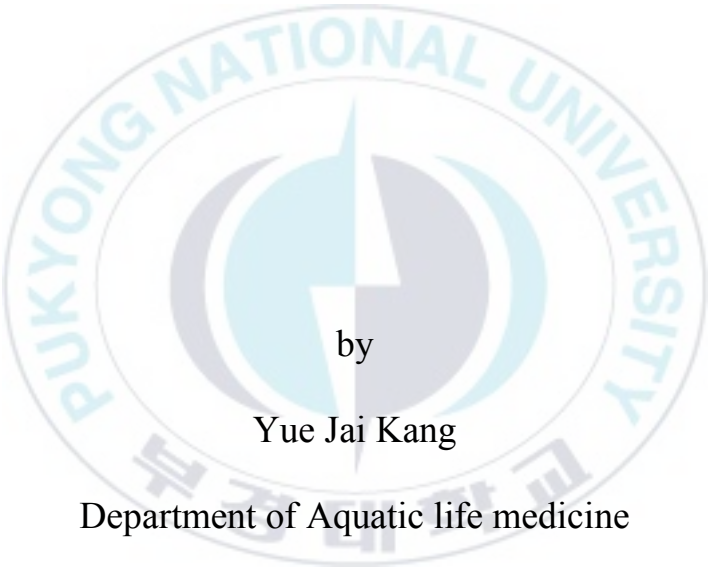
Thesis for the Degree of Doctor of Philosophy

Enhancement of disease resistance of olive flounder (*Paralichthys olivaceus*) using systems producing CpG DNA and double-stranded RNA



by

Yue Jai Kang

Department of Aquatic life medicine

The Graduate School

Pukyong National University

Feb. 2012

Enhancement of disease resistance of olive flounder (*Paralichthys olivaceus*) using systems producing CpG DNA and double-stranded RNA

CpG motif DNA 및 double-strand RNA 생성 system 을 이용한 넙치의 질병 저항성 증강

Advisor: Prof. Ki Hong Kim

by

Yue Jai Kang

A thesis submitted in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

in Department of Aquatic Life Medicine, The Graduate School,
Pukyong National University

Feb. 2012

Enhancement of disease resistance of olive flounder (*Paralichthys olivaceus*) using systems producing CpG DNA and double-stranded RNA

A dissertation

by

Yue Jai Kang

Approved by:

(Chairman) Yoon Kwon Nam

(Member) Do Hyung Kim

(Member) Joon Bum Jeong

(Member) Se Ryun Kwon

(Member) Ki Hong Kim

Feb. 2012

CONTENTS

Enhancement of disease resistance of olive flounder (*Paralichthys olivaceus*) using systems producing CpG DNA and double-stranded RNA

Yue Jai Kang

Department of Aquatic life medicine, The Graduate School

Pukyong National University

ABSTRACT

Unmethylated CpG motifs in DNA and double-stranded RNA (dsRNA) are recognized as pathogen-associated molecular patterns (PAMPs) by toll like receptor 9 (TLR9) and TLR3, respectively, which trigger strong proinflammatory responses in vertebrates. In the present study, effects of CpG DNA and dsRNA on disease resistance of olive flounder were investigated, and various forms of CpG DNA and dsRNA were developed for practical use of them in aquaculture.

Firstly, we found that CpG-ODNs belonging to different classes have different abilities to increase resistance of olive flounder against different pathogens, in which fish administered CpG-ODN 2216 (A-class) showed the highest resistance against VHSV infection and the strongest expression of Mx and ISG15 genes, whereas fish administered CpG-ODN 1668 (B-class) induced the strongest scuticocidal activity of serum and the highest survival rate against *Miamiensis avidus* infection. Secondly, we have firstly demonstrated that plasmids harboring multiple copies of CpG 1668 motifs enhanced resistance of olive flounder against *M. avidus* infection. The use of plasmids containing multiple CpG motifs instead of artificially synthesized CpG-ODNs would be

a way to low the cost. And, we constructed plasmids that harbor multi-copy of CpG motifs that belonging to different classes, and verified that administration of the constructed plasmids enhanced disease resistance of olive flounder against both VHSV and *M. avidus* infections. Furthermore, delivery of plasmids harboring CpG motifs by auxotrophic *Escherichia coli* mutant also enhanced disease resistance of olive flounder. Thirdly, we have newly cloned TLR3 cDNA of olive flounder, and analyzed in vivo expression of the TLT3/TLR22 in response to poly (I:C). Furthemore, using short-hairpin RNAs (shRNA) targeting TLR3 and TLR22 in HINAE cells, we revealed the critical role of TLR22 in induction of type I interferon response. Fourthly, we demonstrated that olive flounder leucocytes stimulated with poly (I:C) showed increased scuticocidal activity in the presence of inactivated immune serum, and in vivo administration of poly (I:C) increased survival rates of olive flounder agasinst *M. avidus* infection. Furthermore, dsRNA containing CpG motifs sequences induced higher resistance in olive flounder against M. avidus and VHSV infections when compared to dsRNA without CpG motif sequences.

Conclusionly, the present resutls indicate that plasmids and dsRNAs harboring CpG motifs can be used as effective immunostimulants that can simultaneously enhance resistance against viral and parasitic diseases.

LIST OF TABLES

LIST OF FIGURES

Acronym

Name	Characteristics	Source or reference
Pathogen		Laboratory stock
M. avidus	*Miamiensis avidus*, isolated from ascitic fluid of a diseased olive flounder	
VHSV	Viral Hemorrhagic Septicemia Virus, *i*solated from a moribund olive flounder	
E. coli	delivery vehicle for CpG motifs-containing plasmids	
HT115	lacks RNase III gene and possess a cassette for expression of T7 RNA polymerase	
χ7213	*asd* mutated auxotrophic mutant	
CpG-ODNs	(Underlined nucleotides indicate phosphorothioate linkage)	
ODN **2216**	CpG-ODN 2216, 5'-GGGGGACGATCGTCGGGGGG-3'	
ODN *2243*	CpG-ODN 2243, 5'-GGGGGAGCATGCTGGGGGGG-3' (control of ODN 2216)	
ODN 1668	CpG-ODN 1668, 5'-TCCATGACGTTCCTGATGCT-3'	
ODN 1720	CpG-ODN 1720, 5'-TCCATGAGCTTCCTGATGCT-3' (control of ODN 1668)	
ODN 2395	CpG-ODN 2395, 5'-TCGTCGTTTTCGGCGCGCGCCG-3'	
Plasmids		
Lit	pLitmus28i vector	NEB
Litself	Fragment encoding the T7 promoter region (Coordinates 2620-2603) in the pLitmus28i was removed	
Lit-CpG15	pLitmus28i-CpG-ODN 1668 x 15 (15 copies of CpG 1668 sequence)	
Lit-CpG30	pLitmus28i- CpG-ODN 1668 x 30 (30 copies of CpG 1668 sequence)	
Lit-CpG60	pLitmus28i- CpG-ODN 1668 x 60 (60 copies of CpG 1668 sequence)	
Lit-GFP	pLitmus28i- Green fluorescent protein (GFP)	
Lit-CpGMix	pLitmus28i-CpGMix (fragment corresponding to randomly arranged 6 copies of each CpG-ODN sequence (CpG-ODN 1668, 2216 and 2395))	

Plasmids	
p-fTLR22	olive flounder TLR22 ORF expression cassette cloned into pGEM T-easy vector
pFuguU6shfTLR22 903-fTLR22	siRNA targeting fTLR22 903 and olive flounder TLR22 ORF expression cassette
pFuguU6shfTLR22 1726-fTLR22	siRNA targeting fTLR22 1726 and olive flounder TLR22 ORF expression cassette
pFuguU6shfTLR22 1726control-fTLR22	siRNA targeting fTLR22 1726 control and olive flounder TLR22 ORF expression cassette
pNeo	Neomycin expression cassette cloned into pGEM T-easy vector
pNeoFuguU6shfTLR22 1726	Neomycin and siRNA targeting fTLR22 1726 expression
pNeoFuguU6shfTLR22 1726control	Neomycin and siRNA targeting fTLR22 1726 control expression
pNeoFuguU6shfTLR3 939	Neomycin and siRNA targeting fTLR3 939 expression

GENERAL INTRODUCTION

Various infectious diseases have largely affected on the aquaculture industry, and recently production losses caused by infectious diseases in cultured fish in Korea has been estimated approximately 25-30%. Although various control measures have been applied to lessen the loss, to date, effective control measures do not exist for most infection diseases, which are the main constraint on aquaculture animal production. Furthermore, as the restriction on the use of chemotherapeutics is becoming strengthened world-widely, development of countermeasures based on the bio-medicinal products is recommended or encouraged.

Immunoprophylactics using vaccines or immunostimulants would be a way to control infectious diseases without using chemotherapeutics. As fish possess innate and adaptive immune systems, modulation of immunity specifically by vaccines or nonspecifically by immunostimulants is possible (Sohn et al., 2000). Although several vaccines have been commercially used in aquaculture, their efficacy is too weak to prevent infectious diseases in aquaculture. Also, there is still a need to develop effective vaccines for many aquatic pathogens. Thus, at present, immunostmulant-mediated enhancement of resistance against overall infectious diseases would be more favorable than vaccine-mediated steno-protection.

An interest in the use of immunostimulants in farm-reared fish for enhancing the activity of innate immune factors and conferring protection against disease has increased in recent years. Various immunomodulators have been reported to enhance the non-specific immunity in fish, including killed bacteria and bacterial products (Anderson, 1992; Kodaina et al., 1993; Goetz et al., 2004), chitin (Sakai et al., 1992; Anderson and Siwicki, 1994), glucans (Yano et al., 1989;

Nikl et al., 1993; Duncan and Klesius, 1996; Ogier de Baulny et al., 1996; Santarem et al., 1997), saponin (Ninomiya et al., 1995), glycyrrhizin (Jang et al., 1995), certain vitamins (Blazer, 1992), (Kitlen et al., 1997), and herbal extracts (Yuan et al., 2997, 2008).

It has been reported that fish has various toll-like receptors (TLRs) that are corresponding to mammalian TLRs, and several agonist molecules that are recognized by TLRs as pathogen-associated molecular patterns (PAMPs) have been utilized as immunostimulants for vertebrates including fish (Akira et al., 2006). Among them, recent studies indicate that unmethylated cytidine-phosphate-guanosine (CpG) motifs flanked by two 5' purines and two 3' pyrimidines are recognized by TLR9 and are immunostimulatory in mammals (Klinman et al., 1996; Krieg et al., 1995, 2000). Due to a combination of CpG suppression and CpG methylation, these sequence motifs are rarely present in eukaryotic genomes but are common in prokaryotic genomes (Bird, 1980; 1987). Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs mimic the activity of bacterial DNA, and are recognized as a danger signal in mammalian immune cells (Yamamoto et al. 1992, Krieg et al. 1995, Ballas et al. 1996, Klinman et al. 1996). In fish, there is limited information concerning the biological effects of CpG ODN. Kanellos et al. (1999) reported that plasmids co-injected with a recombinant protein potentiated antibody responses to the protein in goldfish (*Carassius auratus*). Recently, Jørgensen et al. (2001a,b) demonstrated that plasmid DNA and synthetic CpG-ODN induced production of IFN-like cytokine and IL-1β in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) leucocytes. Oumouna et al. (2002) demonstrated activation of nonspecific cytotoxic cells of catfish (*Ictalurus punctatus*) with synthetic ODN and bacterial genomic DNA. Tassakka and Sakai (2002) reported that intraperitoneal injection of CpG-ODN to carp (*Cyprinus carpio*) enhanced the nonspecific immune responses including phagocytic and nitroblue tetrazolium (NBT) activity in kidney phagocytes and serum lysozyme activity. Meng et al. (2003) observed increase of respiratory burst

activity, acid phosphatase and bactericidal activity of grass carp (*Ctenopharyngodon idellus*) head-kidney macrophages by in vitro incubation with CpG ODN. Recently, CpG motifs constructed in plasmids have a strong immunostimulatory ability like CpG-ODFNs and are actively used as an adjuvant for DNA vaccines in mammals and fish (Liu et al., 2010).

Double-stranded RNAs (dsRNAs) derived from a replication intermediate of viruses is one of the major PAMP, which trigger innate immune responses in vertebrates (Akira et al., 2006). In mammalian cells, endosomal TLR3 recognizes dsRNA (Alexopoulou et al., 2001; Wang et al., 2004; Schulz et al., 2005), and recruits adaptor protein called TRIF via interaction of its TIR domains (Yamamoto et al., 2002). TRIF then indirectly activates several transcription factors IRF3, NFkB and AP-1, which modulate the transcription of interferon β and inflammatory cytokines (Sharma et al., 2003; Yamamoto et al., 2003; Hoeba et al., 2003; Meylan et al., 2004; Matsuo et al., 2008). Similar dsRNA recognizing mechanism through the TLR3 was also reported recently in fish species (Matsuo et al. 2008). The secreted IFN regulates the expression of numerous genes encoding antiviral proteins including dsRNA dependent eukaryotic initiation factor kinase (PKR) and Mx proteins (de Veer et al., 2001).

The aim of the present study was to evaluate the ability of CpG DNA and dsRNA in enhancement of resistance against a parasitic infection (*Miamiensis avidus*) and a viral infection (viral hemorrhagic septicemia virus; VHSV). Scuticociliate parasites are histophagous ciliated protozoa that cause diseases in marine and freshwater finfish worldwide (Harikrishnan et al., 2010). Although several scuticociliate species are known to involve in scuticociliatosis in cultured marine fishes (Dragesco et al., 1995; Munday et al., 1997; Iglesias et al., 2001; Jee et al., 2001; Sacks et al., 2002; Kim et al., 2004a,b), the disease caused by infection of *M. avidus* (=*Philasterides dicentrarchi*) has been the most fatal parasitic disease in farm-reared olive flounder (*Paralichthys olivaceus*) in Korea, which usually lead to high cumulative mortalities

(Kim et al., 2004; Jung et al., 2007). Since *M. avidus* can invade into all internal organs including brain, treatment with chemotherapeutics would have little effect on systematically infected fish. Therefore, immuno-prophylaxis using enhancement of adptive or/and innate immunity would be an effective way to control scuticociliatosis. Viral hemorrhagic septicemia (VHS) caused by infection with VHSV is one of the major causes of morbidity and mass mortality in both cultured and wild fish worldwide (Schlotfeldt & Ahne 1988, Schlotfeldt et al. 1991, Mortensen et al. 1999, Isshiki et al. 2003, Skall et al. 2005). VHSV is an enveloped negative-strand RNA virus and belongs to the genus *Novirhabdovirus* of the family Rhabdoviridae (Lenoir & de Kinkelin 1975, Walker et al. 2000, Tordo et al. 2005). The first epizootic of VHSV in Korea was reported from farmed olive flounder (*Paralichthys olivaceus*) in 2001 (Kim et al. 2003), and since then outbreaks of VHS have been responsible for high mortalities in cultured olive flounder from winter to spring (Kim et al. 2009).

The contents of this thesis are as follows. In chapter I, the effects of CpG-ODNs belonging to different classes on resistance of olive flounder against *M. avidus* and VHSV infections have been investigated. In chapter II, to know whether CpG motif-containing plasmids can be used as immunostimulants for fish instead of CpG-ODNs, effects of plasmids containing various copies of CpG 1668 motif on resistance against *M. avidus* was investigated. Furthermore, in vivo experiments using plasmids containing mixed classes of CpG 1668, 2216 and 2395 motifs were conducted to enhance disease resistance of olive flounder simultaneously against both *M. avidus* and VHSV infections. Delivery of CpG motif-containing plasmids using *Escherichia coli* mutants was also investigated. In chapter III, TLR3 of olive flounder was cloned and the role of TLR3 and TLR22 in inducing type I interferon response was analyzed using RNA interference technology. In chapter IV, the effects of long dsRNA containing CpG motifs on disease resistance were analyzed.

Chapter I

Effect of CpG-ODNs belonging to different classes on resistance of olive flounder (*Paralichthys olivaceus*) against viral hemorrhagic septicemia virus (VHSV) and *Miamiensis avidus* (Ciliata; Scuticociliatia) infections

INTRODUCTION

Recognition of pathogen-associated molecular patterns (PAMP) by innate immune cells via pattern recognition receptors (PRR), such as Toll-like receptors (TLRs), leads to generation of optimal immune responses to pathogens (Krieg et al., 1995; Takeda et al., 2003; Iwasaki and Medzhitov, 2004). As the bacterial DNA contains a higher frequency of unmethylated deoxycytidyl-deoxyguanosine (CpG) dinucleotides than vertebrates DNA, unmethylated CpG motifs are recognized as a PAMP by TLR9 (Ahmad-Nejad et al., 2002; Akira and Takeda, 2004). Synthetic oligodeoxynucleotides with CpG motifs (CpG-ODNs) mimic the bacterial DNA, and stimulate immune cells via recognition by TLR9, which evoke a range of immunostimulatory effects in vertebrates (Krieg, 1999, 2003; Hemmi et al., 2000; Jørgensen et al., 2001a).

Three different classes of CpG-ODNs (A, B, and C) have been characterized based on a structural difference such as sequence and backbone composition, and each class of CpG-ODN has unique immunostimulatory properties (Krieg, 2002). The A-class CpG-ODNs contain a central palindromic phosphodiester CpG motif and phosphorothioated poly G motifs at the 5' and 3' ends. They are known to strongly stimulate type I interferon production and natural killer (NK) cell activation, but are poor in B cell stimulation (Krug et al., 2001; Verthelyi et al., 2001; Ulmann and Vollmer, 2003). The B-class CpG-ODNs contain one or more CpG motifs and are stabilized by entirely phosphorothioated backbone. They are much more potent in B cell stimulation than activation of type I interferon responses (Verthelyi et al., 2001; Kerkmann et al., 2003). The C-class CpG-ODNs are known to possess features of both class-A and -B not only in structure but also in functions. They have a central palindromic sequence, two or more CpG motifs, and

entirely phosphorothioated backbone. The C-class ODNs were demonstrated to stimulate both B and NK cells, and type I interferon responses (Marshall et al., 2003; Vollmer et al., 2004). Although most of these immunostimulatory properties of CpG-ODNs belonging to different classes were based on experiments using immune cells of human and mouse, recently Strandskog et al. (2007) reported that Atlantic salmon leukocytes responded to different classes of CpG ODNs in a similar way to mammalian immune cells. However, little is known regarding effects of different classes of CpG-ODNs on the resistance of fish against different pathogens.

To effectively defense against various infectious agents, immune responses that are adequate to each pathogen should be elicited. Since different classes and different CpG motifs possess abilities to evoke different immune responses, selection of CpG-ODNs that can yield optimal immune responses to the confronting pathogen would be one of the most important factors for use of CpG-ODNs as efficient immuno-prophylactic measures against infectious agents in aquaculture. Olive flounder (*Paralichthys olivaceus*) is the most commercially important marine species in Korea, however, diseases caused by viral hemorrhagic septicemia virus (VHSV) and *Miamiensis avidus* (=*Philasterides dicentrarchi*) infections have been the most serious obstacles for production of olive flounder (Kim et al., 2003, 2004, 2009; Jung et al. 2007). The aim of the present study was to investigate the effects of the three CpG-ODN classes on in vivo resistance of olive flounder against VHSV and *M. avidus* infections.

MATERIALS AND METHODS

1. CpG-ODNs

ODNs were synthesized from Bioneer Corporation (Korea). The sequence of ODN 1668 was 5'-TCCATGACGTTCCTGATGCT-3', ODN 2216 was 5'-GGGGGACGATCGTCGGGGGG-3', and ODN 2395 was 5'-TCGTCGTTTTCGGCGCGCGCCG-3'. ODN 1720 (5'-TCCATGAGCTTCCTGATGCT-3') and ODN 2243 (5'-GGGGGAGCATGCTGGGGGGG-3') were synthesized to use as controls of ODN 1668 and ODN 2216, respectively. The underlined nucleotides indicate phosphorothioate linkage.

2. Pathogens and growth conditions

VHSV KJ2008 isolated from a moribund olive flounder in a natural outbreak of VHS disease on a commercial farm in Korea was propagated in monolayer of *Epithelioma papulosum cyprini* (EPC) cells cultured in Leibovitz medium (L-15, Sigma) at 15°C in the presence of 2% fetal bovine serum (FBS, Gibco) and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml, Sigma). Cultures displaying extensive cytopathic effect (CPE) were harvested and centrifuged 4000 g for 10 min at 4°C, and the supernatants were stored at −80°C.

Ciliates were isolated from ascitic fluid of a diseased olive flounder obtained from a marine aquaculture farm in Korea, and were identified as *M. avidus* using a species specific

oligonucleotide primer pair (Kim et al., 2004). Chinook salmon epithelia (CHSE)-214 cells incubated at 20°C in Eagle's minimum essential medium (MEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) were used to maintain and proliferate the ciliates.

3. Effects of CpG ODNs on resistance of olive flounder against VHSV and M. avidus

Olive flounder fingerlings weighing approximately 10 g were obtained from a local fish hatchery and were acclimated at least for 2 weeks prior to the experiments.

3.1. VHSV challenge

Fish were transferred into six 50 L tanks (15 fish/tank) equipped with a refrigerating apparatus, then, adapted to 15°C by gradual decrease of water temperature. Fish were intraperitoneally (i.p.) injected with 10 μg of ODN 1668, ODN 1720, ODN 2216, ODN 2243 or ODN 2395. Fish in control group were i.p. injected with 50 μl of phosphate buffered saline (PBS). On 24 h post-injection, three fish from each group were randomly sampled for analysis of Mx and ISG15 genes expression. After 3 days of the injection, all remaining fish in each group were intramuscularly (i.m.) challenged with the VHSV KJ2008 at 10^3 PFU/fish. Mortality was recorded daily for 3 weeks post-challenge.

3.2. M. avidus challenge

Fish were randomly divided into 6 groups and reared in six 50 L tanks (15 fish/tank) at 22-23°C. Fish were i.p. injected with each ODN or PBS as the same regime of the above VHSV

challenge groups. At 3 days post-injection, 3 fish in each group were randomly sampled for collection of serum, and the remnant fish were challenged by i.p. injection with 5 x 10^4 cells of CHSE-cultured *M. avidus* per fish, and the mortality was monitored for 3 weeks.

4. Semi-quantitative RT-PCR analysis

Total RNA was isolated from the kidney using RNAiso plus reagent (Takara) at 24 h post-injection. To obtain complementary DNA (cDNA), 1 μg of purified RNA was incubated with 1 μl of random primer (0.5 μg/ml) (Promega) at 70°C for 10 min, and further incubated at 42°C for 60 min in reaction mixture containing 4 μl of 5× reaction buffer, 4 μl of 10 mM dNTP mix (Takara), 1 μl of M-MLV reverse transcriptase (Promega) and 0.5 μl of RNase inhibitor (Promega) in a final reaction volume of 20 μl. PCR was done with primers Mx, ISG15 and 18S ribosomal RNA of olive flounder, which was served as RNA normalization (Table 1). PCR was done in a 10 μl reaction containing 5 μl of 2× Prime Taq Premix (GeNet Bio, Korea), 0.5 μM each primer and 1 μl of 10^{-1} diluted cDNA, and distilled water. The amplification procedure included 1 cycle of 4 min at 95°C, 16 (for 18S rRNA) or 30 cycles (for Mx and ISG15) of 30 s at 95°C, 30 s at 60°C, 30 s at 72°C, with a final extension step of 7 min at 72°C. PCR samples to be compared were electrophoresed on the same 1.0% agarose gel, stained with ethidium bromide (EtBr), and pictured with Gel Doc XR (Biorad). The intensity of bands was estimated using Quantity One software (Biorad). Expression of Mx and ISG15 genes was calculated as relative expression to 18S rRNA gene.

