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ORIGINAL ARTICLE

RNA Aptamers Inhibit the Growth of the Fish Pathogen Viral Hemorrhagic Septicemia Virus (VHSV)

Porntep Punnarak · Mudjekeewis D. Santos · Seong Don Hwang · Hidehiro Kondo · Ikuo Hirono · Yo Kikuchi · Takashi Aoki

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Abstract Viral hemorrhagic septicemia virus (VHSV) is a serious disease impacting wild and cultured fish worldwide. Hence, an effective therapeutic method against VHSV infection needs to be developed. Aptamer technology is a new and promising method for diagnostics and therapeutics. It revolves around the use of an aptamer molecule, an artificial ligand (nucleic acid or protein), which has the capacity to recognize target molecules with high affinity and specificity. Here, we aimed at selecting RNA aptamers that can specifically bind to and inhibit the growth of a strain of fish VHSV both in vitro and in vivo. Three VHSV-specific RNA aptamers (F1, F2, and C6) were selected from a pool of artificially and randomly produced oligonucleotides using systematic evolution of ligands by exponential enrichment. The three RNA aptamers showed obvious binding to VHSV in an electrophoretic mobility shift assay but not to other tested viruses. The RNA aptamers were tested for their ability to inhibit VHSV in vitro using hirame natural embryo (HINAE) cells. Cytopathic effect and plaque assays showed that all aptamers inhibited the growth of VHSV in HINAE cells. In vivo tests using RNA aptamers produced by *Rhodovulum sulfidophilum* showed that extracellular RNA aptamers inhibited VHSV infection in Japanese flounder. These results suggest that the RNA aptamers are a useful tool for protection against VHSV infection in Japanese flounder.

Keywords RNA aptamer · Japanese flounder · VHSV · *Rhodovulum sulfidophilum*

P. Punnarak · M. D. Santos · S. D. Hwang · H. Kondo · I. Hirono · T. Aoki (✉)
Laboratory of Genome Science,
Graduate School of Marine Science and Technology,
Tokyo University of Marine Science and Technology,
Konan 4-5-7, Minato-ku,
Tokyo 108-8477, Japan
e-mail: aoki@kaiyodai.ac.jp

Y. Kikuchi
Department of Ecological Engineering,
Toyohashi University of Technology,
1-1 Hibarigaoka, Tempaku-cho,
Toyohashi 441-8580, Japan

M. D. Santos
Genetic Fingerprinting Laboratory,
National Fisheries Research and Development Institute,
940 Kayumanggi Bldg., Quezon Ave.,
Quezon 1103, Philippines

Introduction

Viral hemorrhagic septicemia is a serious systemic disease of fish. The causative agent, viral hemorrhagic septicemia virus (VHSV), is an enveloped, bullet-shaped particle about 180 nm in length and 60 nm in diameter. This negative-sense single-stranded RNA virus (approximately 11.1 kb) belongs to the genus *Novirhabdovirus* in the family Rhabdoviridae (Smail 1999; Lorenzen et al. 1999). VHSV has been detected in many kinds of wild and marine-cultured fish in Europe, North America, and Japan and exhibits four distinct lineages based on sequence variations in its membrane proteins: genotype I in continental Europe, genotype II in the Baltic Sea, genotype III in the British Isles, and genotype IV in North America and Japan (Oshima et al. 1993; Benmansour et al. 1997; Nishizawa et al. 2002; Einer-Jensen et al. 2004; Einer-Jensen et al. 2005; Byon et al. 2006; Nishizawa et al. 2006; Kim and Faisal 2011).

Several methods have been used to inhibit VHSV infection, including the use of monoclonal antibodies against the cell surface that block viral entry (Lorenzen et al. 1988; Mourton et al. 1992), recombinant DNA vaccines based on the transmembrane glycoprotein (G protein) (Lorenzen et al. 1999; Chico et al. 2009), and recombinant proteins such as

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Mx protein (Caipang et al. 2003), but many of these methods, while showing promise, have not been commercially developed because of various limitations such as the induction period in adaptive immunity, stress to injected fish, labor intensity, and operational costs (Evensen 2009).

Aptamer technology is a relatively new technology that uses artificial nucleic acids or protein ligands that bind tightly to a specific target (Bunka and Stockley 2006). The technology was described by Ellington and Szostak in 1990. "Aptamer" is derived from the Latin word "aptus", meaning "to fit". Aptamers are generated from a library of nucleotides or proteins containing approximately $>10^{15}$ different molecules and are selected in vitro by systematic evolution of ligands by exponential enrichment (SELEX) (Tuerk and Gold 1990). Aptamers can bind to many kinds of targets such as nucleic acids, biological cofactors, antibiotics, peptides and proteins, whole viruses and bacteria, and even multicellular organisms such as yeast and trypanosoma (Stoltenburg et al. 2007). They have been utilized in numerous applications including cytokine assays (Guthrie et al. 2006), diagnostics, environmental and food analyses (Tombelli et al. 2007; Kärkkäinen et al. 2011), biosecurity (Fischer et al. 2007), detection of pathogens (Torres-Chavolla and Alocilja 2009), and cancer cell biology (Phillips et al. 2008). In particular, aptamers have been used in numerous virus studies such as analysis of the molecular biology of virus replication, diagnostic biosensors, and in the development of antiviral agents (James 2007; Torres-Chavolla and Alocilja 2009).

