Protective immunogenicity of the G protein of hirame rhabdovirus (HIRRV) in flounder using DNA vaccine

넙치 렙도 바이러스의 당단백질을 이용한 DNA 백신의 보호 면역력에 관한 연구

Advisor : Tae-Jin Choi

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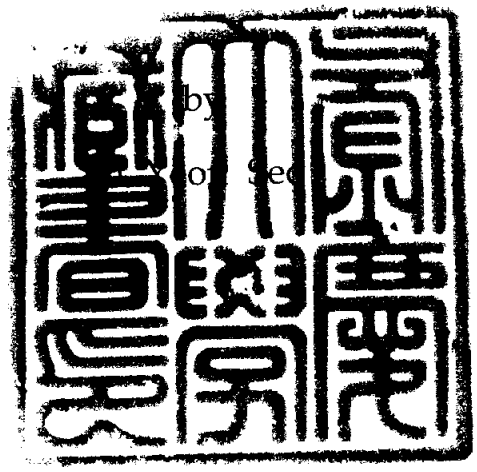

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in the Department of Microbiology, Graduate School, Pukyong National University

August 2003

서지연의 이학석사 학위논문을 인준함

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A Dissertation

by

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June 2003

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ABSTRACT

Hirame rhabdovirus (HIRRV) is one of the important viruses of cultured flounder. The protective immunogenicity of DNA based vaccine was tested against this virus. Genes encoding the nucleocapsid protein (N) and the C-terminal two third of the glycoprotein were amplified by RT-PCR and separately cloned into the expression vector pcDNA 3.1. Expression of the cloned genes in transfected cell line was confirmed by western blot analysis. PCR analyses showed the presence of the injected plasmids in the fish muscle tissue up to 14 days post injection. Expression of the proteins in the injected muscle tissue was confirmed by immunocytochemistry with corresponding antibodies. In addition, higher expression of Mx gene responsible for non-specific immune

responses was detected from the DNA vaccinated fish than nonvaccinated fish. Flounder fry with average weight of 2g and length of 3cm were injected with 5ug of plasmid DNA and challenged 21 day post-immunization. Fish injected with the vector DNA or PBS showed over 95% cumulative mortality 16 day after inoculation. In contrast, fish injected with plasmid containing the N gene, G gene or N+G gene mixture showed 70%, 5%, and 2.5% cumulative mortality, respectively. These results show that the G gene is effective for inducing protective immunity in the injected fish against HIRRV infection.

INTRODUCTION

Hirame rhabdovirus (HIRRV), a fish pathogenic rhabdovirus, was first isolated from Japanese flounder (*Padalichthw olivaceus*) and from ayu fish (*Plecoglossus altivellis*) from Japan(Bjorklund *et al.*, 1996). Subsequently, HIRRV was isolated from diseased flounder in Southern Sea, Korea (Oh *et al.*, 1997). The clinical signs of HIRRV infection are septicemia which exhibits serve bleeding in the internal organ. The HIRRV genome consists of approximately 11,000 bases, and encodes five structural proteins, nucleocapsid protein(N), matrix protein(M1 and M2), envelope glycoprotein(G), RNA polymerase(L) and one non viral(NV) protein.(Kurath *et al.*, 1985; Oh and Choi, 1998).

Neutralizing antibodies are most important component of the protective immune response against virus and exclusively directed only against the G protein (Engelking and Leong 1989; Lorenzen *et al.*, 1990). The glycoprotein(G) is a spike protein which spans the viral envelope and protrudes toward the exterior of virion and according to previous studies, its surface protein has antigenicity that determines the serological properties of rhabdovirues (Hill 1975).

Flounder is the major aquaculture species that compromises more than 60% of rasing fish in Korea. Flounder diseases caused by bacteria such as *Flexibacter maritimus*, *Edwardsiella tarda*,

Streptococcus sp., *Vibrio anguillarum* can be controlled using chemical and antibiotics reagents. However, there is no chemotherapeutical agent for the control of viral diseases. Despite of the importance in Korea aquaculture industry, researches on vaccines for flounder is very limited.

Recently, antiviral DNA vaccine carrying a gene for a major antigenic viral protein have proved to be significantly effective in fish (Scott *et al.*, 2001; Lorezen *et al.*, 2002) contrast to traditional vaccines based on attenuated or killed virus as well as recombinant protein vaccines that have limited success in their safety and environmental, economic circumstance.

DNA immunization with antigen-encoding plasmid DNA can produce the foreign protein and elicit both humoral and cellular immune responses by the host immune system (Harriet *et al.*, 1997). This approach also offers economic, environmental and safety advantages (Donnelly *et al.*, 1996; Hassett and Whitton, 1996; Davis 1997), which are particularly attractive for the aquaculture industry. Most DNA vaccine experiments have been carried out in mammals, but a few studies demonstrates successful DNA-based vaccine encoding viral G gene against major fish viral pathogen belong to the rhabdoviridae family, infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) (Lapatra *et al* ., 2001; Lorezen *et al.*, 2001; Pierre *et al.*, 1998). In this study, C-terminal

half of the HIRRV G gene was tested as a DNA vaccine against HIRRV.