Table 1. Oligonucleotide primer pairs for semi-quantitative RT- PCR of Olive flounder

Name of primer		Sequence (5' to 3')
rf18S	F	AGTTGCTGCAGTTAAAAAGC
	R	TGGCATCGTTTACGGTCGGAACTA
rfISG15	F	CTCCATGTAATCTGCAGCAA
	R	AGATCTAGTGCAGGTGTGA
rfMx	F	AACAGCCAAGGCAAAGATTG
	R	AATGTCCAGCTCCTCCTTCA

5. Scuticocidal activity of serum

Scuticocidal activity of the collected serum was analyzed using 96-well flat-bottomed micro- titration plates. All the sera were serially diluted ranging from 1/4 to 1/1024 in Hank's balanced salt solution (HBSS, Sigma). *M. avidus* were collected after 3 day of CHSE-214 cells depletion in the culture vessels by centrifugation at 200 g for 5 min, washed three times with HBSS and resuspended in the buffer. The ciliates were added to the wells (4×10^2 ciliates/well) of the plate, incubated at 20°C and observed every one hour for 24 h to analysis scuticocidal activity of the sera. The titer of each serum was the last dilution at which 100% of the ciliates were lysed or non-motile, which was observed under an inverted microscope at 40-100x magnification. In all assays, control wells containing heat-inactivated pooled sera (at 50°C for 30 min; 1/4 dilution) of each experimental group and wells containing no serum were included.

6. *Statistical analysis*

Data of semiquantitative RT-PCR and serum scuticocidal activity were analyzed by the Student's *t*-test. Significant differences were determined at $P<0.05$.

RESULTS

1. Effects of CpG-ODNs on resistance of olive flounder against VHSV infection

The group of fish administred CpG-ODN 2216 showed no mortality against a challenge with VHSV (Fig. 1A). On the other hand, olive flounder i.p. injected with CpG-ODN 1668 showed no enhanced resistance against VHSV challenge. Fish administered CpG-ODN 2395 (C-class ODN) showed intermediate resistance against VHSV between ODN 1668 and ODN 2216.

2. Effects of CpG-ODNs on resistance of olive flounder against M. avidus infection

Fish injected CpG-ODN 1668 showed the highest survival rate (Fig. 1B). As in the VHSV infection experiment, administration of CpG-ODN 2395 elicited secondly higher survival rate. Although fish administered CpG-ODN 2216 eventually showed 100% mortality, the cumulative mortality until 2 weeks post-challenge was similar to the group of fish injected CpG-ODN 1668.

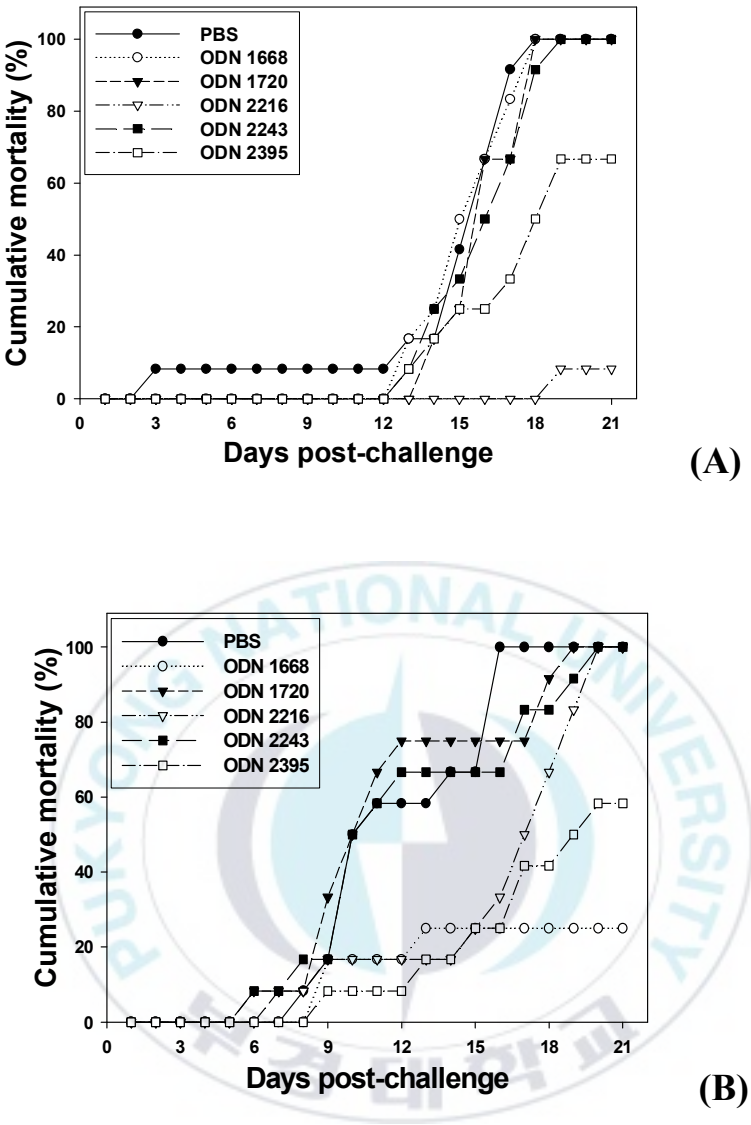
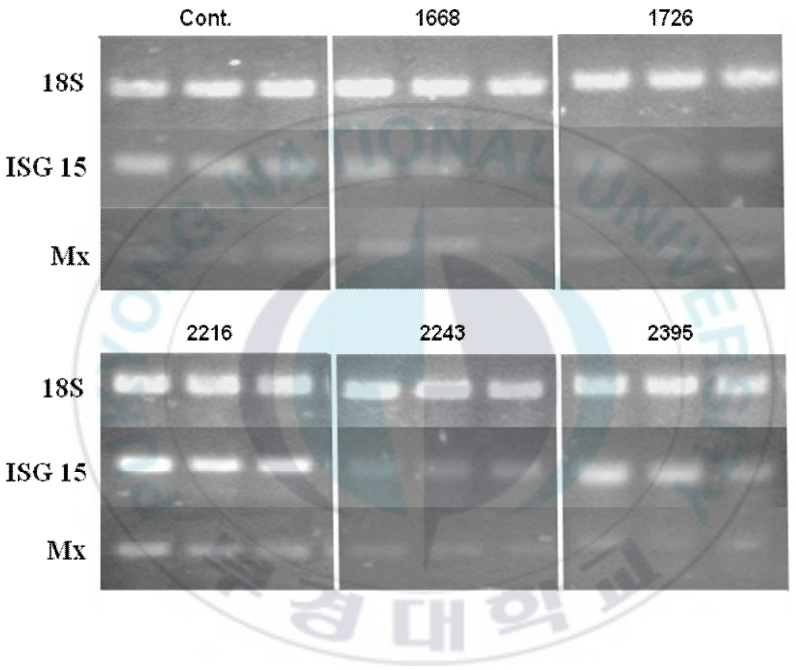


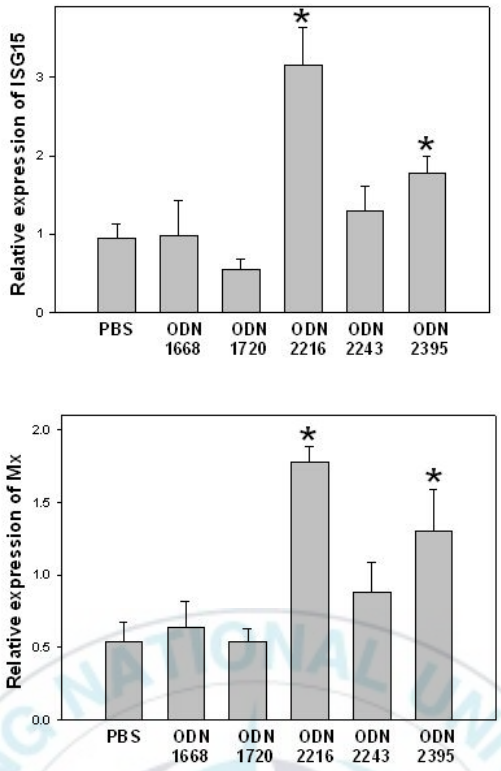
Fig. 1. Cumulative mortality of olive flounder (*Paralichthys olivaceus*) intraperitoneally (i.p.) injected with phosphate buffered saline (PBS), CpG-ODN 1668, 2216, 2395, or control ODN 1720, 2243 (10 μg/fish). At 3 days post-injection, fish in each group were (A) intramuscularly (i.m.) challenged with the VHSV KJ2008 at 10^3 PFU/fish, or (B) challenged by i.p. injection with 5 x 10^4 cells of *M. avidus* per fish.

3. Expression of genes

The effect of each CpG-ODN on the type I interferon response of olive flounder was investigated by analyzing expression of Mx and ISG15 genes at 24 h post-administration. Fish administered CpG-ODN 2216 showed the highest expression of Mx and ISG15 genes compared to fish in other groups (Fig. 2A and 2B). Fish injected with CpG-ODN 2395, also, showed significantly higher expression of Mx and ISG15 genes than fish in the control group.



(A)



(B)

Fig. 2. RT-PCR amplification of ISG15 and Mx genes in olive flounder (*Paralichthys olivaceus*) intraperitoneally (i.p.) injected with phosphate buffered saline (PBS), CpG-ODN 1668, 2216, 2395, or control ODN 1720, 2243 (10 μg/fish). Total RNA was isolated from kidney of olive flounder at 24 h post-injection. Expression of ISG15, Mx, and 18S ribosomal RNA (18S) genes was PCR amplified from reverse-transcribed cDNA samples using primers specific for each gene. (A) Stained gel image showing the band of 18S, ISG15, and Mx. (B) A semi-quantitative analysis of expression of ISG15 and Mx. Data was presented as mean value of relative expression to 18S with standard deviation (n=3). Bars with an asterisk indicate statistically differences at $P < 0.05$, when compared to the control PBS.

4. Scuticocidal activity of serum

Fish injected with CpG-ODN 1668 showed the highest serum scuticocidal activity (Fig. 3). CpG-ODN 2395 also induced significantly higher scuticocidal activity than fish injected with PBS, CpG-ODN 2216, control ODNs 1720 and 2243.

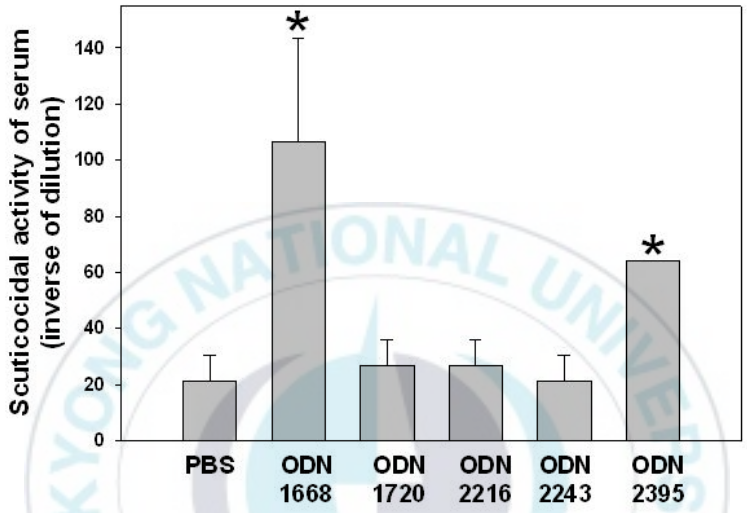


Fig. 3. Scuticocidal activity of sera collected from olive flounder (*Paralichthys olivaceus*) intraperitoneally (i.p.) injected with phosphate buffered saline (PBS), CpG-ODN 1668, 2216, 2395, or control ODN 1720, 2243 (10 μg/fish). At 3 days post-injection, 3 fish in each group were randomly sampled and bled to obtain serum. Values (inverse of serum dilution) are means and T-bars indicate standard deviation. (As the CpG-ODN 2395 injected group showed no differences in the scuticocidal activity of sera, T-bars are not shown). Bars with an asterisk indicate statistically differences at $P < 0.05$, when compared to the control PBS.

DISCUSSION

In the present study, we have firstly demonstrated that CpG-ODNs belonging to different classes have different abilities to increase resistance of olive flounder against different pathogens. Although immunostimulatory effects of various CpG-ODNs on various fish species have been reported (Tassakka and Sakai, 2005; Carrington and Secombes, 2006), most of the experimented CpG-ODNs were B-class ODNs, and a few data on the effects according to different classes of ODNs are available. Recently, Strandskog et al. (2007) demonstrated the different effects of ODNs on fish according to their classes, in which A-class CpG-ODN induced strong IFN α/β activity from leukocytes of Atlantic salmon (*Salmo salar*), B-class ODN stimulated leukocytes proliferation, and C-class CpG-ODN induced both interferon and cell proliferation responses.

Little information is available on in vivo antiviral effects of CpG-ODNs against fish viruses. Jørgensen et al. (2001a, b) reported that leukocytes isolated from Atlantic salmon or rainbow trout secreted antiviral cytokines against infectious pancreatic necrosis virus (IPNV) by stimulation with CpG-ODNs (ODN 1668, 1670, and 1651) that belong to the B-class CpG-ODNs. Later, Jørgensen et al. (2003) reported in vivo enhancement of resistance against IPNV challenge in Atlantic salmon by i.p. injection of CpG-ODN 1681 (B-class ODN). In the present results, CpG-ODN 2216 (A-class ODN) induced distinctively high resistance against VHSV infection in olive flounder, and administration of CpG-ODN 2395 (C-class ODN) led to slightly high protection against VHSV infection. On the other hand, olive flounder i.p. injected with CpG-ODN 1668 (B-class ODN) showed no enhanced resistance against VHSV challenge. It is well-known that type I interferon responses are important for the innate immune-mediated defense against viral infections.

In this study, fish treated with CpG-ODN 2216 showed the strongest expression of Mx and ISG15 genes. The results suggest that CpG-ODN 2216 is a potent stimulator of type I interferon in olive flounder, which might allow the fish to defense efficiently against VHSV infection. Furthermore, in contrast to the results of Jørgensen et al. (2003), the present no protective effect of the B-class CpG-ODN against VHSV infection indicates that the immuno-prophylactic effect of CpG-ODNs might be influenced by fish species and ODN sequences.

Previously, we had demonstrated that olive flounder administered CpG-ODN 1668 showed significantly higher survival against *M. avidus* infection (Lee and Kim, 2009). In the present study, also, fish injected with CpG-ODN 1668 showed the highest survival rate. As in the VHSV infection experiment, administration of CpG-ODN 2395 elicited secondly higher survival rate against *M. avidus* infection. The delayed mortality of fish administered CpG-ODN 2216 suggests that type I interferon-mediated immune responses might partly participate in defense against *M. avidus* infection. The present strongest scuticocidal activity of serum in fish treated with CpG-ODN 1668 indicates that B-class ODNs might have high potential to elicit humoral immune responses in olive flounder. Recently, Piazzon et al. (2011) reported that humoral immune responses played more important role than cellular immune responses in protecting fish against scuticociliates infection. Therefore, CpG-ODN 1668 would be appropriate to use as an immunostimulant for protection against *M. avidus* infection in olive flounder.

In summary, the present study demonstrates that treatment of olive flounder with a CpG-ODN 1668 and CpG-ODN 2216 can enhance resistance of olive flounder against VHSV and *M. avidus* infections, respectively, which suggests that selection of a CpG-ODN appropriate to the characteristics of a certain pathogen is crucial to induce optimal immunostimulation-mediated defense in fish.

Chapter II

Effects of plasmid-based CpG motifs on defense of olive flounder (*Paralichthys olivaceus*) against *Miamiensis avidus* and viral hemorrhagic septicemia virus (VHSV)

INTRODUCTION

Synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG dinucleotides (CpG motif) are known as the strong stimulants for enhancing innate immunity of vertebrates (Krieg et al., 1995, 2000; Krieg, 2002), in which CpG motifs are recognized as a pathogen-associated molecular pattern (PAMP) by the Toll-like receptor 9 (TLR9) of immune cells. TLR9-mediated signal cascades induce diverse immune responses such as production of pro-inflammatory cytokines and type I interferon responses (Aderem and Ulevitch, 2000; Akira and Takeda, 2004). However, in an economical aspect, CpG-ODNs are too expensive to use in aquaculture farms.

Plasmids with stimulatory CpG motifs in their backbone also have potential to be used as immunostimulants and vaccine adjuvants in vertebrates. According to the results of Cornélie et al. (2004), murine TLR9 specifically binds plasmids that contain unmethylated CpG motifs. In fish, Chen et al. (2007) had reported that plasmids harboring tandemly arranged 10 CpG-ODNs sequence (ODN 1681, 1669, 2133, 2102, 2143, 2006, 1826, 1670, 1668, and 1651) enhanced in vitro innate cellular immune responses such as respiratory burst and bactericidal activities of head kidney macrophages of Crucian carp (*Carassius auratus*) and Japanese bass (*Lateolabrax japonicus*). Lately, Liu et al. (2010) also demonstrated that a plasmid (pCN6) with sequences of four ODNs (209, 2133, 201, and 203) significantly enhanced respiratory burst, acid phosphatase, bactericidal activities of head kidney macrophages, serum bactericidal activity, and resistance against *Aeromonas hydrophila* and *Edwardsiella tarda* infections in olive flounder. Therefore, in this study, we adopt bacterial plasmids as a method to deliver CpG motifs.

It has been reported that the innate immune responses play an important role in enhancing the resistance of fish against parasitic ciliates infections. In our previous immunization experiment (Lee and Kim, 2008), olive flounder administered Freund's adjuvant alone showed higher protection against *M. avidus* challenge, suggesting the importance of innate immunity in defense against scuticociliatosis. And, a little is known about the effects of CpG-ODNs on the resistance of fish against parasites infections. So far as we know, there are only two reports on the protective effects of CpG-ODNs against fish parasitic diseases; amoebic gill disease caused by *Neoparamoeba permaquidensis* infection in Atlantic salmon (Bridle et al., 2003) and scuticociliatosis caused by *M. avidus* infection in olive flounder (Lee and Kim, 2009). Effects of CpG-ODN against fish viruses has been poorly reported. It has been reported that Atlantic salmon by i.p injection of CpG-ODN 1681(B-class) has protected against IPNV challenge (Jørgensen et al. 2001a, b, 2003).

Not only different CpG motif sequences but also different copy number of CpG motif can influence on the potency of immune responses in a species (Krieg, 2002; Pontarollo et al., 2002). Therefore, selection of immunostimulatory CpG motif sequences and optimizing the copy number of CpG motif sequences are essential for induction of favorable immune responses using plasmids. In this study, to know whether the plasmid-based CpG 1668 motifs and the copy number of CpG 1668 sequences in plasmids influence on the resistance of olive flounder against *M. avidus* infection, fish were injected with plasmids containing various copy numbers of the CpG 1668 motif, and the effects were analyzed by investigating serum scuticocidal activity and survival rate of fish against *M. avidus* challenge.

In the previous experiments, we have verified that CpG-ODN 2216 (A-class ODN) elicited strong type I interferon response and induced high resistance against viral hemorrhagic septicemia virus (VHSV), whereas CpG-ODN 1668 (B-class) elicited significantly high serum scuticocidal

activity and survival rate against *Miamiensis avidus* challenge. Furthermore, CpG-ODN 2395 (C-class) showed abilities intermediate between ODN 2216 and ODN 1668 in inducing resistance against VHSV and *M. avidus*. As CpG-ODNs belonging to each class possess peculiar abilities to induce different host immune responses, we postulated that co-administration of CpG-ODNs belonging to different classes would elicit combined immune responses, which would simultaneously confer resistance against both viral and parasitic pathogens. Therefore, in this study, we constructed plasmids that harbor multi-copy of CpG-ODN 2216, 1668, and 2395 sequences, and evaluated potential of the constructed plasmids to stimulate immune responses and to protect of fish against VHSV and *M. avidus* infections.

A burdensome procedure is required to isolate plasmids with stimulatory CpG motifs from cultured *Escherichia coli*, and cost for conducting the procedure is too high to use in aquaculture farms. Furthermore, as CpG DNAs can be easily degraded by nucleases, their in vivo use has been limited. Therefore, in this study, to overcome these problems, we used two kinds of *E. coli* mutants, *E. coli* HT115 and *E. coli* χ7213, as the delivery vehicle for CpG motifs-containing plasmids. In the mutant *E. coli* HT115, a gene encoding RNase III that can digest dsRNAs is deleted and *lac* promoter-driven T7 RNA polymerase gene is inserted into the genome. Therefore, if CpG motifs in a plasmid vector are located between two T7 promoters that are arranged in a head to head fashion and the constructed vectors were transferred to *E. coli* HT115, long dsRNA encoding the CpG motifs can be generated without degradation by RNase III. Furthermore, as the plasmids harboring CpG motifs can be maintained in the *E. coli* cytoplasm, CpG DNA-mediated effects also can be expected. In the mutant *E. coli* χ7213, a gene encoding aspartate semialdehyde dehydrogenase (*asd*) is deleted, and the *asd* mutant bacteria obligatory require diaminopimelic acid (DAP), an essential constituent of bacterial cell wall, and will undergo lysis unless provided with DAP. Thus CpG motifs-containing plasmids can be easily exposed to the immune cells of

hosts through burst of the mutant bacteria in the hosts due to the lack of DAP. In this study, in vivo experiments were conducted to know whether delivery of the CpG motifs-containing plasmids by the two mutants *E. coli* can induce protection of fish against *M. avidus* infection.

MATERIALS AND METHODS

1. Ciliates and VHSV

M. avidus were isolated from diseased olive flounders obtained from local fish farms in South Korea. Identification of ciliates species was done by PCR using species specific oligonucleotide primer pairs (Kim et al. 2004). *M. avidus* were grown using Chinook salmon epithelia (CHSE)-214 cells incubated at 20°C in Eagle's minimum essential medium (MEM, Gibco) supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Gibco).

VHSV KJ2008 isolated in 2008 from moribund olive flounder in a natural outbreak of VHS disease on a commercial farm in Korea was propagated in monolayer of EPC cells [culture condition: Leibovitz medium (L-15, Sigma) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS, Gibco)] at 15°C in the presence of 2% FBS and antibiotics. Cultures displaying extensive cytopathic effect (CPE) were harvested and centrifuged 4000 g for 10 min at 4°C, and the supernatants were stored at −80°C.

