Development of DNA vaccine against Red Sea Bream Iridovirus (RSIV)

Red Sea Bream Iridovirus (RSIV)에 대한 DNA vaccine 개발

by

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Development of DNA vaccine against Red Sea Bream Iridovirus (RSIV)

A Dissertation

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요 약

Red sea bream iridovirus (RSIV)는 대표적으로 참돔 (*Pagrus major*)에 감염되는 어류 병원성 바이러스의 일종이다. RSIV에 감염된 어류는 일반적으로 행동이 둔해지고, 심한 빈혈, 아가미의 점상출혈, 비장과 신장 증대 등의 증상을 보인다. RSIV는 일본 (1992)에서 처음 보고되었으며, 우리나라에서는 최근 남해안 일원 해산가두리 양식장에서 RSIV의 감염으로 돌돔이 대량폐사된 바가 있다.

본 연구에서는 RSIV의 integrin binding sites를 이용한 DNA vaccines을 제작하고 그 기능을 검증하기 위해 mouse를 이용한 *in vivo* test와 어류 세포주인 bluegill fry-2 (BF-2) cell을 이용한 *in vitro* test를 실시하였다. 먼저 돌돔에서 분리한 RSIV를 BF-2 cell에 접종하여 2~3일만에 cytopathic effect (CPE)를 관찰할수 있었고, BF-2 cell에서 다량의 RSIV stock을 확보한후 50% tissue culture infectious dose ($TCID_{50}$)를 정하였다. DNA vaccine을 개발하기 위해서 RSIV의 integrin binding sites인 ORF 023L, ORF 037L, ORF 055L, ORF 093L 유전자를 NCBI에서 infectious spleen and kidney necrosis virus (ISKNV)의 염기서열에서 찾아 primer를 제작하여 PCR을 실시하였다. PCR products를 pGEM-T Easy vector에 subclonig을 하여 염기서열 분석 후 RSIV와 ISKNV, RBIV의 염기서열을 비교하였다. 그 결과 ORF 023L의 경우 약 75%, 나머지 gene 3개는 모두 95% 이상의 homology를 가졌다.

목적 단백질발현을 알아 보기위해 ORF 023L gene을 pET28a vector에, 그리고 나머지 3개의 gene을 pGEX4T-1 vector에 cloning한 후 SDS-PAGE결과 약 60 kDa의 ORF 055L 단백질 발현을 확인하였다 따라서 ORF 055L gene을 강력한 CMV promoter를 가진 pcDNA 3.1 (+) 발현 벡터에 cloning하여 DNA vaccine으로서 pcDNA-055을 제작하였다. 제작되어진 DNA vaccine을 생후 4~5주된 BALB/c mice에 1 mg/ml 농도로 주사한 후 2주간격으로 2 mg/ml 농도로 각각 3번씩 주사를 하였다. 항체포집을 위하여 마지막 boosting후 일주일뒤에 1×10^7 cells/ml 농도의 복수암 유발세포인 sarcoma cell을 주사하였고, 7~10일 후에 다량의 항체를 얻을 수 있었고 Western blotting을 통해 항체생성유무를 확인한 결과 ORF 055L에 대한 항체가 생성된 것을 확인 할 수 있었다. 최종적으로 BF-2 cell을 이

용한 중화반응실험에서, pcDNA-055 DNA vaccine에 대한 antisera와 $10^{5.5}TCID_{50}/ml$ 바이러스 mixtures가 접종된 well 모두에서는 CPE가 관찰되지 않았다. 반면에 RSIV (positive control)만을 접종한 well의 60%에서 CPE가 관찰되었다.

결론적으로 pcDNA-055 DNA vaccine을 주사한 마우스에서 얻은 antibody가 RSIV와의 면역효과를 나타냄을 검증하였다.

I. INTRODUCTION

Iridoviruses are icosahedral cytoplasm DNA viruses that have been isolated from invertebrate and vertebrate host species. The virus has a large double-stranded DNA genome and size of 120~300 nm in diameter. The virus contains a spherical deoxyribonucleoprotein core surrounded by a lipid membrane containing protein subunits. Characterization of iridoviruses has been hindered because of the difficulty in isolating and propagating them in tissue cultures [1].

Red sea bream iridovirus (RSIV) is a piscine iridovirus and causes an acute and highly contagious disease designated as red sea bream iridoviral disease (RSBID). Since 1990, outbreaks of RSBID have resulted in high mortality in cultured red sea bream, *Pagrus major,* in the southwestern part of Japan, primarily in the summertime [2]. In addition, many outbreak cases by RSIV have been reported in different aquatic farms of Korea beginning in 1998. Fishes infected by RSIV have severe anemia and show petechia of the gills, congestion of the liver and hypertrophy of the spleen and kidney [14]. Therefore, these viruses have been widely studied due to their ecological and economical impacts to the aquaculture farm in the world. *Edwardsiella tarda, Streptococcus spp., Vibrio anguillarum* can

be controlled using chemicals and antibiotics. However, there is no chemotherapeutical agent for the control of viral diseases. Despite the importance of virus infection in Korea aquaculture industry, researches on vaccines for RSIV have been very limited.

Recently, antiviral DNA vaccine carrying a gene or a major antigenic viral protein has shown significant effects in fish compared totraditional vaccines based on attenuated or killed virus as well as recombinant protein vaccines that have limited success in their safety and environmental and economical circumstances [21, 22]. Immunization with antigen-encoding plasmid DNA can produce the foreign protein and elicit both humoral and cellular immune responses by the host immune system [21]. This approach also offers economical, environmental and safety advantages, therefore, DNA vaccines are particularly attractive for the aquaculture industry.

Integrins are membrane-bound, cell-surface heterodimeric glycoproteins that anchor cells to their surroundings through cell-cell and cell-matrix interactions. Adhesive functions of integrin showimportant roles in viral diseases wound healing, the immune system, cancers, inflammatory responses, and other diseases [18-20].

In this study, integrin binding sites of the RSIV were tested

to develop DNA vaccines against RSIV. Four integrin binding sites of the ORF 023L, ORF 037L, ORF 055L and ORF 093L from infectious spleen and kidney necrosis virus (ISKNV) [9] were selected and amplified using RSIV by PCR. Then, a valuable gene was selected and cloned pcDNA 3.1 (+) vector [24] as a DNA vaccine. Then, the effect of DNA vaccine was evaluated by using neutralization test [32, 33] with BF-2 cells [2, 3, 8].

II. MATERIALS AND METHODS

1. Fish cell line and Virus

1-1. Fish cell line

The Bluegill fry-2 (BF-2) cells were maintained in Eagle's minimum essential medium (EMEM, Sigma, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and antibiotic antimycotic solution (100 units/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin, Sigma, USA) [2, 3, 8]. The cells were cultured at 25℃ and the medium was changed daily.

1-2. Virus samples

Tissue specimens of the spleen, kidney and liver obtained from the moribund rock bream affected by RSIV infection were homogenized in 10 volumes of phosphate-buffered saline (PBS). The tissue homogenates were centrifuged at 8000 rpm for 5 min at 4℃, and the supernatants as a RSIV sample were passed through a 0.45 μm filter membrane. Then, the RSIV was propagated in BF-2 cells at 25℃. Infected cells showing cytopathic effect (CPE) were collected by scraping and the

solution was passed through a 0.45 μm filter membrane. The permeate was stored at -80℃ as a virus stock until use [2, 4].

