhesus macaque LAMP3 (NP_001028044.1), Human LAMP3 (NP_055213.2).

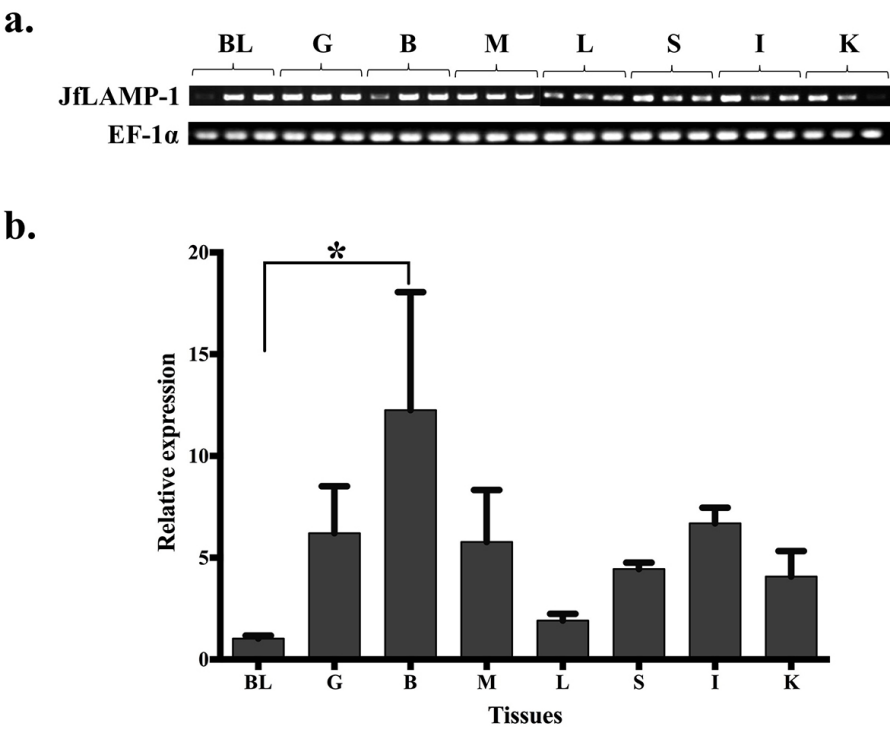

Figure 5. Tissue expression of JfLAMP-1 mRNAs in Japanese flounder. a) Tissue samples were analyzed by RT-PCR. EF1α was used as internal control. Lanes: BL, Blood; G, Gills; B, Brain; M, Muscle; L, Liver; S, Spleen; I, Intestine; K, Kidney, 25 cycles. b) qPCR analysis of JfLAMP-1 expression in Japanese flounder tissues. All data were normalized to EF-1α and blood expression set as 1. Error bars indicate standard error of the mean. Asterisks indicate statistical difference (*$p<0.05$).

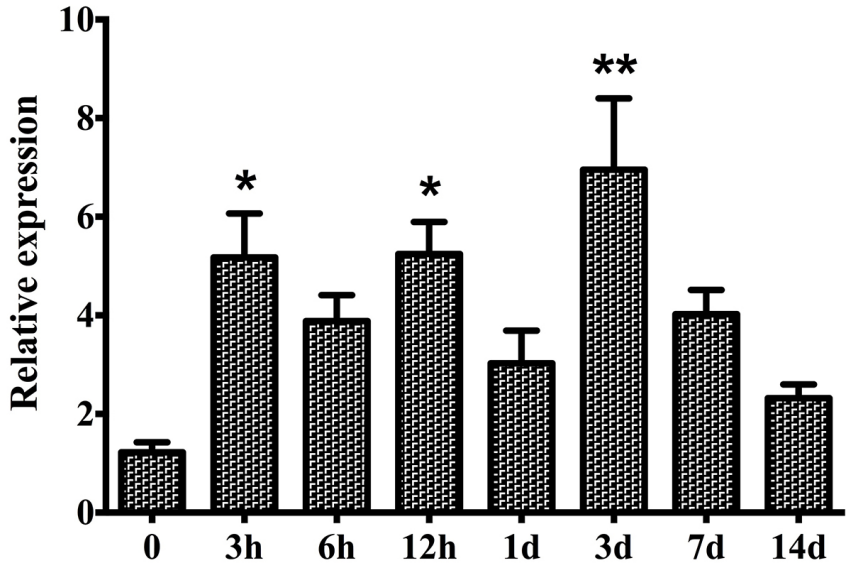

Figure 6. qPCR analysis of JfLAMP-1 expression in spleen from *Edwardsiella tarda* FKC-injected Japanese flounder at different time points at 22℃. All data were normalized to EF-1α. Error bars indicate standard error of the mean. Asterisks indicate statistical difference respect to 0h (*$p<0.05$; **$p<0.01$).

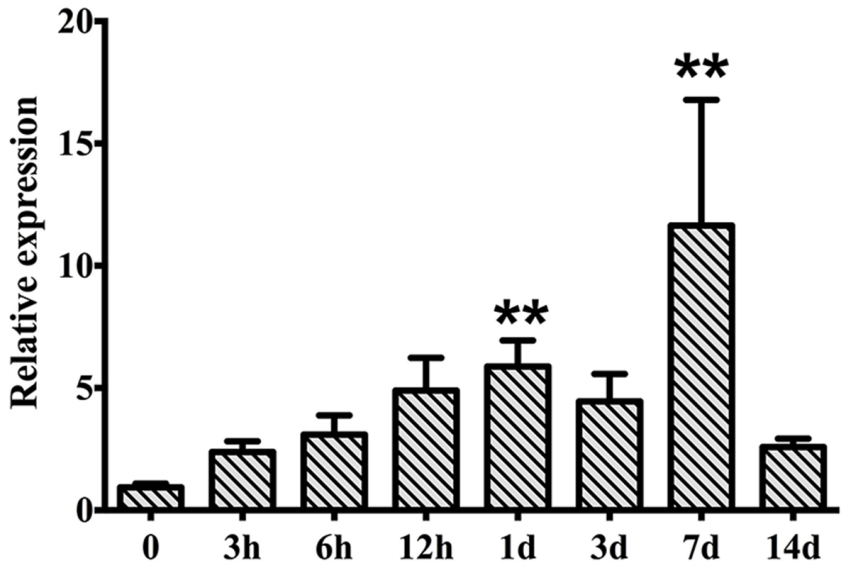

Figure 7. qPCR analysis of JfLAMP-1 expression in spleen from *Edwardsiella tarda* FKC-injected Japanese flounder at different time points at 15℃. All data were normalized to EF-1α. Error bars indicate standard error of the mean. Asterisks indicate statistical difference respect to 0h (**p<0.01).

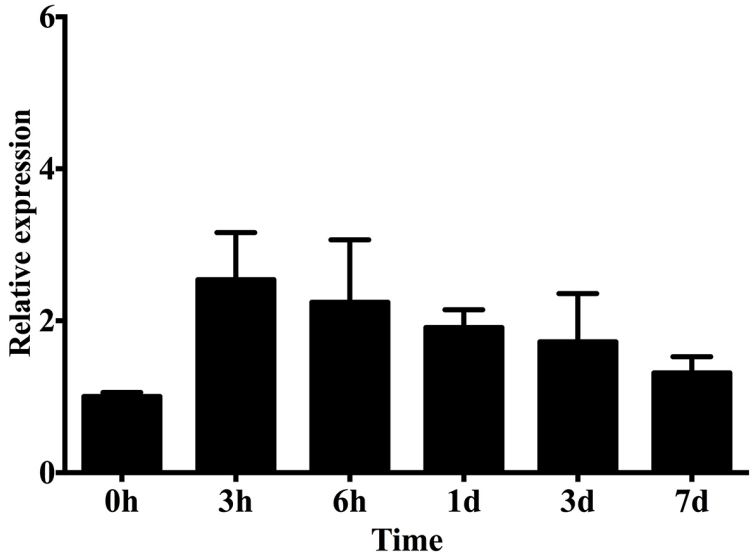

Figure 8. qPCR analysis of JfLAMP-1 expression in spleen from Poly I:C-injected Japanese flounder at different time points at 22℃. All data were normalized to EF-1α. Error bars indicate standard error of the mean.

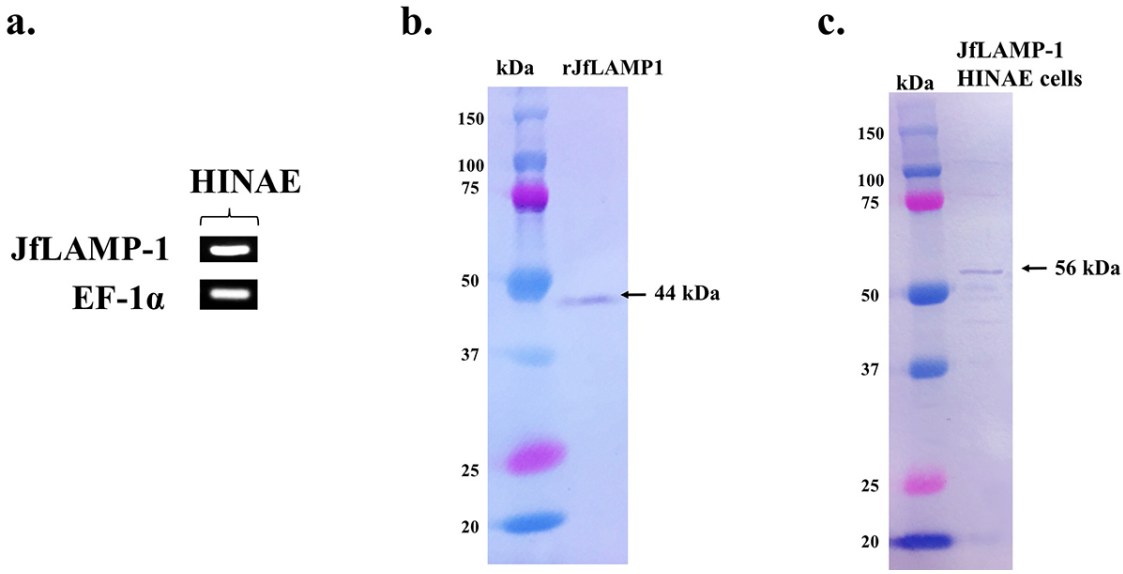

Figure 9. Expression of JfLAMP-1 in HINAE cells. a) JfLAMP-1 mRNAs in HINAE cells. EF1α was used as internal control (25 cycles). b) Western blot analysis of the expression of rJfLAMP-1. rJfLAMP-1 appeared as a band of 44 kDa. c) Western blot analysis of the expression of JfLAMP-1 in HINAE cells. JfLAMP-1 appeared as a band of 56 kDa,

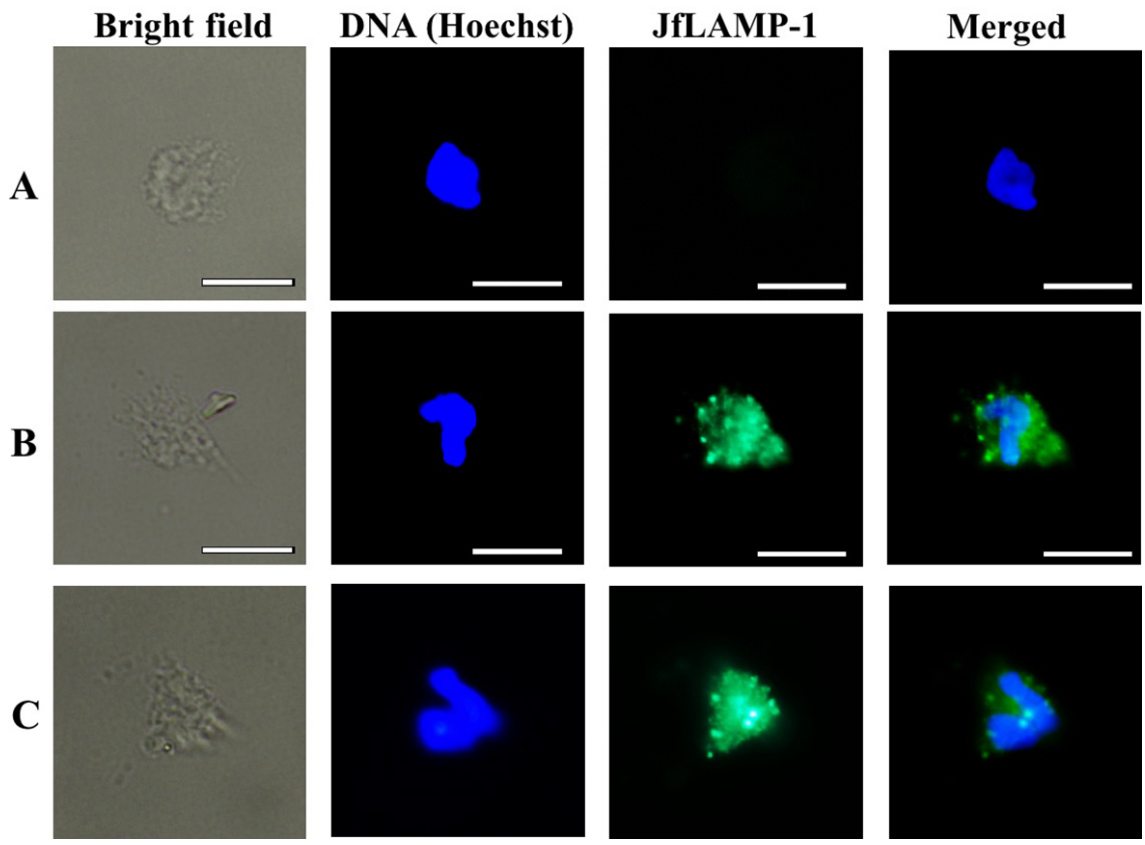

Figure 10. Expression of JfLAMP-1 in HINAE cells. Immunofluorescence staining for JfLAMP-1 in HINAE cells. A. Negative control without anti-JfLAMP-1 primary antibody. B & C. Expression of JfLAMP-1 in HINAE cells, JfLAMP-1 appears distributed in the cytoplasmic granules of different sizes and frequently grouped at the perinuclear zone. Hoechst stain was used to stain the nucleus. Bar: 10 μm.