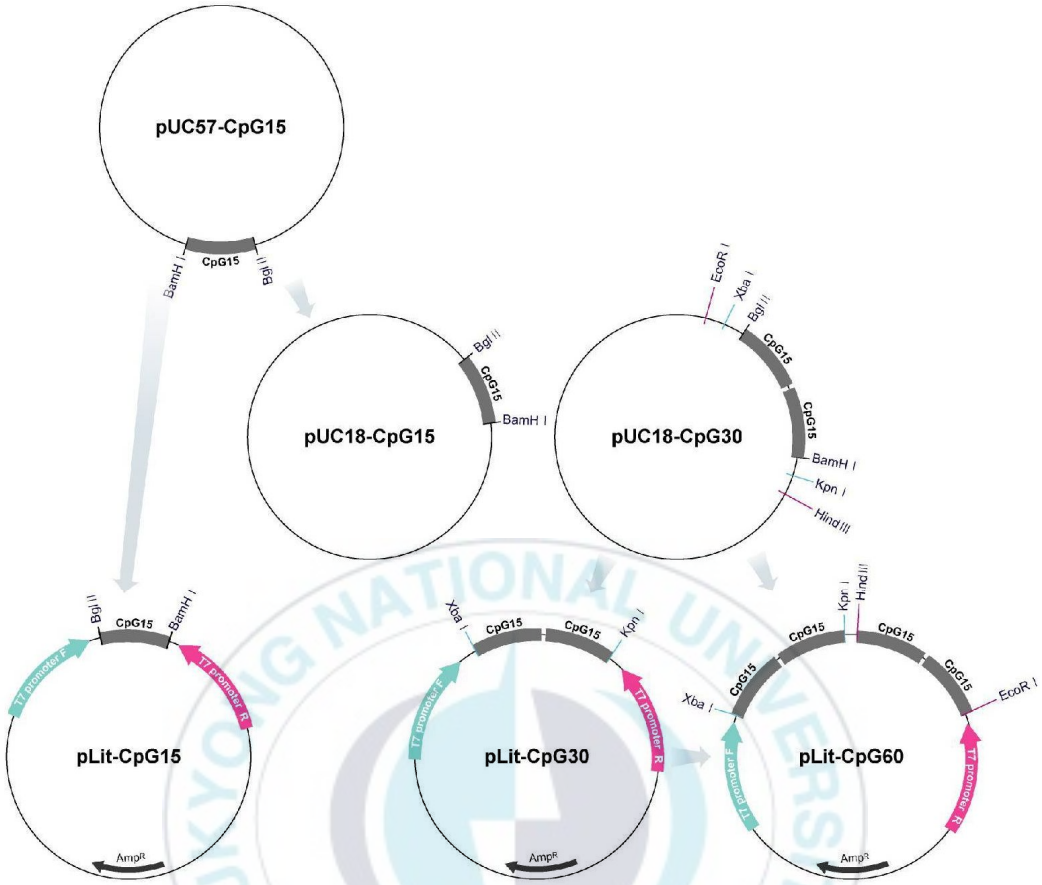
2. Plasmids construction and transfomation

Fifteen copies of CpG-ODN 1668 sequence were artificially synthesized and inserted into pUC57 vector (Cosmo Genetech, Korea). The fragment containing 15 copies of CpG 1668 sequence was isolated by digestion of the vector with *Bgl*II and *BamH*I, and then ligated into Litmus28i vector (NEB) and pUC18 vector that were pre-digested with the same restriction

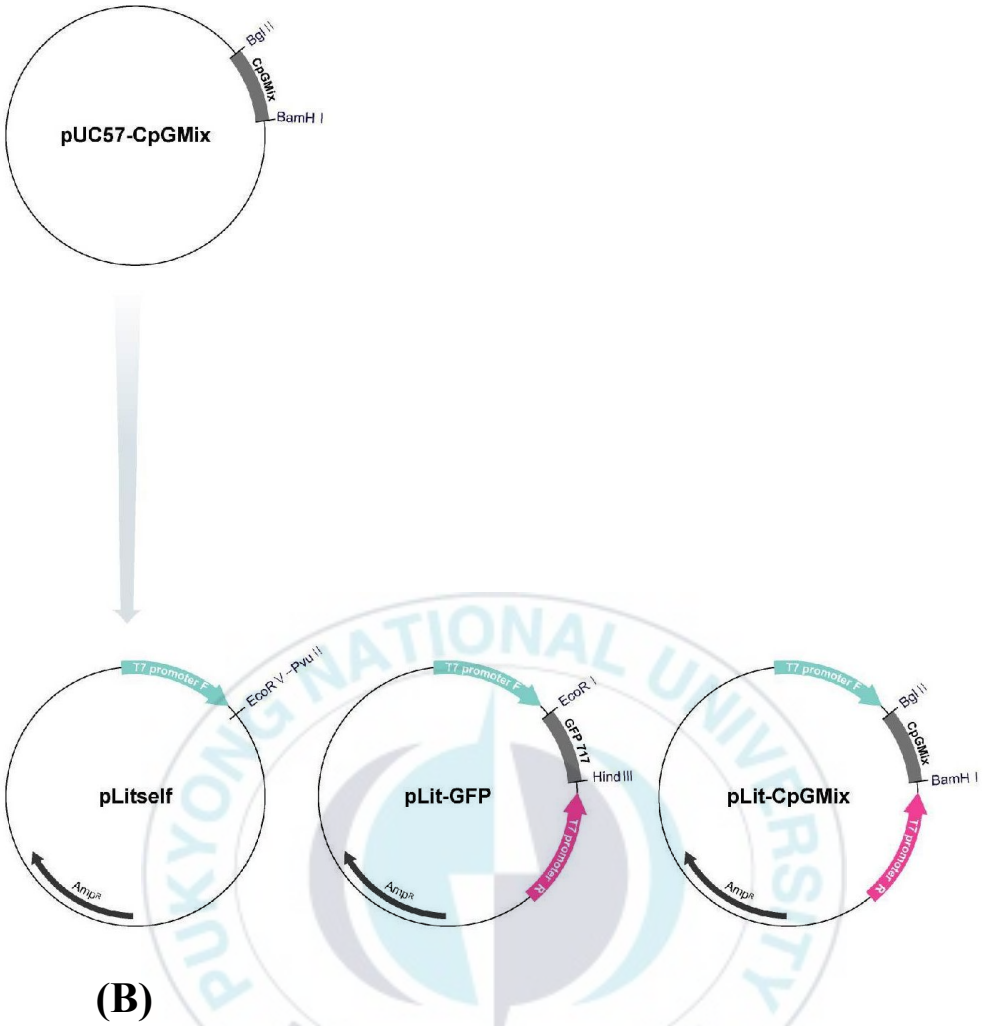
enzymes, then designated it as pLitmus28i-CpG1668 15 (pLit-CpG15) and pUC18-CpG1668 15 (pUC18-CpG15), respectively. The CpG15 fragment in the pLit-CpG15 vector was isolated by digestion of the vector with *Bgl*II and *BamH*I, and ligated into pUC18-CpG15 vector that was pre-digested with *BamH*I, resulting in a vector with 30 copies of the CpG 1668 motif (pUC18-CpG1668 30 : pUC18-CpG30). After digestion of the vector with *Xba*I and *Kpn*I, the CpG30 fragment was inserted into pLitmus28i vector that was pre-digested with the same enzymes, resulting in pLit-CpG30. The CpG30 fragment was also isolated by digestion of the pUC18-CpG30 with *Hind*III and *EcoR*I, and was ligated into the pLit-CpG30, resulting in a vector with 60 copies of CpG 1668 motif, pLitmus28i-CpG1668 60 (pLit-CpG60) (Fig. 4A).

Fragment encoding the T7 promoter region (Coordinates 2620-2603) in the pLitmus28i vector (NEB) was removed by digestion with *EcoR*V and *Pvu*II, and designated as pLitself. Green fluorescent protein (GFP) gene was PCR amplified using primers GFP-F (5'-GAATTCatgagtaaaggagaagaac-3' with *EcoR* Ⅰ site), GFP- R (5'-AAGCTTttatttgtagagctcatc-3' with *Hind* III site), and pGFP as a template. The amplified product (717 bp) was cloned into the pGEM T-easy vector (Promega) and sequenced using an automatic sequencer (Applied biosystems). The cloned fragment was digested with *EcoR* I and *Hind* III, and subcloned into the pLitmus 28i vector that was pre-digested with the same restriction enzymes, resulting in pLit-GFP. A fragment corresponding to randomly arranged 6 copies of each CpG-ODN sequence (CpG-ODN 1668, 2216 and 2395) was artificially synthesized and inserted into pUC57 vector (pUC57-CpGMix)(Cosmo Genetech, Korea). After digestion with *Bgl*II and *BamH*I, the CpG fragment was ligated into pLitself that was pre-digested with the same restriction enzymes, then designated it as pLitmus 28i-CpGMix (Lit-CpGMix) (Fig. 4B).

pLitself, pLit-GFP, Lit-CpGMix were used to transform *Escherichia coli* HT115 *and E. coli* χ7213.


pUC57-CpG15
BamH I
CpG15
Bgl II
pUC18-CpG15
Bgl II
CpG15
BamH I
pUC18-CpG30
EcoR I
Xba I
Bgl II
CpG15
CpG15
BamH I
Kpn I
HindIII
pLit-CpG15
Bgl II
CpG15
BamH I
T7 promoter F
T7 promoter R
AmpR
pLit-CpG30
Xba I
CpG15
CpG15
Kpn I
T7 promoter F
T7 promoter R
AmpR
pLit-CpG60
Xba I
CpG15
Kpn I
HindIII
CpG15
CpG15
CpG15
EcoR I
T7 promoter F
T7 promoter R
AmpR

(A)



(B)

Fig. 4. Process of vector construction. (A) Fifteen copies of CpG-ODN 1668 sequence inserted into pUC57 vector (pUC57-CpG15). CpG-ODN1668 15 and 30 copies fragment was inserted into pUC18 vector (pUC18-CpG15 and pUC18-CpG30), CpG-ODN1668 15, 30 and 60 copies fragment was inserted into pLitmus28i vector (pLit-CpG15, pLit-CpG30 and pLit-CpG60), respectively. (B) Fragment encoding the T7 promoter region (Coordinates 2620-2603) in the pLitmus28i vector was removed by digestion with *EcoR*V and *Pvu*II(pLitself). GFP fragment was inserted into pLitmus28i vector(pLit-GFP), A fragment corresponding to randomly arranged 6

copies of each CpG-ODNs sequence (CpG-ODN 1668, 2216 and 2395) (CpGMix) inserted into pUC57 vector (pUC57- CpGMix), then designated it as pLitmus 28i-CpGMix (Lit-CpGMix). Amp^R, ampicillin resistance gene; BglII-BamHI, XbaI-KpnI, EcoRI-HindIII or EcoRV-PvuII, restriction sites used for the plasmid construction.

3. in vivo experiment

3.1 Effects of plasmids harboring various copies of CpG 1668 motifs on resistance of fish against M. avidus infection

Olive flounder fingerlings weighing approximately 6~7 g were obtained from a local fish farm, divided into twelve 50 L tanks (20 fish/tank), and were acclimated at least for 2 weeks before the experiment. Sea water temperature was maintained between 21-22°C during whole experimental period. Fish in the 12 tanks were divided into two replicates, and each replicate consisted of 6 groups. Fish in group I were intraperitoneally injected (i.p.) with 50 μl of PBS; group II were i.p. injected with 10 μg of CpG-ODN 1668; group III were i.p. injected with 100 μg of Litmus 28i vector; group IV, V, and VI were i.p. injected with 100 μg of pLit-CpG15, pLit-CpG30, and pLit-CpG60, respectively. At 3 d post-injection, 5 fish in each tank were randomly sampled and bled to isolate serum. The rest of fish in one replicate were i.p. challenged with 1 x 10^4 ciliates, and fish in the other replicate were challenged with 2.5 x 10^4 ciliates. The mortality was monitored for 20 days, and dead fish were necropsied to confirm the presence of ciliates.

3.2 Effects of plasmids harboring multi-copies of mixed CpG motifs on resistance of olive flounder against VHSV and M. avidus

Olive flounder fingerlings weighing approximately 4~5 g were obtained from a local fish

farm in Gu-jae Gyung-nam, Korea, and were acclimated at least for 2 weeks before the experiment. Fish in each experimental group were divided into 2 subgroups, either *M. avidus* or VHSV challenge group. For *M. avidus* challenge experiment, fish were divided into 4 groups with 2 replicates in eight 50 L tanks (18 fish/tank). Fish in group 1, 2, 3 and 4 were intraperitoneally injected (i.p.) with 100 μl of PBS (control), 100 μg of pLitself, 100 μg of pLit-GFP, and 100 μg of pLit-CpGMix, respectively. Water temperature was maintained at 21-22°C. At 3 days post-injection, 4 fish in each tank were randomly sampled and bled to isolate serum. The rest of fish in one replicate were i.p. challenged with 2 x 10^4 ciliates, and fish in the other replicate were challenged with 2 x 10^5 ciliates. The mortality was monitored for 17 days, and dead fish were necropsied to confirm the presence of ciliates. The same experimental regime was used for VHSV challenge experiment except no replicated groups. At 24 h post-injection, 3 fish in each tank were randomly sampled for isolation of the kidney. At 3 d post-injection, the rest of fish were intramuscularly (i.m) injected with VHSV KJ2008 at 10^3 PFU/fish. The mortality was monitored for 14 days, and dead fish were PCR-analyzed for detection of VHSV.

3.3 Effects of E. coli mutants carrying CpG-plasmids on resistance of olive flounder against M. avidus

Olive flounder fingerlings weighing approximately 7~8 g were obtained from a local fish farm and were acclimated at least for 2 weeks before the experiment. Water temperature was maintained at 22°C during whole experimental period. Fish were divided into 8 groups (18 fish/group), and were intraperitoneally (i.p.) injected with 50 μl of PBS, *E. coli* χ7213 harboring pLitself (Litself/χ7213), *E. coli* χ7213 harboring pLitmus28i-GFP (Lit-GFP/χ7213), *E. coli* χ7213 harboring pLitmus28i-CpGMix (Lit-CpGMix /χ7213), *E. coli* HT115 harboring pLitself/HT115, *E. coli* HT115 harboring pLitmus28i-GFP (Lit-GFP/HT115), *E. coli* HT115 harboring pLitmus28i-

CpGMix (Lit-CpGMix/HT115) and mixed *E. coli* HT115 and χ7213 harboring pLitmus28i-CpGMix (Lit-CpGMix/HT115+χ7213), respectively, at a dose of $5x10^6$ cfu/fish. At 3 d post-injection, 3 fish in each tank were randomly sampled and bled to isolate serum. The rest of fish were i.p. challenged with 5 x 10^4 ciliates. The mortality was monitored for 14 days, and dead fish were necropsied to confirm the presence of ciliates.

4. Serum scuticocidal activity

The analysis of scuticocidal activity of serum was performed using 96-well flat-bottomed plates. All the sera were serially diluted ranging from 1/4 to 1/4096 in Hank's balanced salt solution (HBSS, Sigma). The CHSE-cultured ciliates were collected after 3 d of CHSE cells depletion in the culture vessels by centrifugation at 200 g for 5 min, washed three times with HBSS. The ciliates were added to the wells (1×10^2 ciliates/well) of the plate, incubated at 20°C and observed every one hour for 24 h to analysis scuticocidal activities of the sera. The titer of each serum was the last dilution at which 100% of the ciliates were lysed or non-motile, which was observed under an inverted microscope at 40-100 x magnification. In all assays, control wells containing no serum were included.

5. Semi-quantitative RT-PCR analysis

Total RNA was extracted from the kidney using RNAiso plus reagent (Takara) at 24 h post-immunization. The complementary DNAs (cDNAs) synthesized from these total RNAs using M-MLV reverse transcriptase and Oligo(dT)15 Primer (Promega) were used in reverse-transcription polymerase chain reaction (RT-PCR). To amplify cDNA of Mx gene, PCR was done with primers MxF (5'-AACAGCCAAGGCAAAGATTG-3') and MxR (5'- AATGTCCAGCTCCTCCTTCA-3'). ISG15 gene was PCR-amplified using primers ISG15F (5'-CTCCATGTAATCTGCAGCAA-3') and ISG15R (5'-AGATCTAGTGCAGGTGTGA-3'). Two primers 18SF (5'-AGTTGCTGCAGTT AAAAAGC-3') and 18SR (5'-TGGCATCGTTTACGGTCGGAACTA-3') were used to amplify 18S ribosomal RNA gene of olive flounder. PCR was performed using 2× Prime Taq Premix (GeNet Bio, Korea). The amplification procedure included 1cycle of 4 min at 95°C,16 (for 18S rRNA) or 30 cycles (for Mx and ISG15) of 30 s at 95°C, 30 s at 60°C, 30 s at 72°C, with a final extension step of 7 min at 72°C. PCR samples to be compared were electrophoresed on the same 1.0% agarose gel, stained with ethidium bromide (EtBr), and pictured with Gel Doc XR (Biorad). Expression of Mx and ISG15 genes was calculated as relative expression to 18S rRNA gene using Quantity One software (Biorad).

6. Statistical analysis

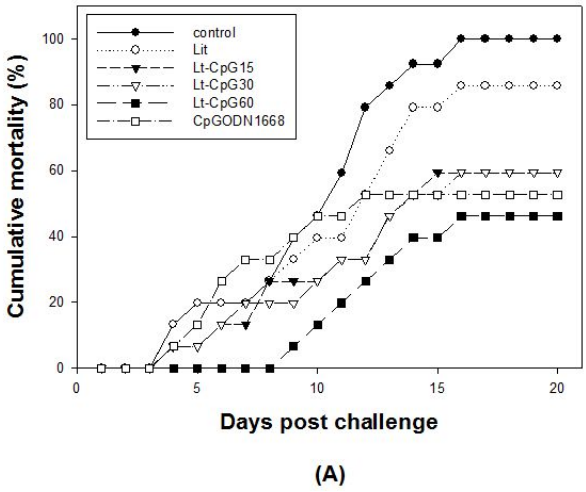
Data of serum scuticocidal activity were analyzed by the Student's *t*-test. Significant differences were determined at $P<0.05$.

RESULTS

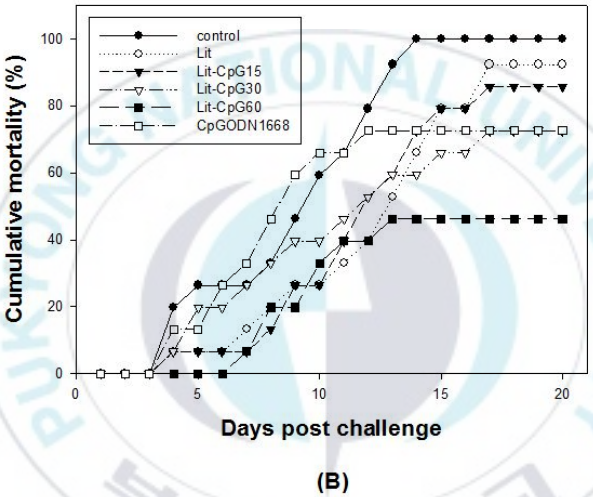
1. Effects of plasmids harboring various copies of CpG 1668 motifs on resistance of olive flounder

1.1 Survival of fish after challenge against M. avidus

In a replicate challenged with 1 x 10^4 ciliates, the cumulative mortalities of fish injected with CpG-ODN 1668 or plasmids harboring CpG 1668 motifs were clearly lower than fish injected with PBS alone or Litmus 28i vector (Fig. 5A). Although the group injected with pLit-CpG60 showed the highest survival rate, there were no great differences in survival rate among groups of fish injected with CpG-ODN 1668 or plasmids harboring CpG 1668 motifs. In another replicate challenged with 2.5 x 10^4 ciliates, only the group of fish injected with pLit-CpG60 showed clearly higher survival rate (Fig. 5B). The ciliates were recovered from the internal organs of all dead fish.



(A)



(B)

Fig. 5. Cumulative mortality of olive flounder (*Paralichthys olivaceus*) fingerling immunized by intraperitoneal injection of phosphate buffered saline (control), pLitmus28i (Lit), pLitmus28i-CpG15 (Lit-CpG15), pLitmus28i-CpG30 (Lit-CpG30), pLitmus28i-CpG60 (Lit-CpG60) or CpG-ODN 1668. Each fish in each group was challenged by i.p. injection of 1 x 10^4 (A) CHSE-cultured ciliates, and fish in the other replicate were challenged with 2.5 x 10^4 (B) CHSE-cultured ciliates. The mortality was monitored for 20 days.

1.2 Serum scuticocidal activity

Fish injected with CpG-ODN 1668 or plasmids harboring CpG 1668 motif showed significantly higher serum scuticocidal activity than fish injected with PBS alone or Litmus 28i vector (Fig. 6). Furthermore, the serum scuticocidal activity was increased proportion to the number of CpG 1668 motif in the plasmid. Serum scuticocidal activity of fish injected with pLit-CpG60 was similar to that of fish injected with CpG-ODN 1668. No scuticocidal activity was observed in the wells containing heat inactivated sera or HBSS alone.

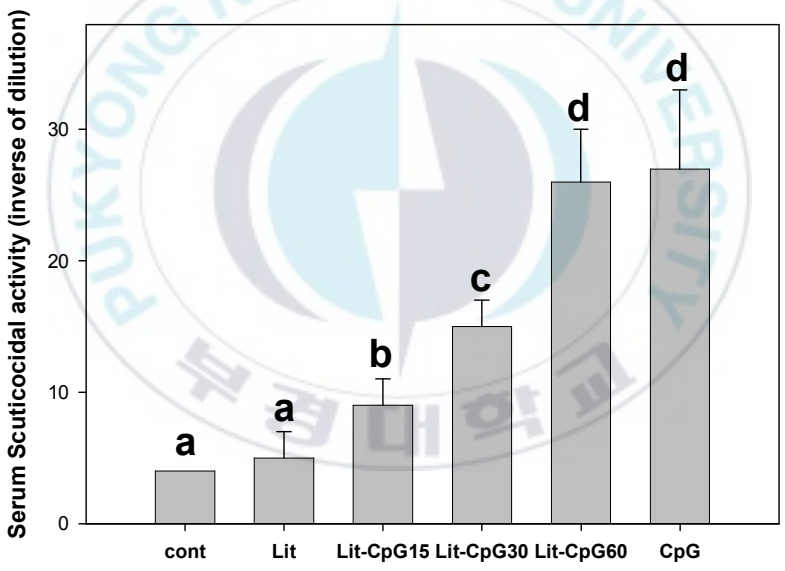


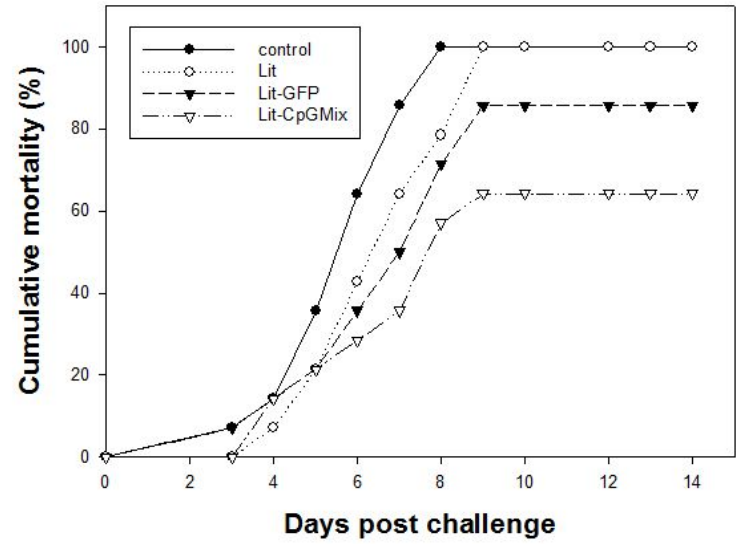
Fig. 6. The scuticocidal activity of sera collected from olive flounder (*Paralichthys olivaceus*) injected with PBS, pLitmus28i (Lit), pLitmus28i-CpG15 (Lit-CpG15), pLitmus28i-CpG30 (Lit-CpG30), pLitmus28i-CpG60 (Lit-CpG60) or CpGODN 1668 (CpG).