1-3. Virus titration

BF-2 cells (1×10^5 cells/ml) were cultured in 25 ㎠ T-flasks. For the virus titration, the cells were transferred to a 96-well plate. After incubation of the cells for 24 h, 100 μl of the virus sample with a log dilution range from 10^0 to 10^{10} was prepared in PBS and the diluted virus samples were added into the 96-well plate and incubated at 25℃. The cultures were observed daily for the detection of CPE. The virus titers were determined using the 50% tissue culture infectious dose (TCID50) assay on confluent BF-2 cell monolayers by Spearman-Karber method [34]. The average of triplicate tests was used as a $TCID_{50}$ result.

2. Cloning of DNA vaccines

2-1. PCR

2-1-1. Oligonucleotide primers

Four oligonucleotide primer sets were based on the nucleotide sequences of the ORF 023L, ORF 037L, ORF 055L and ORF 093L of the infectious spleen and kidney necrosis

virus (ISKNV) [9] registered in the Genebank (Table 1.).

2-1-2. Extraction of RSIV genomic DNA

For the isolation of RSIV genomic DNA, 20 mg samples of spleen, kidney and liver obtained from rock bream infected by RSIV were prepared, and the genomic DNA of RSIV was extracted with Genomic DNA Extraction Kit (Bioneer, Korea).

2-1-3. PCR amplification

PCR amplification was carried out in a 50 μl reaction mixture containing the extracted genomic DNA of RSIV, 2.5 mM dNTP, 5 μM of each primer and 5 units/μl Ex Taq polymerase (Takara Shuzo, Japan) with a Takara thermal cycler (Takara Shuzo, Japan). The ORF 023L gene was obtained after predenaturation at 94℃ for 5 min and incubation for 30 cycles at 94℃ for 30 sec, 55℃ for 30 sec and 72℃ for 3 min, followed by an extension period at 72℃ for 7 min. The ORF 037L gene was obtained after predenaturation at 94℃ for 5 min and incubation for 30 cycles at 94℃ for 30 sec, 55℃ for 30 sec and 72℃ 2 min, followed by an extension period at 72℃ for 7 min. The ORF 055L gene was obtained after predenaturation at 94℃ for 5 min and incubation for 30 cycles at 94℃ for 30 sec, 58℃ for 30 sec and 72℃ 1 min 30 sec, followed by an extension

period at 72℃ for 7 min, and the ORF 093L gene was obtained after predenaturation at 94℃ for 5 min and incubation for 30 cycles at 94℃ for 30 sec, 55℃ for 30 sec and 72℃ 1 min 30 sec, followed by an extension period at 72℃ for 7 min. The PCR products were analysed in 1.0% agarose gel containing ethidium bromide (0.5 μg/ml) and visualized with a UV transilluminator.

Table 1. Oligonucleotide primers.

Genomic region	Primer	Oligonucleotide sequences (5′ to 3′ direction)	Remark	Expected size of amplicons
ORF 023L	1FH	AAGCTTATGGCGACCCTGCT	Forward primer containing HindIII	3138 bp
	1RB	GGATCCTCATGGTTCAGTAG	Reverse primer containing BamHI	
ORF 037L	1FH	AAGCTTATGGTGGACGCTAT	Forward primer containing HindIII	1350 bp
	1RX	CTCGAGCTAGAAGCATATCA	Reverse primer containing XhoI	
ORF 055L	1FH	AAGCTTATGCCGAGCACCAC	Forward primer containing HindIII	942 bp
	1RX	CTCGAGTTACTGCTGGCCAC	Reverse primer containing XhoI	
ORF 093L	1FH	AAGCTTATGATTTCGAGTGC	Forward primer containing HindIII	927 bp
	1RX	CTCGAGTTAGGCCATAATGC	Reverse primer containing XhoI	

2-2. Cloning

2-2-1. pGEM-T Easy vector subcloning

PCR products were eluted from the agarose gel with Gel Extraction Kit (Nucleogen, Korea) and inserted in pGEM-T Easy vector system (Promega Co., USA). *E. coli* DH5α strain was transformed with the ligated DNA and the transformed cells were cultured using Luria-Bertani (LB) broth containing ampicillin (100 μg/ml). The plasmid DNA were extracted with Plasmid Purification Kit (Nucleogen, Korea).

2-2-2. Sequencing analysis

The plasmid DNA was sequenced in the Macrogen. Ltd. (Seoul, Korea) and the analyzed sequences were compared with those of the ISKNV [9] and the RBIV [10] sequences.

2-2-3. Cloning and protein expression

The ORF 023L gene was inserted into pET-28a, and ORF 037L, ORF 055L and ORF 093L gene were inserted into pGEX4T-1. The recombinant plasmids were named as pET28-023, pGEX-037, pGEX-055 and pGEX-093 and the host *E. coil* BL21(DE3) were transformed with the recombinant plasmids. Then, the transformed cells were cultured and 1 mM IPTG was added at 4 h of culture for the expression of target

protein.

2-2-4. SDS-PAGE analysis

Expressed proteins were separated by SDS-PAGE. Samples were boiled for 5 min in a gel-loading buffer and samples were centrifuged at 8000 rpm for 10 min before running and the protein samples were loaded to gel. After electrophoresis at 90 V, loaded gel was stained with Brilliant blue G (Sigma, USA).

2-2-5. Production of DNA vaccine

The constructed genes of integrin binding site were prepared for the development of DNA vaccine by using pcDNA 3.1 (+) that contains the human cytomegalovirus (CMV) promoter for high-level expression in a wide range of mammalian cells for construction of a DNA vaccine (Invitrogen, USA) [24].

2-2-6. Large-scale preparations of DNA vaccine

The constructed DNA vaccine plasmid and the vector plasmid pcDNA 3.1 (+) were amplified in *E. coli* DH5α strain and plasmid DNA was extracted with alkaline lysis method [16]. The concentration of purified plasmids was determined by a spectrophotometer (λ = 260 nm).

3. Antibody production

3-1. Mouse immunization

BALB/c mice (5 mice/one group, 4 to 5 weeks old) were prepared for an immunization with DNA vaccine.

At first, one group of mice was injected 200 μl of PBS as a negative control and the other groups were injected 200 μl of DNA vaccine with a concentration of 1 mg/ml in PBS, respectively. Then, three booster injections to all groups of the mice were given at 2 weeks intervals [3] with the decreased amount 100 μl (2 mg/ml vaccine in PBS) to reduce the damage to the mice. At 7 days after final injection, 1 ml of sarcoma cells containing 1×10^7 cells [28-30] was injected intraperitoneally to all groups of mice, and the ascitic fluid was collected after 2 weeks. Finally, antisera were obtained by the centrifugation of ascitic fluid in 2000 rpm for 15 min at 4℃ and kept in 20℃ until use.

3-2. Western Blot analysis

Expressed protein was separated by SDS-PAGE. The sample was boiled for 5 min in a gel-loading buffer and the sample was centrifuged at 8000 rpm for 10 min before running and the sample was loaded to two gels. After electrophoresis at

90 V, one loaded gel was stained with Brilliant Blue G (Sigma, USA). The other gel was transferred onto a 0.45 μm pore nitrocellulose membrane (BioTrace, PALL, USA) at 100 V for 1 h in a Bio-Rad mini Trans-Blot electrophoretic transfer cell for Western blot analysis. The blotted membrane was rinsed with TTBS (0.02 M Tris-HCl, 0.5 M NaCl, 0.05% Tween-20, pH 7.5) three times for 10 min and then blocked in TBS (0.02 M Tris-HCl, 0.5 M NaCl, pH 7.5) containing 3% (w/v) BSA for 2 h at room temperature and then overnight at 4℃. Then, the membranes were rinsed with TTBS for three times for 10 min and then cut into several strips according to the sample lane and these strips were put into mouse polyclonal antisera diluted 1 : 50 in TTBS containing 1% BSA, and incubated for 2 h at room temperature. After three times washing for each 15 min in TTBS, the membrane was treated for 1 h with alkaline phosphatase conjugated anti-mouse IgG (1 : 2000, Santa Cruz Biotechnology, USA) in TTBS containing 1% BSA. Then, the membrane was washed three times for 10 min in TTBS and developed by BCIP/NBT (Sigma, USA) for 1 to 5 min. The development reaction was stopped by rinsing strips with distilled water [24].

