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Development of DNA Vaccine Against Red Sea Bream Iridovirus (RSIV)

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Abstract Red sea bream iridovirus (RSIV) obtained from infected rock bream was propagated by Bluegill fry-2 (BF-2) cell culture. The virus titer was determined as $10^{5.5}$ $TCID_{50}$/ml on confluent BF-2 cell monolayers. The integrin binding site of ORF 055L of infectious spleen and kidney necrosis virus (ISKNV) was selected for the construction of a primer to obtain the RSIV ORF 055L gene. The genes were amplified using RSIV gene lyzate by PCR. The homologies of the ORF 055L sequence of RSIV with ISKNV and rock bream iridovirus (RBIV) were approximately 96% and 100%, respectively. DNA vaccine was constructed by cloning the ORF 055L of RSIV into pcDNA 3.1 (+), containing a cytomegalovirus (CMV) promoter. For antibody production, pcDNA-055 DNA vaccine was injected to BALB/c mice. The production of antibodies against pcDNA-055 DNA vaccine was confirmed by the Western blot analysis. The antibodies produced by the pcDNA-055 DNA vaccine showed efficacy to neutralize the RSIV in the neutralization test in BF-2 cell culture.

Key words: Red sea bream iridovirus (RSIV), BF-2 cells, DNA vaccine, integrin binding site, pcDNA-055 DNA vaccine

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 a 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 [23].

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 [8]. In addition, beginning in 1998, many outbreak cases by RSIV have been reported in different aquatic farms of Korea. Fishes infected by RSIV have severe anemia and show petechia of the gills, congestion of the liver, and hypertrophy of the spleen and kidney [6]. Therefore, these viruses have been widely studied due to their ecological and economical impacts on aquaculture farming worldwide. *Edwardsiella tarda*, *Streptococcus* spp., and *Vibrio anguillarum* can be controlled using chemicals and antibiotics. However, there is no chemotherapeutic agent for the control of viral diseases. Despite the importance of virus infection in Korea's 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 on fish, compared to traditional 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 [1, 2, 19, 25, 26]. Immunization with antigen-encoding plasmid DNA can produce the foreign protein and elicit both humoral and cellular immune responses by the host immune system [19]. 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 play important roles in viral diseases wound healing, the immune system, cancers, inflammatory responses, and other diseases [3, 10, 22].

In this study, an integrin binding site of infectious spleen and kidney necrosis virus (ISKNV) [7] was selected and amplified using RSIV by PCR. Then, the gene was inserted

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into pcDNA 3.1 (+) vector [20] as a DNA vaccine, and the effect of DNA vaccine was evaluated using a neutralization test [12, 13, 24] with BF-2 cells [8, 15, 17].

MATERIALS AND METHODS

Fish Cell Line

The Bluegill fry-2 (BF-2) cells were maintained in Eagle's minimum essential medium (EMEM, Sigma, U.S.A.) supplemented with 10% fetal bovine serum (FBS, Gibco, U.S.A.) and antibiotic antimycotic solution (100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin, Sigma, U.S.A.) [8, 15, 17]. The cells were cultured at 25°C, and the medium was changed daily.

Virus Samples

Tissue specimens of the spleen, kidney, and liver obtained from the moribund rock bream infected with RSIV were homogenized in 10 volumes of phosphate-buffered saline (PBS). The tissue homogenates were centrifuged for 5 min at 8,000 rpm and 4°C, 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°C. 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°C as a virus stock until used [8, 16].

Virus Titration

BF-2 cells (1×10^5 cells/ml) were cultured in 25-cm^2 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 samples with a log dilution ranging from 10^0 to 10^{10} were prepared in PBS, and the diluted virus samples were added into the 96-well plate and incubated at 25°C. The cultures were observed daily for the detection of CPE. The virus titers were determined using the 50% tissue culture infectious dose ($TCID_{50}$) assay on confluent BF-2 cell monolayers by the Spearman-Karber method [14]. The average of triplicate tests was used as a $TCID_{50}$ result.

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 a genomic DNA extraction kit (Bioneer, Korea).

