The Protective Effect of Recombinant Glycoprotein Vaccine Against HIRRV Infection

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ABSTRACT—Hirame rhabdovirus (HIRRV) was first reported from flounder (Paralichthys olivaceus) in Japan, and has recently been isolated from an aquaculture farm in the Tongyoung area of Korea. Total RNA isolated from RTG-2 cells infected with HIRRV was used in RT-PCR to obtain a cDNA coding for the glycoprotein of HIRRV Korean strain, CA-9703. Around half sized G protein in the C-terminal region was expressed as glutathione-S-transferase fusion protein, and used as viral vaccine against HIRRV. Flounder fry were vaccinated with low (50 µg/mL) and high (300 µg/mL) doses of vaccine by immersing them for 7 min in vaccine solution. After 1 month, the fish were challenged with HIRRV either by immersion or intraperitoneal injection. Specific losses of fish treated with low and high vaccine doses by intraperitoneal injection challenge were both 6.7%, in contrast to 43.3% in non-vaccinated fish. Losses of vaccinated fish by immersion challenge were 1.7% and 0% for low and high vaccine doses, respectively, and 15% for the non-vaccinated control.

Key words: hirame rhabdovirus, HIRRV, glycoprotein, recombinant vaccine, Paralichthys olivaceus, flounder

Although vaccines have been developed for several important pathogens, vaccination against fish viral diseases has been limited primarily because of high production costs and problems with the logistics of vaccinating large populations of fish (Leong, 1993). However, a recent development in molecular biology has made it possible to produce a large amount of recombinant viral protein as a sub-unit vaccine that can be used for vaccination by immersion or injection of individual fish (Munn, 1994; Lorenzen, 1999). In addition, viral genes cloned into an expression vector and introduced into fish tissue as DNA vaccines have been proved to be effective in indoor experiments and small-scale field experiments (Corbell et al., 1999; Kim et al., 2000).

The pathogenic rhabdovirus of fish, hirame rhabdovirus (HIRRV), was first isolated from Japanese flounder (Paralichthys olivaceus) and ayu fish (Plecoglossus altivelis) (Kimura et al., 1986). Subsequently, HIRRV was isolated from diseased flounder in Korea (Oh and Choi, 1998). The clinical sign of HIRRV infection is septicemia that manifests as severe bleeding of internal organs. The electrophoresis pattern of structural proteins originally suggested that HIRRV was closely related to viruses of the genus Lyssavirus (Nishizawa et al., 1991). More recently, it has been classified as belonging to the genus Novirhabdovirus, family Rhabdoviridae (van Regenmortel et al., 2000). Kurath et al. (1985) reported that the HIRRV genome consists of approximately 11,000 bases encoding six viral proteins: RNA polymerase, envelope glycoprotein (G), nucleocapsid protein, two matrix proteins, and non-viral protein.

The glycoprotein is a spike protein that spans the viral envelope and protrudes towards the exterior of the virion (Björklund et al., 1996). This protein is the antigen that determines the serological properties of a rhabdovirus and functions as the antigen eliciting neutralization antibody (Hill et al., 1975). The glycoproteins of vesicular stomatitis virus (VSV) and rabies virus are also responsible for eliciting both neutralizing antibody and protective immune responses (Kelly et al., 1972; Witkor et al., 1984). In the infectious hematopoietic necrosis virus (IHNV), the G protein is the only viral protein necessary to elicit neutralizing antibody (Engelking and Leong, 1989). It has also been reported that an
antigenic determinant of the glycoprotein gene of IHNV expressed as fusion protein with trpE protein of Escherichia coli induced protective immunity (Gilmore et al., 1988). Recent studies on DNA vaccines for fish rhabdovirus glycoprotein also shown that the glycoprotein is sufficient to induce protective immunity against the corresponding virus and even other rhabdoviruses in the same genus (Corbeil et al., 1999; Kim et al., 2000).

Recently, our laboratory has cloned and sequenced the whole genome of a Korean strain of HIRRV that consists of 11034 nucleotides (Oh, 2000). In the present study, we expressed the C-terminal portion of HIRRV glycoprotein as a glutathione-s-transferase (GST)-glycoprotein fusion protein and evaluated the protective immunogenicity of the purified fusion protein.

Materials and Methods

Cell and virus

Rainbow trout gonad (RTG-2) cells were used for primary isolation and routine propagation of the virus. They were grown at 15°C in minimal essential medium (Sigma, USA) supplemented with fetal bovine serum and antibiotics. The HIRRV CA-9703 strain was isolated from an aquaculture farm in Tongyoung, Korea. Monolayers of RTG-2 cells grown in 75 mL tissue culture flasks were inoculated with the virus at a low multiplicity of infection (0.01 to 0.001) and incubated at 15°C. After 7 days, or when the monolayers showed complete lysis, the culture fluid was collected. Virus titer was determined by plaque assay (Reed and Muench, 1938).