3-3. Virus neutralization test in BF-2 cell culture

The neutralization test was carried out with 24-well cell culture plates. BF-2 cells were cultured in 24-well plates for 2 days for forming cell monolayers and the antisera were treated at 56℃ for 30 min then 300 μl of the antisera with a log dilution range from 10^0 to 10^3 in PBS were mixed with 300 μl of RSIV predetermined $TCID_{50}$. For neutralization reaction, the virus-antisera mixture was incubated at 25℃ for 1 h. Then, 300 μl of each mixture was added to BF-2 monolayers. The BF-2 cells were incubated at 25℃ for 1 h. After removing of the mixtures, the plates were washed with PBS, then 1 ml of EMEM containing 10% of FBS was added to the plates and the plates were incubated at 25℃ for 7 days. The plates were observed under the microscope everyday for detection of CPE [13, 32, 33]. Neutralization effect (%) was defined as follows:

$$\text{Neutralization effect (\%)} = \frac{\text{A number of wells without CPE}}{\text{Total number of well}} \times 100$$

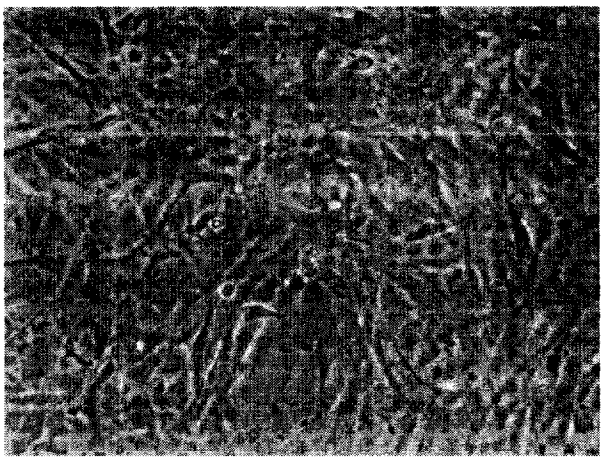
All of the data points were the average of triplicate test.

III. RESULTS AND DISCUSSION

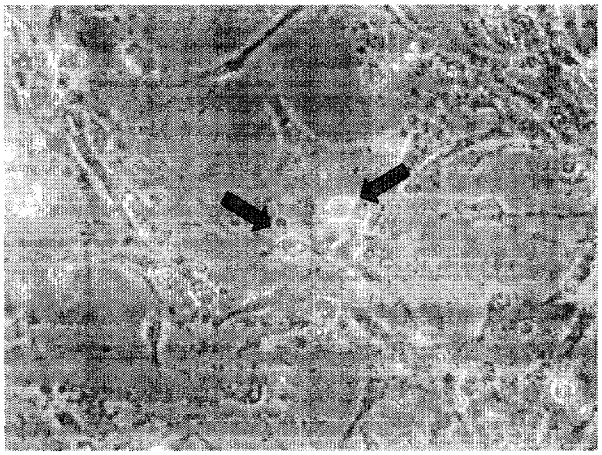
1. Cell culture and Virus infection

1-1. Detection of CPE in BF-2 cell culture

At 2 to 3 days after starting of BF-2 cell culture, cell monolayers was observed as shown in Fig. 1, a. After infection of RSIV, CPE in the culture were detected within 2 to 3 days of post-infection (Fig. 1, b) and BF-2 cells were completely lysed within 5 to 7 days of post-infection. CPE was distinguished by rounding and enlargement in the infected cells.



(a)



(b)

Fig. 1. Bluegill fry-2 (BF-2) cell culture and infection of RSIV. (a) Monolayers culture of BF-2 cells in 3 days of culture, (b) CPE in 3 days of post-infection (x 100).

1-2. Titration of RSIV

A growth curve of BF-2 cells were shown in Fig. 3. After 7 days, BF-2 cells reached the stationary phase, thus, the cells were probably maintained in mild condition without oxygen and nutrients limitation for 7 days. Therefore, viral infectivity was determined by limiting dilution assay at 7 days of post-infection. The data shown in Table 2 was used to determine $TCID_{50}$ by Spearman-Karber method [34].

Briefly, the calculation method was followed by:

$$TCID_{50} = \text{Highest dilution giving 100\% CPE} + \frac{1}{2} - \frac{\text{Total number of test units showing CPE}}{\text{Number of test units per dilution}}$$

$$-3 + 1/2 - 16/8 = -4.5\ TCID_{50} \text{ or } 10^{4.5}\ TCID_{50} \text{ unit volume}^{-1}$$

The titer, given a volume of 0.1 ml, is therefore :

$$10^{4.5}\ TCID_{50}\ 0.1\ ml^{-1}$$
$$= 10 \times 10^{4.5}\ TCID_{50}\ ml^{-1}$$
$$= 10^{5.5}\ TCID_{50}\ ml^{-1}$$

The calculated $TCID_{50}$ of RSIV in BF-2 cell culture from the observation of CPE was determined by $10^{5.5}$ virus/ml.

Table 2. Determination of $TCID_{50}$ RSIV by Spearman-Karber method.

Virus dilution (log dilution)	Infected test units	Cumulative infected (A)	Cumulative non-infected (B)	Ratio of A/(A+B)	Percent infected
-3	8/8	16	0	16/16	100.0
-4	6/8	8	2	8/10	80.0
-5	2/8	2	8	2/10	20.0
-6	0/8	0	16	0/16	00.0

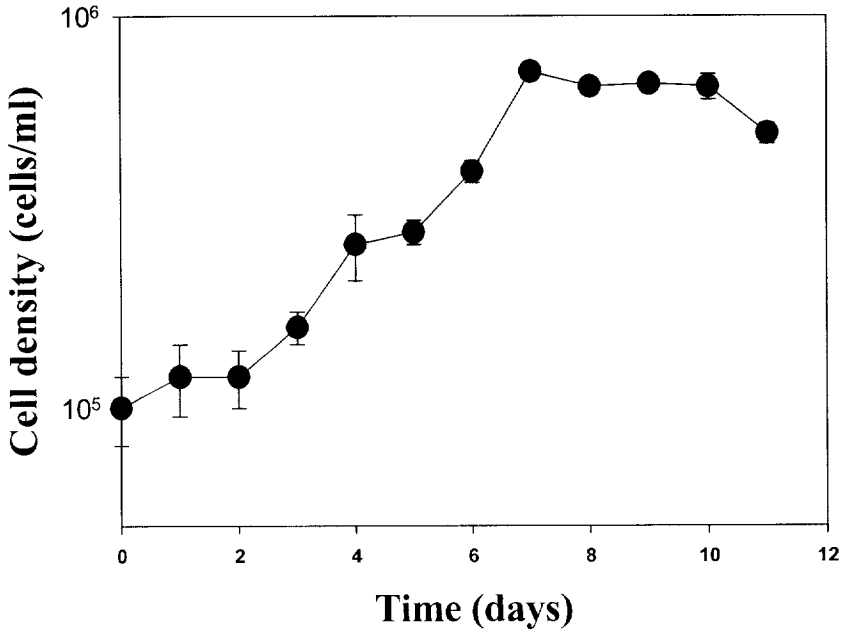


Fig. 2. Growth curve of BF-2 cells.