2. Effects of plasmids harboring multi-copies of mixed CpG motifs on resistance of olive flounder

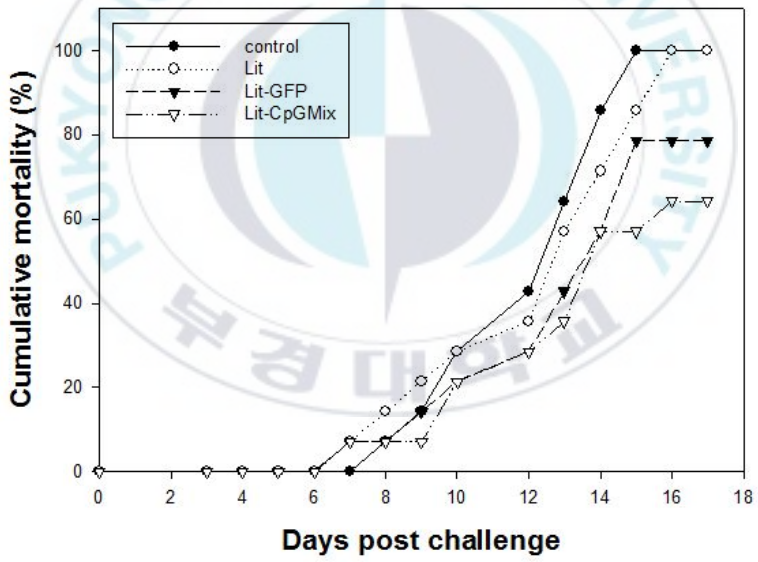
2.1 Survival of fish after challenge against VHSV or M. avidus

At 8 days post-challege, fish in the control (PBS) and pLitself groups showed 100% cumulative mortality. Group injected with pLit-CpGMix showed the highest survival rate (Fig. 7A). The mortality of the pLit-GFP group was 85%, whereas that of the pLit-CpGMix group was 64%.

The cumulative mortality of fish in the control and pLititself-injected groups was 100% by challenge with low level (1 x 10^4) and high level (2 x 10^5) of ciliates. Fish in the pLit-GFP group and the pLit-CpGMix group showed 78% and 64% cumulative mortality, respectively, by low level challenge (Fig. 7B). In the high-level challenge experiment, pLit-GFP and pLit-CpGMix group showed 78% and 71% cumulative mortality, respectively (Fig. 7C).


control
Lit
Lit-GFP
Lit-CpGMix
Cumulative mortality (%)
Days post challenge
0
20
40
60
80
100
0
2
4
6
8
10
12
14

(A)


control
Lit
Lit-GFP
Lit-CpGMix
Cumulative mortality (%)
Days post challenge
0
20
40
60
80
100
0
2
4
6
8
10
12
14
16
18

(B)

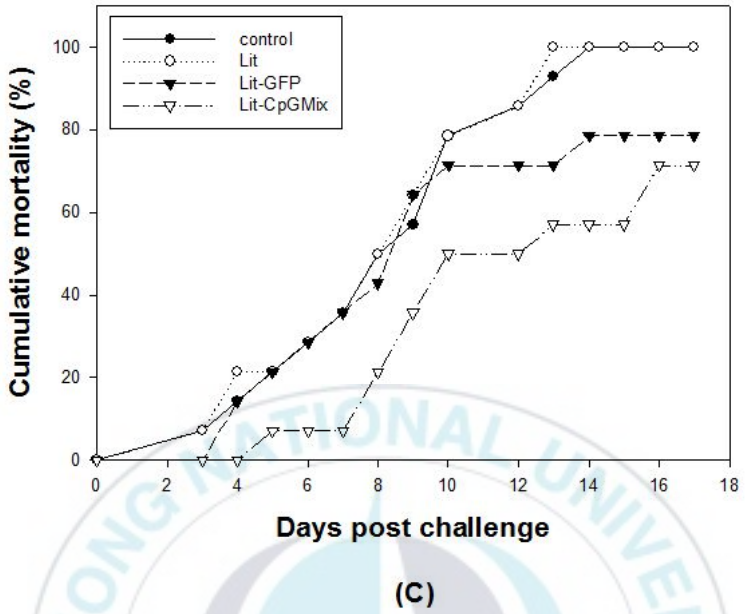


Fig. 7. Cumulative mortality of olive flounder (*Paralichthys olivaceus*) fingerling intraperitoneally injected with phosphate buffered saline (control), pLitmus28i (Lit), pLitmus28i-GFP (Lit-GFP), and pLitmus28i-CpGMix (Lit-CpGMix). (A) After 3 days of the injection, all remaining fish in each group were intramuscularly (i.m.) challenged with the VHSV KJ2008 at 10^3 PFU/fish. Mortality was recorded daily for 14 days. (B) Fish in each group was challenged by i.p. injection with $2x10^4$ of CHSE-cultured ciliates, and (C) the other replicate were challenged with $2x10^5$ of CHSE-cultured ciliates. The mortality was monitored for 17 days.

2.2 Analysis of genes expression in olive flounder using semi-quantitative RT-PCR

The effect of type I interferon response of olive flounder was investigated by analyzing expression of Mx and ISG15 genes at 24 h post-injection. Fish administered pLit-CpGMix showed the highest expression of Mx and ISG15 genes compared to fish in other groups (Fig. 8).

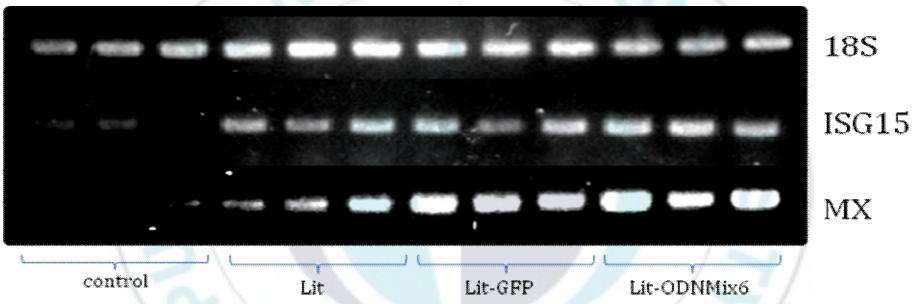


Fig. 8. Analysis of ISG15 and Mx genes expression in olive flounder i.p. injected with phosphate buffered saline (control), pLitmus28i (Lit), pLitmus28i-GFP (Lit-GFP) and pLitmus28i-CpGMix (Lit-CpGMix) by semi-quantitative RT-PCR. Total RNA was isolated from kidney of olive flounder at 24 h post-injection. Expression of ISG15, Mx, and 18S ribosomal RNA (18S) genes was PCR amplified from cDNA samples using primers for each gene.

2.3 Scuticocidal activity of serum

At 3 days post-injection, sera from 4 fish in each tank (total 8 fish/ group) were collected, and the obtained sera of two fish were pooled for measurement. Fish injected with pLit-CpGMix showed the highest serum scuticocidal activity. Fish injected with pLit-GFP group also showed significantly higher scuticocidal activity than fish injected with PBS or pLit (Fig. 9). No scuticocidal activity was observed in the wells containing heat inactivated sera or HBSS alone.

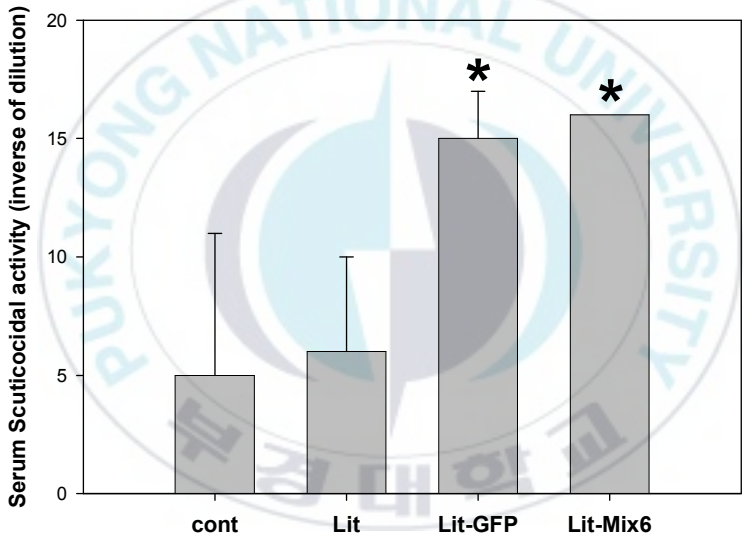


Fig. 9. Scuticocidal activity of sera collected from olive flounder i.p. injected phosphate buffered saline (control), pLitmus28i (Lit), pLitmus28i-GFP (Lit-GFP) and pLitmus28i-CpGMix (Lit-Mix6). Values (inverse of serum dilution) are means and T-bars indicate standard deviation. Bars with an asterisk indicate statistically differences at $P < 0.05$, when compared to the control PBS.

3. Effects of RNase III knock-out or asd knock-out Eschrichia coli harboring CpG motifs-containing plasmids on resistance of olive flounder against M. avidus infection

3.1 Survival of fish after challenge against M. avidus infection

By a challenge with 5 x 10^4 ciliates, fish in the group administered *E. coli* χ7213 harboring Lit-CpGMix showed the highest survival rate (Fig. 10). The groups administered *E. coli* HT115 harboring Lit-CpGMix and mixed *E. coli* χ 7213 harboring Lit-CpGMix + HT115 harboring Lit-CpGMix showed same mortality. The ciliates were recovered from the internal organs of all dead fish.

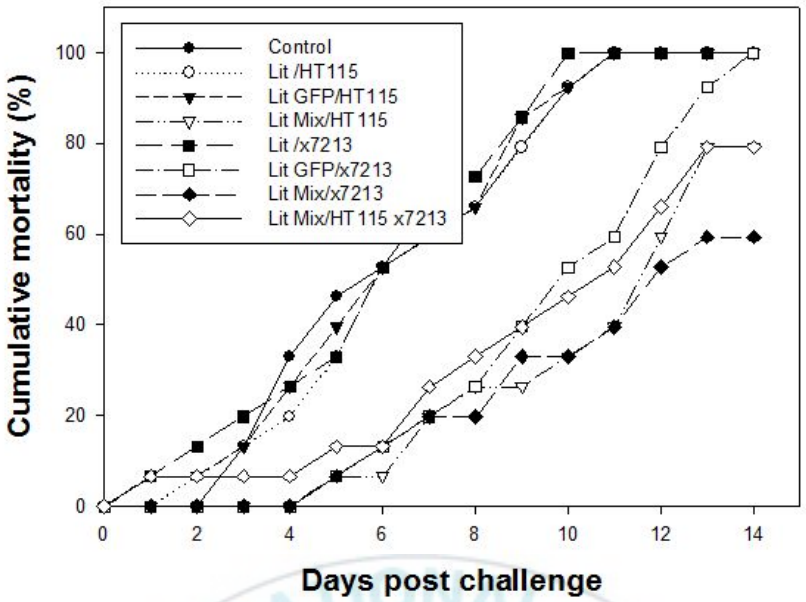


Fig. 10. Cumulative mortality of olive flounder (*Paralichthys olivaceus*) fingerling i.p injected with phosphate buffered saline (control), *E. coli* HT115 harboring pLitself (Lit/HT115), HT115 harboring pLitmus28i-GFP (LitGFP/HT115), HT115 harboring pLitmus28i-CpGMix (Lit Mix /HT115), *E. coli* χ7213 harboring pLitself (Lit/χ7213), χ7213 harboring pLitmus28i-GFP (Lit GFP/χ7213), χ7213 harboring pLitmus28i-CpGMix (Lit Mix/χ7213), and mixed HT115 and χ7213 harboring pLitmus28i-CpGMix (Lit Mix/HT115 χ7213). Fish in each group was challenged by i.p. injection of 5 x 10^4 ciliates. The mortality was monitored for 14 days.

3.2 Scuticocidal activity of serum

Fish injected with pLit- CpGMix/HT115, pLit-GFP/χ7213, pLit- CpGMix/χ7213 and pLit-CpGMix/χ7213+ pLit-CpGMix/HT115 showed higher serum scuticocidal activity than fish injected with PBS, pLit/ HT115, pLit-GFP/ HT115 and pLit-GFP/ χ7213 (Fig. 11). Serum scuticocidal activity of fish injected with pLit-CpGMix/HT115 and pLit-GFP/χ7213 or pLit-CpGMix/χ7213 and mixed pLit-CpGMix/χ7213+ pLit-CpGMix/HT115 were similar each other. No scuticocidal activity was observed in the wells containing heat inactivated sera or HBSS alone.

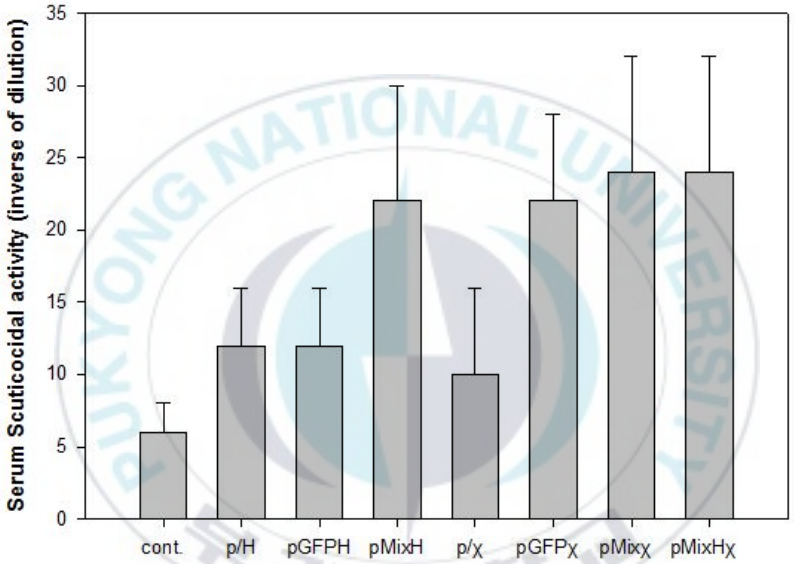


Fig. 11. The scuticocidal activity of sera collected from fish administered phosphate buffered saline (cont.), *E. coli* HT115 harboring pLitself (p/H), HT115 harboring pLitmus28i-GFP (pGFPH), HT115 harboring pLitmus28i-CpGMix (pMixH), *E. coli* χ7213 harboring pLitself (p/χ), χ7213 harboring pLitmus28i-GFP (pGFPχ), χ7213 harboring pLitmus28i-CpGMix (pMixχ), and mixed HT115 or χ7213 harboring pLitmus28i-CpGMix (pMixHχ).

DISCUSSION

Lately, we have compared immunoprophylactic potential of CpG-ODNs belonging to three different classes (A, ODN 2216; B, ODN 1668; C, ODN 2395) against *M. avidus* infection, and found that CpG-ODN 1668 has the highest capability to enhance resistance of olive flounder against the ciliates challenge (Kang and Kim, 2011). However, since the cost for synthesis of CpG-ODNs is too high to use them in aquaculture, the use of plasmids containing multiple CpG motifs instead of artificially synthesized CpG-ODNs would be a way to low the cost. Therefore, the present results suggest that plasmids harboring CpG motifs can be used as immunostimulants in fish instead of costly CpG-ODNs. According to the recent structure-based classification of CpG-ODNs (Krieg, 2002), CpG-ODN 1668 is belonging to the B-class ODN that is characterized by possessing at least one CpG motif and phosphorothioate backbone. The present study showed that the copy number of CpG 1668 in one plasmid exerts an effect on the protective efficacy against *M. avidus* infection. When olive flounder were challenged with a relatively low number of *M. avidus*, the copy numbers in a plasmid shown similar survival rates to CpG-ODN 1668 were 15, 30, and 60. Whereas, when fish were challenged with a high number of *M. avidus*, only fish administered with plasmids harboring 60 copy number of CpG 1668 showed a higher survival rate than fish administered ODN 1668, suggesting that plasmids containing more than 60 copies of CpG 1668 sequence might be required to exceed the immunostimulatory effects of CpG-ODN 1668 in olive flounder.

In the present study, we have firstly demonstrated that plasmids harboring multiple CpG

1668, CpG 2216, and CpG 2395 sequences enhance the resistance of olive flounder against *M. avidus* and VHSV infection, respectively. It has been demonstrated that different classes of CpG-ODNs showed class-specific immunostimulatory effects that are ranging from humoral to cellular immune responses (Krieg, 2002). To effectively defense against infectious agents, cooperation between humoral and cellular immune factors is essential. Thus combination of different CpG motf sequences in a plasmid may stimulate more diverse innate immune factors that can confer more effective protection against various kinds of pathogens.

We have verified that plasmids harboring 15-60 copies of CpG-ODN 1668 sequence enhanced serum scuticocidal activity and resistance against *M. avidus* in olive flounder. In the present *M. avidus* challenge experiment, fish in the group administered plasmids harboring three classes of CpG motifs showed the highest survival rates, suggesting that the plasmid with different CpG motifs has the same potential to induce anti-scuticociliate immune responses as the plasmid with multicopy of CpG-ODN 1668 sequence. Although Piazzon et al. (2011) reported that turbot leucocytes were not helpful to kill *Philasterides dicentrarchi*, in our previous experiments, leucocytes stimulated with poly I:C contributed to killing of scuticociliates in the presence of immune serum. In spite of low copy number (6 copy) of CpG 1668 motif in the present plasmid compared to the previous used plasmid harboring 15-60 copies of CpG 1668 motif, the protective efficacies were similar to each other, suggesting that CpG 2216 motif-mediated stimulation of cellular immune responses including type I interferon responses or CpG 2395 motif-mediated supplemental stimulation might participate in the present enhancement of anti-scuticociliates responses.

Interestingly, in the present study, fish administered plasmids containing different CpG motifs also showed enhanced resistance against VHSV infection. Previously, we had verified that administration of CpG-ODN 2216 induced greatly enhanced survival rate of olive flounder

against VHSV challenge. Thus the presence of CpG 2216 motif in the present vector might play an important role in induction of protective immune responses against VHSV infection.

A-class CpG-ODNs are known to induce type I interferon response that is a key innate immune factor for protection against viral infections, whereas B-class CpG-ODNs are known to enhance humoral immune responses (Verthelyi et al., 2001; Krieg, 2002; Vollmer et al., 2004). In the present study, the simultaneous stimulation of humoral and cellular immune responses was verified by enhanced serum scuticocidal activity and up-regulated Mx gene expression in fish administered plasmids harboring three classes of CpG motifs. The increased serum scuticocidal activity and type I interferon responses might involved in the present increased survival rates against *M. avidus* and VHSV infections. These results suggest that CpG motifs in the plasmids might have capability to class-specifically stimulate immune factors as in CpG-ODNs.

Although fish administered GFP-containing vector showed lower survival rates, lower serum scuticocidal activity, and lower Mx gene expression when compared to mixed CpG motifs-containing vector, the GFP-containing vector elicited higher survival rates against *M. avidus* and VHSV infections, higher serum scuticocidal activity, and higher expression of type I interferon-induced genes compared to control, suggesting that GFP sequence, also, might have some imunostimulating properties. To know this mechanism exactly, further studies are needed.

Delivery of CpG motifs-containing plasmids using *E. coli* has several merits in that the *E. coli* used for vehicle is not virulent to fish, there is no need for isolation of plasmids or dsRNAs because the plasmids can be autonomously amplified in the *E. coli*, the plasmids or produced long dsRNAs are protected from nucleases attacks, and *E. coli* itself can be acted as an immunostimulant. Although attenuated bacteria have been utilized as delivery vehicles for DNA vaccines in mammals (Dietrich et al., 2001; Woo et al., 2001; Mohamed et al., 2002; Schoen et al.,

2004), little is known on the use of live bacteria as a carrier for immunostimulating plasmids or/and long dsRNAs in fish.

In the present study, we used *E. coli* HT115 or χ7213 as the delivery vehicle for CpG motifs-containing plasmids, and investigated their effects on resistance against *M. avidus* infection in olive flounder. The results showed that *E. coli* χ7213 transformed with plasmids harboring mixed CpG motifs induced the highest survival rate against the scuticociliates challenge, and *E. coli* HT115 harboring the plasmids or mix of the two *E. coli* strains harboring CpG plasmids induced higher survival rates than controls. These results suggest that the mutated E. coli has a possibility to be used as a vehicle for in vivo delivery of immunostimulating plasmdis.

As *E. coli* HT115 lacks RNase III gene and possess a cassette for expression of T7 RNA polymerase, long dsRNA might be produced in the cytoplasm of the bacteria transformed with the CpG motifs-containing plasmids. Thus, in the *E. coli* HT115, both CpG plasmids and CpG-containing long dsRNAs would be present simultaneously. However, the effects on the resistance against *M. avidus* infection was not higher than *E. coli* χ7213 that is an *asd* mutated auxotrophic mutant. The serum scuticocidal activity was also increased by administration of fish with the *E. coli* strains harboring CpG motifs-containing plasmids, suggesting that humoral immune responses that are important to defense against *M. avidus* infection (Piazzon et al., 2011) might be effectively induced by delivery of CpG plasmids through *E. coli* mutants.

In the results of this study, the scuticocidal activity of serum was increased in proportion to the copy number of CpG 1668 sequence in a plasmid, and the activity of serum isolated from fish injected with plasmids containing 60 copies of CpG 1668 sequence was equal to that of fish administered CpG-ODN 1668, suggesting that plasmids containing CpG 1668 sequence can stimulate innate humoral immunity of olive flounder as in CpG-ODN 1668 (Kang and Kim, 2011),

which increased resistance of fish against *M. avidus* infection. And, we have verified that plasmids harboring different classes of CpG motifs simultaneously enhanced resistance of olive flounder against *M. avidus* and VHSV infections, and could simultaneously induce each-class-specific immune responses that can synergistically attack the intruding pathogens. In addition to, the results suggest that exposure of immune cells to the CpG motifs-containing plasmids by rupturing of the *E. coli* χ7213 due to inability to synthesize cell wall might be more effective to induce protective immune responses against *M. avidus* than CpG plasmds and long dsRNAs present in the cytoplasm of *E. coli* HT115.