MATERIAL AND METHOD

Virus propagation

The HIRRV used in this study was propagated in the rainbow trout gonad (RTG-2) cell line. The infected cells were incubated at 15℃ in MEM (minimal essential medium) in Hanks' salts (Sigma, USA) with 10% FBS (fetal bovine serum, Hyclone, USA), 100units/㎖ of penicillin (Gibco BRL, USA), 100㎍/㎖ of streptomycine (Gibco BRL, USA), 2㎖/ℓ of fungizone (Gibco BRL, USA) and bufferd to pH 7.5 with sodium bicarbonate.

The medium was harvested when viral cytopathic effects were apparent. Cellular debris was removed by low-speed centrifugation(5,000 rpm), and the resulting clarified medium was used to infect flounder. Virus titers were determined by $TCID_{50}$ assay on confluent RTG-2 cell monolayer.

Plasmid construction

The genes encoding G and N protein of HIRRV have been cloned and sequenced previously (Oh 1999 ; Eou 2000). Using this clones,

the C-terminal two third of G and entire N gene were and amplified with specific primer(Table 1) and cloned separately into pGEM-T vector system (Promega). *E. coli* XL-1 blue strain was transformed with the ligated DNA and the transformed cells were cultured using LB containing ampicillin (80μg/mℓ), and plasmid DNA was extracted with alkaline lysis method. The inserts containing the N and G gene was subcloned into pcDNA 3.1 (+) (Clontech) for DNA vaccine. Each T-vector clone containing the HIRRV N gene and G gene were double digested with EcoR I /Xho I and HindIII/ Kpn I site of plasmid vector, pcDNA 3.1(+), which contain a cytomegalovirus promoter upstream from the inserted genes (Fig. 1), resulted in the clones pcDNA-N and pcDNA G for the N gene and G gene, respectively.

Large-scale preperation of DNA vaccine

All vaccine plasmids and the vector plasmid pcDNA 3.1(+) were amplified in *E. coli* XL-1 blue strain and plasmid DNA was purified with the Qiagen plasmid mega kit(Qiagen, USA). The concentration of purified plasmids was determined by optical density at 260nm.

Table 1. Oligonucleotide primers used in the studies

Name	Sequence	Remark
HRN-N-F	5′ accagaattccatatggcga 3′	N gene forward primer containing EcoR I , Nde I
HRN-N-R	5′ tgaattcctcgagaaggcgacatagat 3′	N gene reverse primer containing EcoR I , Xho I
HIG-FO	5′ aagcttatgattaccttctgtgga 3′	G gene forward primer containig HindIII site
HIG-RO	5′ ggtaccttaccctcgactcgc 3′	G gene reverse primer containig Kpn I site
EXPG	5′-gagctcatatgattaccttc-3′	G gene specific primer
Mx-S	5′-gccagcgtcttgctgaccag -3′	Mx gene forward primer
Mx-R	5′-ctctaacctccattaaaact-3′	Mx gene reverse primer

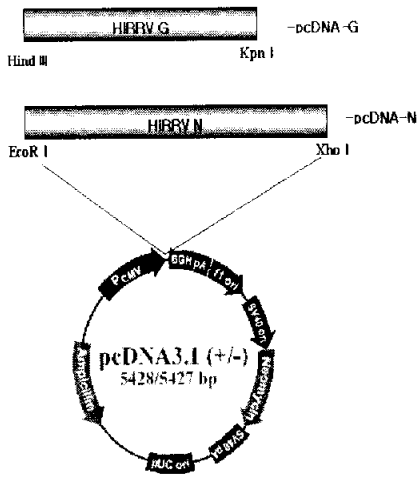

Figure 1. Schematic representation of pcDNA3.1 vector construction of HIRRV N and G gene. pcDNA3.1(+) is 5.4kb vectors derived from pcDNA3 and contains the human cytomegalovirus immediate-early(CMV) promoter for high-level expression in a wide range of mammalian cells. The c-terminal two third of G gene and entire N gene were cloned separately into the vector using HindIII/Kpn I and EcoR I /Xho I restriction endonuclease sites.

Detection of G protein in transfected fish cell line

Rainbow trout gonad (RTG 2) cells were grown in 24-well plates at 16℃ with complete culture medium. When cell were approximately 75~80% confluent, they were transfected with purified pcDNA-G according to calcium-phosphate mediated transfection protocol(Sambrook *et al.*, 2001). After 32 hours incubation, culture medium was removed and cell monolayers were wash with PBS. Cells were then detached by adding trypsin-EDTA solution, and suspension were transferred into 1.5㎖ tubes.