2. Development of DNA vaccines

2-1. Result of PCR

PCR fragments obtained from four different genes of the RSIV isolated in Korea were sequenced and compared with the ISKNV and RBIV. PCR with these four sets of primers for the ORF 023L, ORF 037L, ORF 055L and ORF 093L gene were specific to the RSIV isolated in Korea and produced fragments of the expected sizes, 3138, 1350, 942 and 927 bp, respectively (Fig. 3).

2-2. Gene sequences analysis

The ORF 023L, ORF 037L, ORF 055L and ORF 093L gene of RSIV were cloned in pGEM-T Easy vector system by PCR. The results were shown in Fig. 4. The sequences were compared with ISKNV and RBIV sequences. The ORF 023L sequence homology was approximately 75% (Fig. 5), ORF 037L, ORF 055L and ORF 093L sequence homologies were approximately 95%, 96% and 97%, respectively (Fig. 6 to Fig. 8).

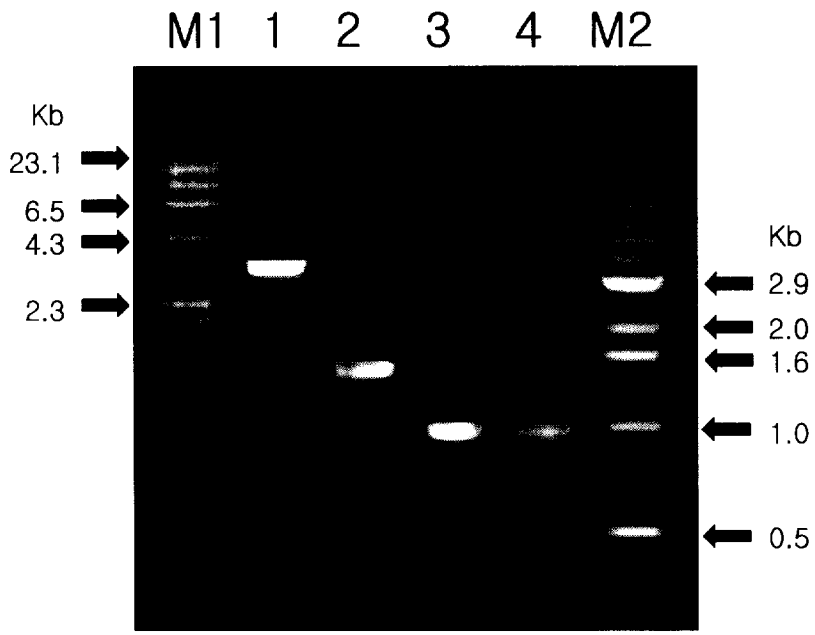


Fig. 3. Detection of the target genes isolated from RSIV by PCR. The ORF 023L, ORF 037L, ORF 055L and ORF 093L were amplified with specific primers listed in table 1. M1: λ /HindIII DNA marker, lane 1: ORF 023L gene by RSIV, lane 2: ORF 037L gene by RSIV, lane 3: ORF 055L gene by RSIV, lane 4: ORF 093L gene by RSIV, M2: 1kb DNA ladder.

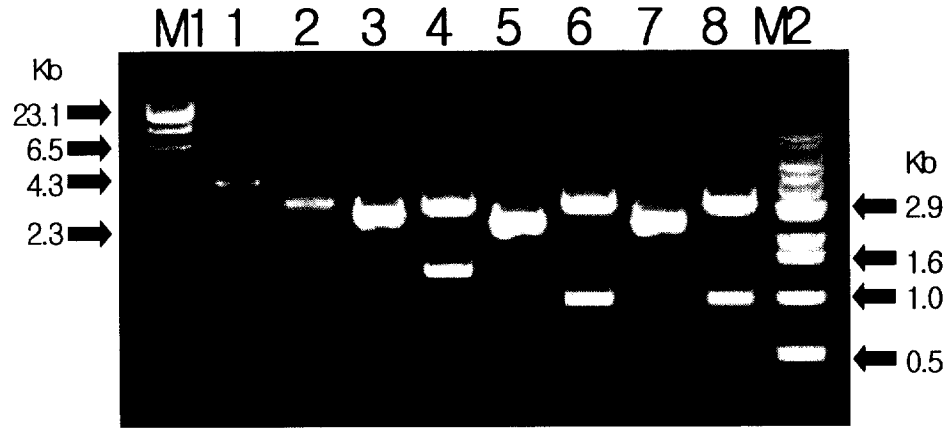


Fig. 4. Subcloning of the ORF 023L, ORF 037L, ORF 055L and ORF 093L gene of RSIV into pGEM-T Easy vector. M1: λ/HindIII DNA marker, lane 1: pGEM-T-023 (control, uncut), lane 2: pGEM-T Easy vector and separated ORF 023L gene (3138 bp), lane 3: pGEM-T-037 (control, uncut), lane 4: pGEM-T Easy vector and separated ORF 037L gene (1350 bp), lane 5: pGEM-T-055 (control, uncut), lane 6: pGEM-T Easy vector and separated ORF 055L gene (942 bp), lane 7: pGEM-T-093 (control, uncut), lane 8: pGEM-T Easy vector and separated ORF 093L gene (927 bp), M2: 1kb DNA ladder.