Chapter III

Molecular cloning of toll-like receptor 3 (TLR3) from olive flounder (*Paralichthys olivaceus*) and transcriptional response to poly I:C in relation to TLR22

INTRODUCTION

Toll-like receptors (TLRs) are a family of transmembrane sensor proteins that recognize pathogen associated molecular patterns (PAMPs), and play crucial roles in both innate and adaptive immune responses (Schnare et al., 2001; Takeda et al., 2003; Iwasaki and Medzhitov, 2004). TLRs contain an extracellular leucine-rich repeat (LRR) domain that is responsible for recognition of PAMPs and an intracellular Toll/Interleukin-1 receptor (TIR) domain that is involved in cellular signaling. The recognition of PAMPs by each TLR is highly specific. Of TLRs, TLR3 recognizes double-stranded RNA (dsRNA) that is generated as a replication intermediate or by-product of many viruses (Jacobs and Langland 1996; Alexopoulou et al., 2001; Sen and Sarkar 2005; Haller et al., 2006). Signaling through TLR3 results in type I interferon responses in mammals (Alexopoulou et al. 2001; Hoebe et al. 2003).

Mammalian TLR3 homologs have been reported in several fish species, such as fugu *Takifugu rubripes* (Oshiumi et al. 2003), zebrafish *Danio rerio* (Meijer et al. 2004; Jault et al. 2004), channel catfish *Ictalurus punctatus* (Bilodeau and Waldbieser 2005), rainbow trout *Oncorhynchus mykiss* (Rodriguez et al., 2005), and grass carp *Ctenopharyngodon idella* (Su et al., 2009). Unlike mammals, however, TLR22 has been identified as an additional dsRNA recognizing TLR in a number of fish species including goldfish (Stafford et al., 2003), zebrafish (Jault et al., 2004), olive flounder *Paralichthys olivaceus* (Hirono et al., 2004), rainbow trout (Rebl et al., 2007), and fugu (Matsuo et al., 2008).

In the present study, we firstly cloned the cDNA of olive flounder TLR3 gene and analyzed

molecular pattern and change in transcription level in response to poly (I:C) injection in olive flounder. Furthermore, to know the functional role of TLR3 and TLR22 in induction of type I interferon response, TLR3 or TLR22 transcriptom in hirame natural embryonic cells (HINAE cells) was knock-downed by short-hairpin RNA (shRNA), and analyzed the effects of each gene knock-down on the type I interferon response.

MATERIALS AND METHODS

1. Fish

Juvenile olive flounder (approximately 10 g in body weight) were obtained from a commercial farm in South Korea. Fish were fed with commercial pellet, and water was renewed once a day. Before experiment, fish were acclimatized for at least 2 weeks.

2. Complementary DNA cloning of Olive flounder TLR3

Total RNA was isolated from the kidney of the fish using RNAiso reagent (Takara), and one μg of purified total RNA was incubated with 1 μl of random primer (0.5 μg/ml) (Promega) at 80°C for 10 min, and further incubated at 42°C for 60 min in a reaction mixture containing 4 μl of 5× reaction buffer, 4 μl of 10 mM dNTP mix (Takara), 1 μl of M-MLV reverse transcriptase (Promega) and 0.5 μl of RNase inhibitor (Promega) in a final reaction volume of 20 μl. Using two degenerate oligonucleotides (fTLR3-Fw: 5’-CTCAGYAAYAAYAAYATYGCMAACAT-3’ and fTLR3-Re: 5’-TCCAGWAGAAYYGRATYCTCCA-3’), a partial fragment of olive flounder TLR3 cDNA was obtained by PCR reaction. The amplified PCR product was run on an agarose gel (1.5%), purified using a gel purification kit (Cosmo Genentech, Korea), subcloned into pGEM-T easy vector (Promega), and sequenced using ABI 3700 (PE Applied Biosystems). To obtain full open reading frame (ORF) sequence, 5'-RACE (primer-1; 5'-AGCTAAGCCTCTCAAACAATCTCCTCAACAGTCTGAAGG-3': primer-2; 5’-GCTGTGATT

TCCCGAATTGCAGTTTGCTGTAAC-3': primer-3; 5'-CCTGGTGAAAGGTCGAACCTCC GCCA-3') and 3'-RACE (primer; 5'-ATCCTGTGGTTTGTGACGTGGTTGAACAAC-3'). PCR reactions were performed separately using the SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions. All RACE PCR products were purified using spin column (Labopass), cloned into pGEM-T easy vector, and sequenced. Multiple sequence alignments were generated using the CLUSTALW 1.8 program, and amino acid sequence identity was verified through BLAST using the protein database in the National Center for Biotechnology Information(NCBI).

3. Expression analysis of TLR3, TLR22, ISG15 and Mx genes in olive flounder after poly (I:C) injection

A total of 18 olive flounder were randomly divided into 3 groups, and fish of each group were intraperitoneally injected with either 1 mg of poly (I:C) (Sigma) suspended in 100 μl of PBS (pH 7.6) or the same volume of PBS only. One group was received no treatment and served as a no handling control. At 12 and 24 h post injection (p.i.), 3 fish were sampled from each group, and total RNA was extracted from kidney using RNAiso reagent. Total RNA (0.5 μg) was then reverse-transcribed to cDNA as mentioned above in order to be used for gene expression assay. Semi-quantitative RT-PCR was carried out to determine the relative mRNA expression level of TLR3. PCR in a reaction volume of 10 μl was performed with 2×Prime Taq Premix (Genet Bio) and 1 μl of 10^{-1} diluted cDNA template. Oligonucleotide primer pairs for TLR3, TLR22, ISG15, Mx and internal control (18S rRNA) genes are shown in Table 4. Thermal cycling conditions were 1 cycle of 4 min at 95°C (initial denaturation) followed by 17 cycles (for 18S rRNA), 26 cycles (for ISG15 and MX), or 32 cycles (for TLR3 and TLR22) of 30 s at 95°C, 30 s at 60°C, 30 s at

72°C, with a final extension step of 7 min at 72°C. PCR samples to be compared were electrophoresed on the same 1% agarose gel and visualized with ethidium bromide (Et-Br) staining.

4. Expression analysis of TLR3, TLR22, IRF3, IRF7 and ISG15 genes in HINAE cells after stimulated poly (I:C)

HINAE cells were sub-cultured in wells (2×10^6cells/well) at 20°C in Leibovitz medium (L-15, Sigma) containing with 10% FBS and penicillin (100 U/ml), streptomycin (100 μg/ml). Total RNA extracted after 6, 12, 24 and 48 h after stimulation with 100 μg/ml of polyinosinic:polycytidilic acid (poly I:C) (Sigma) or with the same volume of PBS (pH 7.6) only. The complementary DNAs (cDNAs) synthesized from these total RNAs using M-MLV reverse transcriptase and Oligo(dT)15 Primer (Promega, USA) were used in reverse-transcription polymerase chain reaction (RT-PCR). Oligonucleotide primer pairs for TLR3, TLR22, IRF3, IRF7, ISG15 and internal control (18S rRNA) genes are shown in Table 4. Thermal cycling conditions were 1 cycle of 4 min at 95°C (initial denaturation) followed by 17 cycles (for 18S rRNA), 30 cycles (for IRF3, 7 and ISG15), or 33 cycles (for TLR3 and TLR22) of 30 s at 95°C, 30 s at 60°C, 30 s at 72°C, with a final extension step of 7 min at 72°C. PCR samples to be compared were electrophoresed on the same 1% agarose gel and visualized with ethidium bromide (Et-Br) staining.

5. Construction of shRNA-producing vector targeting the fTLR22 and fTLR3 of olive flounder

Using Ambion's "siRNA Target Finder and Design Tool", siRNA target sequences for

fTLR22 and fTLR3 genes of Olive flounder were designed (Table 2). The fugu U6 promoter-driven shRNA expression vector (Zenke & Kim, 2008) was used for construction of plasmids producing shRNA targeting the fTLR22 and fTLR3 of olive flounder. Each shRNA-producing cassette was made by following three-step PCR reactions, and amplification conditions were 1 cycle of 3 min at 95°C (initial denaturation), 5 cycles of 30 s at 95°C, 30 s at 48°C, 30s at 72°C, and followed by 30 cycles of 30 s at 95°C, 30 s at 65°C, 30s at 72°C. Using cloned fugu U6 promoter as the template, the first-step PCR was performed with two primers U6F-SphI and each of shRNAR1 which contains the last 16 bps of fugu U6 promoter, shRNA sense and loop. Each amplified product from the first PCR was purified, and used as the template for second-step PCR with U6F-SphI and each of shRNAR2 which contains 10 bps of shRNA sense, loop and 4 bps of shRNA antisense. One-tenth of the second PCR product was then used as the template for the third-step PCR with U6F-SphI and each of shRNAR3-SacII which contains 5 bps of shRNA sense, loop, shRNA antisense and termination. Intermediately, after the U6 promoter sequence, one guanine residue which is predicted to be a transcription start point of shRNA was included in this construct. Each PCR product was gel-purified and cloned into pGEM T-easy vector (Promega) resulting in vector pFuguU6shRNAs (pshTLR22·903, pshTLR22·1726, pshTLR22·1726cont and pshTLR3·939). The pshTLR22·1726cont vector, was used as a two nucleotide sequence different compared with pshTLR22·1726.

To select shRNA clones which effectively suppress target fTLR22 expression, a dual vector harboring each shRNA producing cassette and fTLR22 gene expressing cassette was constructed. The fTLR22 gene ORF which contains *Hind*III and *Bam*HI sites was cloned into pGEM T-easy vector, and then inserted into the pcDNA 3.1(+) vector (Invitrogen)(Table 3). The cassette including CMV promoter, fTLR22 ORF and BGH polyadenylation signal was digested using *Bgl*II and *Sal*I restriction enzymes, and inserted into each above shRNA expression vector,

resulting in pshTLR22·903-fTLR22, pshfTLR22·1726-fTLR22, pshTLR22·1726cont-fTLR22 and control vector is p-fTLR22.

To use as a selection marker, a neomycin resistant cassette, including the SV40 early promoter and the SV40 polyadenylation signal in the pcDNA 3.1 vector, was inserted into the *Apa*I and *Aat*II sites of each shRNA expression vector, and named pNeoshTLR22·1726, pNeoshfTLR22·1726cont, pNeoshTLR3·939 and control vector is pNeo. The integrity of DNA sequence of the vectors was confirmed by sequencing using an automatic sequencer (Applied biosystems).

6. Transfection of EPC cells with shRNA producing vectors and expression analysis of fTLR22 gene

EPC cells were sub-cultured in wells of 6-well plates (3×10^5cells/well) at 28°C in L-15 medium containing 20 mM HEPES and 10% FBS. Cells were grown to about 80% confluence and transfected with p-fTLR22, pshTLR22·903-fTLR22, pshTLR22·1726-fTLR22 and pshTLR22·1726cont-fTLR22 vectors using FuGENE 6 (Roche) according to the manufacturer's instructions.

7. Transfection of HINAE cells with shRNA producing vectors and Selection

HINAE cells were sub-cultured in wells of 6-well plates (2×10^6cells/well) at 20°C in L-15 medium containing 20 mM HEPES and 10% FBS. Cells were grown to about 80% confluence and transfected with pNeo, pNeoshTLR22·1726, pNeoshTLR22·1726cont, pNeoshTLR3·577, pNeoshTLR3·939 vectors using FuGENE 6 (Roche) according to the manufacturer's instructions.

Selection by antibiotics was performed in this study. To select HINAE cells harboring shRNA producing vectors, 400 μg/ml of G-418 was added to the culture medium of cells transfected with each pFuguU6shRNA-Neomycin plasmid. The surviving cells were then trypsinized using TrypLE Express (Gibco) and plated in new T25, and were grown in L-15 medium containing 10% FBS, antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml) and 400 μg/ml of G-418. And the cells were selected for six month.

8. Expression analysis of selected HINAE cell TLR3, TLR22, ISG15 and MX genes after stimulated poly (I:C)

Total RNA extracted after administered polyI:C for 12h of seleted HINAE eclls harboring shRNA producing vector(pNeo, pNeoshTLR22·1726, pNeoshTLR22·1726cont, pNeoshTLR3·577, pNeoshTLR3·939) grown in culture medium containing G-418 (400 μg/ml, Sigma) for six month. Total RNA (0.5 μg) was then reverse-transcribed to cDNA as mentioned above in order to be used for gene expression assay. Semi-quantitative RT-PCR was carried out to determine the relative mRNA expression level of TLR3. PCR in a reaction volume of 10 μl was performed with 2×Prime Taq Premix (Genet Bio) and 1 μl of 10^{-1} diluted cDNA template. Oligonucleotide primer pairs for TLR3, TLR22, IRF3, IRF7, ISG15 and internal control (18S rRNA) genes are shown in Table 4. Thermal cycling conditions were 1 cycle of 4 min at 95°C (initial denaturation) followed by 17 cycles (for 18S rRNA), 25 cycles (for MX and ISG15), or 30 cycles (for TLR3 and TLR22) of 30 s at 95°C, 30 s at 60°C, 30 s at 72°C, with a final extension step of 7 min at 72°C. PCR samples to be compared were electrophoresed on the same 1% agarose gel and visualized with ethidium bromide (Et-Br) staining.

Table 2. For production of various pFuguU6shRNA vectors

Name of primer		Sequence (5' to 3')	Restriction enzyme
U6	F	GCATGCCACTGGATCCATCTGACACTAA	*Sph*I
shfTLR3 577	R1	TCTCTTGAAAAGGAAATAGAAGTCGTCTCAACAGCTAGCAACATC	
	R2	TTTCCTTTCTCTTGAAAAGGAAATAG	
	R3	**CCGCGG**AAAAAAAGACGACTTCTATTTCCTTTCTCTTGAAAAGGAA	*Sac*II
shfTLR3 939	R1	TCTCTTGAACTTGGTCAGGTGCTTGATACAACAGCTAGCAACATC	
	R2	GACCAAGTCTCTTGAACTTGGTCAGG	
	R3	**CCGCGG**AAAAAATATCAAGCACCTGACCAAGTCTCTTGAACTTGG	*Sac*II
shfTLR22 903	R1	TCTCTTGAAGTTAGCGCTGTGATATTGTCAACAGCTAGCAACATC	
	R2	GCTAACTCTCTTGAAGTTAGCGCTG	
	R3	**CCGCGG**AAAAAAACAATATCACAGCGCTAACTCTCTTGAAGTTAG	*Sac*II
shfTLR22 1726	R1	TCTCTTGAAGTTACGAGGCAATTGAGACCAACAGCTAGCAACATC	
	R2	CGTAACTCTCTTGAAGTTACGAGGC	
	R3	**CCGCGG**AAAAAAGTCTCAATTGCCTCGTAACTCTCTTGAAGTTAC	*Sac*II
shfTLR22 1726 control	R1	TCTCTTGAAGTTACGAGGCAtaTGAGACCAACAGCTAGCAACATC	
	R2	CGTAACTCTCTTGAAGTTACGAGGC	
	R3	**CCGCGG**AAAAAAGTCTCAtaTGCCTCGTAACTCTCTTGAAGTTAC	*Sac*II

Underline : U6 promoter 3' region

Bold : restriction enzyme site

Small letter : two substituted nucleotides in target sequence

Table 3. For construction for TLR22 of Olive flounder

Name of primer		Sequence (5' to 3')	Restriction enzyme
fTLR22 ORF	F	AAGCTTATGGGTCCCGGAGGAAAAGGAGATGAAAG	*Hind*III
	R	GCGGCCGCTTACCACTGATTATCCACCACAGTGAGGCG	*Not*I

Underline : restriction enzyme site

Table 4. Oligonucleotide primer pairs for semi-quantitative RT- PCR of Olive flounder or EPC cell

Name of primer		Sequence (5' to 3')
f18S	F	AGTTGCTGCAGTTAAAAAGC
	R	TGGCATCGTTTACGGTCGGAACTA
f TLR3	F	GGCTCATTTCAGTGGCTCACCAGACTTCAGACTC
	R	GCTGCCCCTTTCAGAAACATCACCGGC
f TLR22	F	GACGATGCCTTCAGTTCTCTTCAGAGTCTT
	R	CAAGCCGCGGAATTCTCCATGTTT
f IRF3	F	ACCCTGCTTTGTTTGAGAAGTGCG
	R	TGTGCAGTTCGTTCAGACACTCCT
f IRF7	F	ATTTCACGGCCTTGGATCTGGGTA
	R	TTGCCTCGTAGAAAGTGGGCTGAT
f MX	F	AACAGCCAAGGCAAAGATTG
	R	AATGTCCAGCTCCTCCTTCA
f ISG15	F	CTCCATGTA ATCTGCAGCAA
	R	CAGATCTAGTGCAGGTGTGA
EPC 18S rRNA	F	TAGTTGGTGGAGCGATTTGTC
	R	CACTTGTCCCTCTAAGAAGTTG

RESULTS

1. Complementary DNA and deduced amino acid sequences of olive flounder TLR3

Full length olive flounder TLR3 cDNA (Fig. 12) consisted of 2736 bp ORF encoding 912 amino acids, 25 bp 5' UTR, and 606 bp 3' UTR. Conserved domain analysis using the SMART program revealed that olive flounder TLR3 consisted of 15 leucine-rich repeats (LRR) in addition to a C-terminal cap (LRR-CT), a transmembrane domain, and an intracellular Toll/Interleukin-1 receptor (TIR) domain (Fig. 13). At the amino acid level, olive flounder TLR3 showed high identity with other TLR3s of other fish species.

Fig. 12. The full length cDNA sequence of olive flounder TLR3. The Full length cDNA TLR3 contains a 2736 bp ORF which has both the start codon(ATG) and the stop condon (TGA), 25 bp 5' –UTR and 606 bp 3' –UTR containing 23 bp poly A tail. The start and stop codon is boxed.


Oliver flounder TLR3
Fugu rubripes
zebrafish
Grass carp
Channel catfish
Rainbow trout
house mouse
Human
LRR
LRR
LRR
TM

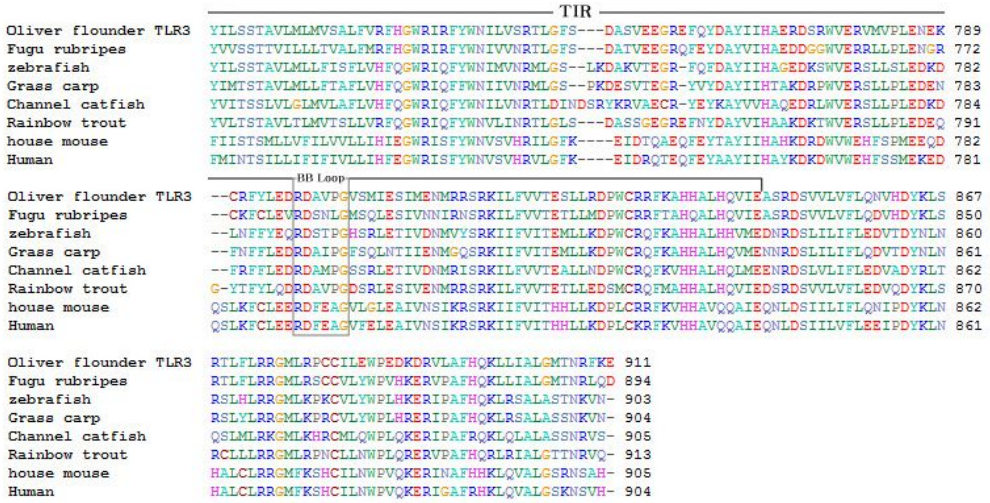


Fig. 13. Alignment of the olive flounder TLR3 TIR domain with those of fugu, zebrafish, grass carp, channel catfish, Rainbow trout, house mouse and human genes. The leucine-rich repeat (LRR) domains are underlined (32 ~ 376, 461 ~ 646), transmembrane (TM) (655 ~ 678), TLR/IL-1 receptor (TIR) (706 ~847).

2. Effect of poly I:C injection on expression of TLR3, TLR22, ISG15, and Mx genes in olive flounder

Expression of TLR3 and TLR22 of olive flounder in response to poly I:C injection was analyzed by semi-quantitative RT-PCR. Expression of ISG15 and Mx genes were also analyzed to measure interferon responses. In the PBS injected control group, no or very weak expression of the above genes was observed throughout the experimental period. Upon injection of poly I:C, although both TLR22 and TLR3 were up-regulated at 12 and 24 h, TLR22 showed higher expression than TLR3. Expression of ISG15 and Mx genes was highly elevated by poly I:C injection (Fig. 14).

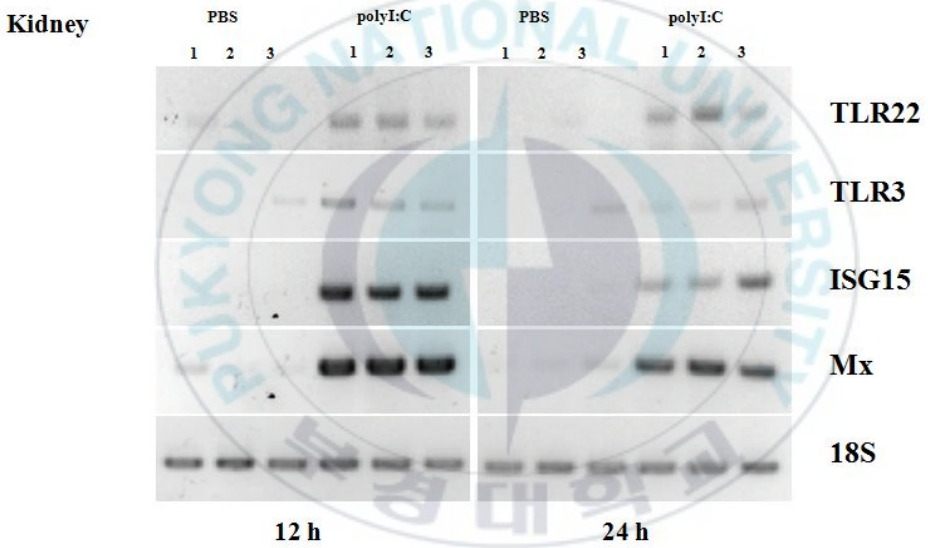


Fig. 14. Effect of poly I:C injection on expression of TLR3, TLR22, ISG15, and Mx genes in olive flounder. At 12 and 24 h post-injection with polyI:C or PBS, the expression of each gene in the kidney was analyzed by semiquantitative RT-PCR.

3. Expression of TLR3, TLR22, IRF3, IRF7 and ISG15 genes in HINAE cells by stimulation with poly (I:C)

Expression of genes in HINAE cells stimulated with poly I:C was analyzed by semi-quantitative RT-PCR using primers specific for fTLR3, TLR22, IRF3, IRF7, ISG15 and 18S. All analyzed genes were up-regulated by poly (I:C) stimulation (Fig. 15). Especially, expression of TLR22, IRF3, IRF7, and ISG15 genes was clearly up-regulated at 6 and 12 h post-stimulation, whereas expression of TLR3 was more increased at 24 and 48 h post-stimulation.