PCR Amplification

The full length of the integrin binding site, ORF 055L, in RSIV genomic DNA was amplified using oligonucleotide primers (forward: aagcttatgccgagcaccac; reverse: ctcgagttactgctggccac) based on the nucleotide sequences of the ORF 055L of the infectious spleen and kidney necrosis virus (ISKNV) [7] registered in GenBank. PCR amplification was carried out in a 50 μl reaction mixture containing the extracted genomic DNA of RSIV, 2.5 mM dNTP, 5 μM each of primers, and 5 units/μl Ex Taq polymerase (Takara Shuzo, Japan) with a Takara thermal cycler (Takara Shuzo, Japan). The ORF 055L gene was obtained after predenaturation at 94°C for 5 min and incubation for 30 cycles at 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min 30 sec, followed by an extension period at 72°C for 7 min. The PCR product was analyzed in 1.0% agarose gel containing ethidium bromide (0.5 μg/ml) and visualized with a UV transilluminator.

pGEM-T Easy Vector Subcloning

PCR product was eluted from the agarose gel with the Gel Extraction Kit (Nuleogen, Korea) and inserted into the pGEM-T Easy vector system (Promega Co., U.S.A.). *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 was extracted with a Plasmid Purification Kit (Nucleogen, Korea). The plasmid DNA was sequenced at Macrogen, Ltd. (Seoul, Korea), and the sequences analyzed were compared with those of ISKNV [7] and RBIV [5].

Production of DNA Vaccine

The ORF 055L gene was used 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 (Invitrogen, U.S.A.) [19]. pGEM-T Easy vector clone containing the ORF 055L gene was digested with both *Hin*dIII and *Xho*I. The ORF 055L gene was inserted into pcDNA 3.1 (+) to construct the pcDNA-055 DNA vaccine.

Large-Scale Preparation of DNA Vaccine

The constructed plasmid vaccine was amplified in the *E. coli* DH5α strain, and the plasmid DNA was extracted by the alkaline lysis method [9]. The concentration of the purified pcDNA-055 DNA vaccine was determined by spectrophotometery (λ=260 nm).

Mouse Immunization

Two groups of BALB/c mice (5 mice/one group, 4 to 5 weeks old) were prepared for immunization with pcDNA-055 DNA vaccine. Group A mice were injected with 200 μl of PBS as a negative control and group B mice were injected with 200 μl of pcDNA-055 DNA vaccine (1 mg/ml in PBS). Then, 100 μl of pcDNA-055 DNA vaccine (2 mg/ml in PBS) was injected to the mice three times at 2-week intervals for boosting [15]. At 7 days after final injection, 1 ml of sarcoma cells containing 1×10^7 cells [4, 11, 18] was injected intraperitoneally to all groups of mice, and then ascitic fluid was collected after 2 weeks. Finally,

antisera were obtained by the centrifugation of ascitic fluid in 2,000 rpm for 15 min at 4°C and kept at −20°C until use.

Cloning and Protein Expression

ORF 055L gene was inserted into pGEX4T-1. The recombinant plasmid was named as pGEX-055, and the host *E. coli* BL21 (DE3) was transformed with the recombinant plasmid. Then, the transformed cells were cultured, and 1 mM IPTG was added at 4 h of culture for the expression of target protein.

SDS-PAGE and Western Blot

Expressed protein was separated by SDS-PAGE. The sample was boiled for 5 min in a gel-loading buffer, the sample was centrifuged at 8,000 rpm for 10 min before running, and the supernatant was finally loaded onto two gels. After electrophoresis at 90 V, one loaded gel was stained with Brilliant Blue G (Sigma, U.S.A.). The other gel was transferred onto a 0.45-μm pore nitrocellulose membrane (BioTrace, PALL, U.S.A.) 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 overnight at 4°C. Then, the membrane was rinsed with TTBS three times for 10 min, and this membrane was put into mice polyclonal antisera diluted 1:50 in TTBS containing 1% BSA, and incubated for 2 h at room temperature. After rinsing of the membrane three times for 15 min each in TTBS, the membrane was treated for 1 h with alkaline phosphatase conjugated anti-mouse IgG (1:2,000, Santa Cruz Biotechnology, U.S.A.) in 1% BSA containing TTBS. The membrane was then washed three times for 10 min each in TTBS and developed by BCIP/NBT (Sigma, U.S.A.) for 1 to 5 min. The development reaction was stopped by rinsing the strips with distilled water [20].