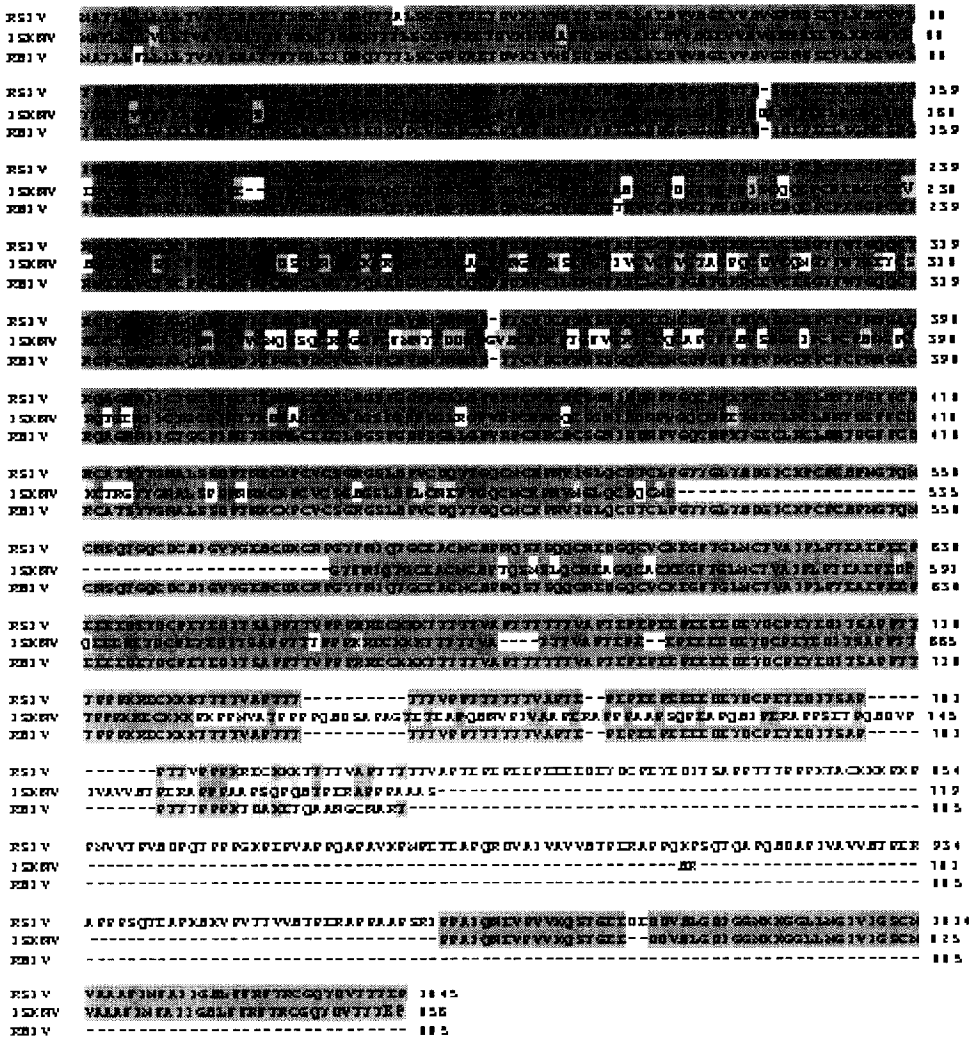


Fig. 5. Comparison of the amino acid sequences of ORF 023L proteins between RSIV, ISKNV and RBIV.

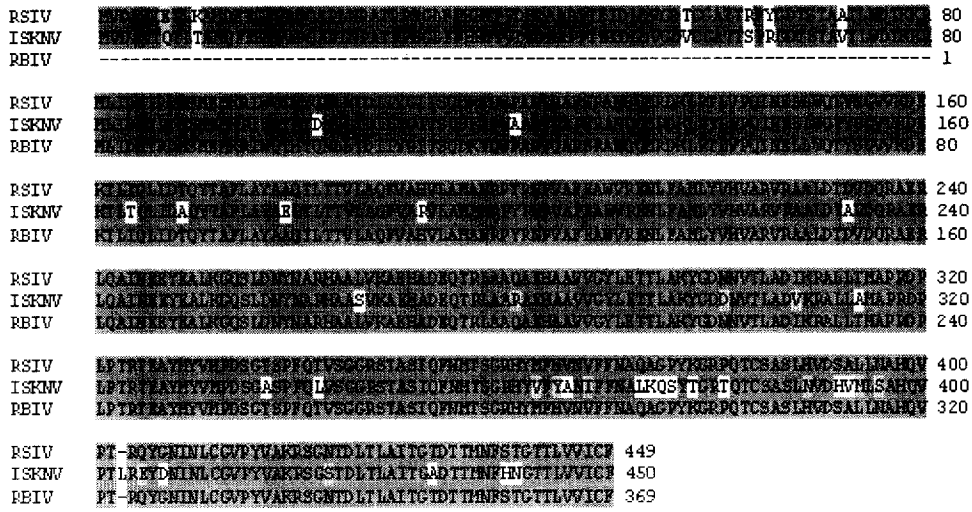


Fig. 6. Comparison of the amino acid sequences of ORF 037L proteins between RSIV, ISKNV and RBIV.

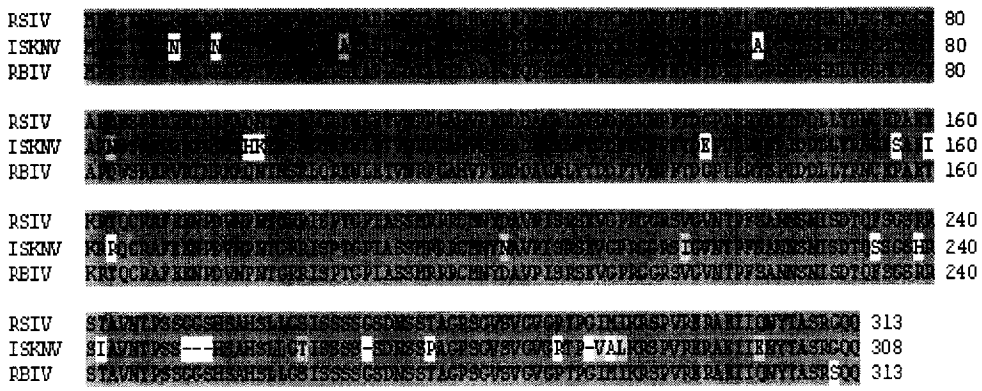


Fig. 7. Comparison of the amino acid sequences of ORF 055L proteins between RSIV, ISKNV and RBIV.

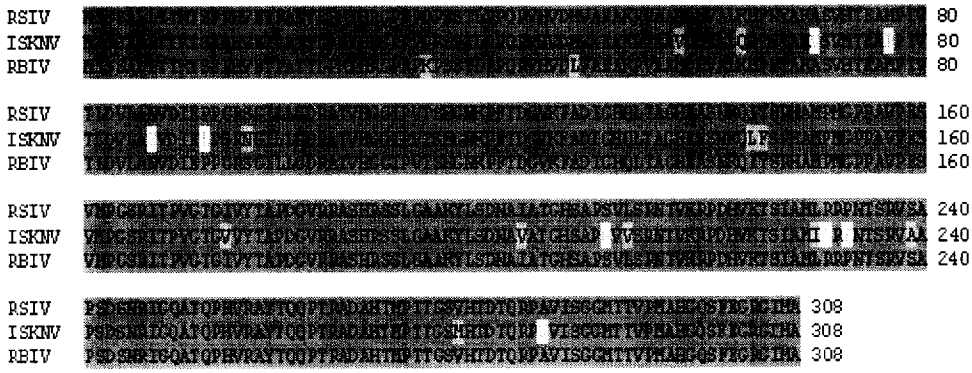


Fig. 8. Comparison of the amino acid sequences of ORF 093L proteins between RSIV, ISKNV and RBIV.

2-3. SDS-PAGE analysis

In SDS-PAGE result, the pGEX-055 protein was expressed with the molecular weight of approximately 60 kDa, but not pET28-023, pGEX-037 and pGEX-093 (Fig. 9). Therefore, ORF 055L only was applied DNA vaccine.

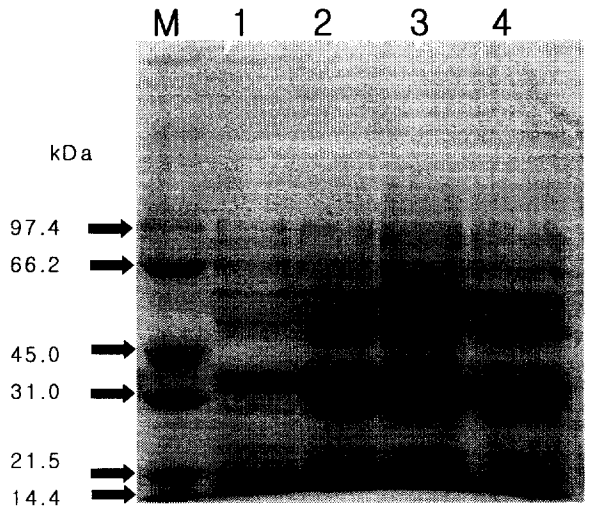


Fig. 9. SDS-PAGE analysis of total proteins. M: molecular weight marker, lane 1: total protein of pET28-023 (115 kDa), lane 2: total protein of pGEX-037 (75 kDa), lane 3: total protein of pGEX-055 (60 kDa), lane 4: total protein of pGEX-093 (60 kDa).

2-4. Construction of DNA vaccine

ORF 055L gene of RSIV was cloned into HindIII and XhoI sites of plasmid pcDNA 3.1 (+) vector under the control of the CMV promoter sequence (Fig. 10).