Aptamers have not yet been used in aquatic animals. There are some limitations that have to be considered for in vivo applications, such as aptamer stabilization, delivery method, and mass production (Osborne et al. 1997). Several methods have been used to enhance the stability of RNA aptamers, such as amino or fluoro modifications at the 2′ position of pyrimidines (Green et al. 1995; Ruckman et al. 1998; Zhou et al. 2009). Various delivery methods have been used, including cell membrane-penetrating peptides, retroviral expression vectors for small RNAs, and naked and liposome-mediated oligonucleotide transfer (Famulok et al. 2007). However, mass production methods need to be developed since the use of T7 RNA polymerase (Milligan et al. 1987) and other chemical synthesis methods (Marshall and Kaiser 2004) are costly and labor intensive. Recently, Suzuki et al. (2010) demonstrated the possibility to produce functional RNA aptamers by using a phototrophic marine bacterium, *Rhodovulum sulfidophilum*, which may be useful for industrial-scale production of aptamers.

Here, we report the identification of RNA aptamers for VHSV and tested their ability to inhibit growth in vitro using hirame natural embryo (HINAE) cells and in vivo using *R. sulfidophilum*-delivered aptamers in Japanese flounder (*Paralichthys olivaceus*). This is the first report to identify RNA aptamers that inhibit a fish pathogenic virus. These results may lead to a new method for improving the health of aquatic animals.

Materials and Methods

Cells and Viruses

Japanese flounder HINAE cells were grown at 25°C as a monolayer in Leibovitz's L-15 (Gibco-BRL, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (JRH Bioscience, Lenexa, KS, USA), 100 IU/ml of penicillin G, and 100 μg/ml of streptomycin (Gibco-BRL). Target virus, VHSV (strain KRRV9822) (Byon et al. 2006), was purified by sucrose gradient ultracentrifugation of infected HINAE cells following Oshima et al. (1993) with minor modifications. The purified virus was then tested for its ability to produce cytopathic effect (CPE) in HINAE cells. Pure isolates were stored in TN buffer (0.01 M Tris HCL, 0.85 % NaCl, pH 7.2) and used for the CPE tests, SELEX, and viral inhibition assay.

RNA Aptamer Selection

RNA aptamers were selected following the SELEX protocol of Pan et al. (1995) with minor modifications. A library of single-stranded DNA oligonucleotide sequences (2.5 mg DNA) was purchased from Operon (Tokyo, Japan). Each sequence consisted of 15-bp 5′ constant sequences (5′-GGGCCAGGCAGCGAG-3′), a central random 40-mer and 20-bp 3′ constant sequences (5′-CCGACCACACGCGTCCGAGA-3′), in that order. The library is estimated to contain about 5×10^{16} sequences. First, a double-stranded DNA library was amplified using the forward primer containing T7 promoter (5′-*TAATACGACTCACTATA*GGGCCAGGCAGCGAG-3′) and reverse primer (5′-TCTCGGACGCGTGTGGTCGG-3′), after which RNA was produced from the resulting dsDNA oligos (containing T7 promoter) using the T7 RiboMAX RNA production system (Promega, Madison, WI, USA) with minor modifications.

In the first cycle of the SELEX procedure, 50 μl (4 mg/ml) of RNA oligos was denatured at 90°C in a heat block for 3 min and place on ice for 5 min to fold them. The folded RNAs were then removed background by passing the folded RNA aptamer-containing solution (50 μl RNA [4 mg/ml]+ 450 μl binding buffer [2.5 mM $MgCl_2$, 100 mM NaCl, 20 mM Tris–HCl, pH 7.5]) through a pre-wetted 0.1-μm nitrocellulose filter (Millipore, USA). The filter was then discarded and the filtrate used to bind to a 50-μl VHSV (pure isolate [2.0×10^6 $TCID_{50}$/ml]) by slow mixing/rotation for 30 min at 37°C in binding buffer to form the RNA

aptamer–VHSV complex. To separate the RNA aptamer–VHSV complex from free RNA molecules, filtration was utilized since the size of the VHSV is known (180 nm in length × 60 nm in diameter). We calculated that VHSV would not pass through a 0.1-μm-pore-size nitrocellulose. The separated RNA aptamer–VHSV complex that remained on the filter was washed with TN buffer into an Eppendorf tube. The RNA aptamers were separated from the VHSV particles by heat treatment at 90°C for 3 min, purified by the phenol/chloroform/isoamyl alcohol (PCI; 25:24:1) method using DEPC water, and converted to cDNA using M-MLV reverse transcriptase system (Life Technologies, Carlsbad, CA, USA). The resulting cDNAs were then amplified using the forward T7 and reverse primers (as mentioned earlier) to produce a T7-containing pool of dsDNA oligos. These were then used as template to synthesize the second RNA pool of oligos for the second cycle of the SELEX procedure. The stringency of the selection was increased by lowering viral concentration through serial dilutions (10% replaced by TN buffer for each cycle) as the number of selection cycles increased. After 6 cycles, the resulting RNA pool was sequenced as described below.