Chapter 3

LAMP1-chimeric DNA vaccine enhances the antibody response in Japanese flounder, *Paralichthys olivaceus*

Abstract

DNA vaccination is an effective alternative against viral and bacterial diseases in fish farming that has shown promising results and gives some advantages compared with the conventional ones. Currently, chimeric antigens in a DNA vaccine have been reported to increase the protection against viral diseases. In this study, the lysosome-associated membrane protein-1 from Japanese flounder, *Paralichthys olivaceus*, (JfLAMP-1) was used in a chimeric DNA vaccine fused with the major capsule protein (MCP) from red seabream iridovirus - RSIV. In the first experiment, JfLAMP-1 and MCP gene were hybridized by replacing the JfLAMP-1 luminal domain with MCP and subsequent cloning in an expression vector (pCIneo). In the second experiment, the luminal domain of JfLAMP-1 gene was kept and the MCP gene as inserted. Fish vaccinated with the chimeric vaccines showed significantly higher antibody levels than fish vaccinated with pCIneo vector harboring the MCP gene ($p<0.05$). This study highlights the opportunity to use LAMP-1-associated chimeric vaccines as an alternative to enhance the immune response against viral pathogens.

Keywords: Antibody response, chimeric antigens, DNA vaccine, iridovirus, Japanese flounder

1. Introduction

In the last decades, global fish, shellfish and algae production has grown simultaneously with the world *per capita* seafood consumption (FAO, 2014; Micha *et al.*, 2015) and fish become an important food supply source for human beings (Godfray *et al.*, 2010; Olesen *et al.*, 2015). However, the intensive culture systems in farmed fish elicit a high vulnerability to infectious diseases and there is not a unique strategy to control them, due to the particular diversity of the environmental conditions for every country and every culture system as well as outbreaks of different strains of pathogens (Kibenge *et al.*, 2012; Shinmoto *et al.*, 2009). In case of viral diseases, the control is more difficult due to the high susceptibility of aquatic animals at an early age, the lack of therapeutics, insufficient knowledge of the pathogenesis of viral infection and limited knowledge of natural resistance mechanisms in aquatic animals (Kibenge *et al.*, 2012). Nevertheless, several strategies are employed in fish farming to prevent and control viral diseases including selective breeding of resistant fish families (Gjedrem, 2015; Olesen *et al.*, 2015), vaccination (Fu *et al.*, 2012; Yasuike *et al.*, 2011; Zheng *et al.*, 2016) and feed additives (Falco *et al.*, 2013; Yeh *et al.*, 2008).

Iridoviridae is one of the more important emerging virus family that affects fish and amphibians (Chinchar, 2002; Chinchar *et al.*, 2009). From this family, Megalocytivirus genus infects a wide range of tropical marine and freshwater fish and includes several species that affect fish such as red sea bream iridovirus (RSIV), infectious spleen and kidney necrosis virus (ISKNV) and orange spotted grouper iridovirus (OSGIV) (Kurita & Nakajima, 2012; Whittington *et al.*, 2010). RSIV has been shown to infect more than 30 species of farmed marine fish (*e.g.* sea bass, Japanese flounder, amberjack, yellowtail) causing mortality and important economic losses in Southeast Asia (Do *et al.*, 2005; Ito *et al.*, 2013; Nakajima & Kurita, 2005; Nakajima *et al.*, 1997). In Japan probably the importation of infected seedlings captured in the South China Sea was responsible for the introduction of the RSIV in the country (Chinchar *et al.*, 2009) and the disease occurs mainly in the summer, in relatively high water temperatures (Kurita & Nakajima, 2012).

For control the RSIV disease, a commercial formalin-inactivated vaccine was developed for fish (Nakajima *et al.*, 1999; Nakajima *et al.*, 1997) which rendered protection against the experimental challenge but has the limitation in the amount of viral copies able to produce in cell culture as well as that most of infected cells fail to express viral structural proteins and lost

the infectivity after several passages in culture (Kurita & Nakajima, 2012). In the same way, formalin-inactivated virus vaccines have shown low induction of cell-mediated immunity and poor immunogenicity (Davis & McCluskie, 1999) and recently were demonstrated different levels of protection against challenge depending on the viral strain (Shinmoto *et al.*, 2009). Alternative inactivation reagents have been tested in iridoviral diseases with similar results (Ou-yang *et al.*, 2012)

Previously, a RSIV DNA-free heat-denatured protein and a vaccine component that was DNA-free but with a structurally altered protein were assessed against RSIV challenge showing lower protection than the formalin-inactivated vaccine but similar neutralizing antibody level and gene expression induction (Caipang *et al.*, 2006a). On the other hand, several studies using vaccination based on recombinant protein antigens (Caipang *et al.*, 2006a; Drennan *et al.*, 2007; Shimmoto *et al.*, 2010) and DNA vaccines (Caipang *et al.*, 2006b; Zheng *et al.*, 2016) have been reported in order to increase the immunity against iridoviral pathogens. Recombinant RSIV's major capsid protein (MCP) subunits (r18P, r351P, and rMCP) were evaluated as subunit vaccines in red sea bream (*Pagrus major*) showing variable protection levels with survival rates from 27-52% after RSIV challenge, but higher that those showed by control group. For DNA vaccines, a DNA vaccine encoding for two different RSIV's ORFs showed similar protection to formalin-inactivated RSIV vaccinated group (Caipang *et al.*, 2006b). Thus, the alternatives to increase the protection against RSIV require new approaches that improve the efficacy at an appropriated cost:benefit ratio

Recently, DNA vaccine-encoding chimeric antigens have shown promising results in animal models against viral and bacterial diseases (Deb *et al.*, 2015; Dhalia *et al.*, 2009; Klucar *et al.*, 2009). Among them, the use of lysosome associated membrane protein (LAMP)-chimeric vaccines have been assessed against viral pathogens rendering enhanced immune response (Arruda *et al.*, 2006; de Arruda *et al.*, 2004; Dhalia *et al.*, 2009; Marques *et al.*, 2003). Lysosomal membrane proteins are highly glycosylated (Fukuda, 1991; Hatakeyama *et al.*, 2014) and it was estimated that lysosome associated membrane proteins (LAMPs) and lysosome integrated membrane proteins (LIMP) constitute about 50% of all proteins in lysosomal membrane (Hunziker *et al.*, 1996; Marsh *et al.*, 1987) and some of them can be expressed on the cell surface (Furuta *et al.*, 1999; Hatakeyama *et al.*, 2014; Tompkins *et al.*, 2006). The sorting of LAMPs resides in a short terminal tail (10-11 amino acids), which is

exposed, on the cytoplasmic side of the membrane (Dahlgren *et al.*, 1995) and their tissue distribution differs between organs (Furuta *et al.*, 1999). Besides of its function as interacting molecules LAMP-1 showed to protect natural killer cells from the degranulation-associated damage (Cohnen *et al.*, 2013). The aim of this study was to show the potential use of Japanese flounder LAMP-1 (JfLAMP-1) as a chimeric DNA vaccine carrier to enhance the antibody response against RSIV.

2. Material and methods

2.1. Plasmid DNA construction and preparation

The cloning and sequence of JfLAMP-1 was described in the chapter 2. The full length of major capsid protein (MCP) gene of red seabream iridovirus was amplified by PCR from a recombinant plasmid containing MCP using specific primers with *Nde*I and *Eco*RI restriction enzymes sites (Table 1), cloned into pGEM T-easy vector (Promega, USA) and transformed in *E. coli* JM109 strain (Wako, Japan). Plasmid was extracted and double digested with corresponding enzymes, purified by electrophoresis and inserted in a pCIneo vector (Promega, USA) digested with the same restriction enzymes. The recombinant plasmids were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with a 3130xl genetic analyzer (Applied Biosystems, USA). pCIneo vector harboring MCP gene (pCMCP) and pCIneo were purified from overnight cultures.

Plasmid was purified by cesium chloride (CsCl) density gradient centrifugation (Noles, 2008; Pollard & Grady, 1967). For each vaccination treatment, *E. coli* JM109 strain-containing a recombinant plasmid was cultured overnight at 37 ℃ in 2 ml of 2x YT broth containing ampicillin. Then, overnight culture was transferred to a flask with 200 ml of 2x YT broth with ampicillin and cultured for 18 h at 37 ℃. After culture, cells were harvested at 5000 rpm for 10 min at 4 ℃. Plasmid was extracted from the bacterial pellet by alkaline lysis method. After, isopropanol and ethanol precipitation, pellet was digested by RNAse at 37 ℃ for 1 h and CsCl (3.88 g) was added after digestion. This mixture was placed into an OptiSeal tube (Beckman Coulter, USA) and ethidium bromide (100 µl) and Tris-EDTA (TE) buffer were added until the tube was filled out completely. Tube was under ultracentrifugation at 65,000 rpm for 20 h at 20 ℃ and the plasmid DNA layer was collected by puncture with a needle. Ethidium bromide was removed with saturated 2-propanol and the sample dialised by using dialysis bag in TE buffer.

2.2. Construction of pCLAMP-MCP and pCLAMP-MCPLumD vaccines

For production of chimeric DNA vaccine of JfLAMP1-MCP (pCLAMP-MCP), primers were designed to hybridize the JfLAMP-1 and iridoviral MCP gene (Table 1), based on an

overlapping of the sequences at the ends (Grandori *et al.*, 1997). MCP gene from iridovirus was inserted into the JfLAMP-1 gene by replacing the luminal domain from JfLAMP-1 and subsequent hybridization of the genes in the remaining domains. For that, signal peptide from JfLAMP-1 was fused with 5'-end of MCP and 3'-end of MCP was hybridized with 5'-end of transmembrane domain/cytoplasmic domain of JfLAMP-1 (Figure 1). Signal peptide, MCP and transmembrane domain/cytoplasmic domain were amplified by separated and fused by pairs. Restriction enzymes sites (*Nde*I and *Eco*RI) were added at the end of JfLAMP-1 and the PCR product was cloned in cloned into pGEM T-easy vector (Promega, USA). After restricted digestion of the plasmid, the chimeric gene insert was cloned in a pCIneo vector (Promega, USA) digested with the same restriction enzymes and its sequence was verified. For the pCLAMP-MCPLumD vaccine, the luminal domain (LumD) of JfLAMP-1 gene was kept and the MCP gene was inserted (Figure 1c).

2.3. Vaccination experiments

For vaccination experiments, Japanese flounder with an average size 10 cm in total length were acclimated and reared at 18 ℃ in artificial seawater for a week prior the start of the experiments. In the pCLAMP-MCP vaccination, experimental animals were distributed in three groups and injected intramuscularly with 10 μg of pcMCP; 10 μg of pCLAMP-MCP, and pCI-neo vector (negative control) in 50 μl of saline buffer near to the dorsal fin. On the other hand, in the pCLAMP-MCPLumD vaccination, experimental animals were distributed in six groups and injected intramuscularly with 10 μg of pcMCP; 10 μg of pCLAMP-MCP, 10 μg of pCLAMP-MCPLumD and as negative control pCI-neo vector, pCLAMP, PBS were used, in 50 μl of saline buffer.