The physical map of insert was analyzed by restriction enzyme digestion. As shown in Fig. 11, lane 1 and 2 show the band of uncut pcDNA-055 and 6.3 kb pcDNA-055 digested with HindIII, respectively. Lane 3 shows the separated pcDNA 3.1 and ORF 055L gene digested by HindIII and XhoI. This indicates that ORF 055L gene was correctly inserted into the pcDNA 3.1 (+) and named as pcDNA-055 DNA vaccine.

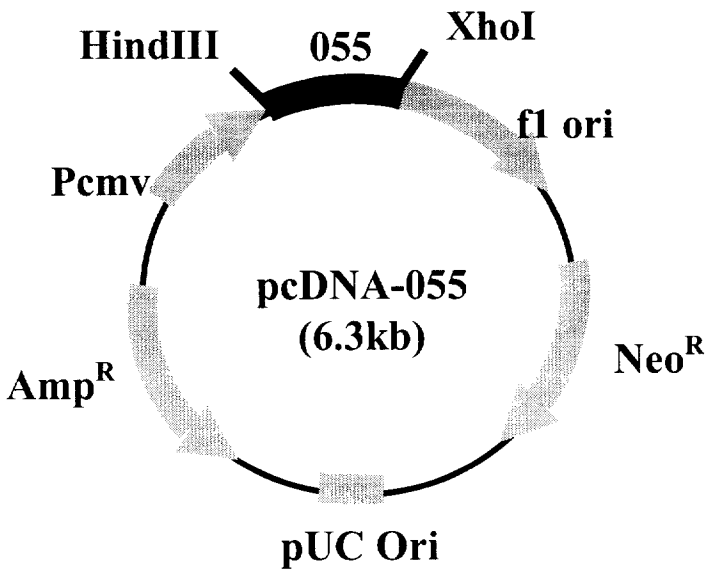


Fig. 10. Schematic diagram of constructed a DNA vaccine by insertion of the ORF 055L gene of RSIV into pcDNA 3.1 (+) vector.

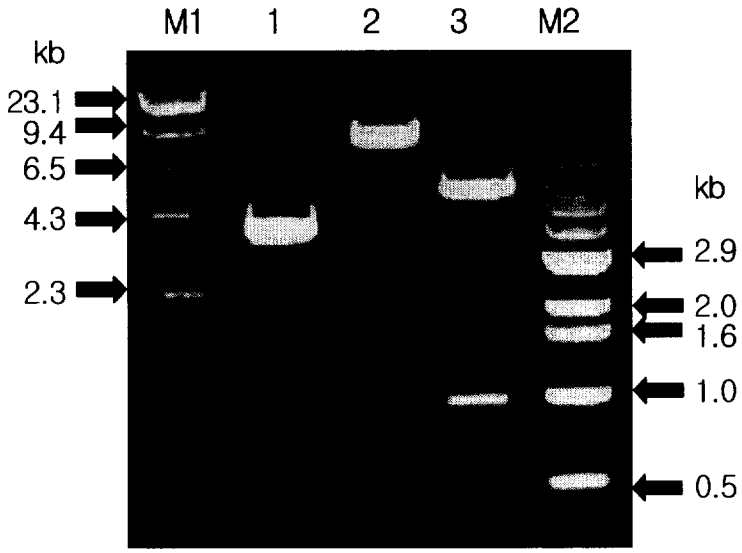


Fig. 11. Cloning of the ORF 055L gene of RSIV into pcDNA 3.1 (+) vector. M1: λ/HindIII DNA marker, lane 1: pcDNA-055 (control, uncut), lane 2: pcDNA-055 was digested with HindIII, lane 3: pcDNA 3.1 and separated ORF 055L gene, M2: 1 kb DNA ladder.

3. Production of antibody against DNA vaccine

3-1. Western blot analysis

Western blotting was carried out using the antisera produced by mice infected with pcDNA-055 DNA vaccine to identify the immune-reaction ability of antibody against pcDNA-055 DNA vaccine. As a result, the transferred pGEX-055 protein band could be detected as the molecular weight of 60 kDa as shown in Fig. 12. This shows that the antibody in the antisera against pGEX-055 protein was produced in mice immunization test by pcDNA-055 DNA vaccine and the antibody had the reaction ability against pGEX-055 protein as antigen.

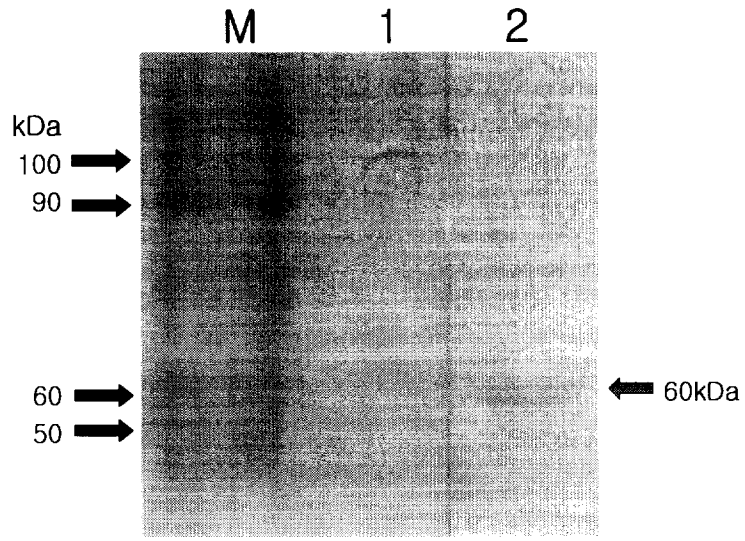


Fig. 12. Western blot analysis. The nitrocellulose membrane was probed with polyclonal antibodies raised against the RSIV 055 protein. M: 10 kDa prestained protein size marker (Elpisbiotech, Korea), lane 1: negative control (antisera from PBS), lane 2: transferred protein after antigen-antibody reaction.

3-2. Virus neutralization test in BF-2 cell culture

Neutralization test was carried out with antisera obtained by mice group A and B that were boostered with PBS and pcDNA-055 DNA vaccine, respectively. CPE was detected from BF-2 cells infected by the RSIV-only in 2 to 3 days of post-infection (Fig. 13, a). All of the infected cells were died in 5 to 7 days of post-infection. BF-2 cells infected with the mixture of RSIV and antisera obtained by mice group A also showed CPE in all plates (Fig. 13, c). Whereas, BF-2 cells infected with the mixture of RSIV and antisera obtained by mice group B with dilutions of 10^0 to 10^3 in PBS were healthy showing few CPE (Fig. 13, d) similar with the negative control (without RSIV, Fig. 13, b). The neutralization result was shown in Fig. 14. The neutralization effects were observed in the cells with the diluted mice group B antisera (10^0 to 10^3) comparing to those with mice group A antisera. BF-2 cells with the mixture of RSIV and antisera obtained by mice group B (pcDNA-055 DNA vaccine) without dilution (10^0) was healthy, showing 100% of the neutralization effect. In addition, BF-2 cells with the mixture of RSIV and antisera obtained by mice group B (pcDNA-055 DNA vaccine) with dilution 10^1, 10^2 and 10^3 were also healthy, showing 84%, 75% and 67% of neutralization effect, respectively. However the low value of

neutralization effect could be observed in BF-2 cell with the mixture of RSIV and antisera from mice group A (PBS), showing below 50% of the neutralization effect with (10^1 to 10^3) and without dilution (10^0).

Therefore, the antisera obtained from mice boostered with pcDNA-055 DNA vaccine showed efficacy to control RSIV. This shows the possibility that pcDNA-055 DNA vaccine can be used to control RSIV *in vivo*.