RNA Aptamers Sequence Analysis

The resulting cDNA pool was then used as template for amplification with the same primers as in the SELEX steps. The PCR conditions were initial denaturation at 95°C for 1 min, 10 cycles (95°C: 1 min, 50°C: 15 s, 72°C: 3 min) and final elongation at 72°C for 5 min. PCR products were purified by the PCI method, ligated into pGEM®-T-easy vector (Promega, Madison, WI, USA), and transformed into competent *Escherichia coli* JM109 cells. DNA sequencing of the PCR products was performed using a 16-capillary array genetic analyzer (Applied Biosystems ABI 3130xl, Hitachi, Japan). DNA sequences were analyzed with the Genetyx-Win Ver. 7 (Software Development Co. Ltd, Japan) computer program, aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and clustered using MEGA 4 (http://www.megasoftwarenet/index.html). RNA folding and stability was predicted using *S*fold application web server (http://sfold.wadsworth.org/cgi-bin/srna.pl).

Electrophoretic Mobility Shift Assay

To confirm that the candidate RNA aptamers indeed bind to the VHSV, we performed an electrophoretic mobility shift assay (EMSA). Candidate RNA aptamers at 10, 12.5, 15, 17.5, 20, 22.5, and 25 μg were incubated with VHSV (10^6 $TCID_{50}$/ml) in binding buffer at 37°C for 30 min. After incubation, the mixed samples were subjected to 5 % nondenaturing polyacrylamide gel and stained with SYBR® Green EMSA following the manufacturer's instructions (Life Technologies). The gels were visualized and documented at 312 nm using UV epi-illumination (AE-6932GXCF-U Print chart, ATTO, Japan). The specificity of VHSV–RNA aptamers was also confirmed through EMSA by incubation of VHSV–RNA aptamers (12.5 μg) with adequate amounts of another VHSV strain (strain Obama, 1.9×10^6 $TCID_{50}$/ml) and other viruses (koi herpesvirus [KHV], 3.6×10^3 $TCID_{50}$/ml; viral nervous necrosis [VNN], 2.0×10^7 $TCID_{50}$/ml). Subsequently, the mixed products were also loaded to the gel and stained as detailed earlier.

In Vitro Inhibition of VHSV Infection by RNA Aptamers

Viral inhibition of candidate RNA aptamers was tested by treating HINAE cells with the RNA aptamers–VHSV complex. Candidate RNA aptamers amounting to either 25 or 12.5 μg each could be folded and bound with VHSV (concentration is 10^4 $TCID_{50}$/ml) in binding buffer and incubated at 37°C for 30 min, after which the mixtures were added to 96-well plates containing a monolayer of HINAE cells in triplicate for each treatment. HINAE in L15 treated with VHSV and VHSV+binding buffer were used as positive controls while the L15 only and the L15+binding buffer served as negative controls. The treated cells were incubated at 15°C. After 4 days, the supernatant of the culture media was collected to perform the plaque assay, while the remaining cells were stained with crystal violet for checking CPE. For plaque assay, the supernatant was added into the HINAE cells in six-well plates after washing with PBS. After adsorption at 15°C for 1 h, cells were again washed with PBS and overlaid with 0.5 % agarose in L-15 medium and incubation was continued at 15°C. After 3 days, cells were fixed with formaldehyde and stained with 0.5 % crystal violet for counting the plaque numbers. One-way ANOVA was used to determine significant differences in plaque numbers between treatments and controls and between each treatment with different concentrations.