The presence of MCP in muscle at 3rd day post-injection was confirmed by RT-PCR using muscle cDNA as template and RSIV MCP specific primers (Table 1). For this, total RNA was extracted from muscle of vaccinated Japanese flounder using RNAiso plus reagent (Takara Bio. Inc., Japan). cDNA was synthesized using SuperScript™ III First Strand synthesis system M-MLV (Life Technologies, USA). RT-PCR was performed with cDNA templates (5 times diluted) from each tissue using specific primer sets under the following PCR conditions: initial denaturation at 95 °C for 4 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C

for 1 min and a final extension of 72 °C for 7 min. EF-1α gene was used as an internal control. The PCR products were electrophoresed on 2% agarose gels.

2.4. Analysis of antibody response

At day 30 post-vaccination, fish from each group were assayed for antibody response against MCP by enzyme-linked immunosorbent assay (ELISA) according to Taechavasonyoo *et al.* (2013). Briefly, a 96-well plate was coated 2 h at 25 °C with rMCP (20 μg/ml) in 100 μl of PBS. The plate was washed three times with PBS-T (PBS containing 0.05% Tween 20) and then blocked overnight with 5% bovine serum albumin (BSA, Sigma-Aldrich, Germany) in PBS at 4 °C. After washing three times with PBST, the diluted sera (1:20) obtained from individually vaccinated fish were added to wells of the plate and incubated at 25 °C for 1 h. The plate was then washed thrice and rabbit anti-Japanese flounder immunoglobulin M serum (1:3000) was added to the plate, and incubated at 25 °C for 1 h. After washing thrice, alkaline phosphatase-conjugated goat anti-rabbit IgG (1:1000) (Promega, Madison, WI) was added to the wells and incubated at 25 °C for 1 h. The reaction was developed by addition of AP substrate (Sigma-Aldrich, Germany) and stopped by addition of 3 M NaOH. The absorbance was then measured with a microtiter plate reader (Multiskan Go, Thermo Scientific, Finland) at the wavelength of 405 nm.

2.4. Statistical analysis

A statistical analysis for antibody response was performed by one-way ANOVA and Tukey's multiple comparisons of the means were done (GraphPad Prism Version 6.0 for Windows, USA). Differences were considered statistically significant when $p < 0.05$.

3. Results

At the 3rd day of vaccination, MCP gene was detected by RT-PCR, in the pCMCP, pCLAMP-MCP and the pCLMPA-MCPLumD groups. The pCLAMP-MCPLumD showed a weaker band compared with the other 2 groups (Figure 3a). The muscle samples from PBS, pCIneo and pCLAMP groups were negative to MCP gene.

3.1. Antibody response in vaccination

Antibody levels against RSIV MCP in serum showed that pCLAMP-MCP elicited significant higher antibody response compared with pCMCP and the pCIneo (Figure 2). In the second experiment, the inclusion of the LumD in the chimeric vaccine showed to induce higher antibody titers against RSIV MCP compared with pCMCP, pCLAMP, pCIneo and PBS groups, however the antibody levels were higher but not statistically different compared with the pCLAMP-MCP (Figure 3b).

4. Discussion

Vaccination is one of the prophylactic strategies applied in aquaculture to increase the protection against diseases (Newman, 1993; Sommerset *et al.*, 2005). In fish, several vaccines have been tested with variable results (Byon *et al.*, 2005; Byon *et al.*, 2006; Hølvold *et al.*, 2014; Ou-yang *et al.*, 2012; Penaranda *et al.*, 2011; Shin *et al.*, 2013; Sommerset *et al.*, 2005; Sun *et al.*, 2010; Tonheim *et al.*, 2008) and DNA vaccines have shown some advantages and high level of protection compared with the conventional modified/inactivated antigen-based vaccines (Byon *et al.*, 2006; Martinez-Lopez *et al.*, 2014; Tonheim *et al.*, 2008; Yasuike *et al.*, 2007), including that are relatively inexpensive and easy to produce, and can be manufactured using identical production processes (Tonheim *et al.*, 2008). Recently, chimeric antigens in DNA vaccines were proposed as an alternative to increase the immune response against parasitic, bacterial and viral pathogens (Grigera *et al.*, 1996; Nawaratna *et al.*, 2015; Starodubova *et al.*, 2010). In this study, a JfLAMP-1-MCP DNA vaccines (pCLAMP-MCP and pCLAMP-MCPLumD) were tested for their ability to induce antibody response in Japanese flounder. The results showed that the pCLAMP-MCP and pCLAMP-MCPLumD chimera DNA vaccine elicited significant higher antibody levels in serum against MCP than the pCMCP. These results are in agreement with studies in which LAMP-chimeric antigens in a DNA vaccine induced higher antibody titers than the single plasmid-encoding antigen in murine models (Arruda *et al.*, 2006; de Arruda *et al.*, 2004; Marques *et al.*, 2003).

In the present study, pCLAMP-MCPLumD induced higher anti-MCP antibodies compared with the pCMCP, however the antibody titers were not higher than pCLAMP-MCP. Previously, Marques *et al.* (2003) the inclusion of LumD of LAMP-1 in a chimeric vaccine induce higher antibody titer than one without it. This higher induction was correlated with a resistance to the early proteolysis (Arterburn *et al.*, 1990) or biased trafficking of the antigen through major histocompatibility complex (MHC) class II pathway (Arruda *et al.*, 2006; Marques *et al.*, 2003)

DNA vaccines have the ability to stimulate both cellular and humoral immunity (Wang *et al.*, 1998). However, since the antigens expressed in DNA vaccine are produced inside the cells commonly they are processed in MHC class I pathway which induce mainly cytotoxicity

response (Tonheim *et al.*, 2008). By using LAMP as a carrier molecule is possible to drive the traffic of the antigen to the MHC class II processing pathway, to expose cryptic epitopes (Arruda *et al.*, 2006; Starodubova *et al.*, 2010) and to enhance the CD4+ T cell priming/activation which is vital for the function of genetic vaccines (Arruda *et al.*, 2006). The co-localization of LAMP-chimeric antigen from a DNA vaccine and MHC class II has been demonstrated in previous studies (Anwar *et al.*, 2005; Arruda *et al.*, 2006; de Arruda *et al.*, 2004; Marques *et al.*, 2003). The optimization antigen expression/traffic will allow to reduce the amount/concentration of DNA vaccine dose used in *in vivo* immunization (Dhalia *et al.*, 2009) and the "labeling" of the antigens with specialized signal sequences can be an alternative in order to enhance the immune response against pathogens in fish.

In conclusion, the use of JfLAMP-1 as an antigen carrier in a DNA vaccine showed to enhance the antibody immune response against iridoviral MCP, compared with the conventional DNA vaccine and can be an alternative to study in the design of new approaches to fish vaccination.

References

Anwar, A., Chandrasekaran, A., Ng, M. L., Marques, E., & August, J. T. (2005). West Nile premembrane-envelope genetic vaccine encoded as a chimera containing the transmembrane and cytoplasmic domains of a lysosome-associated membrane protein: increased cellular concentration of the transgene product, targeting to the MHC II compartment, and enhanced neutralizing antibody response. *Virology, 332*(1), 66-77.

Arruda, L. B., Sim, D., Chikhlikar, P. R., Maciel, M., Jr., Akasaki, K., August, J. T., & Marques, E. T. (2006). Dendritic cell-lysosomal-associated membrane protein (LAMP) and LAMP-1-HIV-1 gag chimeras have distinct cellular trafficking pathways and prime T and B cell responses to a diverse repertoire of epitopes. *Journal of Immunology, 177*(4), 2265-2275.

Arterburn, L. M., Earles, B. J., & August, J. T. (1990). The disulfide structure of mouse lysosome-associated membrane protein 1. *Journal of Biological Chemistry, 265*(13), 7419-7423.

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 Immunology, 18*(2), 135-147.

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*(7), 921-930.

Caipang, C. M., Hirono, I., & Aoki, T. (2006a). Immunogenicity, retention and protective effects of the protein derivatives of formalin-inactivated red seabream iridovirus (RSIV) vaccine in red seabream, *Pagrus major*. *Fish & Shellfish Immunology, 20*(4), 597-609.

Caipang, C. M., Takano, T., Hirono, I., & Aoki, T. (2006b). Genetic vaccines protect red seabream, *Pagrus major*, upon challenge with red seabream iridovirus (RSIV). *Fish & Shellfish Immunology, 21*(2), 130-138.

Chinchar, V. G. (2002). Ranaviruses (family Iridoviridae): emerging cold-blooded killers. *Archives of Virology, 147*(3), 447-470.

Chinchar, V. G., Hyatt, A., Miyazaki, T., & Williams, T. (2009). Family Iridoviridae: poor viral relations no longer. *Curr Top Microbiol Immunol, 328*, 123-170.

Cohnen, A., Chiang, S. C., Stojanovic, A., Schmidt, H., Claus, M., Saftig, P., Janssen, O., Cerwenka, A., Bryceson, Y. T., & Watzl, C. (2013). Surface CD107a/LAMP-1 protects natural killer cells from degranulation-associated damage. *Blood, 122*(8), 1411-1418.

Dahlgren, C., Carlsson, S. R., Karlsson, A., Lundqvist, H., & Sjolin, C. (1995). The lysosomal membrane glycoproteins Lamp-1 and Lamp-2 are present in mobilizable organelles, but are absent from the azurophil granules of human neutrophils. *Biochemical Journal, 311 (Pt 2)*, 667-674.

Davis, H. L., & McCluskie, M. J. (1999). DNA vaccines for viral diseases. *Microbes and Infection, 1*(1), 7-21.

de Arruda, L. B., Chikhlikar, P. R., August, J. T., & Marques, E. T. (2004). DNA vaccine encoding human immunodeficiency virus-1 Gag, targeted to the major histocompatibility complex II compartment by lysosomal-associated membrane protein, elicits enhanced long-term memory response. *Immunology, 112*(1), 126-133.

Deb, R., Dey, S., Madhan Mohan, C., Gaikwad, S., Kamble, N., Khulape, S. A., Gupta, S. K., Maity, H. K., & Pathak, D. C. (2015). Development and evaluation of a *Salmonella typhimurium* flagellin based chimeric DNA vaccine against infectious bursal disease of poultry. *Research in Veterinary Science, 102*, 7-14.

Dhalia, R., Maciel Jr., M., Cruz, F. S. P., Viana, I. F. T., Palma, M. L., August, T., & Marques Jr., E. T. A. (2009). Membrane and envelope virus proteins co-expressed as lysosome associated membrane protein (LAMP) fused antigens: a potential tool to develop DNA vaccines against flaviviruses. *Anais da Academia Brasileira de Ciências, 81*, 663-669.

Do, J. W., Cha, S. J., Kim, J. S., An, E. J., Lee, N. S., Choi, H. J., Lee, C. H., Park, M. S., Kim, J. W., Kim, Y. C., & Park, J. W. (2005). Phylogenetic analysis of the major capsid protein gene of iridovirus isolates from cultured flounders *Paralichthys olivaceus* in Korea. *Diseases of Aquatic Organisms, 64*(3), 193-200.

Drennan, J. D., LaPatra, S. E., Swan, C. M., Ireland, S., & Cain, K. D. (2007). Characterization of serum and mucosal antibody responses in white sturgeon (*Acipenser transmontanus* Richardson) following immunization with WSIV and a protein hapten antigen. *Fish & Shellfish Immunology, 23*(3), 657-669.

Falco, A., Miest, J., Pionnier, N., Pietretti, D., Forlenza, M., Wiegertjes, G. F., & Hoole, D. (2013). β-glucan supplemented diets induce high and broad expression levels of TLR3 what explains protection conferred by these additives against viral infections in fish. *Fish & Shellfish Immunology, 34*(6), 1706.

FAO. (2014). *The state of world fisheries and aquaculture: Opportunities and challenges* (Food and Agriculture Organization of the United Nations Ed.). Rome: Food and Agriculture Organization of the United Nations.

Fu, X., Li, N., Lai, Y., Liu, L., Lin, Q., Shi, C., Huang, Z., & Wu, S. (2012). Protective immunity against iridovirus disease in mandarin fish, induced by recombinant major capsid protein of infectious spleen and kidney necrosis virus. *Fish & Shellfish Immunology, 33*(4), 880-885.

Fukuda, M. (1991). Lysosomal membrane glycoproteins. Structure, biosynthesis, and intracellular trafficking. *Journal of Biological Chemistry, 266*(32), 21327-21330.