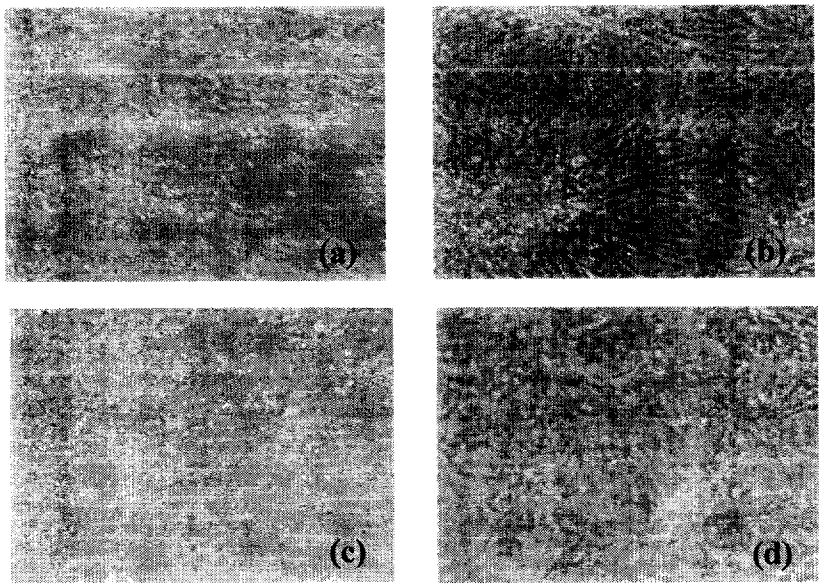


Fig. 13. *In vitro* test of neutralization in BF-2 cell culture. (a): cells infected by RSIV-only as a positive control, (b): cells infected by medium-only as a negative control, (c): cells infected with the mixture of RSIV and antisera obtained by mice group A (dilution of 10^0), (d): cells infected with the mixture of RSIV with antisera obtained by mice group B (dilution of 10^0) (× 100).

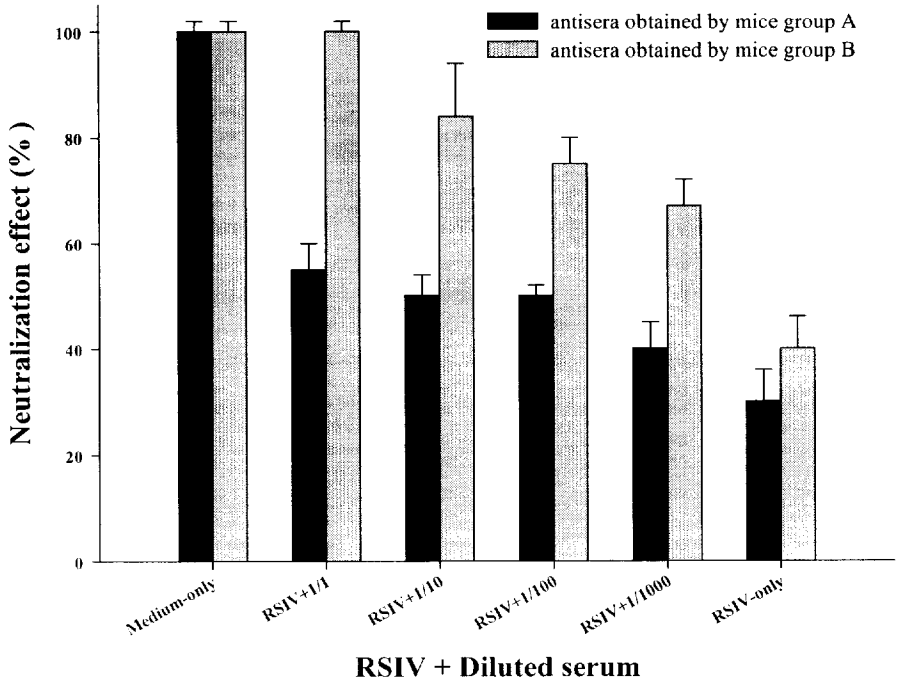


Fig. 14. Neutralization effect *in vitro* test. This graph shows the neutralization effect with dilution of antisera. With dilution of antisera from 10^0 to 10^3, obtained by mice boostered with PBS (group A) and pcDNA-055 DNA vaccine (group B).

IV. CONCLUSIONS

BF-2 cells were used for propagation of the RSIV. CPE caused by RSIV infection was detected within 2 to 3 days post-infection and the cells were completely lysed within 5 to 7 days post-infection. The virus titers were determined as $10^{5.5}TCID_{50}$/ml on confluent BF-2 cell monolayers.

For the development of a DNA vaccine, four integrin binding sites of the ORF 023L, ORF 037L, ORF 055L and ORF 093L of ISKNV were selected and cloned in pGEM-T Easy vector system by PCR. The results of sequences were compared with ISKNV and RBIV. The ORF 023L sequence homology was approximately 75%, the ORF 037L, ORF 055L and ORF 093L sequence homologies were approximately 95%, 96% and 97%, respectively.

For expression of target proteins, the each genes were cloned into pET28a and pGEX4T-1 vectors. In SDS-PAGE result, ORF 055L protein was expressed with a molecular weight of approximately 60 kDa.

pcDNA-055 DNA vaccine was constructed by cloning of the ORF 055L into pcDNA 3.1 (+) containing of a cytomegalovirus (CMV) promoter. BALB/c mice were injected the pcDNA-055 DNA vaccine with each concentration of 1 mg/ml in PBS, and three booster injections to the mice were given at 2 weeks

intervals with each concentration of 2 mg/ml in PBS. Then, 1 ml of 1×10^7 sarcoma cells were injected intraperitoneally to all of the mice at 1 week after final boosting and after 2 weeks, all antisera were obtained from ascitic fluid.

In the result of Western blot, the production of antibodies against pcDNA-055 DNA vaccine was confirmed. In neutralization test, the antibodies against pcDNA-055 DNA vaccine showed efficacy to control RSIV. This shows the possibility that pcDNA-055 DNA vaccine can be used to control RSIV *in vivo*.

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언제나 옆에서 힘이 되었던 손말배움터 15기 동기들과 특히 많은 고민을 나눴던 은희, 은숙, 미선, 경태에게도 고마운 마음을 전하며, 이제 내인생에서 없어서는 안될 친구 진희와 나리에게도 앞날의 무한한 발전이 있기를 바랍니다.

가족이라는 둥지 안에서 철없고 부족한 딸을 언제나 믿고 보살펴주신 마음속에 힘이 되었던 아빠와 늘 우리를 걱정해주시고 생각해주시는 엄마, 그리고 언니와 동생 상우에게도 사랑과 감사의 마음이 전해지기를 바라고 영원한 행복이 가득하기를 바라며 이 한권의 논문을 바칩니다.

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VI. REFERENCES

1. Van Regennortel, M. H. V., C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo., D. J. McGeoch, C. R. Pringle and E. B. Wickner. 2000. Virus Taxonomy. Seventh report of the international committee on taxonomy of viruses. *Academic Press, New York.*

2. Inouye, K., K. Yamano, Y. Maeno, K. Nakajima, M. Matsuoka, Y. Wada and M. Sorimachi. 1992. Iridovirus infection of cultured red sea bream, *Pagrus major. Fish Pathol.* 27: 19-27.

3. Nakajima, K. and M. Sorimachi. 1994. Biological and physico-chemical properties of the iridovirus isolated from cultured red sea bream, *Pagrus major. Fish Pathol.* 29: 29-33.