Preparation of *R. sulfidophilum*-Containing RNA Aptamers

The plasmid pHSR2 and the phototrophic marine bacteria *R. sulfidophilum* strain DSM 1374^T were used to prepare the bacteria producing RNA aptamers. The plasmid containing the *rrn* promoter and *puf* terminator for RNA transcription and the designed aptamer insert was flanked on both sides by self-cleaving hammerhead ribozyme sequences which allowed digestion by restriction enzymes *Aat*I and *Eco*T22I (Suzuki et al. 2010). First, the VHSV–RNA aptamer candidates were

Table 1 Identification of VHSVRNA aptamers

Aptamer	Random sequences	Frequency (%)
F1	UGAGUUUCGUUCCCGUAUAGUAAACUUCUGACGGGAAUGUU	3 (20)
F2	UGGUGCUCUGAGUCCAUCAGUAUAUUUCCUGUAUAAGGGGACG	2 (13)
F3	AAAUGAUUUUGUGUAUUAGGUCUCUAUCAUCUGAAAGGGC	1
C2	UACAAUUGCUGGGUUAUUUCGCCGUGUAAAAAUGUGCGCU	1
C3	ACGGUUAGGCUAGUGUGAGAUUGUGCAUUAGUUUAGAUUG	1
C4	CUUAAGUGUUUGAUGGCGCUUGUUGUUUGCUAGUUUGGAA	1
C5	GACAAUUGCUGGGGUAUUUCACCUUGUAAAAUUGGGCGCU	1
C6	AGUUGUGAUGUUGGGUGGACUGUGUGGAUUCUGCACAGUUUA	1
C7	AUGCGUUUCUUCUUGGUUCCCUUGUGUGGUGUGGAUGUCU	1
C8	UGAAUUAGCUGUGAUGAGAUGGUGGAAUAAGACGUGAAGA	1
C9	UCCUGGAGCUUGUUGAUUCACUAGUUGCUGCUCGUGUUCC	1
C10	GAAAUAGCUACUGGUAGGUAGUGUGUACCGCUGCAUGUGG	1

After six rounds of selection, 15 clones of the selected RNA pool were isolated and sequenced. Only the random sequences of the aptamers are indicated. Values are given as *n* (%)

converted to cDNA using M-MLV system (Life technologies) and then amplified using specific primers containing the restriction sites (forward primer [VHSV-AatI F]: 5′-GTGAT*AGGCCT*GGGCCAGGCAGC-3′ and reverse primer [VHSV-EcoT22I R]: 5′-GGTAC*ATGCAT*GACGCGGTG TGGT-3′. (The restriction enzyme sites are italicized.) Purified PCR products were digested by restriction enzymes, *Aat*I and *Eco*T22I (Toyobo, Co., Ltd., Osaka, Japan), ligated to the pre-digested plasmid (pHSR2), using Ligation high ver. 2 (Toyobo Co. Ltd.), and transformed to *E. coli* (JM109). The alignment of the inserted aptamers to the *rrn* promoter region was checked by cloning and sequencing. Recombinant plasmids (pHSR2) containing VHSV–RNA aptamers were extracted and transformed to *R. sulfidophilum* by heat-shock method (Fornari and Kaplan 1982).

Detection of Extracellular RNA Aptamers Produced by *R. sulfidophilum*

In order to confirm that *R. sulfidophilum* can produce extracellular VHSV–RNA aptamers, *R. sulfidophilum* transformed with RNA aptamers (*R. sulfidophilum*-Apt$^+$) or empty vector (*R. sulfidophilum*-SR2) were cultured in PYS medium containing kanamycin (30 μg/ml) and incubated in aerobic/dark condition at 25°C for 16 h. The culture media were centrifuged (12,000 rpm, 5 min) and the nucleic acid fractions of the supernatants were precipitated with ethanol. Unexpected proteins and DNAs were then removed by PCI and DNaseI (RNase-free grade; Promega). Total extracellular RNA was reverse-transcribed to cDNA using M-MLV system (Life Technologies) and amplified with specific primers VHSV-AatI F and VHSV-Ecot22I R (as described in the previous section). The arrangement of the extracellular VHSV–RNA aptamers was checked by cloning and sequencing.

In Vivo Inhibition of VHSV Infection by *R. sulfidophilum*-Generated RNA Aptamers

Juvenile Japanese flounder (average size 9.8 cm and average weight 6.4 g) was acclimated at 13°C for 7 days before use.

Three groups of treatments were performed: (1) treatment group: fish treated with VHSV+*R. sulfidophilum*-Apt$^+$, (2) positive control group: fish treated with only VHSV and VHSV+*R. sulfidophilum*-SR2, and (3) negative control group: no treatment. *R. sulfidophilum*-Apt$^+$ and *R. sulfidophilum*-SR2 were pre-cultured in PYS medium (containing 30 μg/ml kanamycin) under aerobic/dark condition at 25°C for 3 days, transferred into tanks containing 18 l of sea water (salinity 30 ppt.) and incubated at 13°C for 3 more days. Fish (ten fishes/tank) were immersed with VHSV (final concentration, 1.65×10^4 $TCID_{50}$/ml) in the new tanks containing 1.8 l of previously cultured bacteria (as described earlier) and 0.2 l of fresh pre-cultured bacteria. After incubation at 13°C for 3 h, fish were returned to previous tanks and culture continued at 13°C with daily supplement of fresh pre-cultured bacteria. Fish mortality was recorded daily for 10 days.