After centrifugation (3min at 10,000 rpm), supernatant were discarded and the pellets were resuspended in 10㎕ of PBS and 15㎕ of sample loading buffer [1mM EDTA, 250mM Tris-Cl(pH 6.8), 4% SDS, 2% β Mercaptoethanol, 0.2% bromophenyl blue, 50% Glycerol] . Samples were boiled for 3min before loading on a 12.5% SDS polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane at constant current of 250mA for 1.5hrs in transfer buffer (120mM glycine, 15.6mM Tris · Base, and 20% methanol). The membrane was blocked using 5% skim milk in TTBS buffer (0.8% NaCl, 0.2% KCl, and 0.05% Tween 20 in 20mM Tris · Cl, pH 7.4). The membrane was washed for 5 min in TTBS then treated for 1.5 hrs with Glutathione S-transferase(GST) HIRRV G protein polyclonal antibodies diluted 1:1,000 in TTBS containing 5% skim milk. After

three times washing for 5 min (each) in TTBS, the membrane was treated for 1hr with alkaline phosphatase conjugated anti-mouse IgG in TTBS containing 5% skim milk. The membrane was washed three times for 5 min (each) in TTBS and developed by NBT and BCIP solution in alkaline phospatase buffer (0.1M Tris · Cl, pH 9.5, 0.1M NaCl, 5mM $MgCl_2$).

Preperation of samples for electron microscopy

Muscle tissues from pcDNA-G injected and PBS injected fish were cut into about 2×2㎝ in site of injection. Tissue were cut again into $1mm^3$ and fixed with 2.5% glutaraldehyde for 2 hrs at 4℃. Sample were washed three times with 0.1M phosphate buffer(pH 7.4) to replace the primary fixation buffer. Second fixation was performed with 1% osmium tetroxide(O_5O_4) for 2 hrs at 4℃ and then washed three times with the phosphate buffer. The fixed samples were dehydrated with serial 10min changes of 50, 70, 80, 90, 95, 100% ethanol at room temperature. Then, ethanol was replaced twice with propylene oxide for 30 min and infiltrated for 2 hrs with propylene oxide:epon mixture. After infiltration step, the samples were embedded using Epon 812 and polymerized. The temperature setting of EM oven for polymerization was as follows; 37℃, 12 hrs→45℃,12

hrs→60℃, 48hrs. Ultrathinsection of 60~90nm were made with a LKB ultramicotome(Nova, Sweden) and transferred onto a 200 mesh copper grid.

Protein detection by immunocytochemistry

Ultrathinsections on copper grid from pcDNA-G injected or PBS injected fish were observed after immunogold labeling. The grid was placed on a drop of 5% FPG [5% fetal bovine serum(FBS) in phosphate buffered saline(PBS, pH7.0) containing 0.02M glycine] to block non-specific binding. The grid was transferred on a drop of 1 : 200 diluted mouse anti HIRRV G protein polyclonal antibody diluted in 5% FPG and incubated for 2 hr at room temperature. The unbound antibody was washed off four times by transferring the grid on a drop of 10% FPG and incubating for 5 min. Excess liquid was removed from the grid by touching a dry filter paper. Then the grid was placed on a drop of gold labeled goat anti-mouse IgG 1 : 10 diluted in 5% FPG and incubated for 1 hr at room temperature, wash four times as above. All treated grids were double stained with 5% uranyl acetate and 0.04% lead citrate for 30min and 2~5 min, respectively. The stained grids were observed using transmmition electron microscopy(JEM 1200EX-II, JEOL, Japan).

DNA extraction from muscle tissue and PCR assay

Muscle tissues from pcDNA-G vaccinated fish were taken at 38hr, 7 days and 14 days postinjection, pulverized to powder in liquid nitrogen and dissolved in 3ml of genomic DNA isolation buffer (1.0% SDS, 100mM NaCl, 50mM Tris-Cl, 100mM EDTA pH 8.0, 20㎍/㎖ RNase), and incubated for 1h at 37℃. Proteinase K was added to 150㎍/㎖, and the sample was incubated at 60℃ overnight. The DNA was extracted with phenol:chloroform:isoamyl alchol (25:24:1) and precipitated from the aqueous phase with 1 volume of isopropanol. The precipitate was pelleted by centrifugation (30min at 13,000 rpm), washed with 70% ethanol, and dried at room temperature. The DNA pellet was dissolved in 200㎕ of TE buffer pH 8.3. DNA was subjected to 40 cycles of PCR composed of 1 min denaturation at 96℃, 30s annealing at 55℃, 1 min extension at 72℃ with HIRRV G specific forward and reverse primers (Table 1). The PCR products were electrophored on a 1% agarose gel and analyzed.

Detection of flounder Mx gene mRNA by RT-PCR

Total RNA was extracted from kidney using Trizol (Gibco BRL.

USA) 14 days post injection for analysis. Flounder kidney were homogenized in 1.5 ml tube by adding 1ml of Trizol reagent and incubated for 5 min at room temperature to permit complete dissociation of nucleoprotein complex. 0.2ml of chloroform was added to the lysate and the tube was vigorously shaken by hand for 15 seconds. The sample was centrifuged at 14,000 rpm for 15 min at 4 ℃. The aqueous phase was transferred to a new tube and the RNA was precipitated by adding same volume of isopropyl alcohol for 10 min at -70℃. RNA was precipitated by centrifugation at 14,000 rpm for 10min and washed with 70% ethanol. The pellet was dried in vacuum decicator for 5min and resuspended in diethylpyrocarbonate (DEPC) treated water. The purified total RNA (10μg) was resuspended in 25$\mu\ell$ of reverse transcripton reaction mixture containing 5$\mu\ell$ of 5× buffer (250mM Tris Cl, pH 8.3, 250mM KCl, 50mM $MgCl_2$, 2.5mM sepermidine, 50mM DTT), 0.5$\mu\ell$ of dNTP, 1$\mu\ell$ of $MgSO_4$, 0.5$\mu\ell$ of AMV RTase (10u/$\mu\ell$) and 50 pmol of antisense primer. The reaction mixture was incubated for 42min at 48℃ and the RTase was inactivated for 2min at 94℃. The reverse-transcribed sample (2$\mu\ell$) was amplified by addition of 48$\mu\ell$ of PCR mixture containing 5$\mu\ell$ of 10× buffer (100mM Tris,Cl, pH 8.3, 500mM KCl, 15mM $MgCl_2$), 2.5mM dNTP (each), 0.5$\mu\ell$ of Taq DNA polymerase (5u/$\mu\ell$), and 50 pmole of forward and reverse primer(Table 1). The PCR primers, designed from the 3′ region of the ORF were described