Virus Neutralization Test

The neutralization test was carried out in 24-well cell culture plates. BF-2 cells were cultured in 24-well plates for 2 days to form cell monolayers. Three-hundred μl of antisera with a log dilution ranging from 10^0 to 10^3 in PBS were added to 300 μl of RSIV predetermined $TCID_{50}$. For the neutralization reaction, the virus-antisera mixture was incubated at 25°C for 1 h. Then, 300 μl of each mixture were added to BF-2 monolayers. The BF-2 cells were incubated at 25°C for 1 h. After removing the mixtures, the plates were washed with PBS, then 1 ml of 10% FBS containing EMEM was added to the plates, and the plates were incubated at 25°C for 7 days. The plates were observed under a microscope everyday for the detection of CPE [13, 21, 24]. Neutralization effect (%) was defined as follows:

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

All data points were the average of triplicate tests.

Results and Discussion

Detection of CPE in BF-2 Cell Culture

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

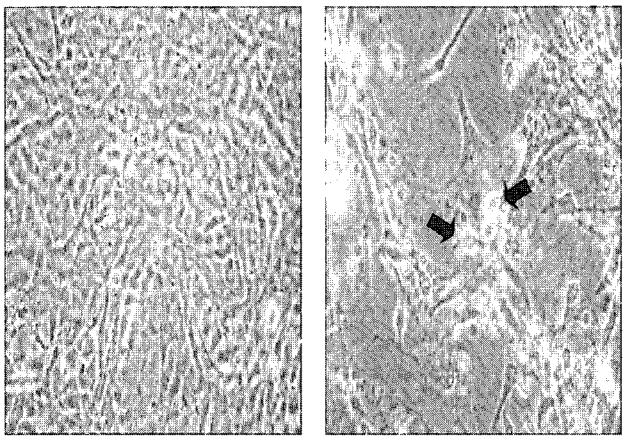
$TCID_{50}$ of RSIV

BF-2 cell growth reached the stationary phase after 7 days, indicating that the cells were probably maintained in a mild condition without oxygen and nutrients limitation for 7 days. Therefore, viral infection was determined by a limiting dilution assay before 7 days post-infection. The data shown in Table 1 were used to determine $TCID_{50}$ by the Spearman-Karber method [14].

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

PCR of ORF 055L

The PCR product, using primers for the ORF 055L gene, was produced as a DNA fragment of 942 bp size, as shown in Fig. 2. The ORF 055L gene of RSIV was cloned in the pGEM-T Easy vector system by PCR and the sequence was analyzed



(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 culture 3 days post-infection (×100).

Table 1. Determination of $TCID_{50}$ RSIV by the Spearman- Karber method.

Virus dilution (log dilution)	Infected test units	Cumulative infected (A)	Cumulative noninfected (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

The calculation method was

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

$-3+1/2-16/8=-4.5$ $TCID_{50}$ or $10^{-4.5}$ $TCID_{50}$ unit $volume^{-1}$.
The titer, given a volume of 0.1 ml, is therefore
$10^{4.5}$ $TCID_{50}$ 0.1 $ml^{-1}=10\times10^{4.5}$ $TCID_{50}$ $ml^{-1}=10^{5.5}$ $TCID_{50}$ ml^{-1}.

(Macrogen. Ltd., Seoul, Korea). The analyzed sequence was compared to those of ISKNV and RBIV (Fig. 2B). The ORF 055L sequence homologies of RSIV with ISKNV and RBIV were approximately 96% and 100%, respectively.

Construction of DNA Vaccine

The ORF 055L gene of RSIV was cloned into the *Hin*dIII and *Xho*I sites of plasmid pcDNA 3.1 (+) vector under the control of the CMV promoter sequence (Fig. 3A). The physical map of insert was analyzed by restriction enzyme digestion. As shown in Fig. 3B, lanes 1 and 2 show the band of uncut pcDNA-055 and 6.3 kb pcDNA-055 digested with *Hin*dIII, respectively. Lane 3 shows the separated pcDNA 3.1 and ORF 055L gene digested by *Hin*dIII and *Xho*I. This indicates that the ORF 055L gene was correctly inserted into the pcDNA 3.1 (+). It was named as pcDNA-055 DNA vaccine.