Results

Characteristics of Candidate RNA Aptamers

RNA aptamers that bound to VHSV were isolated by the SELEX procedure from a pool of ~10^{16} different molecules, each consisting of 75 nucleotides with a middle region consisting of about 40 random RNA oligonucleotide sequences. After six rounds of SELEX, 15 clones were generated and sequenced (Table 1). Two types of sequences

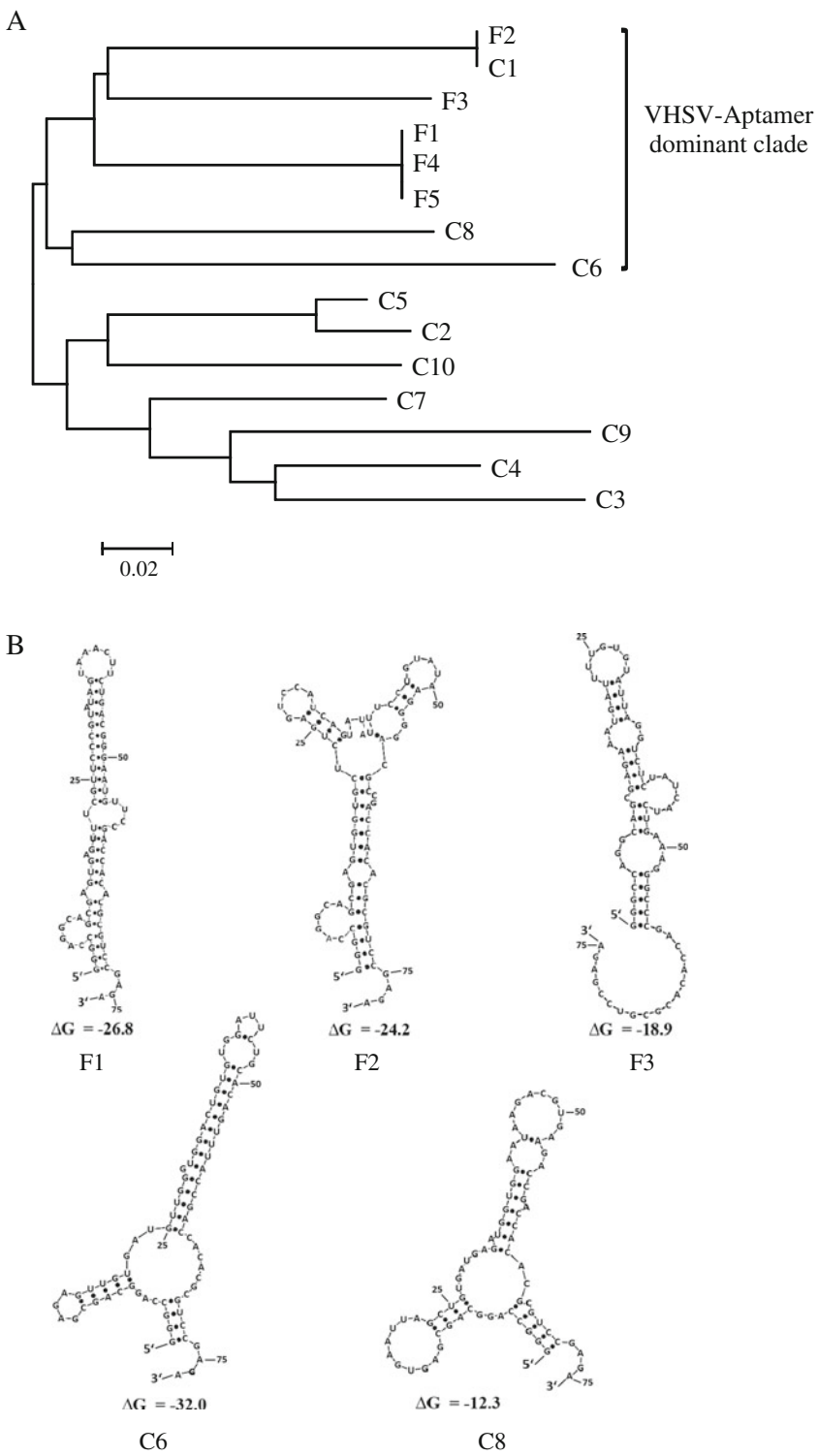

Fig. 1 Structural relationship of the 15 VHSV–RNA aptamers. **a** Cluster analysis of the sequences shows that the dominant sequences (F1 and F2) and the unique sequence F3 are closely related. The C6 and C8 sequences also clustered in the VHSV aptamer dominant clade. **b** The predicted stable secondary structure of aptamers in the VHSV aptamer dominant clade. ΔG, free energy value

(F1 and F2) dominated the RNA pool at 3/15 (20 %) and 2/15 (13 %), respectively. The rest were unique sequences. Cluster analysis of the RNA sequences revealed that the dominant types (F1 and F2) and the unique sequence F3 are closely related and also clustered with the unique sequences C6 and C8 (Fig. 1a).