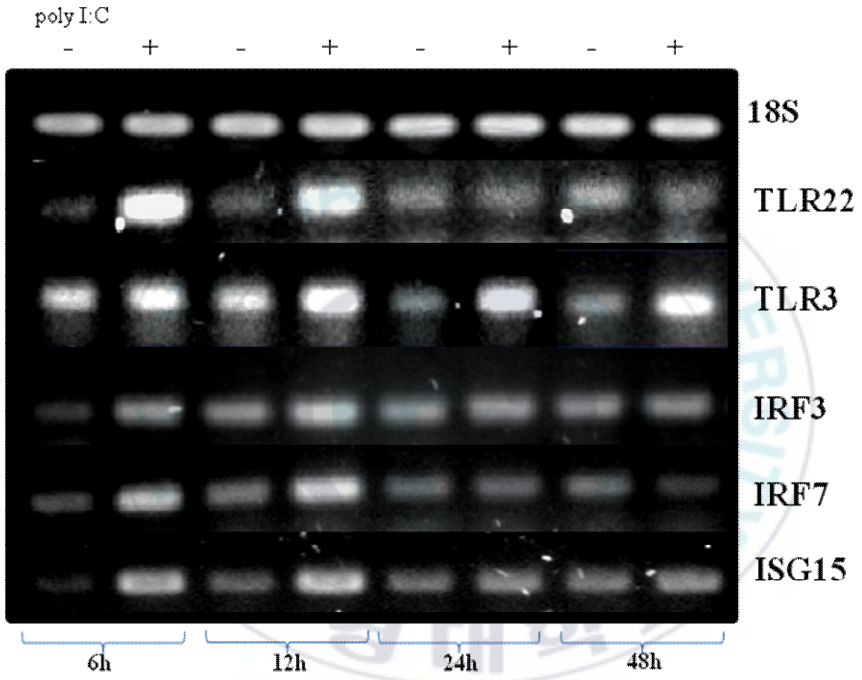


Fig. 15. Effect of poly I:C on the expression of TLR3, TLR22, IRF3, IRF7, and ISG15 genes of HINAE cells. At 6, 12, 24, 48 h post-stimulation, expression of above genes and 18s rRNA gene (control) was analyzed by RT-PCR. Mark '-' is non-stimulated and mark '+' stimulated by poly I:C.

4. Selection of an effective shRNA clone targeting TLR22

To select effective shRNA sequence targeting TLR22, EPC cells were transfected with plasmids p-fTLR22, pshTLR22·903-fTLR22, pshTLR22·1726-fTLR22 and pshTLR22·1726cont-fTLR22, and inhibitory effect on TLR22 expression was analyzed by semiquantitative RT-PCR. Expression of TLR22 gene was clearly reduced in EPC cells by transfection with pshTLR22 ·1726-fTLR22. However, cells transfected with pshTLR22·939-fTLR22, pshTLR22·1726cont-fTLR22 showed no reduction in expression of TLR22 gene (Fig. 16).

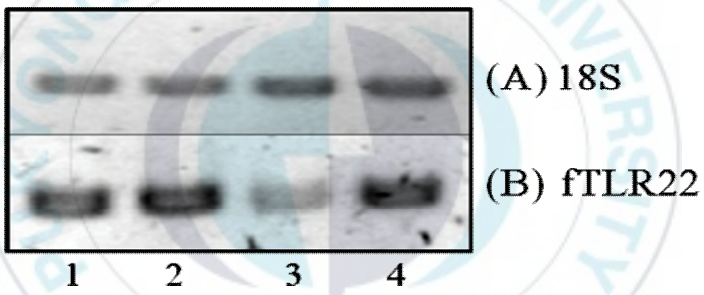


Fig. 16. Effect on expression of fTLR22 and 18S genes in each transfected EPC cells. EPC cells transfected with p-fTLR22 (lane 1), pshTLR22·903-fTLR22 (lane 2), pshfTLR22 ·1726-fTLR22 (lane 3) or pshTLR22·1726cont-fTLR22 (lane 4) vectors. (A) 18S rRNA of EPC cell (18S), (B) TLR22 of olive flounder (fTLR22).

5. Effect of shRNAs targeting TLR22 and TLR3 on expression of type I interferon-induced genes in HINAE cells

HINAE cells harboring pNeo, pNeoshTLR22·1726, pNeoshTLR22·1726cont, pshTLR3·577 and pNeoshTLR3·939 were selected using G-418 supplementation. The selected cells were stimulated with poly I:C, and at 12 h post-stimulation, expression of 18S rRNA, TLR22, TLR3, ISG15 and MX genes were analyzed by semi-quantitative RT-PCR (Fig. 17). Expression of TLR22 gene was clearly reduced in HINAE cells harboring pfTLR22 1726, whereas cells harboring pfTLR22 1726cont showed no decrese in TLR22 expression. Both ISG15 and Mx genes were highly down-regulated by knock-down of TLR22, however, the effect of TLR3 knock-down on Mx and ISG15 genes was not evident.

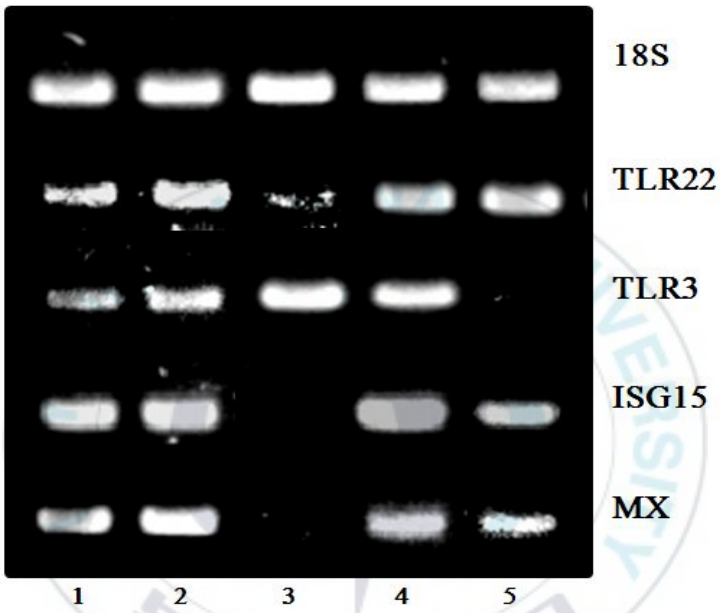


Fig. 17. Effects of shRNAs targeting TLR22 and TLR3 on expression of TLR22, TLR3, ISG15, and Mx genes were analyzed by semi-quantitative RT-PCR. At 12 h post-stimulation of HINAE cells with poly I:C, 18S rRNA, TLR22, TLR3, ISG15 and Mx mRNA were PCR amplified. Lane 1, HINAE cells (no handling); lane 2, HINAE cells harboring pNeo; lane 3, HINAE cells harboring pNeoshTLR22·1726; lane 4, HINAE cells harboring pNeoshTLR22 ·1726cont; lane 5, HINAE cells harboring pNeoshTLR3·939.

DISCUSSION

In mammals including human, TLR3 is located in the endosomal membrane or the cell surface, participates in recognition of dsRNA. However, in fish, an additional TLR, TLR22, is also known to respond to dsRNA. Recently, Matsuo et al. (2008) reported that fish TLR22 might play a role that was conducted by human cell-surface TLR3. In the present study, we analyzed in vivo transcriptional change of TLR3 and TLR22 genes after poly IC treatment, and found that olive flounder TLR3 was up-regulated by extrinsically administered poly IC. However, the increased level of TLR3 was lower than that of TLR22. These results suggest that olive flounder TLR22 is a more susceptible receptor for extracellular dsRNA than TLR3.

In the present results, HINAE cells stimulated with poly I:C showed highly up-regulated TLR22 at relatively early time (6 and 12 h post-exposure), while TLR3 expression was stronger at 24 and 48 h post-exposure, suggesting that TLR22 might recognize extracellular dsRNA more quickly than TLR3 and/or TLR22 might be located cell surface, while TLR3 might respond slowly to extracellular dsRNA and/or TLR3 might be located on endosomal membrane.

Recently, plasmid vector based short hairpin RNAs (shRNAs) are widely used to induce RNA interference (RNAi) in vertebrate cells, and provide a tool to create continuous cell lines in which suppression of a target gene is stably maintained by RNAi. Previously, we had demonstrated successful transcription of shRNA by fugu U6 promoter in bluegill fry (BF-2) cells and efficient knock-down of a target gene by the shRNA in BF-2, grunt fin (GF), and Chinook salmon embryo (CHSE) cells (Zenke and Kim, 2008). In the present study, we established HINAE

cells that stably express shRNAs by transfection with the fugu U6 promoter-driven shRNA expressing vector and selection with G-418 (geneticin). Through the present RNAi experiment, the important role of TLR22 in recognition of extracellular poly (I:C) and in induction of type I interferon response was verified. HINAE cells harboring TLR22-targetting shRNA vector showed greatly reduced TLR22 expression, which resulted in large reduction of ISG15 and Mx genes expression, suggesting that TLR22 might play an important role in induction of type I interferon response to extracellular dsRNAs. While inhibition of TLR3 expression by transfection of HINAE cells with TLR3-targeting shRNA vector weakly reduced the expression of ISG15 and Mx genes by poly IC exposure, suggesting that TLR3 partially participate in the induction of type I interferon response in cells stimulated with extracellular poly (I:C).

Chapter IV

Protective effect of long double-stranded RNA with CpG motifs against *Miamiensis avidus* and viral hemorrhagic septicemia virus (VHSV) infections in olive flounder (*Paralichtys olivaceus*)

INTRODUCTION

Double-stranded RNA (dsRNA), a by-product of viral replication, is recognized as a pathogen-associated molecular pattern (PAMPs) by toll like receptor 3 (TLR3), which trigger strong proinflammatory response in vertebrates (Alexopoulou et al., 2001; Schulz et al., 2005; Wang et al., 2004). Binding of dsRNA to TLR3 recruits the adaptor protein TRIF (Yamamoto et al., 2002), and activates transcription factors, such as IRF3, NF-κB and AP-1, which induce transcription of type I interferon and inflammatory cytokines (Sharma et al., 2003; Yamamoto et al., 2003; Hoebe et al., 2003; Meylan et al., 2004). Unlike mammals, another dsRNA recognizing TLR, TLR22, has been reported in several fish species (Stafford et al., 2003; Jault et al., 2004; Rebl et al., 2007; Matsuo et al., 2008) including olive flounder *Paralichthys olivaceus* (Hirono et al., 2004).

Unmethylated CpG motifs in DNA of various pathogens are also recognized as a PAMP by TLR9 in vertebrates including fish (Ahmad-Nejad et al., 2002; Akira and Takeda, 2004). Recognition of CpG DNA by TLR9 triggers recruitment of the adaptor molecule MyD88, which activates signal cascade that results in the production of various cytokines and chemokines (Krieg, 2002; Klinman, 2004). Artificially synthesized CpG oligodeoxynucleotides (CpG-ODNs) also can be recognized by TLR9, and are categorized into three classes (A, B, and C) based on the structural and functional characteristics (Krieg, 1999, 2003; Hemmi et al., 2000; Jørgensen et al., 2001a). It has been demonstrated that the immunostimulatory effects of each CpG-ODN class are class-specific (Krieg, 2002). Recently, the protective effect of CpG-ODN 2216 (A-class) and CpG-ODN 1668 (B-class) against viral hemorrhagic septicemia virus (VHSV) and *Miamiensis*

avidus infection, respectively, has been demonstrated by our previous experiments (Kang and Kim, 2011), and we have further demonstrated that olive flounder administrated with plasmids harboring mixed CpG motifs of class A, B, and C showed high survival rates against VHSV and *M. avidus* infections.

Artificially synthesized polyinosinic–polycytidylic acid (poly I:C), an analog of dsRNA (Alexopoulou et al., 2001), is well-known to stimulate antiviral immune responses via induction of type I interferon responses in mammals (Heitmeier et al., 1998; Auch et al., 2004; Kulka et al., 2004) and in fish species (Fernandez-Trujillo et al., 2008; Plant et al., 2005; Jensen et al., 2002b; Saint-Jean and Pérez-Prieto, 2006; Jensen and Robertsen, 2002a; Jensen et al., 2002c). However, no information is available concerning the ability of dsRNA to increase resistance of fish against diseases caused by extracellular parasites. Furthermore, whether specific sequence motifs in dsRNAs can modulate immunostimulatory effects has not been reported. Thus, the aims of the present study were i) to know whether dsRNA can enhance disease resistance of olive flounder against *M. avidus* infection, and ii) to investigate whether CpG motifs in dsRNA can modulate resistance against VHSV and *M avidus* infections.

MATERIALS AND METHODS

1. Vector construction

The fragment GFP (ORF 717bp) sequence was ligated into Litmus 28i vector (NEB) that was pre-digested with *EcoR*I and *Hind*III, then designated it as pLit-GFP. A fragment corresponding to randomly arranged 6 copies of each CpG-ODN sequence (CpG-ODN 1668, 2216, and 2395) was artificially synthesized and inserted into pUC57 vector (pUC57-CpG Mix)(Cosmo Genetech, Korea). After digestion with *Bgl*II and *BamH*I, the CpG fragment was ligated into Litmus 28i vector that was pre-digested with the same restriction enzymes, then designated it as pLit-CpGMix (Fig. 18).

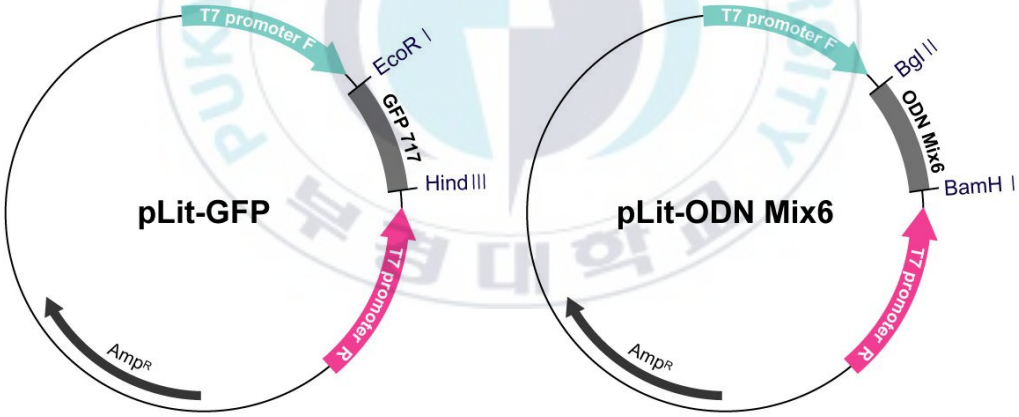


Fig. 18. Map of vectors with the double T7 promoter-driven long double-stranded RNA expression cassette.

2. dsRNA purification

To produce long dsRNA, *E. coli* HT115(ΔRNase III) was transformed with the constructed vectors, pLit-GFP or pLit-CpGMix, inoculated in 200 ml of LB with 0.1mg/ml ampicillin in 300 ml flask, and incubated at 37°C overnight. The cultured samples were centrifuged at 8,000 rpm, for 10min at 4°C, removed supernatant and suspended with 0.5% SDS at 95°C for 2 min. After 10 min incubation on ice, the lysate was digested with RNase (Promega) and DNase (ELPIS) at 37°C for 10 min. The dsRNAs were purified by RNAiso (Takara), electrophoresed on 1% agarose gel, stained with ethidium bromide (EtBr), and pictured with Gel Doc XR (Biorad) (Fig. 19).

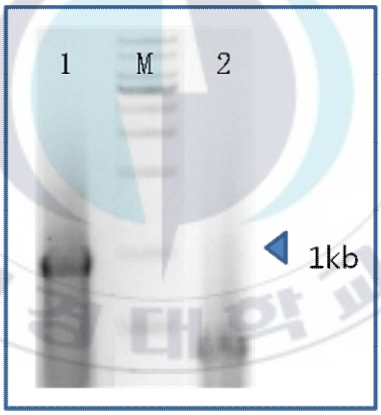


Fig 19. Purification of dsRNA in *E. coli* HT115 harboring dsRNA producing vectors. Lane 1 is dsRNA of CpGMix, lane 2 is dsRNA of GFP, lane M is 1 kb ladder (EPIS, Korea).

3. Iimmuno-stimulation and challenge test

Olive flounder fingerlings weighing approximately 4~5 g were obtained from a local fish farm, divided into twelve 50 L tanks (18 or 17 fish/tank), and were acclimated at least for 2 weeks before the experiment. Fish in each experimental group were divided into 2 subgroups, either *M. avidus* or VHSV challenge experiment. For *M. avidus* challenge experiment, fish in the 8 tanks were divided into two replicates, and each replicate consisted of 4 groups. Fish in group 1, 2, 3 and 4 were intraperitoneally injected (i.p.) with 50 μl of PBS, with 50 μg/50 μl/fish of poly I:C, with 50 μg/ 50 μl/ fish of dsRNAs (GFP or CpGMix), respectively. Water temperature was maintained at 21-22°C during whole experimental period. At 3 d post-injection, 4 fish in each tank were randomly sampled and bled to isolate serum. The rest of fish in one replicate were i.p. challenged with 2 x 10^4 ciliates, and the other replicate were challenged with 2 x 10^5 ciliates. The mortality was monitored for 14 days, and dead fish were necropsied to confirm the presence of ciliates. The VHSV challenge experiment had same groups with *M. avidus* experiment. Fish in group 1, 2, 3 and 4 were i.p. injected with the same regime of above. Water temperature was maintained at 15°C. At 24 h post-injection, 3 fish in each tank were sampled for isolation of kidney. The rest of fish were intramuscularly (i.m) challenged with VHSV KJ2008 at 10^3 PFU/fish. The mortality was monitored for 14 days.

4. Serum scuticocidal activity

The sera collected from fish in the *M. avidus* challenge experiment were used to examine scuticocidal activity. The analysis of scuticocidal activity was performed using 96-well flat-bottomed plates. All the sera were serially diluted ranging from 1/4 to 1/4096 in Hank's balanced salt solution (HBSS, Sigma). And diluted sera put in 50ul to the wells of the plate, The ciliates were collected after 1 days of CHSE cells depletion in the culture vessels by centrifugation at 200 g for 5 min, washed three times with HBSS. The ciliates were added to the wells (1×10^2 ciliates/well) of the plate, incubated at 20°C and observed every one hour for 24 h to analysis scuticocidal activities of the sera. The titer of each serum was the last dilution at which 100% of the ciliates were lysed or non-motile, which was observed under an inverted microscope at 40-100 x magnification.

5. Semi-quantitative RT-PCR analysis

Total RNA was extracted from the kidney using RNAiso plus reagent (Takara) at 24 h post-immunization. The complementary DNAs (cDNAs) synthesized from these total RNAs using M-MLV reverse transcriptase and Oligo(dT)15 Primer (Promega) were used in reverse-transcription polymerase chain reaction (RT-PCR). To amplify cDNA of Mx gene, PCR was done with primers MxF (5'-AACAGCCAAGGCAAAGATTG-3') and MxR (5'- AATGTCCAGCTCCTCCTTCA-3'). ISG15 gene was PCR-amplified using primers ISG15F (5'-CTCCATGTAATCTGCAGCAA-3') and ISG15R (5'-AGATCTAGTGCAGGTGTGA-3'). Two primers 18SF (5'-AGTTGCTGCAGTTAAAAAGC-3') and 18SR (5'-TGGCATCGTTTACGGTCGGAACTA-3')

were used to amplify 18S ribosomal RNA gene of olive flounder. PCR was performed using 2× Prime Taq Premix (GeNet Bio, Korea). The amplification procedure included 1cycle of 4 min at 95°C,16 (for 18S rRNA) or 30 cycles (for Mx and ISG15) of 30 s at 95°C, 30 s at 60°C, 30 s at 72°C, with a final extension step of 7 min at 72°C. PCR samples to be compared were electrophoresed on the same 1.0% agarose gel, stained with ethidium bromide (EtBr), and pictured with Gel Doc XR (Biorad). Expression of Mx and ISG15 genes was calculated as relative expression to 18S rRNA gene using Quantity One software (Biorad).

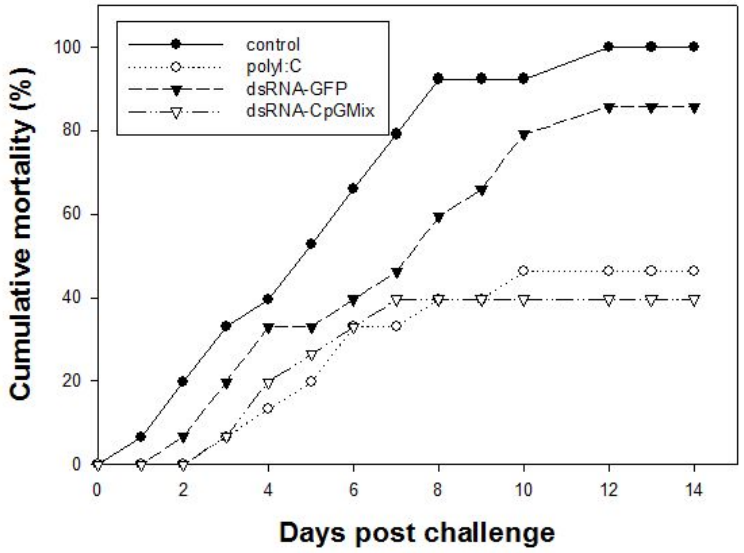
RESULTS

1. Effects of long double-stranded RNA containing CpGMix motif *on resistance of olive flounder against VHSV infection*

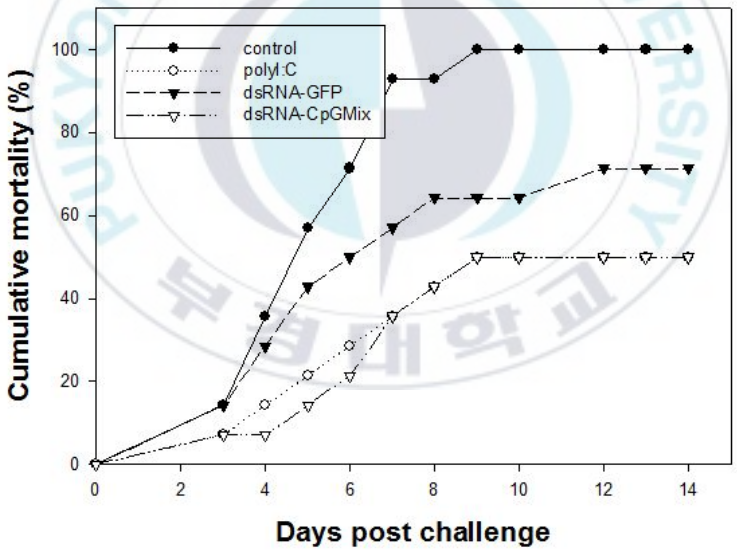
Cumulative mortality of fish in the control group was 100% by a challeng with VHSV at 10^3 PFU/fish (Fig. 20A). The group of fish administered the dsRNA encoding GFP sequence showed lower mortality than control group. Groups of fish injected with dsRNA containing CpGMix motifs or with poly I:C showed greatly significantly increased survival rates compared to the control group.

