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Neutralization of infectivity of porcine circovirus type 2 (PCV2) by capsid-binding 2′F-RNA aptamers

Sorah Yoon[a,c,1], Garam Lee[a,1], Dongun Han[b], Jae-Young Song[a], Kyung-Sun Kang[c], Yong-Soon Lee[c,*]

[a] Virology Division, National Veterinary Research and Quarantine Service, 480, Anyang 6-dong, Manan-gu, Anyang, 430-757, Republic of Korea
[b] Animal Care Division, Cheonan Yonam College, 3-1 Suhyang-ri, Seonghwan-eup, Cheonan, 331-709, Republic of Korea
[c] Department of Veterinary Public Health, Seoul National University, San 56-1, Shillim-dong, Gwanak-gu, Seoul, 151-742, Republic of Korea

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ABSTRACT

Porcine circovirus type 2 (PCV2) is the main causative agent of porcine circovirus-associated diseases (PCVD), which is responsible for economic losses in the swine industry. The capsid protein of PCV2 has important role for virus neutralization that blocks viral infection. To develop the therapeutic agents, two 2′F-RNA aptamers that bound to the PCV2 capsid protein with nanomole affinity were isolated from a 2′F-RNA library by the Systematic Evolution of Ligands by EXponential enrichment (SELEX). The binding affinity of aptamers was analyzed by Electrophoretic Mobility shift assay (EMSA) and surface plasmon resonance (SPR) analysis. The RNA aptamers have been shown to exhibit high affinity and specificity to PCV2 capsid protein and to neutralize PCV2 infectivity in PK-15 cells in dose dependent manner.

Neutralizing aptamers such as this could be promising candidates in developing efficacious anti-PCV2 drugs as well as therapeutic delivery reagent.

1. Introduction

PCV2 is unenveloped, single-stranded circular DNA virus and is a main causative agent of PCV2 associated disease (PCVD) such as PMWS, featuring lymphocytes depletion, histiocytes infiltration, and weight loss (Allan et al., 1999; Sanchez et al., 2004). The PCV2 genome contains two major open reading frames (ORFs). ORF1 encodes the replicase protein (Rep) involved in rolling-circle PCV2 DNA replication (Mankertz et al., 1998). ORF2 encodes the immunogenic capsid protein (Nawagitgul et al., 2000). The PCV2 capsid proteins are well known to be essential to virus neutralization that blocks viral infection (McNeilly et al., 2001).

Although the PCV2 vaccines are evaluated effective to reduce losses of PCVD in the growing pigs, the viral DNA is detected in serum (Kyle P. Horlen, 2008). Based on fact that the PCV2 DNA load in the animal is correlated with disease (Brunborg et al., 2004), the detection of viral DNA in vaccinated animal indicates that active PCV2 infection still existed. Therefore, it is imperative to develop antiviral agents against the virus.

Aptamers that were identified using systematic evolution of ligands by exponential enrichment (SELEX) as an *in vitro* selection strategy can adopt complex structures to bind target proteins with high affinities and specificities (Ellington and Szostak, 1990; Tuerk and Gold, 1990). RNA aptamers may become an important source of therapeutic agents (Mori et al., 2004). RNA aptamers have been isolated against several viral proteins, such as human immunodeficiency virus gp120 (Khati et al., 2003) and influenza hemagglutinin (Jeon et al., 2004). However, no studies have been undertaken for the isolation of RNA aptamers to block PCV2 infection.

In present study, we employed a 2′-fluropyrimidine-RNA (2′F-RNA) combinatorial library and isolated 2′F RNA aptamers against PCV2 capsid which is essential for the viral neutralization. We observed that aptamers demonstrate antiviral activity by neutralizing PCV2.

2. Materials and methods

2.1. Expression and purification of the PCV2 capsid protein

PCV2 capsid-PET43 plasmid was constructed by PCR amplification of full-length ORF2 gene (702 bp) from PCK01-2 (Korean strain) DNA using primers 5′-GGATCCATGACGTATCCAAGG-3′ and 5′-CTCGAGGGGGTTAAGTGG-3′ that contain an *Bam*HI and a *Xho*I site respectively. The PCR fragment was ligated into the *Bam*HI/*Xho*I-cleaved pET43 plasmid (Merck, Darmstadt, Germany), which expresses recombinant proteins tagged with a hexahistidine at C-terminus. Proteins were overexpressed in *Escherichia coli* Rosetta-gami2 (DE3) (Merck) strain and purified with nickel-chelate resin, Probond (Invitrogen, Carlsbad, CA) according to the

* Corresponding author. Tel.: +82 2 880 1270; fax: +82 2 876 7610.
E-mail addresses: leeys@snu.ac.kr, yoonsra@snu.ac.kr (Y.-S. Lee).
[1] These authors contributed equally to this paper.