Secondary Structure of the Aptamers

The *S*fold web server was used to predict the folding and secondary structure of the 15 screened oligonucleotides. The predicted structures of the most stable RNA sequences based on minimum free energy for the dominant types (F1 and F2)

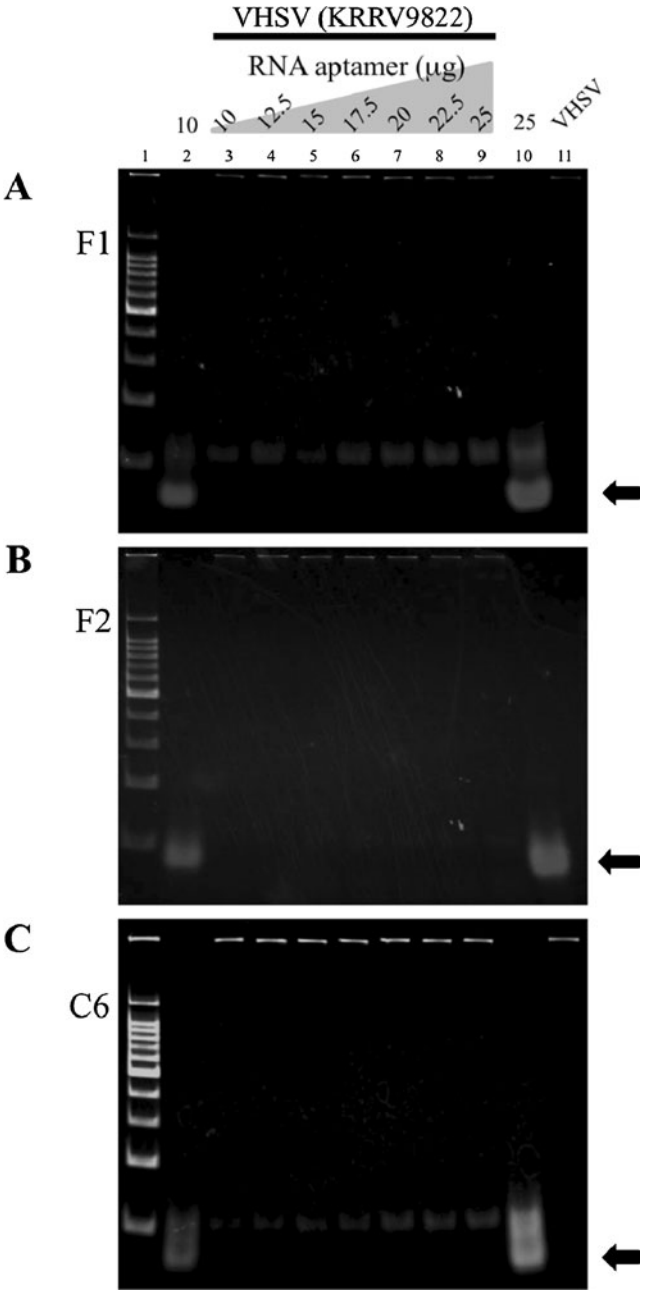

Fig. 2 EMSA of candidate VHSV–RNA aptamers in the presence and absence of VHSV strain KRRV9822. **a** F1, **b** F2, and **c** C6. *Lanes 2* and *10*, aptamer only (*arrows*). *Lanes 3–9*, different amounts of aptamer bound to viral particles