Furuta, K., Yang, X. L., Chen, J. S., Hamilton, S. R., & August, J. T. (1999). Differential expression of the lysosome-associated membrane proteins in normal human tissues. *Archives of Biochemistry and Biophysics, 365*(1), 75-82.

Gjedrem, T. (2015). Disease resistant fish and shellfish are within reach: A review. *Journal of Marine Science and Engineering, 3*(1), 146.

Godfray, H. C. J., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., Nisbett, N., Pretty, J., Robinson, S., Toulmin, C., & Whiteley, R. (2010). The future of the global food system. *Philosophical Transactions of the Royal Society B: Biological Sciences, 365*(1554), 2769-2777.

Grandori, R., Struck, K., Giovanielli, K., & Carey, J. (1997). A three-step PCR protocol for construction of chimeric proteins. *Protein Engineering, 10*(9), 1099-1100.

Grigera, P. R., Garcia-Briones, M., Periolo, O., la Torre, J. L., & Wagner, R. R. (1996). Immunogenicity of an aphthovirus chimera of the glycoprotein of vesicular stomatitis virus. *Journal of Virology, 70*(12), 8492-8501.

Hatakeyama, Y., Hatakeyama, J., Oka, K., Tsuruga, E., Inai, T., Anan, H., & Sawa, Y. (2014). Immunohistochemical study of amelogenin and lysosome-associate membrane proteins (LAMPs) in cartilage. *International Journal of Morphology, 32*, 618-626.

Hølvold, L. B., Myhr, A. I., & Dalmo, R. A. (2014). Strategies and hurdles using DNA vaccines to fish. *Veterinary Research, 45*(1), 21-21.

Hunziker, W., Simmen, T., & Honing, S. (1996). Trafficking of lysosomal membrane proteins in polarized kidney cells. *Nephrologie, 17*(7), 347-350.

Ito, T., Yoshiura, Y., Kamaishi, T., Yoshida, K., & Nakajima, K. (2013). Prevalence of red sea bream iridovirus among organs of Japanese amberjack (*Seriola quinqueradiata*) exposed to cultured red sea bream iridovirus. *Journal of General Virology, 94*(Pt 9), 2094-2101.

Kibenge, F. S. B., Godoy, M. G., Fast, M., Workenhe, S., & Kibenge, M. J. T. (2012). Countermeasures against viral diseases of farmed fish. *Antiviral Research, 95*(3), 257-281.

Klucar, P., Barnes, P. F., Kong, Y., Howard, S. T., Pang, X., Huang, F. F., Tvinnereim, A. R., Samten, B., & Shams, H. (2009). Vaccination strategies to enhance local immunity and protection against *Mycobacterium tuberculosis*. *Vaccine, 27*(12), 1816-1824.

Kurita, J., & Nakajima, K. (2012). Megalocytiviruses. *Viruses, 4*(4), 521-538.

Marques, E. T., Jr., Chikhlikar, P., de Arruda, L. B., Leao, I. C., Lu, Y., Wong, J., Chen, J. S., Byrne, B., & August, J. T. (2003). HIV-1 p55Gag encoded in the lysosome-associated membrane protein-1 as a DNA plasmid vaccine chimera is highly expressed, traffics to the major histocompatibility class II compartment, and elicits enhanced immune responses. *Journal of Biological Chemistry, 278*(39), 37926-37936.

Marsh, M., Schmid, S., Kern, H., Harms, E., Male, P., Mellman, I., & Helenius, A. (1987). Rapid analytical and preparative isolation of functional endosomes by free flow electrophoresis. *Journal of Cell Biology, 104*(4), 875-886.

Martinez-Lopez, A., Garcia-Valtanen, P., Ortega-Villaizan, M., Chico, V., Gomez-Casado, E., Coll, J. M., & Estepa, A. (2014). VHSV G glycoprotein major determinants implicated in triggering the host type I IFN antiviral response as DNA vaccine molecular adjuvants. *Vaccine, 32*(45), 6012-6019.

Micha, R., Khatibzadeh, S., Shi, P., Andrews, K. G., Engell, R. E., & Mozaffarian, D. (2015). Global, regional and national consumption of major food groups in 1990 and 2010: a systematic analysis including 266 country-specific nutrition surveys worldwide. *BMJ Open, 5*(9), e008705.

Nakajima, K., & Kurita, J. (2005). Red sea bream iridoviral disease. *Uirusu, 55*(1), 115-125.

Nakajima, K., Maeno, Y., Honda, A., Yokoyama, K., Tooriyama, T., & Manabe, S. (1999). Effectiveness of a vaccine against red sea bream iridoviral disease in a field trial test. *Diseases of Aquatic Organisms, 36*(1), 73-75.

Nakajima, K., Maeno, Y., Kurita, J., & Inui, Y. (1997). Vaccination against red sea bream iridoviral disease in red sea bream. *Fish Pathology, 32*(4), 205-209.

Nawaratna, S. S., Gobert, G. N., Willis, C., Mulvenna, J., Hofmann, A., McManus, D. P., & Jones, M. K. (2015). Lysosome-associated membrane glycoprotein (LAMP)--preliminary study on a hidden antigen target for vaccination against schistosomiasis. *Scientific Reports, 5*, 15069.

Newman, S. G. (1993). Bacterial vaccines for fish. *Annual Review of Fish Diseases, 3*.

Noles, S. R. (2008). *Traditional methods for CsCl isolation of plasmid DNA by ultracentrifugation*. Retrieved from United States: https://tools.thermofisher.com/content/sfs/brochures/D17309~.pdf

Olesen, I., Bentsen, H., Phillips, M., & Ponzoni, R. (2015). Can the global adoption of genetically improved farmed fish increase beyond 10%, and how?. *Journal of Marine Science and Engineering, 3*(2), 240.

Ou-yang, Z., Wang, P., Huang, X., Cai, J., Huang, Y., Wei, S., Ji, H., Wei, J., Zhou, Y., & Qin, Q. (2012). Immunogenicity and protective effects of inactivated Singapore grouper iridovirus (SGIV) vaccines in orange-spotted grouper, *Epinephelus coioides*. *Developmental & Comparative Immunology, 38*(2), 254-261.

Ou-yang, Z., Wang, P., Huang, Y., Huang, X., Wan, Q., Zhou, S., Wei, J., Zhou, Y., & Qin, Q. (2012). Selection and identification of Singapore grouper iridovirus vaccine candidate antigens using bioinformatics and DNA vaccination. *Veterinary Immunology and Immunopathology, 149*(1–2), 38-45.

Penaranda, M. M., Lapatra, S. E., & Kurath, G. (2011). Specificity of DNA vaccines against the U and M genogroups of infectious hematopoietic necrosis virus (IHNV) in rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology, 31*(1), 43-51.

Pollard, E. C., & Grady, L. J. (1967). CsCl density gradient centrifugation studies of intact bacterial cells. *Biophysical Journal, 7*(2), 205-213.

Shimmoto, H., Kawai, K., Ikawa, T., & Oshima, S. (2010). Protection of red sea bream *Pagrus major* against red sea bream iridovirus infection by vaccination with a recombinant viral protein. *Microbiology and Immunology, 54*(3), 135-142.

Shin, Y. J., Kwon, T. H., Seo, J. Y., & Kim, T. J. (2013). Oral immunization of fish against iridovirus infection using recombinant antigen produced from rice callus. *Vaccine, 31*(45), 5210-5215.

Shinmoto, H., Taniguchi, K., Ikawa, T., Kawai, K., & Oshima, S.-i. (2009). Phenotypic diversity of infectious red sea bream iridovirus isolates from cultured fish in Japan. *Applied Environmental Microbiology, 75*(11), 3535-3541.

Sommerset, I., Krossoy, B., Biering, E., & Frost, P. (2005). Vaccines for fish in aquaculture. *Expert Reviews of Vaccines, 4*(1), 89-101.

Starodubova, E. S., Isaguliants, M. G., & Karpov, V. L. (2010). Regulation of immunogen processing: signal sequences and their application for the new generation of DNA-vaccines. *Acta Naturae, 2*(1), 53-60.

Sun, Y., Liu, C., & Sun, L. (2010). Construction and analysis of the immune effect of an *Edwardsiella tarda* DNA vaccine encoding a D15-like surface antigen. *Fish & Shellfish Immunology, 30*, 273-279.

Taechavasonyoo, A., Hirono, I., & Kondo, H. (2013). The immune-adjuvant effect of Japanese flounder *Paralichthys olivaceus* IL-1β. *Developmental & Comparative Immunology, 41*(4), 564-568.

Tompkins, K., George, A., & Veis, A. (2006). Characterization of a mouse amelogenin [A−4]/M59 cell surface receptor. *Bone, 38*(2), 172-180.

Tonheim, T. C., Bøgwald, J., & Dalmo, R. A. (2008). What happens to the DNA vaccine in fish? A review of current knowledge. *Fish & Shellfish Immunology, 25*(1–2), 1-18.

Wang, B., Godillot, A. P., Madaio, M. P., Weiner, D. B., & Williams, W. V. (1998). Vaccination against pathogenic cells by DNA inoculation. In H. Koprowski & D. B. Weiner (Eds.), *DNA Vaccination/Genetic Vaccination* (pp. 21-35). Berlin, Heidelberg: Springer Berlin Heidelberg.

Whittington, R. J., Becker, J. A., & Dennis, M. M. (2010). Iridovirus infections in finfish - critical review with emphasis on ranaviruses. *Journal of Fish Diseases, 33*(2), 95-122.

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 Immunology, 23*(3), 531-541.

Yasuike, M., Kondo, H., Hirono, I., & Aoki, T. (2011). Gene expression profile of HIRRV G and N protein gene vaccinated Japanese flounder, *Paralichthys olivaceus* during HIRRV infection. *Comparative Immunology, Microbiology and Infectious Diseases, 34*(2), 103-110.

Yeh, S.-P., Chang, C.-A., Chang, C.-Y., Liu, C.-H., & Cheng, W. (2008). Dietary sodium alginate administration affects fingerling growth and resistance to *Streptococcus* sp. and iridovirus, and juvenile non-specific immune responses of the orange-spotted grouper, *Epinephelus coioides*. *Fish & Shellfish Immunology, 25*(1–2), 19-27.

Zheng, F., Liu, H., Sun, X., Zhang, Y., Zhang, B., Teng, Z., Hou, Y., & Wang, B. (2016). Development of oral DNA vaccine based on chitosan nanoparticles for the immunization against reddish body iridovirus in turbots (*Scophthalmus maximus*). *Aquaculture, 452*, 263-271.

Table 1. PCR primers used in this study.

Target	**Usage**	**Primer sequence (5′–3′)***
JfLAMP ORF	Cloning F	AACATATGGAACTCTCTCACACGGT
	Cloning R	GAATTCGATGGTCTGGTATCC
EF1α	RT PCR/qPCR F	CTCGGGCATAGACTCGTGGT
	RT PCR/qPCR R	CATGGTCGTGACCTTCGCTC
MCP	Cloning F	GGGCATATGTCTGCAATCTCAGGTGC
	Cloning R	CCCGAATTCTTAATGATGATGATGAT
	RT-PCR F	ATCAAAACAGACTGGCCATGCTAAT
	RT-PCR R	AAATTATCACACCAGCGAATGTAGC
LAMP-SigPep (MCP)**	Hybridization R	GATCGCAGACATAGCCTGAATACAA
(LAMP-SigPep) MCP	Hybridization F	TTGTATTCAGGCTATGTCTGCGATC
MCP (LAMPTrasDom)	Hybridization R	GGGATGAGCATCAGGATAGGGAAGC
(MCP) LAMPTrasDom	Hybridization F	GCTTCCCTATCCTGATGCTCATCCC
MCP (LAMPLumD)	Hybridization R	GGACCAGGACCAGATGGTACGCAAA
(MCP) LAMPLumD	Hybridization F	CTTTGCGTACCATCTGGTCCTGGTCC

*Restriction enzyme sites (*Nde*I and *Eco*RI) are underlined at the start of the sequence. **Between parentheses appear the position of the gene which nucleotides are added in order to hybridize at the ends of each domain.