previously (Lee *et al.*, 2000). Reaction was performed with initial denaturation step of 10 min at 96℃, and then 30 cycles composed of 1 min denaturation at 96℃, 30 sec annealing at 55℃, and 30 sec extension at 72℃, followed by a 7 min postextension at 72℃. The reaction products were electrophoresed on a 1% agarose gel and sequenced.

Vaccination and challenge

The protective immunogenicity of the constructed DNA vaccines was tested by injection of the DNA vaccine separately or in combination. Five groups of forty flounder fry with mean weight of 3g were prepared for the injection with pcDNA-G, pcDNA-N, pcDNA-G and pcDNA-N mixture, pcDNA3.1(+) vector and PBS. The DNA was adjusted as 5ug/50ul using PBS. The fish was anaesthetised by immerzing in 100 ㎍/㎖ of tricaine methane sulfonate (MS-222) and intramusculary injected with 50ul of DNA or PBS.

The injected fish was kept in 50ℓ tanks at 14℃ before being challenged with the virus. At 21 days postvaccination (dpv), each groups of fish were challenged by addition of HIRRV to the water to a concentration of 1.2×10^{4} TCID㎖$^{-1}$ for 90 min at 15℃. Flounder

mortalities in each tank was recorded, and dead fish were daily for 16 days post-challenge.

RESULT AND DISCUSSION

Plasmid construction

The G and N gene of HIRRV were cloned into EcoR I /Xho I and HindIII/Kpn I sites of plasmid pcDNA3.1 vector, respectively under control of the CMV promoter sequence. To ensure that G and N gene were inserted into the vector correctly, the insert were analyzed by restriction enzyme digestion with corresponding enzyme(Fig. 2) and PCR amplification of the insert(Fig. 3). Sequences of the G and N genes in the pcDNA 3.1 vector were confirmed by DNA sequencing(Figure 4, Figure 5). The inserts for G and N gene were 780bp and 1.2kb and the resulting plasmids were named as pcDNA-G and pcDNA-N for the G gene and N gene, respectively.

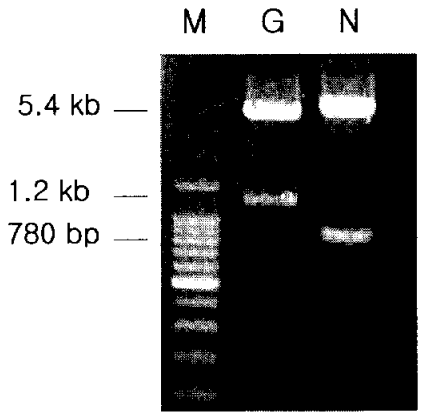

Figure 2 . Cloning of HIRRV G gene and N gene into the pcDNA vactor. pcDNA 3.1 vector contain the HIRRV G and N gene were digested with HindIII/Kpn I and EcoR I /Xho I , respectively. M, 100bp DNA ladder; N, pcDNA-N contain the N gene; G, pcDNA-G contain the G gene.

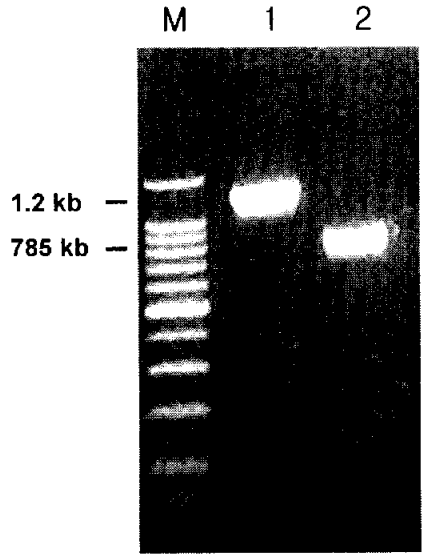

Figure 3. Confirmation of pcDNA-G and pcDNA-N by PCR. The N gene and G gene inserts cloned into pcDNA vector were amplified with specific primers listed in table 1. M, 100bp DNA ladder; lane 1, pcDNA-N PCR product; lane 2, pcDNA-G PCR product.