Antibody Production

The protein from pGEX-055 was expressed by the addition of IPTG. The molecular weight of the gene product was shown to be 60 kDa by SDS-PAGE (Fig. 4A). The protein was used as an antigen for Western blot assay. Western blot was carried out, using the antisera produced by mice infected with pcDNA-055 DNA vaccine, to identify the immune-reaction abilities of antibody against pcDNA-055 DNA vaccine. As shown in Fig. 4B, the transferred pGEX-055 protein band could be detected as the molecular mass of 60 kDa. This shows that the antibody in the antisera against pGEX-055 protein was produced in the mice immunization

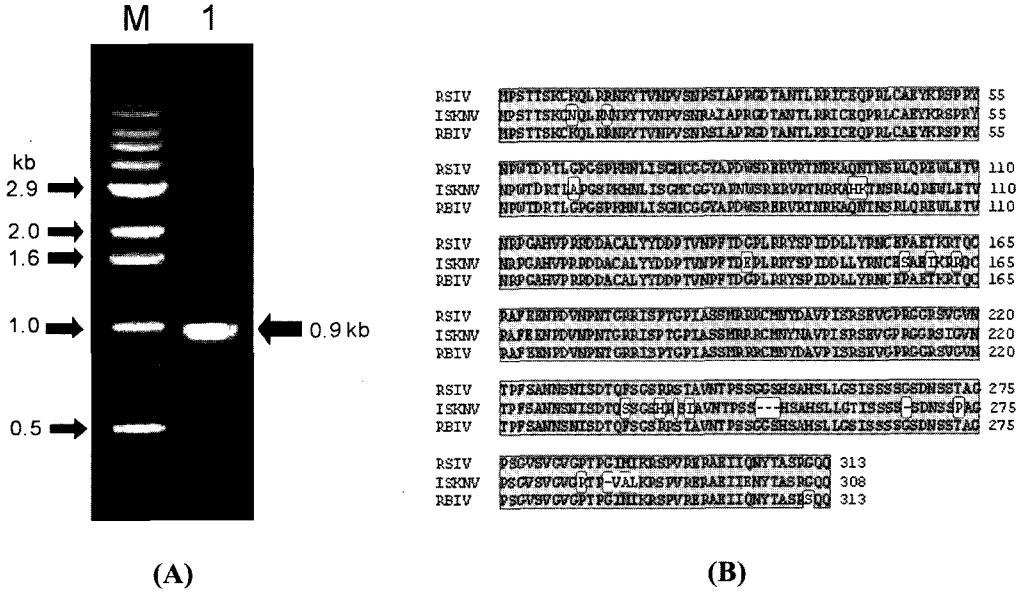


Fig. 2. Detection of the target gene isolated from RSIV by PCR and comparison of the amino acid sequence.
(A) M: 1 kb DNA ladder; lane 1: ORF 055L gene from RISV. (B) Comparison of the amino acid sequence of the ORF 055L protein of RSIV with ISKNV and RBIV.