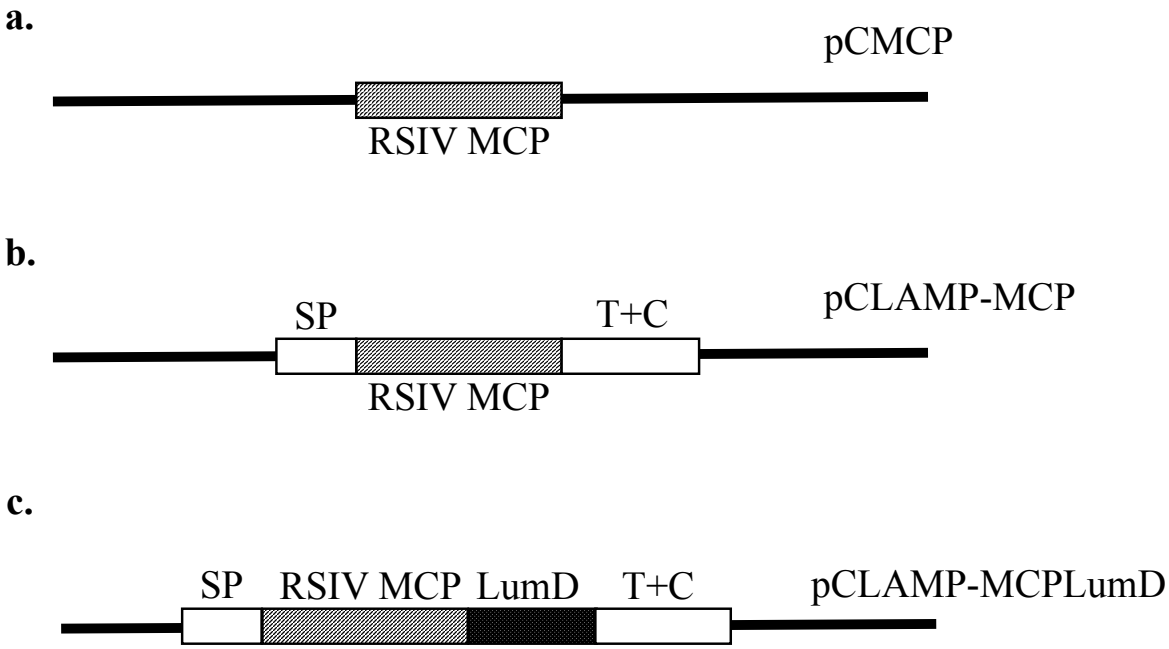

Figure 1. Schematic representation of the expression plasmids used for vaccination experiments. a) pCMCP, pCIneo harboring RSIV MCP gene. b) pCLAMP-MCP, pCIneo harboring the chimeric antigen, luminal domain (LumD) from JfLAMP-1 was replaced with RSIV MCP gene c) pCLAMP-MCPLumD, pCIneo harboring the chimeric antigen, RSIV MCP gene was inserted between LumD and transmembrane domain (T) of JfLAMP-1. SP, signal peptide domain; T+C, Transmembrane +Cytoplasmic domain.

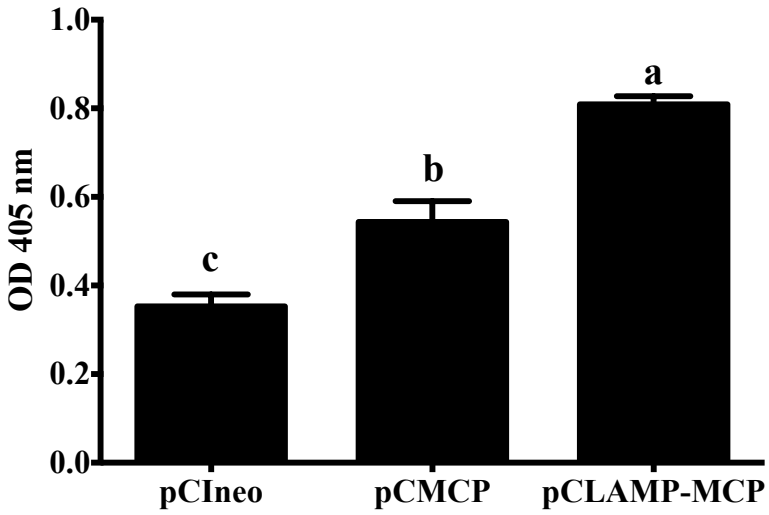

Figure 2. Antibody levels in vaccinated Japanese flounder. Antibody response against MCP in Japanese flounder. Serum samples were taken 30 days after vaccination and anti-MCP antibody level was measured in by ELISA at 405 nm. Different letters denote significant differences. Dilution 1:20. OD, Optical density.

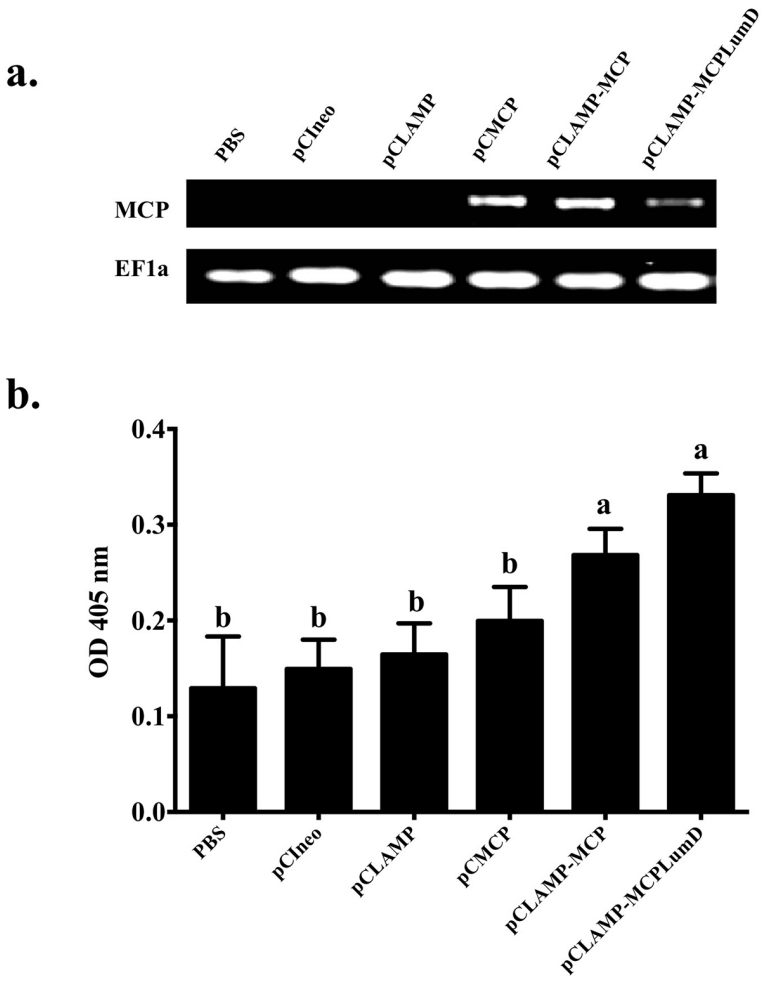

Figure 3. Antibody levels in vaccinated Japanese flounder with chimeric DNA vaccines. a) RSIV MCP expression in muscle sample after 3 days of vaccination detected by RT-PCR. EF1a gene was used as reaction as internal control (28 cycles). b) Antibody response against MCP in Japanese flounder. Serum samples were taken 30 days after vaccination and anti-MCP antibody level was measured in by ELISA at 405 nm. Different letters denote significant differences. Dilution 1:20. OD, Optical density.

Chapter 4

LAMP1-chimeric DNA vaccine protection against fish diseases

Abstract

The use of chimeric antigens in a DNA vaccine that come for the hybridization with sorting signals have shown promising results in animal models, in which sorting motifs drive the movement of the antigens inside the cell. In the present study, lysosome-associated membrane protein-1 from Japanese flounder, *Paralichthys olivaceus*, (JfLAMP-1) which has a high identity with LAMP-1 from striped beakfish, *Oplegnathus fasciatus*, was used as a carrier for the major capsid protein (MCP) from red sea bream iridovirus (RSIV) in order to evaluate its potential as DNA chimeric vaccine. JfLAMP-1 was fused with the MCP from RSIV to produce a chimeric DNA vaccine. For this, striped beakfish individuals were distributed in four experimental groups: PBS, pCIneo, pCMCP and pCLAMP-MCP and after 30 days of vaccination, a challenge test was performed by using RSIV in low and high dose. In the vaccinated group challenged with low dose of RSIV, pCMCP and pCLAMP-MCP showed similar relative percentage of survival of 13%, however in the high dose challenge, pCLAMP-MCP vaccinated group showed relative percentage of survival of 19%, compared with 0% of the pCMCP. In this chapter, a preliminary experiment to evaluate the protection of a chimeric DNA vaccine against *Edwardsiella tarda*. In conclusion, chimeric DNA vaccines using sorting signals from specific molecules can be candidates to enhance the immune response against specific pathogens, for example, by modulation of the traffic of antigen.

Keywords: Chimeric antigens, DNA vaccine, iridovirus, striped beakfish

1. Introduction

Iridoviruses are large double stranded DNA viruses that infect a wide range of vertebrates (Chinchar *et al.*, 2009; Eaton *et al.*, 2010). In marine and freshwater fish, several iridoviruses belonging to the Megalocytivirus genus have been reported (Whittington *et al.*, 2010). From them, red sea bream iridovirus (RSIV) has been shown to infect several species of farmed marine fish causing mortality and important economic losses in Southeast Asia (Do *et al.*, 2005; Ito *et al.*, 2013; Nakajima & Kurita, 2005; Nakajima *et al.*, 1997).

RSIV was first isolated from cultured red sea bream in Shikoku island in Japan, which showed inactive swimming and severe anemia with 20-60% of mortality (Inouye *et al.*, 1992). In Japan probably the importation of infected seedlings captured in the South China Sea was responsible for the introduction of the RSIV in the country (Chinchar *et al.*, 2009) and the disease occurs mainly in the summer, in relatively high water temperatures (Ito *et al.*, 2013; Kurita & Nakajima, 2012). The increase trade and movement of several fish species amplifies the risk of spread the RSIV in asymptomatic latently infected fish (Kyung *et al.*, 2006)

RSIV infection affects mainly juvenile fish and causes anemia, gill hemorrhage and splenomegaly (Inouye *et al.*, 1992; Nakajima & Kurita, 2005). RSIV shows a systemic spread to several organs with hypertrophy of large number of cells often situated near to vascular spaces where the degenerative and necrotic changes occur (Gibson-Kueh *et al.*, 2003). PCR, nested PCR and indirect immunofluorescence assays have been used for the diagnosis (Kyung Choi *et al.*, 2006; Nakajima & Kurita, 2005) Spleen is considered the organ of choice for diagnosis and after infection the quantity of RSIV DNA is higher than other tissues in Japanese amberjack (Ito *et al.*, 2013). Nevertheless, Kyung *et al.* (2006) showed the detection of the virus in heart, stomach, intestine, muscle, eyes and gills rather than spleen in asymptomatic rockbream (*Oplegnathus fasciatus*). This highlights the possibility of differential kinetic of the virus depending on the species.

For control the RSIV disease, a commercial formalin-inactivated vaccine was developed and used in juvenile marine fish (Nakajima *et al.*, 1999; Nakajima *et al.*, 1997) which rendered protection against the experimental challenge but has the limitation in the amount of viral copies able to produce in cell culture as well as that most of infected cells fail to express viral structural

proteins and lost the infectivity after several passages in culture (Kurita & Nakajima, 2012). Recently, it was demonstrated different levels of protection against challenge depending on the viral strain (Shinmoto *et al.*, 2009) which demand the design of vaccine of methods of vaccination that render consistent results.

Chimeric antigens encoded in a DNA vaccine have shown promising results in animal models (Deb *et al.*, 2015; Dhalia *et al.*, 2009; Klucar *et al.*, 2009). Among them, the use of lysosome associated membrane protein (LAMP)-chimeric vaccines have been assessed against viral pathogens rendering enhanced immune response (Arruda *et al.*, 2006; de Arruda *et al.*, 2004; Dhalia *et al.*, 2009; Marques *et al.*, 2003). In the chapter 3, it was demonstrated that JfLAMP-1 used in a chimeric vaccine enhanced the antibody titers against RSIV major capsid protein (MCP). Based on this, the aim of this study was to show the potential use of JfLAMP-1 as a chimeric DNA vaccine carrier to protect striped beakfish from RSIV challenge. In addition, a preliminary experiment of the chimeric vaccine against *Edwardsiella tarda* in Japanese flounder was carried out.