HIRN-N-F

accagaattccatatggcgaaccttaaggaagaatttgcaggactccgaggagtgaagggaggagccctcgaag

actctggcacggagtatgatcccaccaagatcaacctgacgctctacgggacagacaagctctataccctggcg

atcatcaagagggcagtcagtcaggtcggagggtcccaaaccaacaaagcactaggaatcctttgtgctttcag

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gaacatggtggacctggtgaaaggccttctgttcacttgtgccctgatggtcaagtacgatgtggacaagatggc

cacatactgccagcagaagctggagcgactggccaacagtcagggactcaacgagctgaccctgataagcacgag

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ctcacggatcacctgtatcttgatgaacatcaacgagcagtacgatgaggggacaaccggaggactggcaggagt

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gcctcagatcttgtccgccaggccgagcaggcagcgcaagagaccgagggcgaggatgataagacgatgaggagg

cgaggaagacggaggggagggagaggggggagaggacgaagagtactactgagacccggcatctatgctgccttct

cgaggaattcact

HRN N-R

Figure 4. Nucleotide Sequence of HIRRV N gene used in this study. Boldface indicates HIRRV N gene translation start and stop codon and arrow indicates gene specific primer

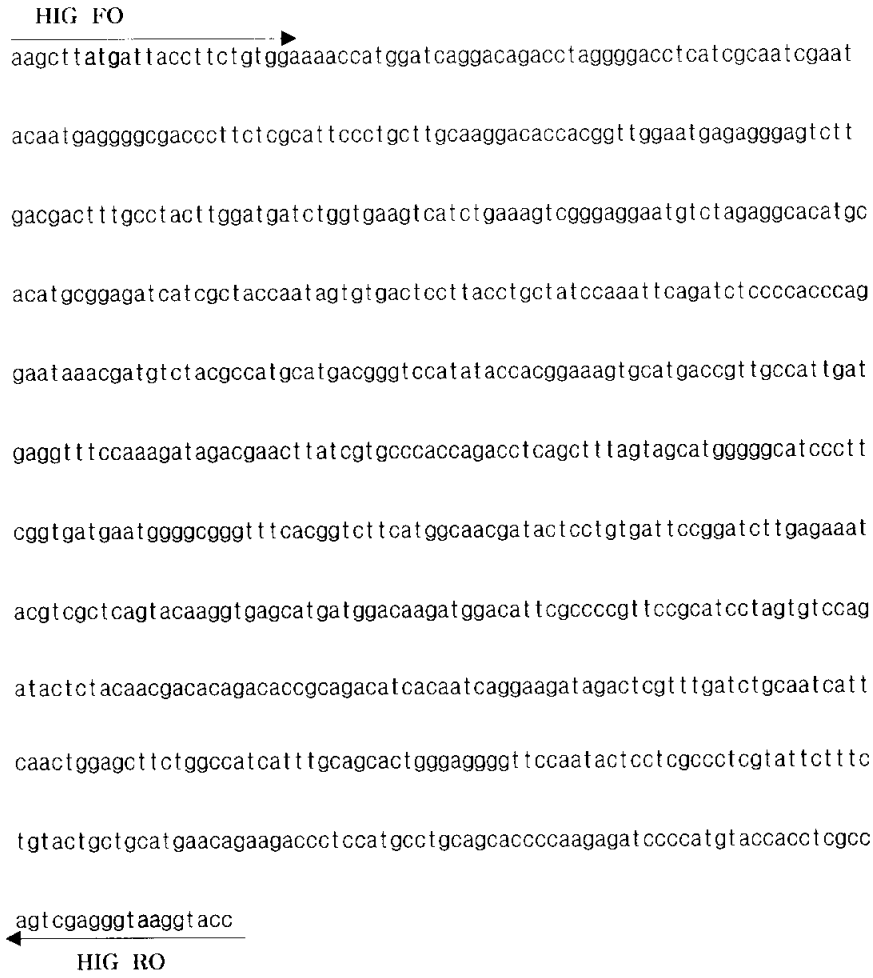

Figure 5. Nucleotide Sequence of part of HIRRV G gene used in this study Boldface indicates HIRRV G gene translation start and stop codon and arrow indicates gene specific primer

In vitro expression of recominant G protein in transfected cells

The G protein were detected in transfected fish cell lysates by Western blot. The apparent size of the G protein (35kDa) (Fig. 6) in RTG 2 cell lysate appeared to be slightly larger than that in the our expectation, which might be due to an glycosylated form of G in the RTG 2 cell preperation. These data shows that the recombinant G protein could be correctly processed by fish cell and mRNA for the recombinant G was transcribed quantitatively in fish cell.

For DNA vaccine, conservation of native structure and thus the antigenicity of protein is very important issue, and correct conformation is essential for the presence or accessibility of certain neutralization epitope (Lorenzen *et al.*, 1990, 1993; Lorenzen 1997). We demonstrated that the native structure of expressed G protein in fish cell with Glutathione S-transferase(GST)-HIRRV G protein polyclonal antibodies and this recombinant G protein that has antigenicity would elicts the neutralization antibodies against the virus.