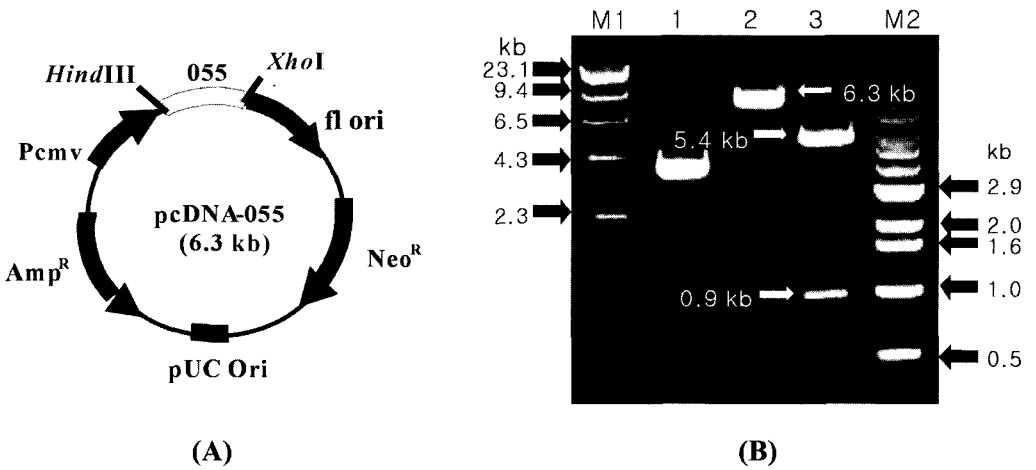


Fig. 3. Construction of DNA vaccine.
(A) ORF 055L gene of RSIV into pcDNA 3.1 (+) vector (DNA vaccine). (B) DNA agarose gel electrophoresis of DNA vaccine. M1: λ/*Hin*dIII DNA marker; lane 1, pcDNA-055 (control, uncut); lane 2, pcDNA-055 digested with *Hin*dIII; lane 3, pcDNA 3.1 and separated ORF 055L gene digest by *Hin*dIII and *Xho*I; M2, 1 kb DNA ladder.

test by the pcDNA-055 DNA vaccine, and that the antibody had reaction against pGEX-055 protein as an antigen.

Virus Neutralization Test in BF-2 Cell Culture

A neutralization test was carried out with antisera obtained by groups A and B mice that were boostered with PBS and pcDNA-055 DNA vaccine, respectively. CPE was detected from BF-2 cells infected by the RSIV only at 2 to 3 days post-infection (Fig. 5Aa). All of the infected cells died 5 to 7 days post-infection. BF-2 cells infected with the mixture of RSIV and antisera obtained by group A mice also showed CPE in all plates (Fig. 5Ac). BF-2 cells infected with the mixture of RSIV and antisera obtained by group B mice with dilutions of 10^0 to 10^3 in PBS were healthy, showing few CPE (Fig. 5Ad), similar to the negative control (without RSIV, Fig. 5Ab). As shown in Fig. 5B, the neutralization effects were observed in the cells with the diluted group B mice antisera (10^0 to 10^3), compared with those from group A mice antisera. BF-2 cells with the mixture of RSIV and antisera obtained by group B mice