2. Effects of long double-stranded RNA containing CpG motifs *on resistance of olive flounder against M. avidus infection*

In the challenge experiment with low level 1 x 10^4 or high level 2 x 10^5 ciliates, the cumulative mortalities of fish injected with dsRNAs were clearly lower than fish injected with PBS alone. The groups injected with dsRNA-CpGMix and poly I:C showed the highest survival rates (Fig. 20B, 20C). The ciliates were recovered from the internal organs of all dead fish.


control
polyI:C
dsRNA-GFP
dsRNA-CpGMix
Cumulative mortality (%)
Days post challenge
0
20
40
60
80
100
0
2
4
6
8
10
12
14

(A)


control
polyI:C
dsRNA-GFP
dsRNA-CpGMix
Cumulative mortality (%)
Days post challenge
0
20
40
60
80
100
0
2
4
6
8
10
12
14

(B)

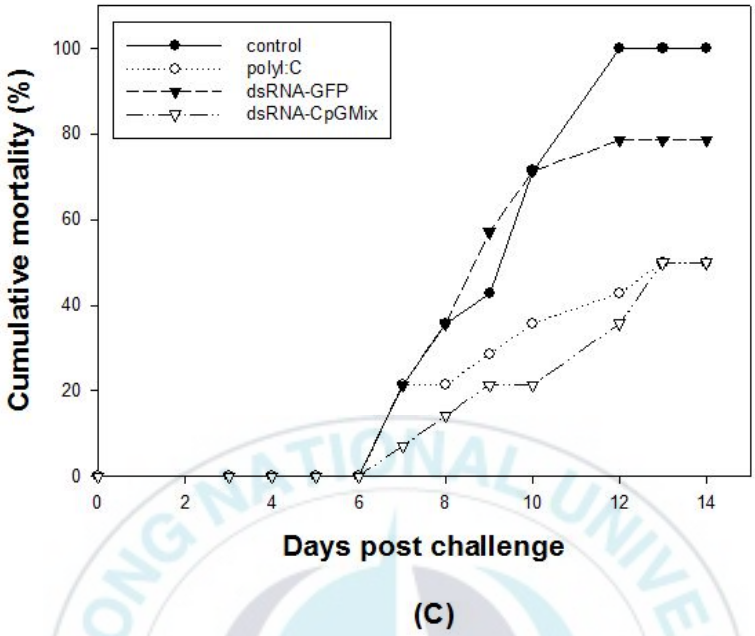


Fig. 20. Cumulative mortality of olive flounder (*Paralichthys olivaceus*) fingerlings intraperitoneally injected with phosphate buffered saline (control), polyI:C, dsRNA-GFP and dsRNA-CpGMix. After 3 days of the injection, all remaining (A)fish in each group were intramuscularly (i.m.) challenged with the VHSV KJ2008 at 10^3 PFU/fish. Mortality was recorded daily for 14 days. (B) Fish in each group was challenged by i.p. injection with $2x10^5$ of CHSE-cultured ciliates, and (C) the other replicate were challenged with $2x10^4$ of CHSE-cultured ciliates. The mortality was monitored for 17 days.

3. Analysis of genes expression in olive flounder using semi-quantitative RT-PCR

The effect of each dsRNA on the type I interferon response of olive flounder was investigated by analyzing expression of Mx and ISG15 genes at 24 h post-administration. Fish administered poly I:C or dsRNA-CpGMix showed higher expression of Mx and ISG15 genes compared to fish in other groups (Fig. 21).

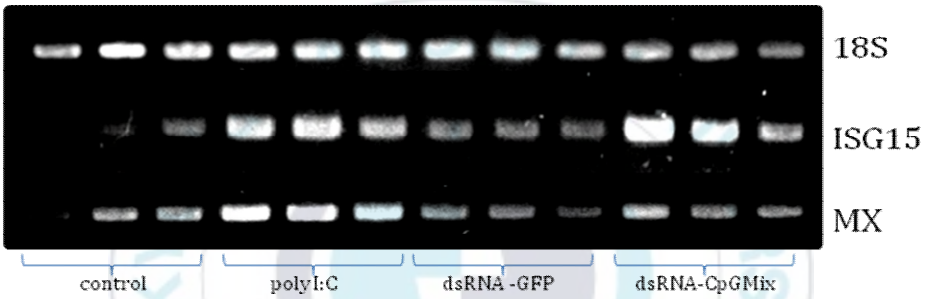


Fig. 21. RT-PCR amplification of ISG15 and Mx genes in olive flounder (*Paralichthys olivaceus*) intraperitoneally (i.p.) injected with phosphate buffered saline (PBS), poly I:C, dsRNA-GFP or dsRNA-CpGMix(50 μg/fish). Total RNA was isolated from kidney of olive flounder at 24 h post-injection. Expression of ISG15, Mx, and 18S ribosomal RNA (18S) genes was PCR amplified from cDNA samples using primers for each gene.

4. Scuticocidal activity of serum

Fish injected with dsRNA-CpGMix showed significantly higher serum scuticocidal activity than fish injected with PBS alone or dsRNA-GFP (Fig. 22). Serum scuticocidal activity of fish injected with dsRNA-CpGMix was similar to that of fish injected with poly I:C. No scuticocidal activity was observed in the wells containing heat inactivated sera or HBSS alone.

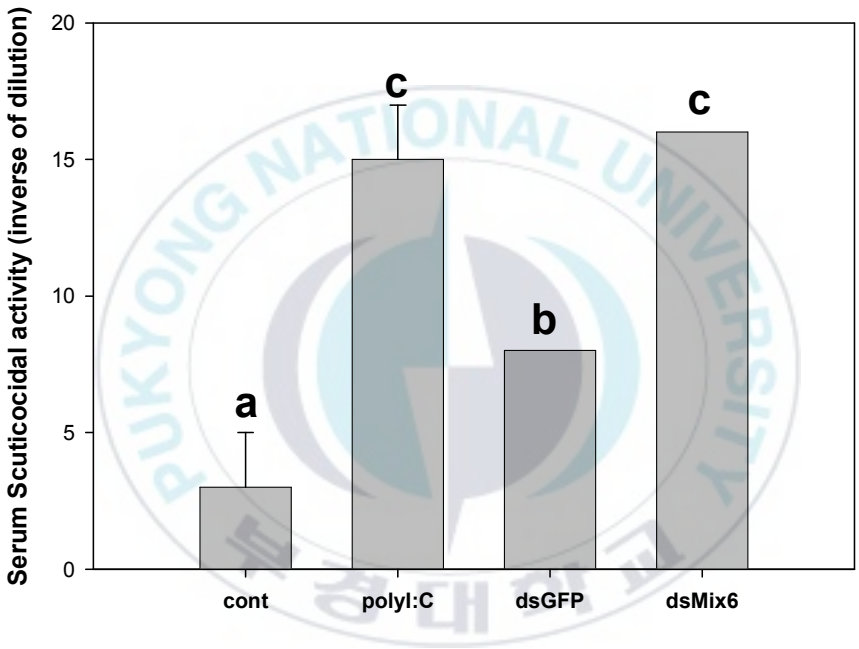


Fig. 22. Scuticocidal activity of sera collected from olive flounder i.p. injected phosphate buffered saline (control), poly I:C, dsRNA-GFP (dsGFP) or dsRNA-CpGMix (dsMix6). Values (inverse of serum dilution) are means and T-bars indicate standard deviation. Different letters on the bar indicate statistically differences at $P < 0.05$.

DISCUSSION

Synthetic poly (I:C) or naturally occurring dsRNAs have been known to enhance immune responses in vertebrates, and have been used to increase resistance to viral infections. Previously, Sugiyama et al. (2005) reported that single-stranded RNA expressing unmethylated CpG motifs and a poly(G) tail stimulated human monocytes and PBMC to produce IL-6, IL-12, and to activate NF-κB, p38 MAPK, which was similar to the immunostimulatory effect of CpG-ODNs. However, there are no reports concerning on the CpG motifs presented in dsRNA can exert further immunostimulatory effects on hosts. In the present study, we firstly demonstrated that dsRNA containing CpG motifs can also further stimulate immune responses of olive flounder.

In this study, CpG motifs-containing dsRNA induced high survivability of fish against VHSV infection, and the survival rate was comparable to that of fish administered poly (I:C). However, GFP sequence-containing dsRNA was not effective to protect fish against VHSV. These results suggest that CpG motifs in the dsRNA were recognized by certain receptor(s) of olive flounder, which led to stimulation of protective immune responses.

In the present study, fish administered poly (I:C) showed enhanced survival rates against *M. avidus* challenge. In a previous study, we have verified that scuticocidal activity of olive flounder leucocytes was distinctly increased by stimulation with poly (I:C). These results suggest that interferon-mediated cellular immune responses might actively participate in the protective responses of fish against intruding scuticociliates. Furthermore, in this study, serum scuticocidal activity of fish administered poly (I:C) was stronger than that of control fish suggesting that not

only cellular but also humoral immune responses might be stimulated by poly (I:C).

In the present results, fish administered dsRNA containing mixed CpG motifs showed high survival rates against *M. avidus* challenges, and the survival rates were equal to those of fish administered poly (I:C). This result suggests that olive flounder can recognize CpG motifs that are present in dsRNA. Although it has been demonstrated that fish can recognize dsRNA and unmethylated CpG DNA through TLR3 (TLR22) and TLR9, respectively, which receptor(s) can bind to CpG motfs on dsRNA is not known, even in human. Furthermore, fish administered dsRNA encoding partial GFP showed higher survival rates than control fish, but lower survival rates than fish administered dsRNA containing CpG motifs, suggesting that differences in sequence, structure, or presence of certain motifs in dsRNA might be important factors for modulating immune responses.

This is the first report to show that sequences of CpG motifs in dsRNA might be important to enhance immune responses and disease resistance against *M. avidus* and VHSV infections in olive flounder. Further studies concerning on the receptors that can recognize CpG motifs on dsRNA are needed to elucidate the immunostimulatory mechanism of dsRNA containing CpG motifs.

CpG motif DNA 및 double-stranded RNA 생성 system을 이용한 넙치의 질병 저항성 증강

강 예 재

부경대학교 대학원 수산생명의학과

요 약

unmethylated CpG motif 와 double-stranded RNA (dsRNA)는 척추동물에서 Pathogen-associated molecular patterns (PAMPs)로 인식되며, 각각 TLR9 과 TLR3 에 의해 인식된 후 여러 신호전달단계를 거쳐 다양한 면역반응을 자극한다. 본 연구에서는 dsRNA 와 3 가지 type 의 CpG DNA 를 넙치에 면역자극제로 사용하여 기생충성질병인 *Miamiensis avidus* 와 바이러스성 질병인 VHSV 에 대한 방어력에 미치는 영향을 분석하였다. 또한 CpG motif 를 지닌 dsRNA 를 제작하여 넙치에서 실질적으로 질병 방어에 영향을 주는지 알아보았다.

1. 3 가지 class CpG-ODN 의 면역자극과 질병에 대한 방어효과

CpG-ODN 2216 (A-class)을 주사한 넙치는 Mx 와 ISG15 유전자의 강한 발현과 VHSV 감염에 대한 높은 생존율을 보여준 반면, CpG-ODN 1668 (B-class)을 주사한 어류는 *M. avidus* 감염에 대한 높은 생존율과 증가된 serum scuticocidal activity 를 보여주었다. 또한 CpG-ODN 2395 (C-class)는 VHSV 와 *M. avidus* 감염에 중간정도의 효과를 나타내었다. 이로부터 각각의 class 에 속하는 CpG-ODNs 은 서로 다른 병원체에 대한 방어능력을 나타냄을 알 수 있었다.

2. CpG 1668 motif 를 여러 개 가진 plasmid 와 CpG motif 3 개의 class 가 mix 된 plasmid 를 통한 VHSV 및 *M. avidus* 에 대한 방어효과 와 이를 delivery 할수 있는 mutant E. coli 를 이용한 *M. avidus* 에 대한 방어효과 분석

CpG 1668 motifs 를 15 개, 30 개 및 60 개를 가진 plasmid 를 제작하여 이들을 넙치에 투여한 후 *M. avidus* 감염에 대한 저항성을 분석한 결과 60 개의 motif 를 지닌 plasmid 가 가장 높은 serum scuticocidal activity 및 높은 생존율을 유도하였다. 이로부터 비교적 제작비용이 고가인 인공 합성 CpG-ODNs 대신에 경제적으로 합성이 가능한 multiple CpG motifs 를 탑재한 plasmid 를 사용하여도 같은 면역증진 효과를 얻을 수 있음을 알 수 있었다.

또 3 종류의 class 에 속하는 CpG motif 를 탑재한 plasmid 를 제조하여 넙치에 투여한 결과 VHSV 와 *M.avidus* 질병 둘다의 감염에 대해 증가된 방어력을 보여주었으며, 또한 높은 serum scuticocidal activity 와 Mx, ISG15 유전자의 높은 발현을 보여주었다.

Delivery system 으로 CpG motif 가 탑재된 plasmid 를 auxotrophic *Escherichia coli* mutant 에 transformation 하여 넙치에 투여한 결과 *M. avidus* 에 대한 방어력이 증진됨을 알 수 있었다.

3. 넙치 TLR3 cloning 및 in vivo 와 in vitro 에서 TLR3/ TLR22 의 역할 분석

넙치 TLR3 cDNA 를 cloning 하였으며, poly (I:C) 자극에 의한 넙치의 TLR3/TLR22 의 발현을 분석하였다. 또한 shRNA 를 이용해 HINAE cell 에서의 TLR3 혹은 TLR22 의 발현을 저해하는 실험을 통해 TLR22 가 외부에서 투여된 dsRNA 에 의한 type I interferon 반응유도에 중요한 역할을 함을 알 수 있었다.

4. CpG motif 를 지닌 dsRNA 의 질병 방어능 증진 효과

CpG motif 를 지닌 dsRNA 가 CpG motif 가 없는 dsRNA 에 비해 *M. avidus* 와 VHSV 모두에 대해 높은 저항성을 유도함을 넙치 실험을 통해 알 수 있었으며, 또한

CpG motif 를 지닌 dsRNA 는 Mx 와 ISG15 유전자의 강한 발현유도 및 serum scuticocidal activity 도 증진됨을 보여주었다.

ACKNOWLEDGMENT

먼저 부족한 저에게 이런 능을 주시고 무사히 박사졸업을 할 수 있게 허락하신 하나님께 감사드립니다.

이제 비로소 박사 논문의 모든 과정을 마치고 감사의 글을 적으려 하니, 지난 대학원 박사과정 4년동안 실험실 생활의 좌절과 성취의 기쁨이 주마등처럼 떠오릅니다. 이렇게 연구과정 동안 여러모로 부족한 저를 이끌어주시고, 아낌없는 성원을 보내주신 많은 분들께 지면을 빌어 이 글로나마 감사의 마음을 대신 하고자 합니다.

연구과정 중에 여러 번 실험의 실패로 인해 쓰러져 힘들 때 일으켜 세워주시고, 저의 부족한 인격을 배려로 저의 부족한 연구과정을 사랑으로 채워주신 존경하는 김기홍선생님과 부족한 저를 학부과정부터 항상 챙겨주시고 인자하게 바라봐 주신 존경하는 김영길교수님께 감사드립니다. 또 논문 심사를 통해 아낌없는 조언을 해주신 주심이신 남윤권교수님과 부심이신 김도형교수님, 정준범교수님, 권세련교수님 또 수산생명의학에 대한 학문적 가르침을 주신 박수일교수님, 정준기교수님, 정현도교수님, 허민도교수님, 강주찬교수님께 머리숙여 진심으로 감사드립니다.

그리고 저의 30 대를 보낸 부경대학교 수산생명의학과 어패류 기생충학 연구실의 석·박사과정의 생활에서 소중한 인연이 된 여러 선후배님들. 먼저 수산생명 의학도로서 멋지게 실험할 수 있게 넙치를 구해주시고 늘 걱정해주신 장명덕선배님, 황윤정선배님 너무나 고마움을 느낍니다. 또 사회의 일선에서 당당히 일하시고 계시는 안경진선배님, 조재범선배님, 김천수선배님, 이찬휘선배님, 정재혁선배님, 김형준박사, 이은혜박사, 성미, 기준이, 수경이, 동진이, 우리, 은숙이, 상효, 상준이, 지윤이 또 일본으로 건너간 Zenke kosuke 박사께도 고마움을 전합니다. 그리고 미처 다 적지 못한 여러 선배님들, 그리고 후배님들에게도 감사드립니다.

가족처럼 느껴지는 실험실 식구들, 실험실이 가장 힘들었던 시기부터 항상 옆에서 서로에게 좋은 충고와 아낌없는 조언을 해줬기에 더 기억에 남으며 보이지 않는 경쟁 속에서 서로에게 윈윈 효과를 주었으며, 늘 함께 실험하고 함께 박사졸업까지 하게 된 최승혁박사, 방장으로서 여러모로 신경쓰는 김민선박사, 컴퓨터 전문가이며 요번에 같이 졸업하는 병철이, 늘 나를

지지해주고 나의 고민을 들어준 고마운 선영이, 우리과 얼짱 대한이, 나의 막바지 실험으로 많이 바쁠 때 도와준 일호와 경수 너무 고맙다, 빨리 건강해져서 함께 밤늦도록 같이 실험하고픈 병우, 생각이 깊고 착한 막내들의 대장 지선이, 귀염둥이 안마쟁이 수진이, 여름방학 알바생이었던 1 학년 새내기 나영이, 이제 막들어온 우리 막내 주성이에게 너네들이 있어서 너무나 즐거웠고 함께여서 기뻤다는 말을 하고 싶습니다.

마지막으로 늦은 나이에도 불구하고 공부를 할 수 있게 정신적으로나 물질적으로 도와주시고 제 옆에서 든든하게 버팀목이 되어주시는 저의 아버지와 지금도 저를 위해 마음속으로 간절히 기도하시고 계실 저의 어머니, 하늘나라에서 흐뭇하게 지켜봐 주실 장인어른, 늘 마르지 않는 기도로 저의 영혼을 일깨워주시는 저의 장모님, 늘 나에게 따뜻한 등산복을 챙겨 선물해주는 처남, 이제는 어엿한 애기 엄마가 된 동생 미정이 그리고 이서방에게 사랑하고 고맙다고 전하고 싶습니다. 또 학교에서 늦게 들어오는 날이 많아 집에서 늘 혼자 밥차려 먹었던 나의 사랑하는 아내, 논문을 집필하는 중에 같이 뜬눈으로 밤을 지새워주고 많은 도움을 준 소영이에게 진심으로 고맙고 잠시나마 기쁨을 줄 수 있어서 감사하게 여깁니다, 끝으로 저의 삶의 원동력 아들 강태승에게 사랑한다고 말하고 싶습니다.

‘꿈, 세상을 살아가는데 복잡하고, 어려운 전략만이 필요한 것은 아니다. 어쩌면 인생을 사는 최고의 생존전략은, 실낱 같은 꿈 하나를 가슴 깊이 심어두는 것이다. 가슴에 심은 꿈은, 아무도 뽑을 수 없다. 그 꿈을 심는 사람도 당신이고, 뽑는 사람도 당신이기 때문이다.’

이러한 말이 있듯이 논문을 쓰면서 저는 꿈을 꾸었고, 졸업을 한 뒤에도 그 꿈을 실현시키기 위해서 저는 앞만 보고 달릴 것입니다. 논문을 완성하면서 희망과 꿈을 가슴에 담았습니다. 그리고 도움을 주셨던 여러분들 또한 가슴에 담았습니다. 이것을 밑바탕으로 고마웠던 분들을 항상 생각하며 살 것 입니다.

모난돌을 다듬어 각지지 않고 부드러운 둥근돌로 거듭나듯이 이제 부족한 새내기였던 제가 이 시대의 진정한 석학으로서 사회에 한 발짝을 내딛으려 합니다. 조금은 떨리는 마음도 가지지만, 기대에 어긋나지 않게 항상 성실한 모습으로 수산생명의학과의 이름을 드높일 수 있게 노력하겠습니다.

REFERENCES

Aderem A, Ulevitch RJ., (2000). Toll-like receptors in the induction of the innate immune response. Nature ., 406,782–7.

Ahmad-Nejad, P., Häcker, H., Rutz, M., Bauer, S., Vabulas, R.M., Wagner, H., (2002). Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. Eur. J. Immunol., 32, 1958-1968.

Akira S, Takeda K., (2004). Toll-like receptor signalling. Nat Rev Immunol., 4,499–511.

Akira Shizuo et al., (2006). Pathogen recognition and innate immunity. Cell., 15, 5-11

Alexopoulou, L., Holt, A.C., Medzhitov, R., Flavell, R.A., (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature., 413, 732-738.

Anderson, D. P. and Siwicki, A. K., (1994). Duration of protection against *Aeromonas salmonicida* in brook trout immunostimulated with glucan or chitosan by injection or immersion. Prog. Fish Cult., 56, 258-261.

Anderson, D. P., (1992). Immunostimulants, adjuvants, and vaccine carriers in fish: applications to aquaculture. Ann. Rev. Fish Dis., 2, 281-307.

Auch, C.J., Saha, R.N., Sheikh, F.G., Liu, X., Jacobs, B.L., Pahan, K., (2004). Role of protein kinase R in double-stranded RNA-induced expression of nitric oxide synthase in human astroglia. FEBS Lett., 563, 223–228.

Ballas, Z.K., W.L. Rasmussen and A.M. Krieg., (1996). Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J. Immunol., 157, 1840-1845.

Bilodeau AL, Waldbieser GC., (2005). Activation of TLR3 and TLR5 in channel catfish exposed to virulent Edwardsiella ictaluri. Dev Comp Immunol., 29(8), 713–721.

Bird, A.P., (1980). DNA methylation and the frequency of CpG in animal DNA. Nucl. Acids Res., 8, 1499-1504.

Bird, A.P., (1987). CpG islands as gene markers in the vertebrate nucleus. Trends Genet., 3, 342-346.

Blazer, V. S., (1991). Piscine macrophage function and nutritional influences: a review. J. Aqua. Anim. Health., 3, 77-86.

Bridle AR, Butler B, Nowak BF., (2003). Immunostimulatory CpG oligodeoxynucleotides increase resistance against amoebic gill disease in Atlantic salmon. Salmo salar L. J Fish Dis., 26, 367-71.

Carrington AC, Secombes CJ., (2007). CpG oligodeoxynucleotides up-regulate antibacterial systems and induce protection against bacterial challenge in rainbow trout (Oncorhynchus mykiss). Fish Shellfish Immunol., 23, 781-92.

Carrington, A.C., Secombes, C.J., (2006). A review of CpGs and their relevance to aquaculture. Vet. Immunol. Immunopathol., 112, 87-101.