In vivo expression of recombinant G protein in muscle tissue

Expression of the G protein from injected fish was detected in

days 3, 7 postinjection. No gold label was observed in the tissues from vector injected fish(Fig. 7). It is known that immunization with DNA vaccine raise immune responses with much lower levels of protein(nanogram levels) (Harriet *et al.*, 1997) than live-attenuated, killed-whole, or subunit vaccines(Lorenzen *et al.*, 1999). Detection of expressed G protein in muscle tissue by immunocytochemistry confirmed the accurate performance of the pcDNA-G construct *in vivo*. Several studies have shown expression of antigen protein from DNA vaccine by immunocytochemistry or immunoenzymatic assay(Lprenzen *et al.*, 1998; Pierre *et al.*, 1998), whereas the majority of reports fail to detect any synthesized protein quantities within the range of the picogram(Wolff *et al.*, 1988; Taubes 1997). Although minute quantities of antigenic protein are expressed from DNA vaccines, earlier studies in mammals have shown this to be adequate to induce very strong and long-lasting immune responses(Harriet *et al.*, 1997). These reports gives possibility to induce similar protective immune responses in fish.

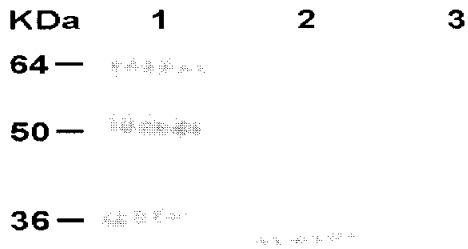

Figure 6. Western blot analysis of HIRRV G protein expressed in transfected fish cell line. The membrane was probed with polyclonal antibody raised against the Glutathione S-transferase(GST)-HIRRV-G protein. Lane1, prestain marker(invitrogen); lane 2, transfected RTG-2 with pcDNA-G; lane 3, negative control

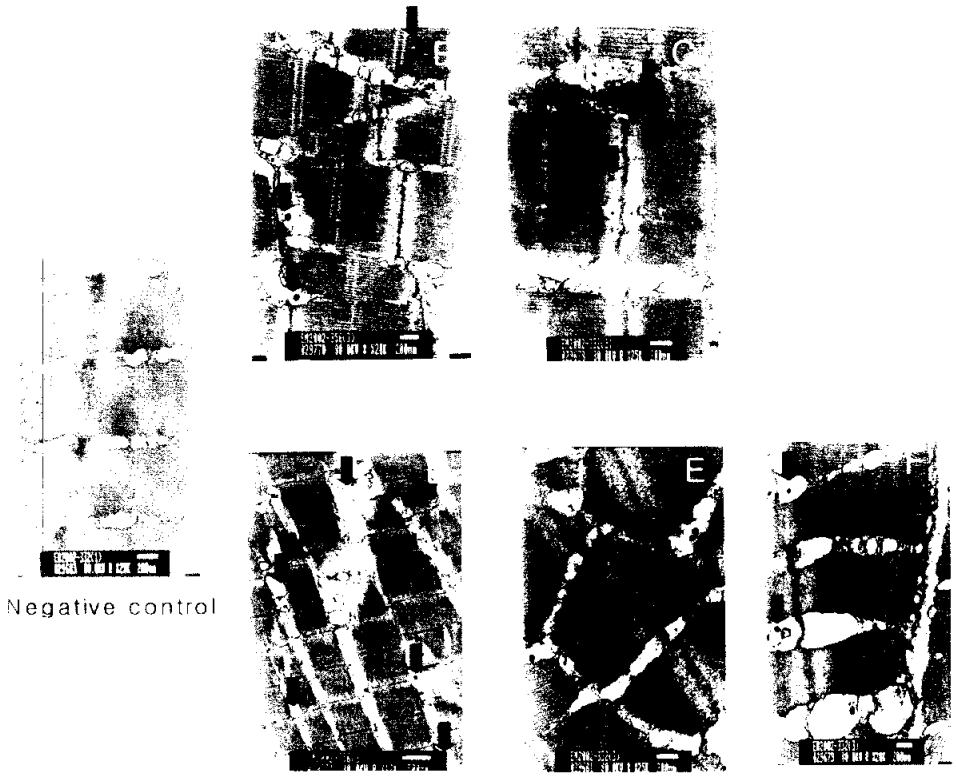

Figure 7. *In vivo* expression of recombinant G protein in muscle tissue

Arrow indicates G protein-gold complex. No G protein-gold complex was observed in negative control(A). The G protein-gold complex appeared in tissue sections from the pcDNA-G injected fish on days 3 (B, C), 7 (D, E, F) after injection.

Persistence of pcDNA G in muscle tissue

In order to confirm the presence of the pcDNA-G plasmid within the tissue taken at different time intervals, PCR was performed with gene specific primer. DNA band of 780bp was amplified on a muscle tissue extract on days 38hr and 7, 14 postinjection. No amplification was detected from noninjected tissue extracts(Fig. 8). An important issue in the DNA vaccination is the site of plasmid uptake and protein synthesis. With plasmid injected intramuscularly, it is generally admitted that the preferential site of plasmid uptake in the muscle cell itself. From DNA vaccine, minute quantities of antigenic protein were expressed and induced very strong and long-lasting immune responses(Wahren 1996; Davis, 1997). Therefore DNA might be dedected for prolonged periods in tissue to occur long-lasting immune responses.