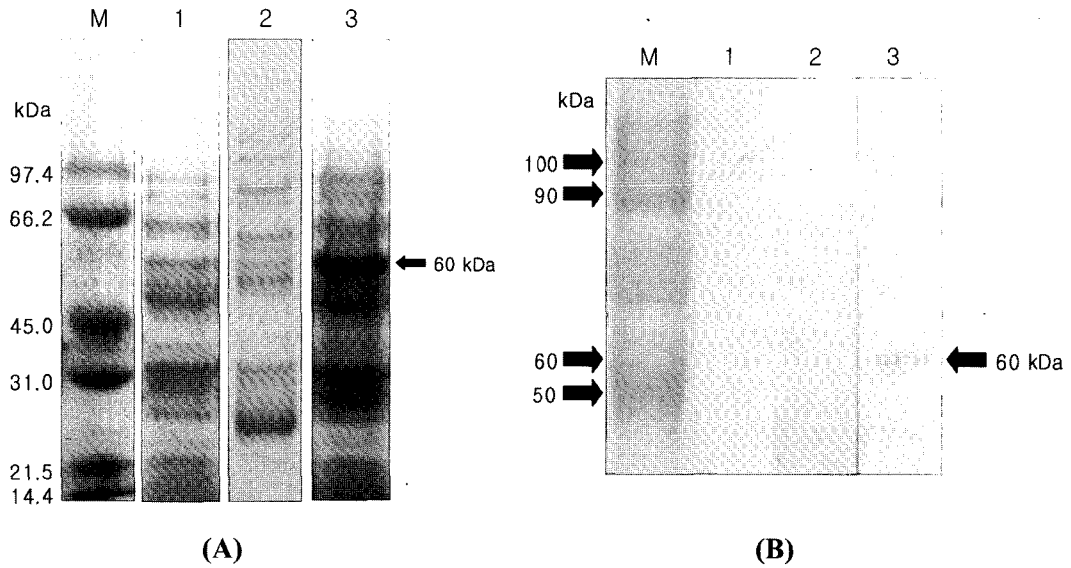


Fig. 4. Antibody production.
(A) SDS-PAGE analysis. M, molecular weight marker; lane 1, total proteins of culture without plasmid (pGEX4T-1); lane 2, total proteins of culture with plasmid (pGEX4T-1); lane 3, total proteins of the culture exhibiting RSIV ORF 055L protein (60 kDa). (B) Western blot analysis. Nitrocellulose membrane was probed with polyclonal antibody against the RSIV-055 protein. M: 10 kDa prestained protein size marker (Elpisbiotech, Korea); lane 1, negative control I (antisera from PBS); lane 2, negative control II (antisera from pcDNA vector); lane 3; pcDNA-055 DNA vaccine.

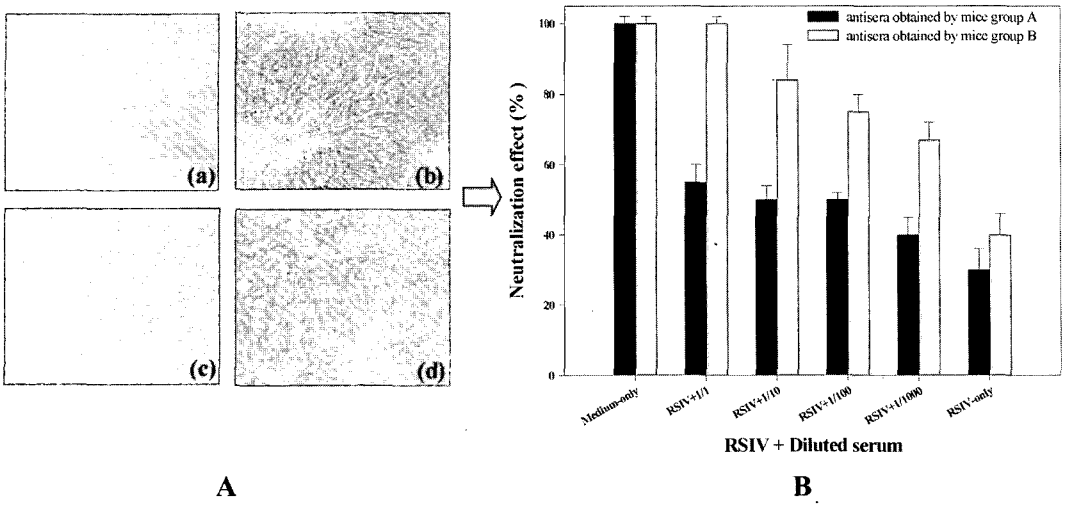


Fig. 5. *In vitro* neutralization effect test.
A. (a) Cells infected by RSIV-only as a positive control, (b) cells infected without RSIV as a negative control, (c) cells infected with the mixture of RSIV and antisera obtained by group A mice (PBS as a negative control, dilution of 10^0), (d) cells infected with the mixture of RSIV with antisera obtained by group B mice (pcDNA-055 DNA vaccine, dilution of 10^0) (×100). B. The neutralization effect with dilution of antisera. The neutralization effect was observed by the detection of CPE for 7 days post-infection under a microscope.

(pcDNA-055 DNA vaccine) without dilution (10^0) were healthy, showing 100% of neutralization effect. In addition, BF-2 cells with the mixture of RSIV and antisera obtained from group B mice (pcDNA-055 DNA vaccine) with dilutions 10^1, 10^2, and 10^3 were also healthy, showing 84%, 75%, and 67% of neutralization effects, respectively. However, the low value of the neutralization effect could be observed in BF-2 cells with the mixture of RSIV and antisera from group A mice (PBS), showing less than 50% of neutralization effect with (10^1 to 10^3) and without (10^0) dilution. Therefore, the antisera obtained from mice boostered with pcDNA-055 DNA vaccine showed an efficacy to control RSIV, suggesting the possibility that pcDNA-055 DNA vaccine can be used to control RSIV *in vivo*.

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