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manufacturer's manual. Proteins were transferred by electroblotting onto transfer nitrocellulose membrane (Bio-Rad, Hercules, CA) using semi-dry transfer cell (Bio-Rad). The membrane was then treated sequentially with blocking solution (phosphate-buffered saline containing 5% non-fat skim milk), with anti-PCV2 antibodies followed by anti-porcine IgG goat antibodies conjugated to peroxidase (KPL, Gaithersburg, MD). Finally, the membrane was soaked in a chromogen/substrate solution (TMB single solution; KPL) for color development.

2.2. Isolation of RNA aptamers against His-tagged PCV2 capsid protein

In vitro selection was carried out essentially as described (Hwang et al., 2009), with a few modifications. The 2′F-RNA aptamers were selected from randomized sequences. A random library of RNA oligonucleotides of sequence 5′-GGGAGAGCGGAAGCGUGCUGGGCC-N_{40}-CAUAACCCAGAGGUGAUGGAUCCCCC-3′ [N40 represents 40 nucleotide (nt) sequences formed by equimolar incorporation of A, G, C, and U at each position] was constructed by *in vitro* transcription of synthetic DNA templates with NTPs (2′F UTP, 2′F CTP, GTP, ATP, Epicentre Biotechnologies, Madision, WI) and T7 RNA polymerase. To remove RNAs that bind nonspecifically to agarose beads, 1.44 μM of the RNA library was preincubated with 20 μl of Ni-NTA agarose beads in 100 μl binding buffer (30 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM dithiothreitol, and 1% BSA) for 30 min at room temperature with shaking, precipitated by centrifugation, and discarded. The precleared supernatant was transferred to a new tube and incubated with 333 nM of his-tagged PCV2 capsid for 30 min at room temperature. RNAs which bound to PCV2 capsid were recovered, amplified by RT-PCR and *in vitro* transcription, and used in the following selection rounds. In subsequent rounds, capsid concentration was reduced by 2-fold at every 3 round for more stringent condition. After 12 rounds of SELEX, the resulting cDNA was amplified. The amplified DNA was cloned and individual clones were identified by DNA sequencing. Structures of aptamers were predicted using MFOLD (Zuker, 2003), available at http://www.bioinfo.rpi.edu/applications/mfold/ using a salt correction algorithm and temperature correction for 25 °C.

2.3. Electrophoretic mobility shift assay (EMSA)

The binding affinity of the RNA to PCV2 capsid was assessed by EMSA. The purified RNAs were incubated with various concentrations of PCV2 capsid in 20 μl of binding buffer containing 1 μg of tRNA and the RNA-capsid complexes were run on a 7% native polyacrylamide gel containing 2% glycerol. Bands were visualized by SYBR Gold (Invitrogen).

2.4. Biosensor assays

A BIAcore 2000 (GE Healthcare, Uppsala, Sweden) was used to measure binding parameters by surface plasmon resonance (SPR) technique as described previously (Oguro et al., 2003). Briefly, the aptamer templates were amplified and tagged at the 39-end with dT16 by PCR using 5′-template primer (5′-TAATACGACTCACTATAGGGAGACAAGAATAAACGCTCAA-3′) and dT16 tagging 3′-template primer (5′-TTTTTTTTTTTTTTTTGCCTGTTGTGAGCCTCCTGTCGAA-3′). These DNA templates were then transcribed to poly (A)-tailed RNAs. A 5′-biotinylated dT16 oligomer was bound to the surface of the streptavidin sensor chip (GE Healthcare) of flow cells 1 and 2. The poly(A)-tailed RNA was immobilized to about 1000 RUs in flow cell 2 by complementary hybridization to the dT16 oligomer. Capsid solution of different concentration between 500 and 15.7 nM was injected to the flow cells 1 and 2 of the sensor chip. Data was obtained by subtracting the flow cell 1 data from the flow cell 2 data, thereby showing the net interaction between RNA and protein. To regenerate the sensor chip, bound material was completely removed by injecting 50 mM NaOH. Kinetic constants were estimated by using BIAevaluation 3.0 software (GE Healthcare).