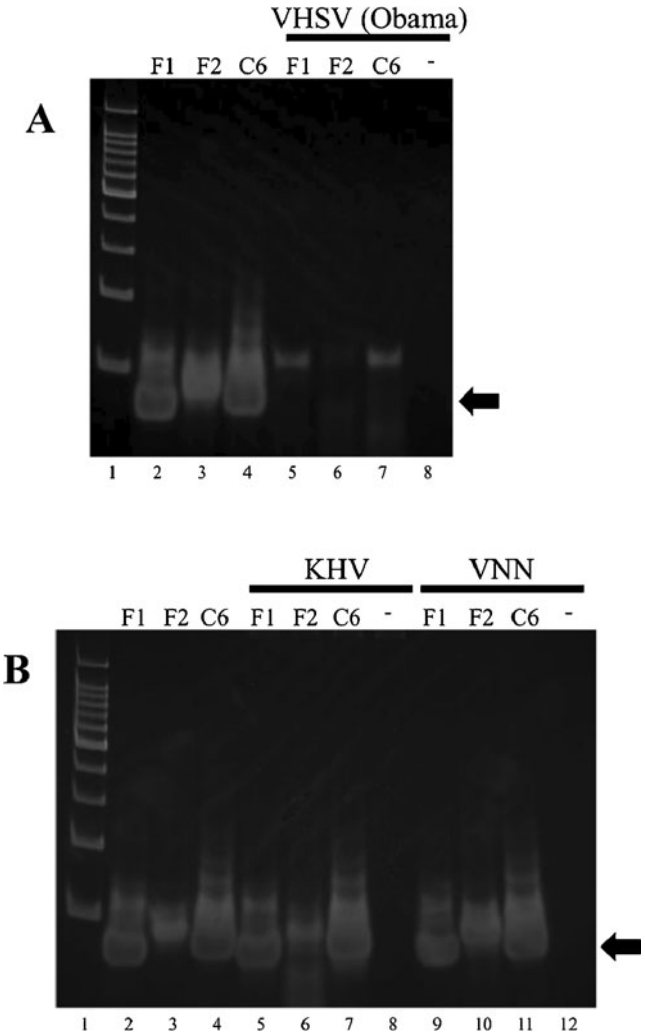

Fig. 3 EMSA of VHSV–RNA aptamers against other viruses. **a** VHSV strain Obama. **b** KHV and VNN. *Lanes 2–4* are control viruses that do not bind to RNA aptamers F1, F2, and C6, respectively. The residual free RNAs are indicated by *arrows*

and related sequences (F3, C6, and C8) are shown in Fig. 1b. Aptamers C6 and C8 can be grouped into a VHSV aptamer dominant cluster, although their structures were different from those of the dominant types F1 and F2. The predicted structures of C6 and C8 seemed to be similar, but the secondary structure of C6 was more stable than that of C8 because it had a lower free energy (ΔG). Therefore, the dominant sequences (F1 and F2) and the unique sequence C6 were selected as candidate RNA aptamers for viral inhibition experiments.

Binding of Selected VHSV–RNA Aptamers

The ability of each of the three selected RNA aptamers to bind to VHSV particles was determined by EMSA. RNA aptamers alone appeared as bands at the bottom of the gel (lanes 2 and 10) but, in the presence of viral particles, these bands disappeared, even when the amount of RNA aptamer per lane was as high as 25 μg (Fig. 2a–c, lanes 3–9), indicating that all candidate RNA aptamers definitely bound to VHSV. The three aptamers also bound to a different strain of VHSV (strain Obama) (Fig. 3a), but not to KHV or VNN (Fig. 3b).

Inhibition of VHSV Infection by RNA Aptamers in HINAE Cells

The three aptamers were tested in vitro by treating HINAE cells with an RNA aptamer–VHSV complex. The three