2. Material and methods

2.1. Stripped beakfish LAMP-1 Cloning

Based on EST analyses of Stripped beakfish transcripts primers were designed to amplify the open reading frame (ORF) of Stripped beakfish (SbLAMP-1) (Table 1) and cDNA from spleen was used as a template. PCR products were ligated and cloned into pGEM T-easy vector (Promega, USA) and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with a 3130xl genetic analyzer (Applied Biosystems). Putative signal peptide was predicted using SignalP (http://www.cbs.dtu.dk./services/SignalP/) and InterproScan was used to predict the domains (luminal, transmembrane and cytoplasmic) (http://www.ebi.ac.uk/interpro/). NetNGlyc and NetOGlyc programs were used for prediction of N-linked and O-linked glycosylation sites (http://www.cbs.dtu.dk./services/NetNGlyc/, http://www.cbs.dtu.dk./services/NetOGlyc/). Sequence alignments were generated using GENETYX-MAC 16.0.7 software (Software Development Co., Ltd., Tokyo, Japan) and Geneious® 8.1.7 (Biomatters Limited) (Kearse *et al.*, 2012). Previously it was probed that Japanese flounder lysosome-associated membrane protein-1 (JfLAMP-1) works as a carrier in a DNA vaccine to increase the antibody production against RSIV MCP (chapter 3). Because LAMP-1 from stripped beakfish (*Oplegnathus fasciatus*) shows high identity with JfLAMP-1, I hypothesize that JfLAMP-1 can be used as a carrier for a chimeric vaccine in this species.

2.2. Plasmid DNA for RSIV MCP vaccination

The full length of major capsid protein (MCP) gene of red seabream iridovirus was amplified by PCR from a cDNA template using specific primers with *Nde*I and *Eco*RI restriction enzymes sites (Table 1), cloned into pGEM T-easy vector (Promega, USA) and transformed in *E. coli* JM109 strain (Wako, Japan). Plasmid was extracted and double digested with corresponding enzymes, purified by electrophoresis and inserted in a similar restricted pCIneo vector (Promega, USA). The recombinant plasmids were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with a 3130xl genetic analyzer (Applied Biosystems). pCIneo vector harboring MCP gene (pCMCP), pCLAMP-MCP and pCIneo were purified from overnight cultures as described previously in the chapter 3.

2.2.1. pCLAMP-MCP vaccination experiment

The construction of the chimeric vaccines was based on an overlapping of the sequences at the ends (Grandori *et al.*, 1997), same as described in chapter 3. Experimental animals (n=120) were distributed in three groups and injected intramuscularly with 10 µg of pcMCP; 10 µg of pCLAMP-MCP, and pCI-neo vector (negative control) in 50 µl of saline buffer near to the dorsal fin.

2.3. Viral copy number

The copy number was determined by qPCR through standard curve method (Caipang *et al.*, 2003). For this, serial dilutions of a known concentration of plasmid harboring RSIV MCP gene was used to quantify the Ct value of the dilution. Ct values and the concentration of plasmid (in each serial dilution) was plotted and a standard curve was constructed. For qPCR, specific primer set for MCP (Table 1) was designed and the reaction was carried out using SYBR green PCR master mix (Applied Biosystems) on a StepOnePlus Real-time PCR system (Applied Biosystems) according to the manufacturer's protocol.

2.4. Challenge test

30 days after vaccination, fish were separated in two experimental challenge groups (n=60) by each treatment: high dose (viral copy number= 1.83 $x10^4$/ml) and low dose (viral copy number= 1.75 $x10^3$/ml). Animals were i.p. injected with 100 µl of the viral suspension and the mortality was monitored daily.

2.5. pCLAMP-Eta2 vaccination preliminary experiment

A JfLAMP-1-chimeric DNA vaccine was constructed using Eta2 gene from *Edwardsiella tarda* as antigen. For this, Eta2 gene was amplified using specific primers with restriction enzymes sites, cloned into pGEM T-easy vector (Promega, USA) and transformed in *E. coli* JM109 strain (Wako, Japan). Plasmid was extracted and digested with the corresponding enzymes, purified by electrophoresis and inserted in a pCIneo vector (Promega, USA) digested with the same restriction enzymes.

pCLAMP-Eta2 DNA vaccine was constructed by insertion of Eta2 between the regions that code for the luminal domain and the transmembrane domain of JfLAMP-1 gene, by using overlapping of the sequences at the ends (Grandori *et al.*, 1997), as described in chapter 3. In addition, two DNA vaccines were constructed by adding the medaka beta actin promoter (mbac) to the plasmid, *i.e.* pCmbacEta2 and pCmbacLAMP-Eta2 (Figure 2). The recombinant plasmids were sequenced and purified from overnight cultures as described in chapter 3. Specific primer sets are listed in table 1.

2.5.1. Eta2 vaccination and bacterial challenge

Japanese flounder with an average size 10 cm in total length were acclimated and reared at 18 ℃ in artificial seawater for a week prior the start of the experiments. 5 groups were used as follows: pCIneo, pCEta2, pCLAMP-Eta2, pCmbacEta2 and pCmbacLAMP-Eta2. Each group had 10 individuals which were injected intramuscularly with 10 μg of each recombinant plasmid in 50 μl of saline buffer near to the dorsal fin.

After 15 days of vaccination, animals were challenged with *Edwardsiella tarda* strain NUF806 by i.p injection of 100 μL of a bacterial suspension in saline buffer with a total concentration of 6 x 10^7 colony forming units. The mortality was monitored every 12 h.

2.6. Statistical analysis

A statistical analysis for survival rate was performed by Kaplan-Meier analysis (GraphPad Prism v6.0 for MacOS, California, USA). Differences were considered statistically significant when $p < 0.05$. The relative percentage of survival (RPS) was calculated as RPS= (1-(%mortality of immunized group / %mortality of non-immunized group))*100.

3. Results

3.1. SbLAMP-1 cloning

SbLAMP-1 gene has an ORF of 1248 bp that codes for 415 aa with a predicted molecular weight of 43.694 kDa. The 3'UTR had a polyadenylation signal (AATAA) 414 bp downstream of the translation termination codon (TGA). SbLAMP-1 contains a signal peptide and 3 domains: luminal domain, transmembrane domain and cytoplasmic domain. 17 predicted N-glycosylation sites and 46 predicted O-glycosylation sites were detected. Pairwise alignment of SbLAMP-1 with JfLAMP-1 showed 81% of amino acid identity. Transmembrane and cytoplasmic domain were highly conserved and the HAG**Y**QT**I** motif was found at the cytoplasmic domain. In the same way, eight conserved cysteine residues were present. Based on the higher identity and conserved domains among the LAMP-1, JfLAMP-1 was used as a carrier of RSIV MCP in a DNA vaccine on stripped beakfish.

3.2. Viral copy number

The values obtained for the standard curve in the viral copy number were: Slope= 3.211, Y-Inter= 9.519, R^2= 0.985, Efficiency= 104.82 %, Threshold= 1.153 (ΔRn) and Tm= 83.63.

3.3. Challenge test

In the vaccination and challenge experiment with RSIV in striped beakfish at low dose the pCLAMP-MCP vaccinated group showed a RPS of 13% (Figure 3). On the other hand, in the high dose challenge experiment, pCLAMP-MCP vaccinated group showed RPS of 19%, compared with 0% of the pCMCP group (Figure 4).

For Eta2 DNA vaccination in Japanese flounder, after challenge almost all the fish died before 120 h. However, the pCLAMP-Eta2 group showed a slower mortality rate that last until 168 h (Figure 5).

4. Discussion

Vaccination against iridovirus have shown to elicit good protection for viral challenge (Drennan *et al.*, 2007; Ou-yang *et al.*, 2012a; Ou-yang *et al.*, 2012b; Shin *et al.*, 2013; Zheng *et al.*, 2016). In case of RSIV, formalin-inactivated vaccine induced protection in red sea bream, amberjack and seriola species (Nakajima *et al.*, 2002; Nakajima *et al.*, 1999). However, in these studies the supernatant from RSIV-infected grunt fin cell line culture was used which mass production is difficult and expensive (Nakajima *et al.*, 1997). On the other hand, DNA vaccination against RSIV showed similar protection that inactivated vaccines. Caipang *et al.* (2006) found that a RSIV gene encoding a major capsid protein (MCP), and an open reading frame containing a transmembrane domain when used as DNA vaccines were both immunogenic and protective against viral challenge. However, in *Oplegnathus* genus fish species, which are highly susceptible to the RSIV infection, the vaccination efficacy by either type of vaccine is lower or absent (Matsuyama *et al.*, 2016).

Recently, chimeric antigens in DNA vaccines were proposed as an alternative to increase the immune response against pathogens (Grigera *et al.*, 1996; Nawaratna *et al.*, 2015; Starodubova *et al.*, 2010). In the present study, a DNA vaccine coding for a chimeric antigen of JfLAMP-1 and RSIV MCP induced a RPS of 19% which was higher than the conventional DNA vaccine (0 %). This result showed the potential use of chimeric antigens in DNA vaccines using LAMP-1 or molecules containing sorting signals to improve the immunogenicity and protection of the vaccines.

In the chapter 3 was demonstrated that JfLAMP-1 chimeric DNA vaccines induced higher antibody titers in vaccinated fish, which may be one of the mechanisms that render protection. Shimmoto *et al.* (2010) showed that a vaccine based on a chimeric antigen can give protection against the challenge with RSIV and that this protection may be mediated by neutralizing antibodies that block the entry of the virus and the spread to fish organs, since less accumulation of virus in tissues was found in vaccinated animals.

DNA vaccines have the ability to stimulate cellular as well as humoral immunity (Wang *et al.*, 1998). In addition, LAMP as a carrier molecule can drive the traffic of the antigen to the MHC class II processing pathway, enhance the CD4+ T cell priming/activation (Arruda *et al.*, 2006; Starodubova *et al.*, 2010) and co-localize with MHC class II (Anwar *et al.*, 2005; Arruda *et al.*, 2006; de Arruda *et al.*, 2004; Marques *et al.*, 2003).

The LAMP-1 chimeric DNA vaccine against *E. tarda* by using Eta2 as antigen, requires adjustment in the challenge dose, which was higher for the size of the animals. The use of lower dose may allow to find an effect of the vaccine, since the experimental animals are challenged with a concentration that allow the animal to start an immune response and mimic the situation in a natural infection.

In conclusion, the use of JfLAMP-1 as an antigen carrier in a DNA vaccine elicited higher protection against RSIV than the conventional DNA vaccine in striped beakfish.

References

Anwar, A., Chandrasekaran, A., Ng, M. L., Marques, E., & August, J. T. (2005). West Nile premembrane-envelope genetic vaccine encoded as a chimera containing the transmembrane and cytoplasmic domains of a lysosome-associated membrane protein: increased cellular concentration of the transgene product, targeting to the MHC II compartment, and enhanced neutralizing antibody response. *Virology, 332*(1), 66-77.

Arruda, L. B., Sim, D., Chikhlikar, P. R., Maciel, M., Jr., Akasaki, K., August, J. T., & Marques, E. T. (2006). Dendritic cell-lysosomal-associated membrane protein (LAMP) and LAMP-1-HIV-1 gag chimeras have distinct cellular trafficking pathways and prime T and B cell responses to a diverse repertoire of epitopes. *Journal of Immunology, 177*(4), 2265-2275.

Caipang, C. M., Hirono, I., & Aoki, T. (2003). Development of a real-time PCR assay for the detection and quantification of red seabream iridovirus (RSIV). *Fish Pathology, 38*(1), 1-7.

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 Immunology, 21*(2), 130-138.

Chinchar, V. G., Hyatt, A., Miyazaki, T., & Williams, T. (2009). Family Iridoviridae: poor viral relations no longer. *Current Topics in Microbiology and Immunology, 328*, 123-170.

de Arruda, L. B., Chikhlikar, P. R., August, J. T., & Marques, E. T. (2004). DNA vaccine encoding human immunodeficiency virus-1 Gag, targeted to the major histocompatibility complex II compartment by lysosomal-associated membrane protein, elicits enhanced long-term memory response. *Immunology, 112*(1), 126-133.

Deb, R., Dey, S., Madhan Mohan, C., Gaikwad, S., Kamble, N., Khulape, S. A., Gupta, S. K., Maity, H. K., & Pathak, D. C. (2015). Development and evaluation of a *Salmonella typhimurium* flagellin based chimeric DNA vaccine against infectious bursal disease of poultry. *Research in Veterinary Science, 102*, 7-14.

Dhalia, R., Maciel Jr., M., Cruz, F. S. P., Viana, I. F. T., Palma, M. L., August, T., & Marques Jr., E. T. A. (2009). Membrane and envelope virus proteins co-expressed as lysosome associated membrane protein (LAMP) fused antigens: a potential tool to develop DNA vaccines against flaviviruses. *Anais da Academia Brasileira de Ciências, 81*, 663-669.

Do, J. W., Cha, S. J., Kim, J. S., An, E. J., Lee, N. S., Choi, H. J., Lee, C. H., Park, M. S., Kim, J. W., Kim, Y. C., & Park, J. W. (2005). Phylogenetic analysis of the major

capsid protein gene of iridovirus isolates from cultured flounders *Paralichthys olivaceus* in Korea. *Diseases of Aquatic Organisms, 64*(3), 193-200.