4. Nakajima, K. and M. Sorimachi. 1995. Production of monoclonal antibodies against red sea bream iridovirus. *Fish Pathol.* 30: 47-52.

5. Nakajima, K., Y. Maeno, M. Fukudome, Y. Fukuda, S.

Tanaka, S. Matsuoka and M. Sorimachi. 1995. Immunofluorescence test for the rapid diagnosis of red sea bream iridovirus infection using monoclonal antibody. *Fish Pathol.* 30: 115-119.

6. Nakajima, K., Y. Maeno, J. Kurita and Y. Inui. 1997. Vaccination against red sea bream iridoviral disease in red sea bream. *Fish Pathol.* 32: 205-209.

7. Kurita, J., K. Nakajima, I. Hirono and T. Aoki. 1998. Polymerase chain reaction (PCR) amplification of DNA of red sea bream iridovirus (RSIV). *Fish Pathol.* 33: 17-23.

8. Nakajima, K., Y. Maeno, K. Yokoyama, C. Kaji and S. Manabe. 1998. Antigen analysis of red sea bream iridovirus and comparison with other fish iridoviruses. Fish Pathol. 33: 73-78.

9. He. J. G., M. Deng, S. P. Weng, Z. Li, S. Y. Zhou, Q. X. Long, X. Z. Wang and S. M. Chant 2001. Complete genome analysis of the mandarin fish infectious spleen and kidney necrosis iridovirus. *Virology.* 291: 126-139.

10. Do, J. W., C. H. Moon, H. J. Kim, M. S. Ko, S. B. Kim, J.

H. Son, J. S. Kim, E.J. An, M. K. Kim, S. K. Lee, M. S. Han, S. J. Cha, M. S. Park, M. A. Park, Y. C. Kim, J. W. Kim and J. W. Park. 2004. Complete genomic DNA sequence of rock bream iridovirus. *Virology.* 325: 351-363.

11. Imajoh, M., H. Sugiura and S. Oshima. 2004. Morphological changes contribute to apoptotic cell death and are affected by caspase-3 and caspase-6 inhibitors during red sea bream iridovirus permissive replication. *Virology.* 322: 220-230.

12. Jeong, J. B., J. Jun, M. H. Yoo, M. S. Kim, Jack L. Komisar and H. D. Jeong. 2003. Characterization of the DNA nucleotide sequences in the genome of red sea bream iridoviruses isolated in Korea. *Aquaculture.* 220: 119-133.

13. Shi, C., Q. Wei, K. Y. H. Gin and T. J. Lam. 2003. Production and characterization of monoclonal antibodies to a grouper iridovirus. *J. Viol. Methods.* 107: 147-154.

14. Gibson-Kueh, S., P. Netto, G. H. Ngoh-Lim, S. F. Chang, L. L. Ho, Q. W. Qin, F. H. C. Chua, M. L. Ng and H. W. Ferguson. 2003. The pathology of systemic iridoviral disease in fish. *J. Comp. Pathol.* 129: 111-119.

15. Qin, Q. W., C. Shi, K. Y. H. Gin and T. J. Lam. 2002. Antigenic characterization of a marine fish iridovirus from grouper, Epinephelus spp. *J. Viol. Methods.* 106: 89-96.

16. Jeong, J. B. 2003. Comparison of red sea bream iridoviruses by the characterization of DNA nucleotide sequences. *Graduate school, Pukyong Natl. Univ., Korea.*

17. He, J. G., S. P. Wang, K. Zeng, Z. J. Huang and S-M. Chan. 2000. Systemic disease caused by an iridovirus-like agent in cultured mandarin fish, Siniperca chuatsi (Basilewsky), in China. *J. Fish Dis.* 23: 219-222.

18. Johansson, S., G. Svineng, K. Wennerberg, A. Armulik and L. Lohikangas. 1997. Fibronectin-integrin interactions. *Front. Biosci.* 2: d126-146.

19. Shiokawa, S., Y. Yoshimura, H. Sawa, S. Nagamatsu, H. Hanashi, K. Sakai, M. Ando and Y. Nakamura. 1999. Functional role of arg-gly-asp (RGD)-binding sites on β1 integrin in embryo implantation using mouse blastocysts and human decidua. *Biol. Reprod.* 60: 1468-1474.

20. Blystone, S. D. 2004.Integrating an integrin: a direct route to actin. *Biochim. Biophys. Acta.* 1692: 47-54.

21. Robinson, H. L. and C. A. T. Torres. 1997. DNA vaccines. *Semin. Immunol.* 9: 271-283

22. Bergmann-Leitner, E. S. and W. W. Leitner. 2004. Danger, death and DNA vaccines. *Microbes and Infection.* 6: 319-327.

23. Eou, J. I. 2000. Molecular analysis of the hirame rhabdovirus (HIRRV) genomic RNA and application in fish virus vaccine development. *Graduate school, Pukyong Natl. Univ., Korea.*

24. Seo, J. Y. 2003. Protective immunogenicity of the G protein of hirame rhabdovirus (HIRRV) in flounder using DNA vaccine. *Graduate school, Pukyong Natl. Univ., Korea.*

25. Gomez-Chiarri, M. and L. A. Chiaverini. 1999. Evaluation of eukaryotic promoters for the construction of DNA vaccines for aquaculture. *Biomolecular Engineering.* 15: 121-124.

26. McLauchlan, P. E., B. Collet, E. Ingerslev, C. J. Secombes,

N. Lorenzen and A. E. Ellis. 2003. DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout: size, dose, route of infection and duration of protection-early protection correlates with Mx expression. *Fish & Shellfish Immunoligy.* 15: 39-50.

27. Nakajima, K., Y. Maeno, A. Honda, K. Yokoyama, T. Tooriyama and S. Manabe. 1999. Effectiveness of a vaccine against red sea bream iridoviral disease in a field trial test. *Dis. Aqua. Org.* 36:73-75.

28. Bruce., M. P., V. Boyd, C. Duch and J. R. White. 2002. Dialysis-based bioreactor systems for the production of monoclonal antibodies-alternatives to ascites production in mice. *J. Immunol. Methods.* 264: 59-68.

29. Qoi, L. S. M., H. Wang, C. W. Luk, V. E. C. Ooi. 2004. Anticancer and antiviral activities of *Youngia japonica (L.)* DC (Asteraceae, Compositae). *J. Ethnopharmacol.* 94: 117-122.

30. Kaufman, R. J., P. C. Brown and R. T. Schimke. 1981. Loss and stabilization of amplified dihydrofolate reductase genes in mouse sarcoma s-180 cell lines. *Mol. Cell. Biol.* 1: 1084-1093.

31. Kim, G. R. and Y. J. Jeong. 1990. *In vitro* subculture if ascitic sarcoma 180 tumor cell. *J. Kor. Soc. of Virology.* 20: 83-89.

32. Villen, J., E. de Oliveira, J. I. Nunez, N. Molina, F. Sobrino and D. Andreu. 2004. Towards a multi-site synthetic vaccine to foot-and-mouth disease: addition of discontinuous site peptide mimic increases the neutralization response in immunized animals. *Vaccine.* 22: 3523-3529.

33. Lai, Y. S., H. C. Chic, S. Murali, I. C. Guo, S. C. Chen, K. Fang and C. Y. Chang. 2001. *In vitro* neutralization by monoclonal antibodies against yellow grouper nervous necrosis virus (YGNNV) and immunolocalization of virus infection in yellow grouper, *Epinephelus awoara* (Temminck & Schlegel). *J. Fish Dis.* 24: 237-244.

34. Mahy, B. W. J. and H. O. Kangro. 1996. Virology methods manual. *Academic Press, New York.*