2.5. Neutralization assays

The permanent PK-15 cell line, which was free of PCV1, was maintained in D-MEM (Invitrogen) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 5% L-glutamine, 1× antibiotic (Invitrogen) at 37 °C in a humidified 5% CO_2 incubator. PK-15 cells were infected with 1×10^5 50% tissue culture infective dose ($TCID_{50}$)/ml virus inoculums that had been preincubated with initial RNA library pool (N40) or serially diluted aptamers for 30 min at room temperature. After absorption for 2 h, cells were washed with D-MEM to remove residual virus. Then 4 h later, 300 mM glucosamine (Sigma, St. Louis, MO) was treated.

2.6. Real-time quantitative RT-PCR

The extent of virus replication was determined by measuring intracellular viral genomic DNA by quantitative real-time PCR. Total cell nucleic acids were prepared from virus-infected PK15 cells at 24 h and 48 h after preincubation with aptamers by using Trizol (Invitrogen) for reverse transcription. The PCR primers for PCV2 were 5′-AAAGCAAATGGGCTGCTAA-3′ and 5′-TGGTAACCATCCCACCA CTT-3′. The PCR primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-CATTGACCTCCACTACATG-3′ and 5′-TCTCCATGGTGGTGAAGAC-3′. Quantitative real-time PCRs were performed on a LightCycler 480 (Roche, Indianapolis, IN). The amplification protocol followed the instructions of an Express SYBR GreenER qPCR kit (Invitrogen). Each sample was run in triplicates. The relative amount of viral target gene was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample.

2.7. Indirect fluorescence assay (IFA)

PK-15 cells were infected with 1×10^5 $TCID_{50}$/ml virus inoculums that had been preincubated N40 or 1000 nM concentrations of aptamers for 30 min at room temperature. After absorption for 2 h, cells were washed with D-MEM to remove residual virus. Then 4 h later, 300 mM glucosamine was treated. The infected cells were washed and fixed with 80% cold acetone at 48 h postinfection. Monoclonal antibodies against PCV2 was diluted in PBS and incubated with cells for 1 h at 37 °C. After washing with PBS, the cells were incubated with anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody (KPL) diluted in PBS for 1 h at 37 °C. Followed by the incubation with secondary antibody, the cells were stained with DAPI (2,4-diamidino-2-phenylindole) (Invitrogen). The cells were washed and examined using fluorescence microscope.

3. Results

3.1. Expression of recombinant protein in E. coli

PCV2 capsid-PET43 plasmid containing coding regions of PCV2 ORF2 was constructed in order to overexpress capsid protein for utilization as SELEX target antigens (Fig. 1A) 72 kDa histidine tagged capsid proteins were expressed in *E. coli* and purified by nickel-chelate resin. For confirmation, recombinant proteins were analyzed by SDS-PAGE and western blotting with anti-PCV2 antibody (Fig. 1B).