Cloning of glycoprotein gene

Total RNA was extracted from HIRRV cultured in RTG-2 cells using Trizol (Gibco BRL). Two primers for cloning were designed, based on the published sequence of the Japanese isolate (Bjorklund et al., 1999). The forward primer HRG-N (5'-tgttctccatatgacagctgc-3') contains the ATG codon for translation initiation (underlined). The reverse primer HRG-C (5'-tacctgctgctagctgc-3') is located in the 3' untranslated region (UTR) of the G gene. The cDNA was synthesized with the reverse primer and amplified using both primers. PCR conditions were pre-denaturation at 95°C for 5 min, 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 50°C, and 1.5 min extension at 72°C, followed by a 7 min post-extension at 72°C. The 6815 bp RT-PCR product containing the entire open reading frame (ORF) of the HIRRV G gene was analyzed by electrophoresis in 1% agarose gels, eluted, and cloned into pGEM-T vector (Promega). DNA sequencing of the resulting clone, pGTHRG, was carried out in an ABI PRISM™ 310 Analyzer (Perkin Elmer).

Expression of GST-glycoprotein fusion protein

Around one half of the G protein in the C-terminal region was expressed as GTS-glycoprotein fusion protein. The pGTHRG was digested with EcoRI and Xhol, and an 800 bp DNA fragment encompassing 260 amino acids at the C-terminal was cloned into the pGEX4T-1 vector. The E. coli BL21 (DE3) strain was transformed with the resulting clone pECHRG. The transformed E. coli was cultured in LB broth containing ampicillin (100 μg/mL). The cells were induced with IPTG at a final concentration of 1 mM after the OD600 reached 0.5. Three milliliters of cell suspension were harvested at 2, 4, and 6 h after induction, and the protein was analyzed by SDS-PAGE using 12.5% polyacrylamide gels.

Purification of over-expressed glycoprotein

Cells containing pECHRG clones were cultured overnight in 20 mL of LB broth containing ampicillin (100 μg/mL) at 37°C, diluted into 1 L of fresh medium, and grown until the OD600 reached 0.5. The culture was induced with IPTG to 1 mM final concentration and incubated for 6 hours. The cells were harvested by centrifugation at 5,000 × g for 10 min at 4°C and resuspended in 15 mL of lysis buffer (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF). The mixture was sonicated with Vibra Cell™ (Sonics and Materials Inc.) for 2 min with 50% output in an ice-bath and centrifuged at 8,000 × g for 5 min at 4°C. The pellet was resuspended in 20 mL of lysis buffer containing lysozyme (200 μg/mL), 0.1% Triton X-100, and deoxycholic acid (1 mg/mL), and kept on ice for 30 min. After centrifugation at 8,000 × g for 5 min, the pellet was washed twice with lysis buffer containing 0.1% Triton X-100, and deoxycholic acid (1 mg/mL), centrifuged at 8,000 × g for 5 min, and then resuspended in 5 mL of lysis buffer. A 0.5 mL aliquot of the sample was denatured and run on 10% or 12.5% polyacrylamide gels. The gels were stained with 0.3% CuCl2 and the protein band excised. Protein was eluted from the band by electro-separation (S&S Elutrap, Schleicher & Schuell) and quantified by the Bradford assay.

Vaccination and challenge

Flounder fry (mean weight, 1.79 g; mean body length, 5.4 cm) were obtained from a local hatchery that has no viral disease record. The fish were acclimatized to water temperature (18°C) for 7 days in 50 L tanks with 30 fry per tank (30 fry/tank × 12 tanks). The fish were fed commercial diet corresponding to 3% of total body weight, three times per day. The vaccine solutions were prepared by dissolving a known amount of purified protein in filter-sterilized seawater to make final concentrations of 50 μg/mL and 300 μg/mL for low and high doses, respectively. The fry was grouped into three; 120 fry for low dose vaccina-
tion, 120 fry for high dose vaccination and 120 fry for non-vaccinated negative control. Vaccination was by direct immersion of batches of 10 fish in 1 L tanks containing 300 mL of vaccine solution for 7 min. The non-vaccinated control group was immersed in filtered seawater for 10 min. After treatment, the fish were reared in 50 L tanks for 30 days at 18°C before being challenged with the virus. The vaccinated and non-vaccinated fish were divided into three groups. One group of 60 fry was challenged by direct immersion in a virus suspension containing 105.5 TCID50/mL for 1 hour. Another group of 30 fry was challenged by intra-peritoneal injection of 105.3 TCID50/mL/fish. The third group of 30 fry was challenged by peritoneal injection of 1 mL HBSS as a negative control. The challenged fish were reared at 18°C for 30 days and the mortality was recorded.