Drennan, J. D., LaPatra, S. E., Swan, C. M., Ireland, S., & Cain, K. D. (2007). Characterization of serum and mucosal antibody responses in white sturgeon (*Acipenser transmontanus* Richardson) following immunization with WSIV and a protein hapten antigen. *Fish & Shellfish Immunology, 23*(3), 657-669.

Eaton, H. E., Ring, B. A., & Brunetti, C. R. (2010). The genomic diversity and phylogenetic relationship in the family iridoviridae. *Viruses, 2*(7), 1458-1475.

Gibson-Kueh, S., Netto, P., Ngoh-Lim, G. H., Chang, S. F., Ho, L. L., Qin, Q. W., Chua, F. H. C., Ng, M. L., & Ferguson, H. W. (2003). The pathology of systemic iridoviral disease in fish. *Journal of Comparative Pathology, 129*(2–3), 111-119.

Grandori, R., Struck, K., Giovanielli, K., & Carey, J. (1997). A three-step PCR protocol for construction of chimeric proteins. *Protein Engineering, 10*(9), 1099-1100.

Grigera, P. R., Garcia-Briones, M., Periolo, O., la Torre, J. L., & Wagner, R. R. (1996). Immunogenicity of an aphthovirus chimera of the glycoprotein of vesicular stomatitis virus. *Journal of Virology, 70*(12), 8492-8501.

Inouye, K., Yamano, K., Maeno, Y., Nakajima, K., Matsuoka, M., Wada, Y., & Sorimachi, M. (1992). Iridovirus infection of cultured red sea bream, *Pagrus major*. *Fish Pathology, 27*(1), 19-27.

Ito, T., Yoshiura, Y., Kamaishi, T., Yoshida, K., & Nakajima, K. (2013). Prevalence of red sea bream iridovirus among organs of Japanese amberjack (*Seriola quinqueradiata*) exposed to cultured red sea bream iridovirus. *Journal of General Virology, 94*(Pt 9), 2094-2101.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., & Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics, 28*(12), 1647-1649.

Klucar, P., Barnes, P. F., Kong, Y., Howard, S. T., Pang, X., Huang, F. F., Tvinnereim, A. R., Samten, B., & Shams, H. (2009). Vaccination strategies to enhance local immunity and protection against *Mycobacterium tuberculosis*. *Vaccine, 27*(12), 1816-1824.

Kurita, J., & Nakajima, K. (2012). Megalocytiviruses. *Viruses, 4*(4), 521-538.

Kyung Choi, S., Ryun Kwon, S., Kwon Nam, Y., Koo Kim, S., & Hong Kim, K. (2006). Organ distribution of red sea bream iridovirus (RSIV) DNA in asymptomatic yearling

and fingerling rock bream (*Oplegnathus fasciatus*) and effects of water temperature on transition of RSIV into acute phase. *Aquaculture, 256*(1), 23-26.

Marques, E. T., Jr., Chikhlikar, P., de Arruda, L. B., Leao, I. C., Lu, Y., Wong, J., Chen, J. S., Byrne, B., & August, J. T. (2003). HIV-1 p55Gag encoded in the lysosome-associated membrane protein-1 as a DNA plasmid vaccine chimera is highly expressed, traffics to the major histocompatibility class II compartment, and elicits enhanced immune responses. *Journal of Biological Chemistry, 278*(39), 37926-37936.

Matsuyama, T., Minami, T., Fukuda, Y., Sano, N., Sakai, T., Takano, T., & Nakayasu, C. (2016). Passive immunization against red sea bream iridoviral disease in five marine fish species. *Fish Pathology, 51*(1), 32-35.

Nakajima, K., Ito, T., Kurita, J., Kawakami, H., Itano, T., Fukuda, Y., Aoi, Y., Tooriyama, T., & Manabe, S. (2002). Effectiveness of a vaccine against red sea bream iridoviral disease in various cultured marine fish under laboratory conditions. *Fish Pathology, 37*(2), 90-91.

Nakajima, K., & Kurita, J. (2005). Red sea bream iridoviral disease. *Uirusu, 55*(1), 115-125.

Nakajima, K., Maeno, Y., Honda, A., Yokoyama, K., Tooriyama, T., & Manabe, S. (1999). Effectiveness of a vaccine against red sea bream iridoviral disease in a field trial test. *Diseases of Aquatic Organisms, 36*(1), 73-75.

Nakajima, K., Maeno, Y., Kurita, J., & Inui, Y. (1997). Vaccination against red sea bream iridoviral disease in red sea bream. *Fish Pathology, 32*(4), 205-209.

Nawaratna, S. S., Gobert, G. N., Willis, C., Mulvenna, J., Hofmann, A., McManus, D. P., & Jones, M. K. (2015). Lysosome-associated membrane glycoprotein (LAMP)--preliminary study on a hidden antigen target for vaccination against schistosomiasis. *Scientific Reports, 5*, 15069.

Ou-yang, Z., Wang, P., Huang, X., Cai, J., Huang, Y., Wei, S., Ji, H., Wei, J., Zhou, Y., & Qin, Q. (2012a). Immunogenicity and protective effects of inactivated Singapore grouper iridovirus (SGIV) vaccines in orange-spotted grouper, *Epinephelus coioides*. *Developmental & Comparative Immunology, 38*(2), 254-261.

Ou-yang, Z., Wang, P., Huang, Y., Huang, X., Wan, Q., Zhou, S., Wei, J., Zhou, Y., & Qin, Q. (2012b). Selection and identification of Singapore grouper iridovirus vaccine candidate antigens using bioinformatics and DNA vaccination. *Veterinary Immunology and Immunopathology, 149*(1–2), 38-45.

Shimmoto, H., Kawai, K., Ikawa, T., & Oshima, S. (2010). Protection of red sea bream *Pagrus major* against red sea bream iridovirus infection by vaccination with a recombinant viral protein. *Microbiology and Immunology, 54*(3), 135-142.

Shin, Y. J., Kwon, T. H., Seo, J. Y., & Kim, T. J. (2013). Oral immunization of fish against iridovirus infection using recombinant antigen produced from rice callus. *Vaccine, 31*(45), 5210-5215.

Shinmoto, H., Taniguchi, K., Ikawa, T., Kawai, K., & Oshima, S.-I. (2009). Phenotypic diversity of infectious red sea bream iridovirus isolates from cultured fish in Japan. *Applied Environmental Microbiology, 75*(11), 3535-3541.

Starodubova, E. S., Isaguliants, M. G., & Karpov, V. L. (2010). Regulation of immunogen processing: signal sequences and their application for the new generation of DNA-vaccines. *Acta Naturae, 2*(1), 53-60.

Wang, B., Godillot, A. P., Madaio, M. P., Weiner, D. B., & Williams, W. V. (1998). Vaccination against pathogenic cells by DNA inoculation. In H. Koprowski & D. B. Weiner (Eds.), *DNA Vaccination/Genetic Vaccination* (pp. 21-35). Berlin, Heidelberg: Springer Berlin Heidelberg.

Whittington, R. J., Becker, J. A., & Dennis, M. M. (2010). Iridovirus infections in finfish - critical review with emphasis on ranaviruses. *Journal of Fish Diseases, 33*(2), 95-122.

Zheng, F., Liu, H., Sun, X., Zhang, Y., Zhang, B., Teng, Z., Hou, Y., & Wang, B. (2016). Development of oral DNA vaccine based on chitosan nanoparticles for the immunization against reddish body iridovirus in turbots (*Scophthalmus maximus*). *Aquaculture, 452*, 263-271.

Table 1. PCR primers used in this study.

Target	Usage	Primer sequence (5′–3′)*
SbLAMP-1 ORF	Cloning F	ATGAAACTCTGTCACGCTTTGGC
	Cloning R	TCAGATGGTCTGGTATCCGGCGT
MCP	Cloning F	GGGCATATGTCTGCAATCTCAGGTGC
	Cloning R	CCCGAATTCTTAATGATGATGATGAT
	qPCR F	ATCAAAACAGACTGGCCATGCTAAT
	qPCR R	AAATTATCACACCAGCGAATGTAGC
Eta2	Cloning F	CATATGTCATTGACACAACTAGG
Eta2-His	Cloning R	TTAATGATGATGATGATGATGTATAACCTGTTTCA
mbact-Eta2	Cloning F	TCCAGTTTAGCCATGTCATTGACACAACTAGG
Eta2-mbact	Cloning R	GAGAAAGTCTGTTTAATGATGATGATGATGAT
mbact-LAMP-F	Cloning F	TCCAGTTTAGCCATGGAACTCTCTCACACGGT
LAMP-mbact-R	Cloning R	GAGAAAGTCTGTTTAGATGGTCTGGTATCCAG
Eta2-LAMP(lum)-R	Cloning R	GATGGGGATGAGCATATGATGATGATGATG
LAMP(lum)-pCI-F	Cloning F	ATGCTCATCCCCATCATCGT
LAMP(sig)-eta2-F	Cloning F	GGTTGTATTCAGGCTATGTCATTGACACAA
pCI-LAMP(sig)-R	Cloning R	AGCCTGAATACAACCTAGCC

*Restriction enzyme sites (*Nde*I and *Eco*RI) are underlined at the start of the sequence. **Between parentheses appear the position of the gene which nucleotides are added in order to hybridize at the ends of each domain.

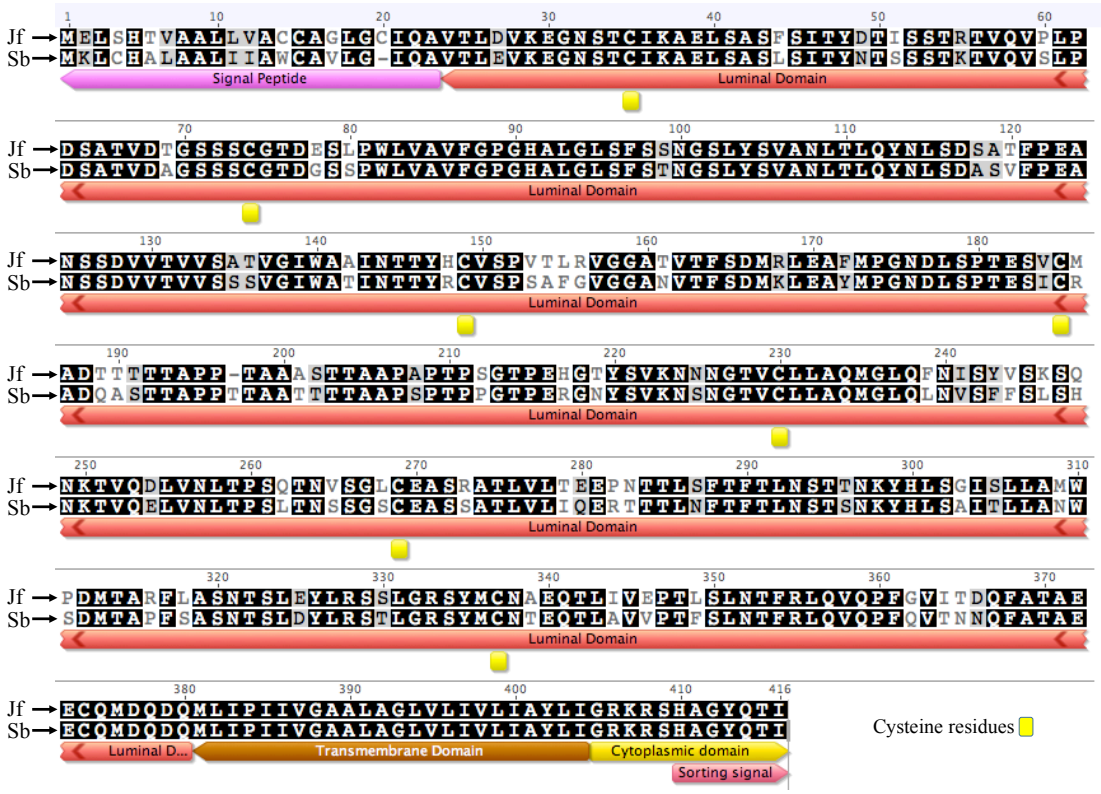

Figure 1. Pairwise alignment of LAMP-1 of Japanese flounder (Jf) and Striped beakfish (Sb). Cytoplasmic and transmembrane domains show high identity among LAMP-1 molecules. The cysteine residues are also conserved.