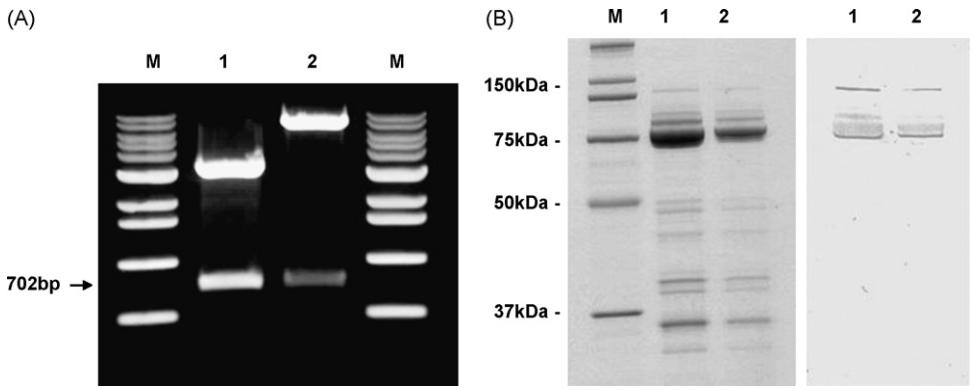

Fig. 1. Expression and purification of the PCV2 capsid protein. (A) PCV2 capsid-pET43 plasmid construction. Full-length ORF2 gene (702 bp) from PCK01-2 strain was amplified using PCV2 ORF2 specific primers and then cloned into pGEMT-easy vector (lane 1) and pET43 vector (lane 2). Arrow marks the position of PCV2 capsid fragment. (B) Purification of recombinant PCV2 capsid protein and immunoblot. IPTG induced proteins were purified with nickel-chelate resin and separated using 12% SDS-PAGE (left) and transferred onto nitrocellulose membrane and incubated with anti-PCV2 antibody followed by anti-porcine IgG goat antibody conjugated to peroxidase. The immunoreaction was visualized by an HRP-based chromogen/substrate system (right). Lanes 1–2 represents two purified recombinant PCV2 capsid proteins.

3.2. *In vitro selection of PCV2 capsid specific RNA aptamers*

To isolate 2′F RNA aptamers to the PCV2 capsid, a library of approximately 10^{14} different 2′F RNA molecules, containing a 40-nt-long sequence derived form random sequence flanked by defined region, was screened by SELEX.

After 12 cycles of selection, the highly enriched aptamer pools were cloned. The nucleotide sequences of 20 clones were determined. Two different aptamers were selected (Fig. 2A). Two aptamers had very different sequences and did not contain a conserved sequence. Aptamer 1 sequences were found four times and the length of the random regions was 39 nucleotides (nt). Aptamer 2 sequences were found two times and the length of the random regions was 40 nt. Structural analysis of the selected aptamers was performed by using Mfold (Zuker, 2003) to predict the most likely minimum energy structures (Fig. 2B and C). The predicted secondary structures of RNA aptamers contained several stem-loop regions.