Mx gene expression

Mx protein is one of several interferon (IFN)-inducible proteins that have been shown to inhibit the replication of different types of viral infection in several studies (Lee *et al.*, 2000; Tkeshi *et al.*, 1998; Grant *et al.*, 1997). In a recently study, the IFN-induced Mx protein

was detected in the liver and kidney tissue of fish injected with the IHNV G DNA vaccine but not in fish injected with formalin-killed or subunit IHNV vaccine or vaccine control, DNA vaccine injected group had protective effects against challenge virus(Kim *et al*, 2000). Even though how Mx protein exerts its specific antiviral effects in different viruses and cell is not known, these data supposed that Mx gene may play a nonspecific antiviral role in the protection produced by the DNA vaccine. RT-PCR analysis of Mx protein mRNA from a healthy flounder showed that the Mx mRNA was predominantly expressed in the kidney, spleen, intestine, brain, peritoneal cavity fluid and gill (Lee *et al.*, 2000).

Approximately 230bp of DNA band were detected from flounder kidney by RT-PCR and increased in DNA vaccine injected sample (Fig. 9). There are previous studies that intramuscular injection of HIRRV into Japanese flounder produced a dramatic increase of Mx mRNA and the expression of Mx mRNA in the HIRRV-injected flounder was approximately three fold higher than that of non infected flounder, positive control (Lee et al., 2000). Taken all these data together, we supposed that intramuscular injection of DNA vaccine into flounder induced Mx mRNA like as virus.

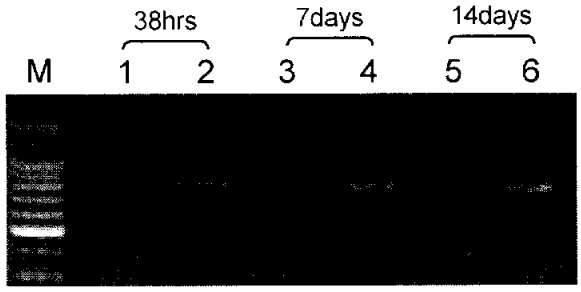

Figure 8. Detection of pcDNA-G in muscle extracts by PCR. pcDNA-G was detected in 38hr and 7, 14 post vaccination. M, 100bp DNA ladder; Lanes 1, 3, 5, pcDNA 3.1 injected group as a negative control; Lanes 2, 4, 5, pcDNA-G injected sample.

Figure 9. RT-PCR of Mx gene from flounder kidney. Mx gene were dedected using PCR with gene specific primer. Lane 1, non-vaccinated healthy fish; lane 2, PBS group; lane 3, pcDNA vector group-1; lane 4, pcDNA-vector group-2; lane5, pcDNA-N group-1; lane 6, pcDNA-N group-2; lane 7, pcDNA-G group-1; lane 8, pcDNA-G group-2; lane 9, pcDNA NG group-1; lane 10, pcDNA NG group-2.

Protection of flounder from viral challenge

Fish injected with the vector DNA or PBS showed 97.5% and 95% cumulative mortality 16 day after inoculation. In contrast, fish injected with plasmid containing the N gene, G gene and N+G gene mixture showed 70%, 5%, and 2.5% cumulative mortality, respectively (Fig. 10). These results show that the G gene is effective for inducing protective immunity in the injected fish against HIRRV infection. Several studies shows that G protein is known as the inducer of neutralizing and protective antibodies. The high prevalence of seropositive fish following vaccination with the G-construct may this way explain the high survival in pcDNA-G and pcDNA G+N njected groups of fish.

High level of protection against HIRRV was induced by injection of 5μg of G-encoding plasmid in this study. However several studies showed that only 0.1μg of DNA was sufficient to provide protection to fish against viral challenge (Serge *et al.*, 2000). Effect of DNA vaccines at such low doses means that they will be useful for aquaculture economically. However, these results shows significant effects of DNA vaccine only laboratory condition. Further studies will be to performed for mass vaccination of flounder fry under farming condition.

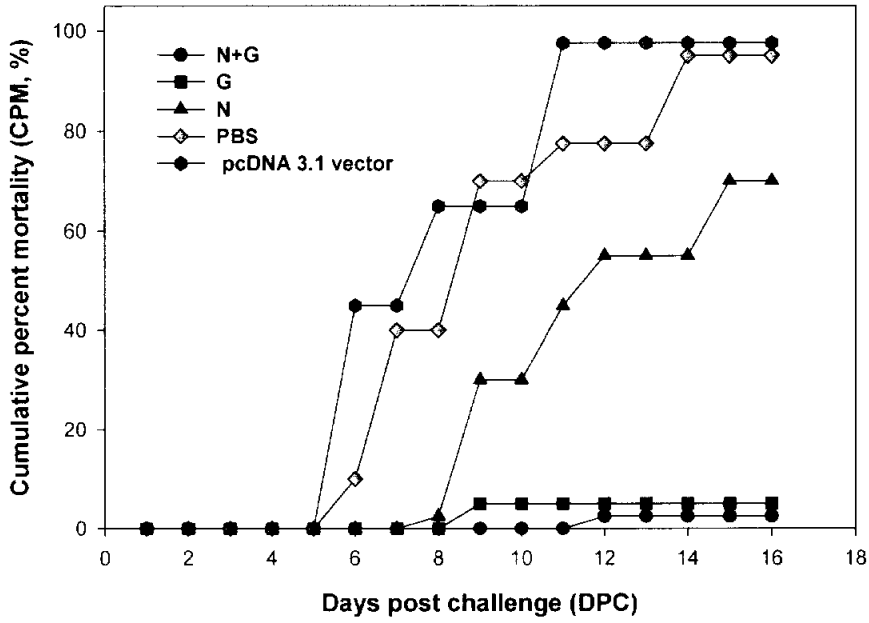

Figure 10. Cumulative % mortality of flounder immunized with DNA vaccine against HIRRV.