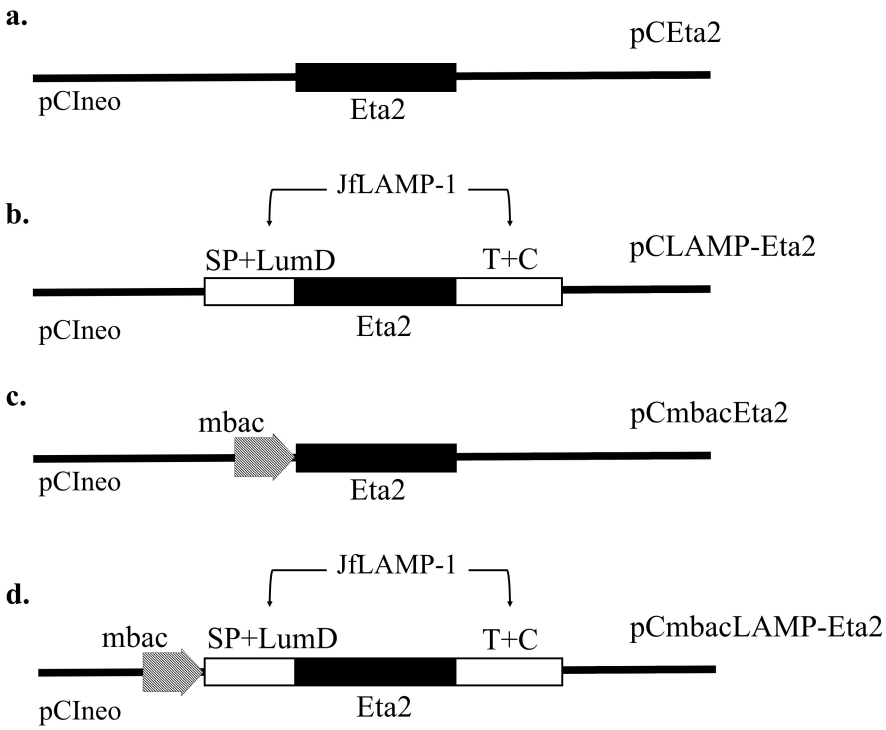

Figure 2. Schematic representation of the expression plasmids used for Eta2 vaccination experiments. a) pCEta2, pCIneo harboring Eta2 gene. b) pCLAMP-Eta2, pCIneo harboring the chimeric antigen. c) pCmbacEta2, pCIneo harboring Eta2 gene with medaka beta actin promoter (mbac). d) pCmbacLAMP-Eta2, pCIneo harboring the chimeric antigen with mbac. SP, signal peptide domain; LumD, luminal domain; T, Transmembrane domain; C, Cytoplasmic domain.

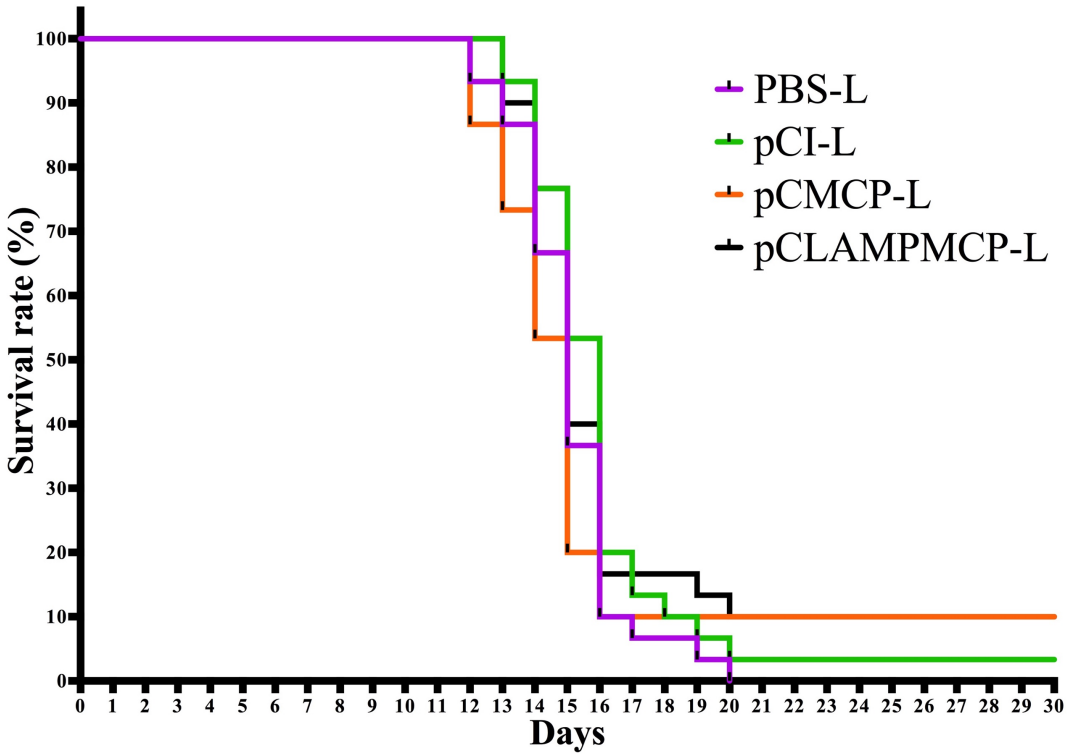

Figure 3. Survival rate of striped beakfish challenged with low dose (mean copy number=1.75 x10^3) of RSIV after 30 days of DNA vaccination.

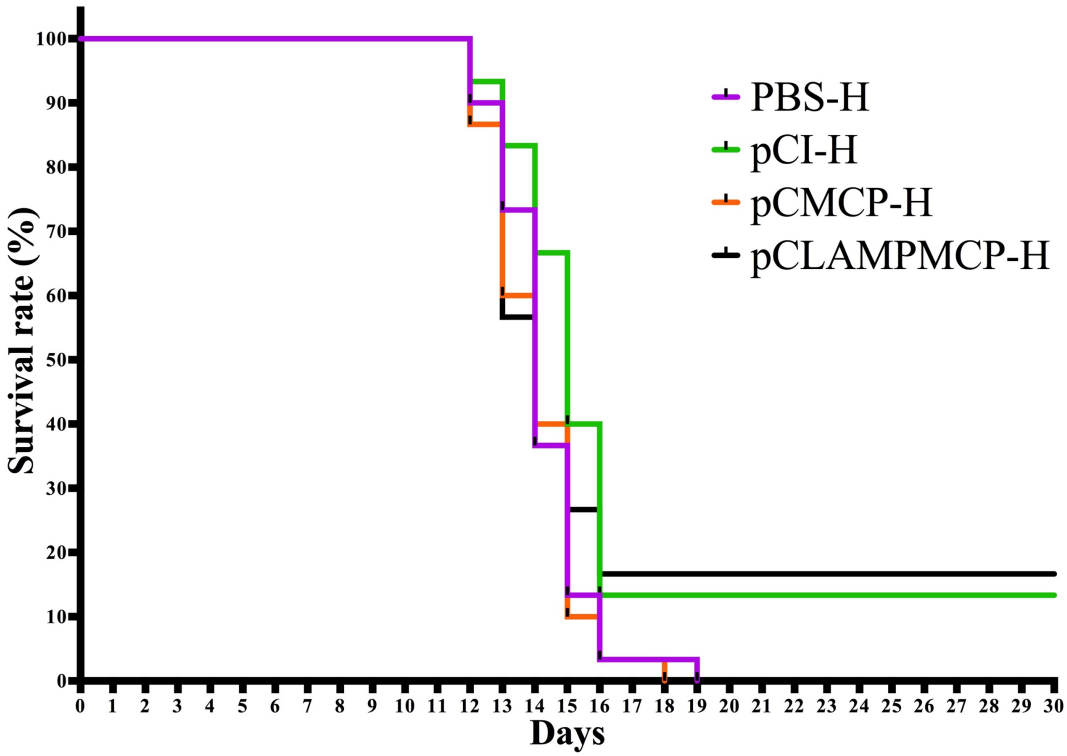

Figure 4. Survival rate of striped beakfish challenged with high dose (mean copy number=1.83 x10^4) of RSIV after 30 days of DNA vaccination.

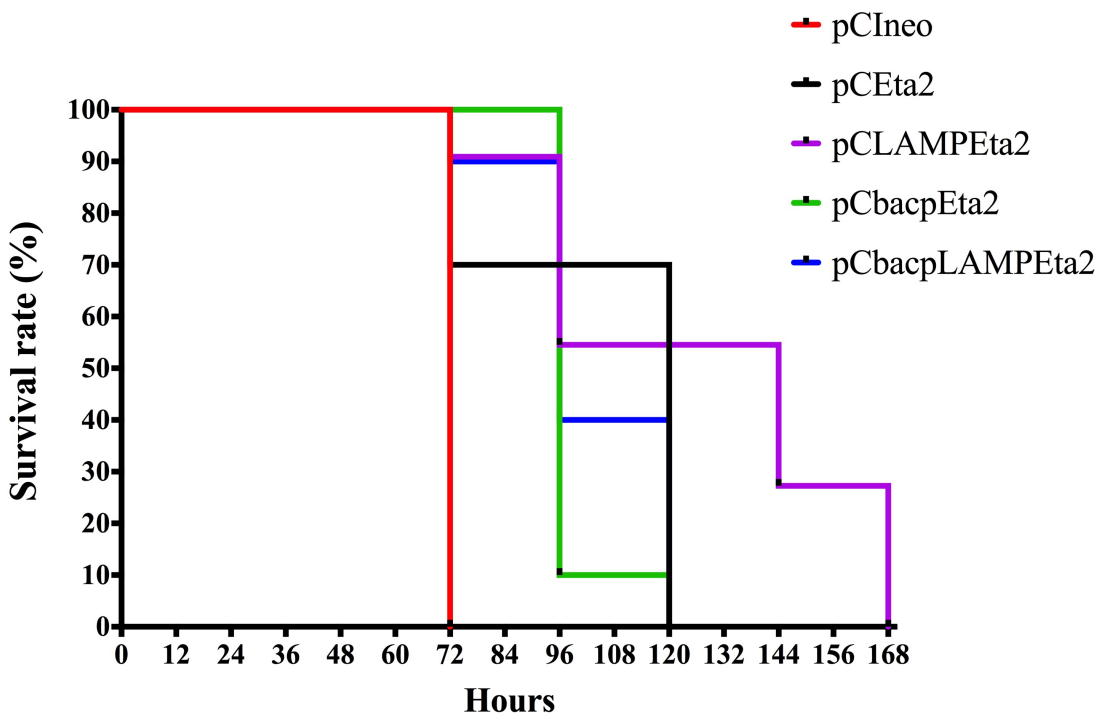

Figure 5. Survival rate of Japanese flounder challenged with *E. tarda* after 15 days of DNA vaccination.

Chapter 5

General conclusion

Vaccination is a prophylactic method to induce immunity against specific pathogens and protect the animals from disease. Antigens coded in DNA vaccines have shown promising result to induce immunity, however for some pathogens the results need to be improved. Thus, genetic bioengineering of DNA vaccines can be an alternative to increase the efficacy of the vaccines. The use of sorting signals from intracellular molecules to construct chimeric antigens may allow to drive the movement of the antigen inside the cell and to modify the immune response, in order to increase the immunogenicity and render more protection in the vaccine.

In my study, I hypothesized that the use of the sorting signal from Japanese flounder LAMP-1 can increase the immunogenicity of the DNA vaccine using RSIV MCP as antigen. For this, JfLAMP-1 was characterized and a chimeric DNA vaccine was constructed and tested.

Thus, JfLAMP-1 gene was cloned and characterized, showing a constitutive expression in tissues and upregulation on FKC stimulation with *E. tarda*. In poly I:C stimulation there was no changes in the expression, what make hypothesize a role of JfLAMP-1 mainly in the antibacterial immunity. At protein level, JfLAMP-1 appears distributed in granules from different sizes group close to the nucleus.

Then, JfLAMP-1 chimeric vaccines were constructed and their efficacy to induce antibody immune response and to protect a RSIV-susceptible fish species was probed. LAMP-1 and RSIV MCP chimeric antigen in a DNA vaccine showed to induce higher titers of antibodies against RSIV MCP in Japanese flounder compared with the conventional DNA vaccine. Furthermore, striped beakfish vaccinated with the chimeric vaccine and then challenged with RSIV, showed higher survival rates than the individuals vaccinated with the conventional DNA vaccine. These findings highlight the potential improvement of the immunogenicity of the antigens by using chimeric DNA vaccines.

In conclusion, chimeric antigens coded in DNA vaccines using sorting signals from specific molecules can be candidates to enhance the immune response against pathogens, *e.g.* by modulation of the traffic of antigen.