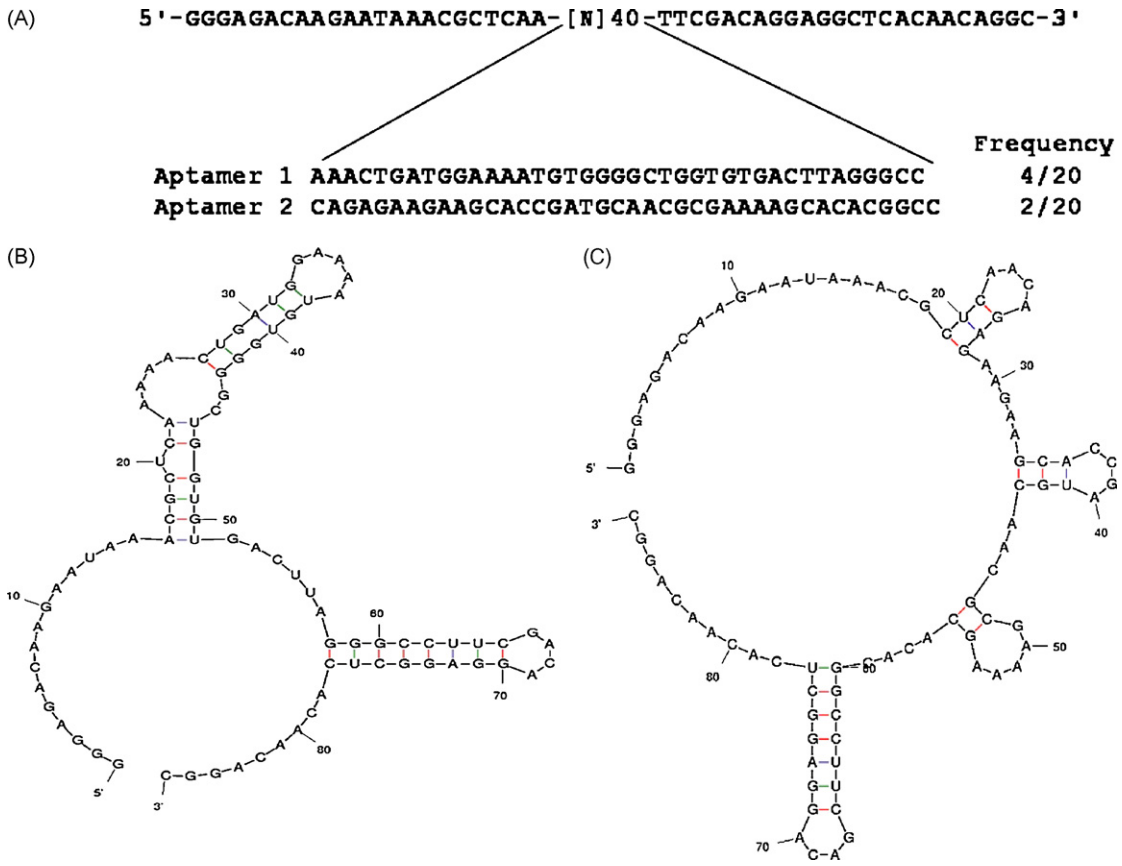

Fig. 2. The sequence and secondary structure of RNA aptamers selected from randomized N40 RNA libraries. (A) The sequence of the parental N40 RNA pool contains 5′ and 3′ constant sequences for primer annealing. After 12 rounds of selection, the sequences of 20 clones were identified and the frequencies of two aptamer clones were shown as number. The secondary structures of aptamer 1 (B) and aptamer 2 (C) were predicted using the Mfold program.