Detection of HIRRV from dead fish

All dead fish were first observed for typical HIRRV symptoms including hemorrhage on the skin and gills, and accumulation of ascitic fluid. Dead fish not showing these symptoms were tested for the presence of HIRRV by using RT-PCR. Three dead fish showing typical symptoms in the high dosage vaccination group were used as a positive control in RT-PCR. Total RNA was isolated from their kidneys using Trizol (Gibco BRL) and RT-PCR was conducted with two HIRRV G protein-specific primers, EXPG (5'-gagctcatatgattaccttc-3') and HRG-C (5'-tatctaggatcctagaggtaag-3') as described above. The PCR products with the expected size of 800 bp were analyzed on 1.0% agarose gels.

Results

The entire G protein gene was first cloned into the E. coli expression vector pET11c. Although colonies containing the cDNA were recovered, there was no expression of glycoprotein after induction with IPTG. Therefore, the C-terminal portion containing amino acids 250 to 508 was fused to the GST gene, and a fusion protein with the expected molecular weight of 55 kDa was detected from E. coli transformed with pECHRG after induction of 1mM IPTG (Fig. 1). This fusion protein reacted with antibody against total HIRRV virion in Western blot analysis, which indicated that the expressed HIRRV fusion protein is in the correct ORF (data not shown).

The purified GST-G fusion protein was used as the antigen for vaccination in low (50 µg/mL) and high (300 µg/mL) doses. The results in Table 1 show that in the non-vaccinated control, the cumulative mortality was 46.7% after intra-peritoneal injection and was 16.7% after immersion inoculation. The cumulative mortalities of fish vaccinated by intra-peritoneal injection were 6.7% for the high dose rate and 20% for the low dose rate.

![Fig. 1. SDS-PAGE analysis of GST-G fusion protein expressed in E. coli.](image)

<table>
<thead>
<tr>
<th>Vaccine dosage</th>
<th>Mode of Infection</th>
<th>Number of Dead Fish (%)</th>
<th>Dead Fish Positive with Symptoms</th>
<th>Specific Lossa (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High dosage (300 µg/mL)</td>
<td>Injection</td>
<td>2/30 (6.7)</td>
<td>2 (2)a</td>
<td>2/30 (6.7)a</td>
</tr>
<tr>
<td></td>
<td>Immersion</td>
<td>1/60 (1.7)</td>
<td>1 (1)a</td>
<td>1/60 (1.7)a</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0/30 (0)</td>
<td>0</td>
<td>0/30 (0)</td>
</tr>
<tr>
<td>Low dosage (50 µg/mL)</td>
<td>Injection</td>
<td>6/30 (20)</td>
<td>2 0/4b</td>
<td>0/30 (0)</td>
</tr>
<tr>
<td></td>
<td>Immersion</td>
<td>0/60 (0)</td>
<td>0</td>
<td>0/60 (0)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0/30 (0)</td>
<td>0</td>
<td>0/30 (0)</td>
</tr>
<tr>
<td>Negative control</td>
<td>Injection</td>
<td>14/30 (46.7)</td>
<td>10 3/4b</td>
<td>13/30 (43.3)</td>
</tr>
<tr>
<td></td>
<td>Immersion</td>
<td>10/60 (16.7)</td>
<td>8 1/2a</td>
<td>9/60 (15)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0/30 (0)</td>
<td>0</td>
<td>0/30 (0)</td>
</tr>
</tbody>
</table>

a: Fish showing symptoms were used in RT-PCR as positive controls; b: Fish not showing symptoms, but positive in RT-PCR; c: Values with the same symbols were compared statistically; significant differences are indicated by the number of symbols; d: Specific Loss = number of fish deaths due to viral infection / total number of inoculated fish.
Fish mortalities after immersion infection were 1.7% and 0% for the high and low doses, respectively.

Post-mortem analysis was carried out to confirm that mortality was due to infection with HIRRV. Fish showing typical HIRRV symptoms, including hemorrhage and accumulation of ascitic fluid, were considered as having been killed by viral infection, and all except for three fish in the high dose vaccine group that were used as a positive control in RT-PCR, were not tested further. Dead fish that did not show any specific symptoms were tested for the presence of HIRRV by RT-PCR. As shown in Fig. 2, HIRRV was detected from all three dead fish in the high dose vaccinated group and from four out of six in the non-vaccinated group. Interestingly, HIRRV was not detected in dead fish from the low dose vaccine group that did not show any clinical symptoms.