국 문 초 록

넙치 랩도 바이러스(HIRRV)는 넙치 양식에 있어 중요한 질병원 중 하나이다. 랩도 바이러스는 일본에서 처음 보고되었으며, 우리나라 통영 연안 지역의 양어장에서 분리되었다. 본 연구에서는 랩도 바이러스의 당단백질을 이용한 DNA 백신을 제작하고 이에 따른 넙치에서의 보호 면역력을 검증하고자 하였다. 랩도 바이러스의 nucleocapsid protein(N)과 glycoprotein의 C 말단의 3분의 2를 암호화하는 유전자를 진핵 발현 벡터인 pcDNA 3.1 벡터에 클로닝하고 이를 넙치에 백신으로서 처리하기전 몇가지 실험들을 통해 백신으로서의 가능성을 검증하였다. 발현벡터에 클로닝 된 G 유전자는 먼저 어류 세포에 transfection하여 western analysis를 통해 단백질의 발현을 확인할 수 있었다. 이렇게 확인된 클론은 넙치 근육에 직접 주사하여 immunocytochemistry를 통해 조직내에서의 단백질 발현을 가시적으로 확인 할 수 있었다. 또한 넙치 근육에 주사한 DNA는 주사한지 14일째까지 PCR을 통해 확인이 가능하였다. 바이러스의 감염에 대하여 비특이적인 면역 반응을 나타낸다고 알려져 있는 Mx 유전자는 백신을 주사하였을때가 그렇지 않을때보다 상대적으로 많은 양이 발현함을 알 수 있었다.

평균 체중이 2g이고 전장이 3cm인 넙치 치어를 그룹별로 나누어 당백신을 5μg의 농도로 주사하고 21일 후에 랩도 바이러스를 일정시간 감염시켰을 때 벡터만 주사하였거나 PBS만을 주사한 그룹에서 95% 이상

의 축적 사망률을 나타내는데 비하여 G gene을 주사하였거나 N gene과 같이 주사하였을때는 5%, 2.5%의 매우 낮은 치사율을 보였다. 이러한 결과로 미루어 랩도 바이러스의 당단백질을 이용한 DNA 백신이 백신을 주사한 넙치로부터 바이러스의 감염에 대해 효과적인 보호 면역력을 나타냄을 알 수 있었다.

ACKNOWLEDGMENTS

많은 시간이 흘렀습니다. 그 동안 부모와도 같은 사랑과 격려로 이끌어 주신 최태진 교수님과 부족한 논문을 끝까지 읽어주신 이훈구 교수님, 수산생명의학과 김기홍 교수님께 감사를 드립니다. 대학원 과정동안 학문적 충고로 부족한 제자를 이끌어주신 이원재 교수님, 김진상 교수님, 이명숙 교수님, 송영환 교수님, 김영태 교수님께도 감사를 드립니다.

힘들고 지칠때마다 격려해주신 은영 언니, 끝까지 제 곁에서 너무나 많은 도움을 준 현화 언니께 진심으로 감사드립니다. 항상 본보기가 되어준 현향 언니, 힘든 속에서도 항상 의지가 되어준 종오 선배께 고마움을 전하며 밝은 앞날이 있길 바랍니다. 부족한 논문을 세심한 편집으로 도와주신 홍묵선배, 부족한 동기 때문에 고생이 많았을 재경이, 경규, 어렵고 힘든일 마다않고 묵묵히 애써준 선미, 경우에게 미안함과 고마움을 전합니다. 부족한 선배 밑에서 실험실 생활하느라 고생 많았던 혜경, 혜진이, 끝까지 지켜주지 못해 항상 미안한 마음뿐인 수미에게 고마움을 전합니다. 오랜 시간을 함께 했기에 저에게는 가족과도 같은 바이러스 실험실의 무궁한 발전을 기원합니다.

대학원 생활 동안 많은 조언과 도움을 주신 각 실험실 선배님들과 후배들에게도 고마움을 전하며 동기라는 이름만으로도 큰 힘이 된 혜진이, 주연이, 대성선배와 경석선배께도 감사의 말을 전합니다. 항상 걱정해주고 격려해준 지연이에게 미안함과 고마움을 전합니다.

끝으로 항상 믿어주시고 끝없는 사랑을 베풀어 주신 할아버지, 할머니, 부모님과 동생들에게 고맙고 사랑한다는 말 전하며 이 작은 결실을 바칩니다.

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