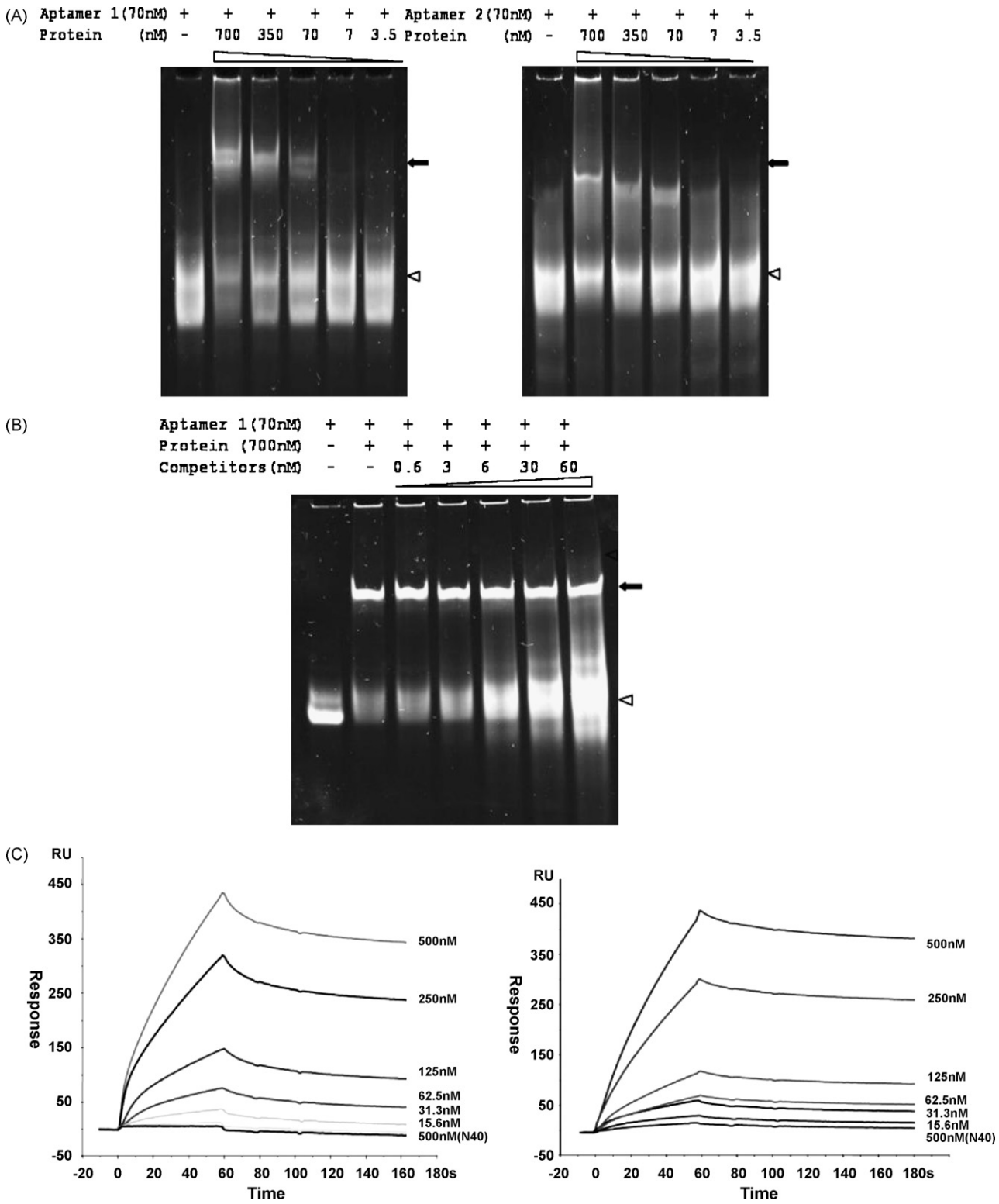

Fig. 3. Electrophoretic Mobility Shift Assay (EMSA). (A) 70 nM of each aptamer was co-incubated with recombinant PCV2 capsid (3.5, 7, 70, 350, 700 nM) and – indicated RNA only. Free RNA was indicated with open arrowhead and RNA-Protein complex was indicated with arrow. Binding ability of aptamer 1 (left) and aptamer 2 (right) was performed using a gel shift assay. Following incubation, RNA-protein complex were resolved on 7% native gels and visualized by SYBR Gold staining. (B) Competition of Aptamer 1 for binding to capsid protein by the original RNA library. Aptamer 1 (70 nM) was incubated with protein (700 nM) along with competitors, N40 (0.6–60 nM) and loaded onto 7% polyacrylamide gel. Free RNA was indicated with open arrowhead and RNA-Protein complex was indicated with arrow. (C) Sensorgrams of SPR. The real-time measurement of the interaction between PCV2 capsid and the aptamer 1 (left) and aptamer 2 (right) was performed using BIAcore 2000. Immobilized RNA aptamer plus poly(A_{16}) tail was treated with His-PCV2 capsid in proportion to the amount (15.6–500 nM). Initial RNA library pool is shown as N40.

Table 1
SPR kinetic constants of aptamers. The aptamers were previously immobilized to a sensor chip and various concentrations of PCV2 capsid (15.7, 31.3, 62.5, 125, 250, and 500 nM) were injected in to the flow cells. Kinetic constants were calculated from the sensorgrams with BIAevaluation software. The association rate constant (K_a) and the dissociation rate constant (K_d) were calculated according to the manufacturer's procedure. The dissociation constant (K_D) is equal to K_d/K_a and the association constant is the reciprocal of K_D.

	K_a (M^{-1} s^{-1})	K_d (s^{-1})	K_A (M^{-1})	K_D (nM)
Aptamer 1	2.81×10^4	1.5×10^{-3}	1.87×10^7	53.4
Aptamer 2	2.77×10^4	7.42×10^{-4}	3.73×10^7	26.8

3.3. Binding affinity of aptamers to PCV2 capsid protein

To estimate the affinity of the RNA aptamer-capsid interaction, a gel shift assay was utilized. The selected RNA aptamer clones efficiently formed a shifted nucleoprotein complex with in a protein concentration-dependent manner (Fig. 3A). Formation of RNA aptamer-capsid complex was not blocked competitively by addition of excess amount of competitor RNA, N40 (Fig. 3B).

To quantify the binding affinities of the RNA aptamers, SPR technique was utilized. The SPR profile clearly demonstrated that the selected RNA aptamer 1 and 2 bound strongly to the PCV2 capsid protein (Fig. 3C). N40 did not bind to PCV2 capsid. Two of the aptamers showed good binding kinetics to capsid. As summarized in Table 1, the dissociation constants (K_D) of aptamer 1 and 2 were 53.4 nM and 26.8 nM, respectively. These aptamers showed selective binding with the PCV2 capsid.