**Discussion**

The entire G protein was not expressed in E. coli and one possible explanation for this is the toxicity of expressed glycoprotein in E. coli. It is known that a short (less than 15 amino acids) stretch of hydrophobic amino acid residues occurs in the signal peptide sequence or transmembrane anchor domain (Brosius, 1984; Steinberg et al., 1995). The signal peptide sequences of many viral glycoproteins have been implicated as possible causes of host toxicity and low expression level in E. coli (Brosius, 1984; Steinberg et al., 1995). The signal sequence and transmembrane domain have also been identified from the G protein of HIRRV (Björklund et al., 1996). There are several reports of the use of recombinant glycoprotein vaccines against fish rhabdoviruses, and most of those were expressed as fusion proteins (Gilmore et al., 1988; Lorenzen et al., 1993) or using insect cells (Lecocq-Xhonneux et al., 1994; Cain et al., 1999).

In epitope mapping of the C-terminal portion of IHNV glycoprotein expressed as trpE fusion protein, three linear epitopes were identified in the region of amino acids 270 to 460 (Xu et al., 1991). In addition, fusion protein containing this region was more effective as a vaccine than those containing amino acids 66-269 and amino acids 454-508. In the present study, detailed epitope mapping was not conducted to identify the neutralizing epitope in the HIRRV G protein. Nevertheless, the C-terminal portion containing amino acids 250 to 508 was expressed as a GST-G fusion protein based on high sequence homology between these two proteins.

In contrast to individual immunization of animals and humans, a large stock of fish can be immunized by immersion in vaccine solution. However, the doses of antigen in reported immersion experiments differ. Gilmore et al. (1988) used 3 μg/mL of purified IHNV G protein in 1 min immersion vaccination. In contrast, Engelking et al. (1989) used 40–60 μg/mL of purified IHNV G protein in immersion.

In our study, the purified GST-G fusion protein was used as the antigen for vaccination in low (50 μg/mL) and high (300 μg/mL) doses, instead of cell lysate. The cumulative mortality of the non-vaccinated control was 46.7% after intra-peritoneal injection and was 16.7% after immersion inoculation, which is comparable to the mortalities reported by Oseko et al. (1988). The fusion protein was effective in intra-peritoneal injection and immersion infection in both doses (Table 1).

RT-PCR analysis was carried out to confirm that mortality was due to infection with HIRRV and specific losses were obtained. For the purpose of analysis, specific loss was defined as the percentage of fish confirmed to have died from viral infection. The results summarized in the last column of Table 1 show there were no statistically significant differences between the specific losses of fish given high or low doses of vaccine by injection or immersion; however, specific losses were significantly lower for vaccinated fish than for the control groups (Tukey's method, post-ANOVA test). These results indicate that the GST-G fusion protein is an effective fish vaccine and that both dose rates and treatment methods were effectively protected flounder against HIRRV infection. Further experimentation is required to determine the optimal vaccine concentrations. The virus concentrations used for intra-peritoneal injection and immersion inoculation in this experiment were $10^{5.3}$ and $10^{5.5}$ TCID$_{50}$/mL, respectively. These are much higher than normal viral concentrations that might be encountered in aquaculture farms. This indicates that in practice, vaccination with low concentrations of antigen would be sufficient to induce a protective immune response.
Another consideration with the GST-G fusion protein vaccine is the vaccination effect of non-glycosylated protein expressed in E. coli. Glycosylation is generally considered to be important for proper activity of viral vaccines. However, trpE fusion protein of IHNV glycoprotein expressed in E. coli induced protective immunity, whereas G protein expressed in insect cells failed to induce antibody-mediated protection in trout fry (Gilmore et al., 1988; Cain et al., 1999). Furthermore, the GST-G fusion protein was purified from a denaturing acrylamide gel and was not renatured before application. This suggests that glycosylation and conformation-independent linear epitope or epitopes are responsible for the induced protective immunity against HIRRV. Linear reduction-resistant neutralizing epitopes have also been identified in rabies (Van Deer Heijden et al., 1993; Ni et al., 1995), VSV (Keil and Wagner, 1989; Grigera et al., 1992), and IHNV (Leong et al., 1995).

The major problems for the application of recombinant sub-unit vaccine in aquaculture are the low efficacy and the high cost of mass production (Winton, 1997). Further attempts are under way to produce a cost-effective and biologically active vaccine using a eukaryotic expression system developed in our laboratory, and to conduct field experiments.

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References


Oseko, N., M. Yoshimizu, and T. Kimura (1988): Effect of water temperature on artificial infection of Rhabdovirus olivaceus (hirame rhabdovirus-HRV) to hirame (Japanese