Chen Y, Xiang L-X, Shao J-Z., (2007). Construction of a recombinant plasmid containing multi-copy CpG motifs and its effects on the innate immune responses of aquatic animals. Fish & Shellfish Immunology., 23, 589-600.

Cornélie S, Hoebeke J, Schacht A-M, Bertin B, Vicogne J, Capron M, Riveau G., (2004). Direct Evidence that Toll-like Receptor 9 (TLR9) Functionally Binds Plasmid DNA by Specific Cytosine-phosphate-guanine Motif Recognition. J Biol Chem., 279, 15124-15129.

de Veer MJ, Holko M, Frevel M, Walker E, Der S, Paranjape JM, et al., (2001). Functional classification of interferon-stimulated genes identified using microarrays. J Leukoc Biol., 69(4), 912-920.

Dietrich G, Kolb-Maurera A, Spreng S, Schartl M, GoebelW, Gentschev I., (2001). Gram-positive and Gram-negative bacteria as carrier systems for DNA vaccines. Vaccine., 19(17–19), 2506–12.

Dragesco A, Dragesco J, Coste F, Gasc C, Romestand B, Raymond J, Bouix G. Philasterides dicentrarchi, n. sp. (Ciliophora, Scuticociliatida)., (1995). a histophagous opportunistic parasite of Dicentrachus labrax (Linnaeus. 1758), a reared marine fish. Eur J Protistol., 31, 327-40.

Duncan, P. L. and Klesius, P. H., (1996). Dietary immunostimulants enhance nonspecific immune response in channel catfish but not resistance to *Edwardsiella ictaluri*. J. Aquat. Anim. Health., 8, 241-248.

Fernandez-Trujillo, A., Ferro, P., Garcia-Rosado, E., Infante, C., Alonso, M.C., Bejar, J., Borrego, J.J., Manchado, M., (2008). Poly I:C induces Mx transcription and promotes an antiviral state

against sole aquabirnavirus in the flatfish Senegalese sole (Solea senegalensis Kaup). Fish Shellfish Immunol., 24, 279-285.

Goetz FW, Iliev DB, McCauley LAR, Liarte CQ, Tort LB, Planas JV, MacKenzie S., (2004). Analysis of genes isolated from lipopolysaccharide-stimulated rainbow trout (*Oncorhynchus mykiss*) macrophages. Mol Immunol., 41, 1199–1210.

Haller O, Kochs G, Weber F., (2006). The interferon response circuit: induction and suppression by pathogenic viruses. Virology., 344, 119–130.

Heitmeier, M.R., Scarim, A.L., Corbett, J.A., (1998). Double-stranded RNA-induced inducible nitric-oxide synthase expression and interleukin-1 release by murine macrophages requires NF-kappaB activation. J. Biol. Chem., 273, 15301–15307.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., Akira, S., (2000). A Toll-like receptor recognizes bacterial DNA. Nature., 408, 740-745.

Hirono I, Takami M, Miyata M, Miyazaki T, Han HJ, Takano T, Endo M, Aoki T., (2004). Characterization of gene structure and expression of two toll-like receptors from Japanese

flounder, Paralichthys olivaceus. Immunogenetics., 56, 38-46.

Hoebe K, Du X, Georgel P, Janssen E, Tabeta K, Kim SO, Goode J, Lin P, Mann N, Mudd S, Crozat K, Sovath S, Han J, Beutler B., (2003). Identification of Lps2 as a key transducer of MyD88-independent TIR signaling. Nature., 424, 743–748.

Iglesias R, Paramá A, Álvarez MF, Leiro J, Fernández J, Sanmartín ML., (2001). Philasterides dicentrarchi (Ciliophora, Scuticociliatida) as the causative agent of scuticociliatosis in farmed turbot Scophthalmus maximus in Galicia (NW Spain). Dis Aquat Org., 46, 47-55.

Iwasaki A, Medzhitov R., (2004). TLR receptor control of the adaptive immune responses. Nat Immun., 5(10), 987–995.

Jacobs BL, Langland JO., (1996). When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. Virology., 219(2)., 339–349.

Jang, S. I., Marsden, M. J., Kim, Y. G., Choi, M. S. and Secombes, C. J., (1995). The effect of glycyrrhizin on rainbow trout, *Oncorhynchus mykiss* (Walbaum), leucocyte responses. J. Fish Dis., 18, 307-315.

Jault C, Pichon L, Chluba J., (2004). Toll-like receptor gene family and TIR-domain adapters in Danio rerio. Mol Immunol., 40, 759–771.

Jee BY, Kim YC, Park MS., (2001). Morphology and biology of parasite responsible for scuticociliatosis of cultured olive flounder Paralichthys olivaceus. Dis Aquat Org., 47, 49–55.

Jensen, I., Albuquerque, A., Sommer, A.I., Robertsen, B., (2002b). Effect of poly I:C on the expression of Mx proteins and resistance against infection by infectious salmon anaemia virus in Atlantic salmon. Fish Shellfish Immunol., 13, 311-326.

Jensen, I., Larsen, R., Robertsen, B., (2002c). An antiviral state induced in Chinook salmon embryo cells (CHSE-214) by transfection with the double-stranded RNA poly I:C. Fish Shellfish Immunol., 13, 367-378.

Jensen, I., Robertsen, B., (2002). Effect of double-strandedRNAand interferon on the antiviral activity of Atlantic salmon cells against infectious salmon anemia virus and infectious pancreatic necrosis virus. Fish Shellfish Immunol., 13, 221–241.

Jørgensen JB, Johansen L-H, Steiro K, Johansen A., (2003). CpG DNA induces protective antiviral immune responses in Atlantic salmon (Salmo salar L.). J Virol., 77, 11471-9.

Jørgensen, J.B., Johansen, A., Stenersen, B., Sommer, A.-I., (2001a). CpG oligodeoxynucleotides and plasmid DNA stimulate Atlantic salmon (Salmo salar L.) leucocytes to produce supernatants with antiviral activity. Dev. Comp. Immunol., 25, 313-321.

Jørgensen, J.B., Zou, J., Johansen, A., Secombes, C.J., (2001b). Immunostimulatory CpG oligodeoxynucleotides stimulate expression of IL-1beta and interferon-like cytokines in rainbow trout macrophages via a chloroquinesensitive mechanism. Fish Shellfish Immunol., 11, 673-682.

Jung SJ, Kitamura SI, Song JY, Oh MJ., (2007). Miamiensis avidus (Ciliophora: Scuticociliatida) causes systemic infection of olive flounder Paralichthys olivaceus and is a senior synonym of Philasterides dicentrarchi. Dis Aquat Org., 73, 227-234.

Kanellos, T.S., I.D. Sylvester, V.L. Butler, A.G. Ambali, C.D. Partidos, A.S. Hamblin and P.H. Russel., (1999). Mammalian granulocyte-macrophage colony-stimulating factor and some CpG motifs have an effect on the immunogenicity of DNA and subunit vaccines in fish. Immunology., 96, 507-510.

Kang Yue Jai, Kim Ki Hong., (2011). Effect of CpG-ODNs belonging to different classes on resistance of olive flounder (*Paralichthys olivaceus*) against viral hemorrhagic septicemia virus (VHSV) and *Miamiensis avidus* (Ciliata; Scuticociliatia) infections. Aquaculture.

Kerkmann, M., Rothenfusser, S., Hornung, V., Towarowski, A., Wagner, M., Sarris, A., Giese, T., Endres, S., Hartmann, G., (2003). Activation with CpG-A and CpG-B oligonucleotides reveals two distinct regulatory pathways of Type I IFN synthesis in human plasmacytoid dendritic cells. J. Immunol., 170, 4465-4474.

Kim SM, Cho JB, Kim SK, Nam YK, Kim KH., (2004a). Occurrence of scuticociliatosis in olive flounder Paralichthys olivaceus by Philasterides dicentrarchi (Ciliophora: Scuticociitida). Dis Aquat Org., 62, 233-238.

Kim SM, Cho JB, Lee EH, Kwon SR, Kim SK, Nam YK, Kim KH., (2004b). Pseudocohnilembus persalinus (Ciliophora: Scuticociitida) is an additional species causing scuticociliatosis in olive flounder Paralichthys olivaceus. Dis Aquat Org., 62, 239-244.

Kim, S.M., Lee, J.I., Hong, M.J., Park, H.S., Park, S.I., (2003). Genetic relationship of the VHSV(viral hemorrhagic septicemia virus) isolated from cultured olive flounder, Paralichthys olivaceus in Korea. J. Fish Pathol., 16, 1-12 (In Korean with English abstract).

Kim, W.S., Kim, S.R., Kim, D., Kim, J.O., Park, M.A., Kitamura, S.I., Kim, H.Y., Kim, D.H., Han, H.J., Jung, S.J., Oh, M.J., (2009). An outbreak of VHSV (viral hemorrhagic septicemia virus) infection in farmed olive flounder Paralichthys olivaceus in Korea. Aquaculture., 296, 165-168.

Kitlen, J. W., Hejbol, E. K., Zinck, T., Varming, K., Byatt, J. C. and McLean, E., (1997). Growth performance and respiratory burst activity in rainbow trout treated with growth hormone and vaccine. Fish Shellfish Immunol., 7, 297-304.

Klinman DM, Ishii KJ, Verthelyi D., (2000). CpG DNA augments the immunogenicity of plasmid DNA vaccines. Curr Top Microbiol Immunol., 247, 131-42.

Klinman DM., (2004). Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat Rev Immunol., 4, 249-258.

Klinman, D., A.K. Yi, S.L. Beaucage, J. Conover and A.M. Krieg., (1996). CpG motifs expressed by bacterial DNA rapidly induce lymphocytes to secrete IL-6, IL-12 and INF. Proc. Nat'l. Acad. Sci. USA., 93, 2879-2883.

Kodaina, H., Hirota, Y., Mukamoto, M., Baba, T. and Azuma, I., (1993). Activation of rainbow trout (*Oncorhynchus mykiss*) phagocytes by muramyl dipeptide. Dev. Comp. Immunol., 17, 129-140.

Krieg AM, Hartmann G, Yi AK., (2000). Mechanism of action of CpG DNA. Curr Top Microbiol Immunol., 247, 1-21.

Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM., (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. Nature., 374, 546-9.

Krieg AM., (2002). CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol., 20, 709-760.

Krieg AM., (2003). CpG motifs: the active ingredient in bacterial extracts. Nat Med., 9, 831–835.

Krieg, A.M., (1999). Mechanisms and applications of immune stimulatory CpG oligodeoxynucleotides. Biochim. Biophys. Acta., 1489, 107-116.

Krieg, A.M., G. Hartmann and A.K. Yi., (2000). Mechanism of action of CpG DNA. Curr. Top. Microbiol. Immunol., 247, 1-21.

Krieg, A.M., Yi, A.K., Matson, S., Waldschmidt, T.J., Bishop, G.A., Teasdale, R., et al., (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. Nature., 374, 546-549.

Krug, A., Rothenfusser, S., Hornung, V., Jahrsdorfer, B., Blackwell, S., Ballas, Z.K., Endres, S.,

Krieg, A.M., Hartmann, G., (2001). Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. Eur. J. Immunol., 31, 2154-2163.

Kulka, M., Alexopoulou, L., Flavell, R.A., Metcalfe, D.D., (2004). Activation of mast cells by double-stranded RNA: evidence for activation throughToll-like receptor 3. J. Allergy Clin. Immunol,. 114, 174–182.

Lee CH, Jeong HD, Chung JK, Lee HH, Kim KH., (2003). CpG motif in synthetic ODN primes respiratory burst of olive flounder Paralichthys olivaceus phagocytes and enhances protection against Edwardsiella tarda. Dis Aquat Org., 56:43-8.

Lee EH, Kim KH., (2008). Can the surface immobilization antigens of Philasterides dicentrarchi (Ciliophora: Scuticociliatida) be used as target antigens to develop vaccines in cultured fish?. Fish Shellfish Immunol., 24, 142-146.

Lee EH, Kim KH., (2008a). Immobilization antigen-independent protection of olive flounder (Paralichthys olivaceus) against Philasterides dicentrarchi (Ciliophora: Scuticociliatia) infection. Aquaculture., 279, 211-213.

Lee, E.H., Kim, K.H., (2009). CpG-ODN increases resistance of olive flounder (Paralichthys olivaceus) against Philasterides dicentrarchi (Ciliophora: Scuticociliatia) infection. Fish Shellfish Immunol., 26, 29-32.

Liu CS, Sun Y, Hu Y-H, Sun L., (2010). Identification and analysis of the immune effects of CpG motifs that protect Japanese flounder (Paralichthys olivaceus) against bacterial infection. Fish &Shellfish Immunology., 29, 279-285.

Liu CS, Sun Y, Hu YH, Sun L., (2010). Identification and analysis of a CpG motif that protects turbot (*Scophthalmus maximus*) against bacterial challenge and enhances vaccine-induced specific immunity. Vaccine., 28, 4153-4161.

Marshall, J.D., Fearon, K., Abbate, C., Subramanian, S., Yee, P., Gregorio, J., Coffman, R.L., Van Nest, G., (2003). Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. J. Leukoc. Biol., 73, 781-792.

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. J Immunol., 181, 3474-3485.

Meijer AH, Krens SFG, Rodriguez IAM, He S, Bitter W, Snaar-Jagalska BE, Spaink HP., (2004). Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish. Mol Immunol., 40.773–783.

Meng, Z., J. Shao and L. Xiang., (2003). CpG oligodeoxynucleotides activate grass carp (*Ctenopharyngodon idellus*) macrophages. Dev. Comp. Immunol., 27, 313-321.

Meylan, E., Burns, K., Hofmann, K., Blancheteau, V., Martinon, F., Kelliher, M., Tschopp, J., (2004). RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. Nat. Immunol., 5, 503-507.

Mohamed TS, Marvin SR, Anthony LD, George KL, David MH., (2002). Mucosal and systemic HIV-1 Env-specific CD8+ T-cells develop after intragastric vaccination with a Salmonella Env DNA vaccine vector. Vaccine., 20(3–4), 623–9.

Munday BL, O'Donoghue PJ, Watts M, Rough K, Hawkesford T., (1997). Fatal encephalitis due to the scuticociliate Uronema nigricans in sea-caged, southern bluefin tuna Thunnus maccoyii. Dis Aquat Org., 30, 17–25.

Nikl, L., Evelyn, T. P. T. and Albright, L. J., (1993). Trials with an orally and immersion-administered β-1,3-glucan as an immunoprophylactic against *Aeromonas salmonicida* in juvenile chinook salmon *Oncorhynchus tshawytscha.* Dis. Aquat. Org., 17, 191-196.

Ninomiya, M., Hatta, H., Fujiki, M., Kim, M., Yamamoto, T. and Kusuda, R., (1995). Enhancement of chemotactic activity of yellowtail (*Seriola quinqueradiata*) leucocytes by oral administration of quillaja saponin. Fish Shellfish Immunol., 5, 325-328.

Ogier de Baulny, M., Quentel, C., Fournier, V., Lamour, F. and Le Gouvello, R., (1996). Effect of long-term oral administration of β-glucan as an immunostimulant or an adjuvant on some non-specific parameters of the immune response of turbot *Scophthalmus maximus*. Dis. Aquat. Org., 26, 139-147.

Oshiumi H, Tsujita T, Shida K, Matsumoto M, Ikeo K, Seya T., (2003). Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, Fugu rubripes, genome. Immunogenetics., 54, 791–800.

Oumouna, M., L. Jaso-Friedmann and D.L. Evans., (2002). Activation of nonspecific cytotoxic cells (NCC) with synthetic oligodeoxynucleotides and bacterial genomic DNA: Binding, specificity and identification of unique immunostimulatory motifs. Dev. Comp. Immunol., 26, 257-269.

Piazzon MC, Wiegertjes GF, Leiro J, Lamas J., (2011). Turbot resistance to Philasterides dicentrarchi is more dependent on humoral than on cellular immune responses. Fish Shellfish Immunol., 30, 1339-1347.

Plant, K.P, Harbottle, H., Thune, R.L., (2005). Poly I:C induces an antiviral state against Ictalurid Herpesvirus 1 and Mx1 transcription in the channel catfish (Ictalurus punctatus). Dev. Comp. Immunol., 296, 27-35.

Pontarollo RA, Babiuk LA, Hecker R, Van Drunen Littel-Van Den Hurk S., (2002). Augmentation of cellular immune responses to bovine herpesvirus-1 glycoprotein D by vaccination with CpG-enhanced plasmid vectors. J Gen Virol., 83, 2973-81.

Rebl A, Siegl E, Koller B, Fischer U, Seyfert HM., (2007), Charaterization of twin toll-like receptors from rainbow trout (Oncorhynchus mykiss): Evolutionary relationship and induced expression by Aeromonas salmonicida. Dev Comp Immunol., 31, 499e510.

Rhodes LD, Rathbone CK, Corbett SC, Harrell LW, Strom MS., (2004). Efficacy of cellular vaccines and genetic adjuvants against bacterial kidney disease in chinook salmon (Oncorhynchus tshawytscha). Fish Shellfish Immunol., 16, 461-74.

Sacks David & Trauth Nancy Noben., (2002). The immunology of susceptibility and resistance to *Leishmania major* in mice., 2, 845-858

Saint-Jean, S.R., Pérez-Prieto, S.I., (2006). Interferon mediated antiviral activity against salmonid fish viruses in BF-2 and other cell lines. Vet. Immunol. Immunopathol., 110, 1-10.

Sakai, M., Kamiya, R., Ishii, S., Atsuta, S. and Kobayashi, M., (1992). The immunomodulating effects of chitin in rainbow trout *Oncorhynchus mykiss*. Dis. Asian Aquacult., 1, 413-417.

Santarem, M., Novoa, B. and Figueras, A., (1997). Effects of β-glucans on the non-specific immune responses of turbot (*Scophthalmus maximus* L.). Fish Shellfish Immunol., 7, 429-437.

Schnare M, Barton GM, Holt AC, Takeda K, Akira S, Medzhitov R., (2001). Toll-like receptors control activation of adaptive immune responses. Nat Immunol., 2, 947–50.

Schoen C, Stritzker J, Goebel W, Pilgrim S., (2004). Bacteria as DNA vaccine carriers for genetic immunization. Int J Med Microbiol., 294, 319–35.

Schulz, O., Diebold, S.S., Chen, M., Naslund, T.I., Nolte, M.A., Alexopoulou, L., Azuma, Y.T., Flavell, R.A., Liljestrom, P., Reis e Sousa, C., (2005). Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature., 433, 887-892.

Sen GC, Sarkar SN., (2005). Transcriptional signaling by double-stranded RNA: role of TLR3. Cytokine Growth Factor Rev., 16, 1–14.

Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J., (2003). Triggering the interferon antiviral response through an IKK-related pathway. Science., 300(5622), 1148-1151.

Sohn KS, Kim MK, Kim JD, Han IK., (2000). The Role of Immunostimulants in Monogastric Animal and Fish. Animal Sciences., 13,1178-1187.

Stafford JL, Ellestad KK, Magor KE, Belosevic M, Magor BG., (2003). A toll-like receptor (TLR) gene that is up-regulated in activated goldfish macrophages. Dev Comp Immunol., 27, 685-698.

Strandskog, G., Ellingsen, T., Jørgensen, J.B., (2007). Characterization of three distinct CpG oligonucleotide classes which differ in ability to induce IFN α/β activity and cell proliferation in Atlantic salmon (Salmo salar L.) leukocytes. Dev. Comp. Immunol., 31, 39-51.

Su J, Jang S, Yang C, Wang Y, Zhu Z., (2009). Genomic organization and expression analysis of Toll-like receptor 3 in grass carp (Ctenopharyngodon idella). Fish Shellfish Immunol., 27, 433-439.

Sugiyama T, Gursel M, Takeshita F, Coban C, Conover J, Kaisho T, Akira S, Klinman DM, Ishii KJ., (2005). CpG RNA: Identification of novel single-stranded RNA that stimulates human CD14+CD11c+ monocytes. J Immunol., 174, 2273-2279.

Takeda K, Kaisho T, Akira S., (2003). Toll-like receptors. Annu Rev Immunol., 21, 335–376.

Tassakka AC, Sakai M., (2003). The in vitro effect of CpG-ODNs on the innate immune response of common carp, Cyprinus carpio L. Aquaculture., 220, 27-36.

Tassakka, A.C.M.A.R., Sakai, M., (2005). Current research on the immunostimulatory effects of CpG oligodeoxynucleotides in fish. Aquaculture., 246, 25-36.

Tassakka, A.R. and M. Sakai., (2002). CpG oligodeoxynucleotides enhance the non-specific immune responses on carp, *Cyprinus carpio*. Aquaculture., 209, 1-10.

Uhlmann, E., Vollmer, J., (2003). Recent advances in the development of immunostimulatory oligonucleotides. Curr. Opin. Drug Discov. Dev., 6, 204-217.

Verthelyi, D., Ishii, K.J., Gursel, M., Takeshita, F., Klinman, D.M., (2001). Human peripheral blood cells differentially recognize and respond to two distinct CpG motifs. J. Immunol., 166, 2372-2377.

Vollmer, J., Weeratna, R., Payette, P., Jurk, M., Schetter, C., Laucht, M., Wader, T., Tluk, S., Liu, M., Davis, H.L., Krieg, A.M., (2004). Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. Eur. J. Immunol. 34., 251–262.

Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, and Flavell RA., (2004). Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. Nat Med., 10(12), 1366-1373.

Woo PC, Wong LP, Zheng BJ, Yuen KY., (2001). Unique immunogenicity of hepatitis B virus DNA vaccine presented by live-attenuated Salmonella typhimurium. Vaccine., 19, 2945–54.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., and Akira, S., (2003). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science., 301(5633), 640-643.

Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K., Akira, S., (2002). Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. J. Immunol., 169, 6668-6672.

Yamamoto, S., T. Yamamoto, T. Kataoka, E. Kuramoto, O. Yano and T. Tokunaga., (1992). Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. J. Immunol., 148, 4072-4076.

Yano, T., Mangidaar, R. E. P. and Matsuyama, H., (1989). Enhancement of the resistance of carp *Cyprinus carpio*, to experimental *Edwardsiella tarda* infection, by some β-1,3-glucans. Nippon Suisan Gakkaishi., 55, 1815-1819.

Yuan C, Li D, Chen W, Sun F, Wu G, Gong Y, Tang J, Shen M, Han X., (2007). Administration of a herbal immunoregulation mixture enhances some immune parameters in carp (Cyprinus carpuio). Fish Physiol Biochem., 33, 93–101.

Yuan C, Pan X, Gong Y, Xia A, Wu G, Tang J, Han X., (2008). Effects of Astragalus polysaccharides (APS) on the expression of immune response genes in head kidney, gill and spleen of the common carp, Cyprinus carpio L. Int Immunopharmacol., 8,51–58.

Zenke, K., Kim, K.H., (2008). Novel fugu U6 promoter driven shRNA expression vector for efficient vector based RNAi in fish cell lines. Biochem. Biophys. Res. Commun., 371, 480-483.