3.4. Neutralization of PCV2 infectivity by aptamers

Aptamer 1 was chosen for the further characterization because of their relative abundance. We tested whether the aptamer 1 isolated against PCV2 capsid could prevent or block PCV2 infection in target cells. Pretreatment of PCV2 with 1000 nM of aptamer 1 exhibited significant reduction up to 70%, compared with N40 treated. By pretreatment of PCV2 with different concentrations (250–1000 nM) of aptamer 1, the amount of viral DNA was also reduced up to 30–70% at 24 h postinfection and up to 30–60% at 48 h postinfection, respectively (Fig. 4A). As shown in Fig. 4B, aptamer 1

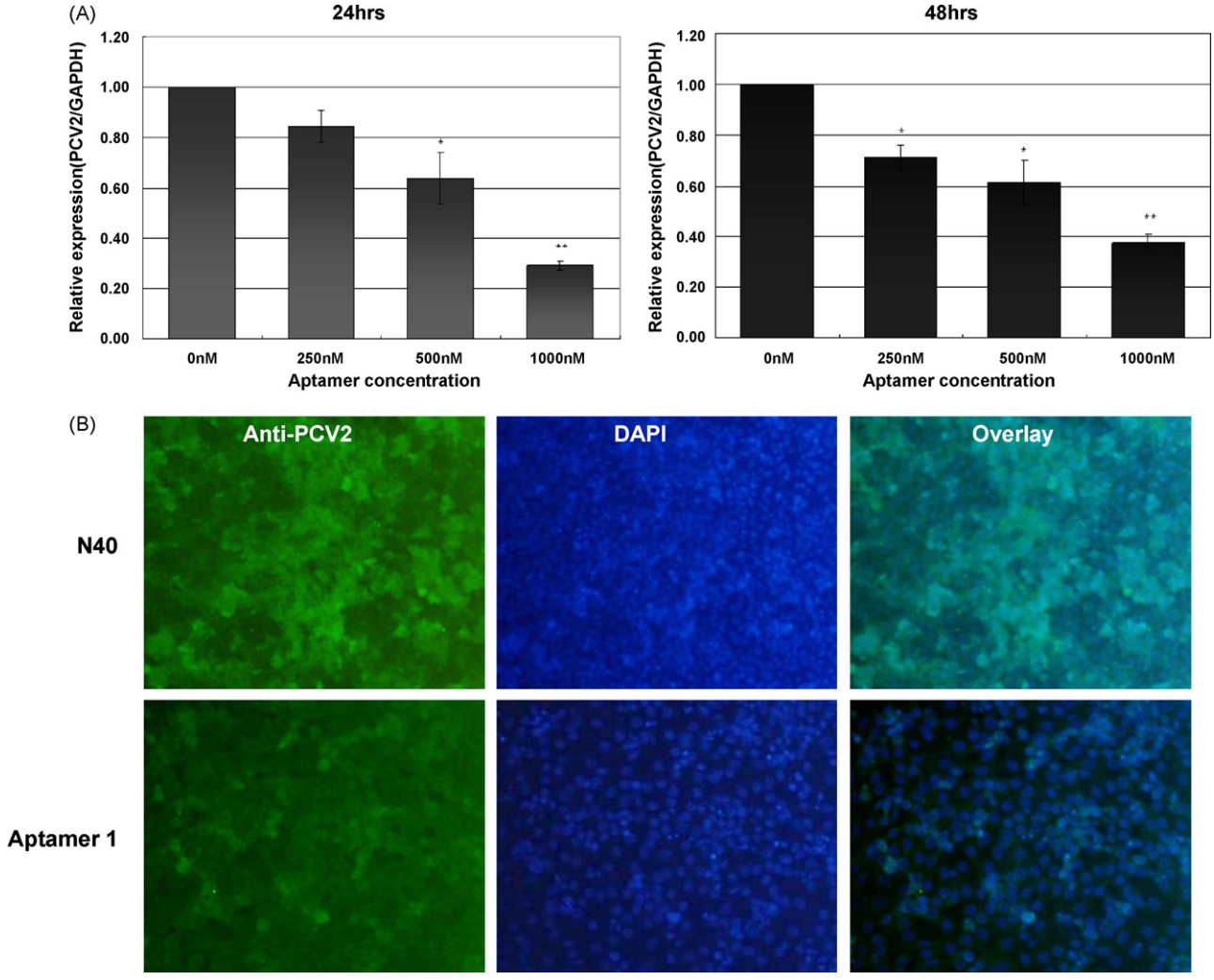

Fig. 4. Neutralization of PCV2 by aptamers. (A) Neutralization assays using quantitative real-time PCR on 24 h (left) and 48 h (right). The PCV2 virus was preincubated with N40 and different concentrations of aptamer 1. The PK-15 cells were infected with PCV2 virus treated with N40 and aptamer 1 and total nucleic acids were extracted. The results are expressed as viral DNA relative to GAPDH and reported as mean ± S.D. Asterisks indicate that the value is significantly different from the value for the virus control in the corresponding assay, with P values of $P = 0.001$(**) to 0.01(*). P values were calculated using a two-tailed, paired t-test with 95% confidence intervals. Data shown are the means of three replicates, and error bars represent the standard errors of the means. (B) Neutralization assays using IFA. PK-15 cells were infected with 1×10^5 $TCID_{50}$/ml virus inoculums that had been preincubated with initial N40 or 1000 nM concentrations aptamer 1. After 48 h postinfection, cells were fixed with 80% cold acetone and stained with PCV2 monoclonal antibody by FITC-conjugated secondary antibody. Nuclei were stained with DAPI. Merged signals were shown. The cells were examined using fluorescence microscope.

also reduced the flurorescence-stained cells compared with those in N40 control in IFA (Fig. 4B). Using quantitative real-time PCR and IFA, we showed that aptamer 1 neutralized the virus in PK-15 cells.

4. Discussion

We have presented a novel strategy for the identifying RNA aptamers that target the PCV2 capsid and demonstrated that the selected RNA aptamers have the antiviral activity by neutralization. The PCV2 capsid is important for virus neutralization that blocks viral infection (McNeilly et al., 2001). Thus, the identification of RNA aptamers that specially target the capsid protein can be relevance for the target of anti-PCV2 drugs. 2′F substituted RNA aptamers are selected in this study because they are not only resistant to nucleases (Kraus et al., 1998) and have higher affinities than do unmodified RNA or NH_2 substituted RNA aptamers (Pagratis et al., 1997). The two species of 2′F RNA aptamers were generated for PCV2 capsid protein by *in vitro* RNA selection. The selected 2′F-RNA aptamers show the nanomole affinity to PCV2 capsid.

For the sake of comparison, a 2′F modified RNA aptamers that is specific for the PCV2 capsid was tested in the gel shift assays using other his-tagged proteins such as PCV2 ORF1 or ORF3. As expected, the selected aptamers did not show binding affinity to the other his-tagged proteins (data not shown), indicating the specific interaction of the aptamers with the target PCV2 capsid. As shown in Fig. 2B, nonspecific competitor RNA could not hamper the binding of aptamer 1 to capsid, suggesting that our selected aptamers recognize the PCV2 capsid protein specifically.

Real-time PCR and IFA were done to test the neutralization of PCV2 infectivity. The level of intracellular viral DNA, determined by real-time PCR, reflects that of viral input during entry. A significant reduction in the level of intracellular PCV2 DNA was observed in cells infected with virus pretreated with aptamers 1, indicating the entry of virus is blocked. These results indicate that the RNA aptamers have antiviral effects by virus neutralization. The IFA also showed the same results of real-time PCR. It is possible that aptamer 1 bind to neutralizing epitopes of capsid protein, given the fact that these aptamers recognize their target through RNA-Protein interactions.

To test the possibilities that aptamer 1 may interact with cellular surface receptor not viral surface molecules, cells were preincubated with 1000 nM of aptamer 1 and washed before being infected with PCV2. No reduction was observed (data not shown). These observations suggest that the observed antiviral effects by aptamer 1 are due to their high affinity binding to the viral capsid protein, not interacting cells.

A previous report suggests the inhibition of PCV2 replication in vitro and in vivo (Feng et al., 2008). To confirm the possibility that the selected aptamers act as antisense agents, we analyzed the sequences of aptamers. The sequences of the selected aptamers have no extensive homology to PCV2 DNA. According to the report that determines the relative efficacy in HIV replication, the aptamers can efficiently inhibit HIV replication at higher multiplicities of infection but shRNA dose not (Joshi et al., 2005). For the direct comparison, the relative efficacy of RNA aptamers and siRNA in PCV2 replication will be determined in the further study. Furthermore, aptamers are frequently reported as potential targeting agents for the delivery of siRNA cargoes (Chu et al., 2006).

This study demonstrates that RNA aptamers isolated from randomized sequences have been shown to exhibit high affinity and specificity to PCV2 capsid protein and to neutralize the virus infectivity effectively. This is the first description of RNA aptamer directed to the PCV2 capsid protein and the inhibitory effect against PCV2. Although clinical use may be some distance away, the PCV2 aptamers described here might be used as antiviral and targeting agent against PCV2 infection.

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