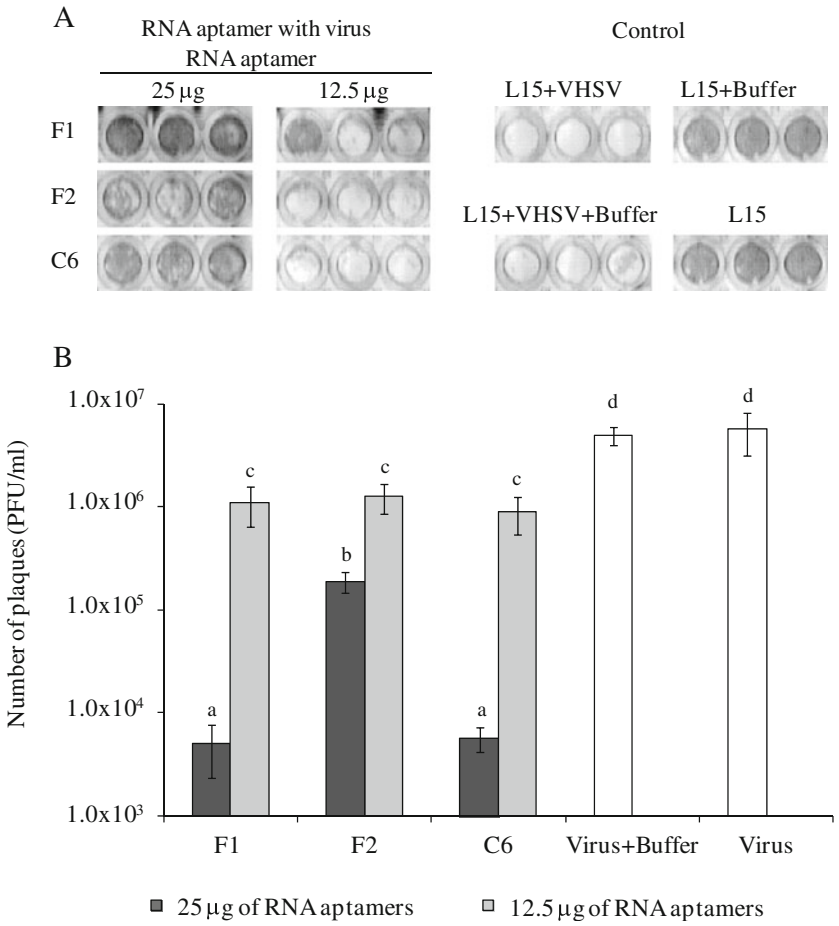

Fig. 4 Inhibition assay of VHSV–RNA aptamer candidates (F1, F2, and C6) in HINAE cells. **a** CPE of the HINAE cells (in a 96-well plate) treated with different concentrations of RNA aptamer–VHSV complex (25 and 12.5 μg) and controls 4 days post-infection. *Bright color* indicated infected cells and *dark color* indicated life cells. **b** Plaque-forming units/ml (PFU/ml) of the HINAE cells (in a six-well plate) treated with the previous supernatant (**a**) at 3 days post-infection. The standard deviation is indicated by error bars and the same superscripts signify values that are not significantly different at *P*-value > 0.01 ($N = 3$)

aptamers showed less CPE than positive controls (L15+VHSV only and L15+VHSV+binding buffer) (Fig. 4a), indicating that they were capable of inhibiting viral infection. Similarly, the plaque numbers were lower in the presence of the aptamers than in the presence of the positive controls ($P<0.01$) (Fig. 4b). The plaque numbers in HINAE cells treated with the RNA aptamer–VHSV complex ranged from 5.0×10^3 to 1.3×10^6 PFU/ml while the cells treated only with VHSV or VHSV+binding buffer were 5.7×10^6 and 5.0×10^6 PFU/ml. HINAE cells treated with 25 μg aptamers were significantly better protected than cells treated with 12.5 μg aptamers ($P<0.01$). At a higher aptamer concentration, the number of PFUs was significantly smaller with F1 and C6 than with F2 ($P<0.01$) (Fig. 4b). These results were consistent with those in Fig. 4a.

Detection of Extracellular RNA Aptamers in Culture Medium

Three pHSR2 plasmids encoding the RNA aptamers were prepared and transformed into *R. sulfidophilum*. The resultant *R. sulfidophilum*-Apt$^+$ strains produced extracellular RNA aptamers that were detected in the culture medium by RT-PCR. The PCR products amplified from the supernatant of the *R. sulfidophilum*-Apt$^+$ culture media were comparable to those of RNA synthesized in vitro using the T7 RiboMAX kit (Fig. 5a). In contrast, no such PCR products were obtained from the supernatants of *R. sulfidophilum* and *R. sulfidophilum*-SR2 cultures (Fig. 5a [right], lanes 5 and 6). The PCR products were confirmed to be VHSV–RNA aptamers by sequencing (data not shown). These results revealed that VHSV–RNA aptamers can be produced by *R. sulfidophilum* and can be released extracellularly.

Inhibition of VHSV Infection in Japanese Flounder by *R. sulfidophilum*-Generated RNA Aptamers

The ability of the VHSV–RNA aptamers to inhibit infection in Japanese flounder in vivo was investigated. At 2 days post-infection, 20 % mortality was observed in Japanese flounder treated with VHSV only and VHSV+*R. sulfidophilum*-SR2 (empty vector control) and cumulative mortality reached 90 % at 10 days post-infection. On the contrary, cumulative mortality was only 10 % in fish treated with

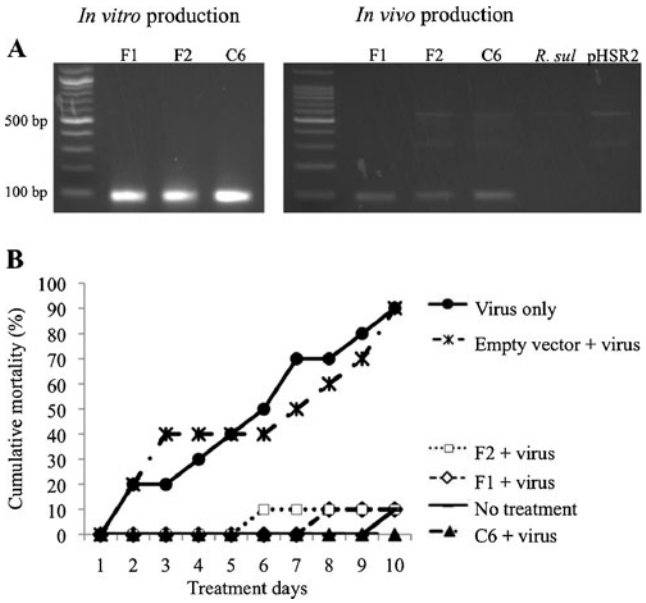

Fig. 5 Production of RNA aptamers by *R. sulfidophilum*. **a** RT-PCR of purified culture medium of *R. sulfidophilum* (*right*) compared with RNA produced in vitro using a T7 RiboMAX kit (*left*). *F1*, *F2*, and *C6*—*R. sulfidophilum*+corresponding aptamer; *R. sul*—*R. sulfidophilum* only; *empty*—culture medium only. **b** In vivo mortality test of *R. sulfidophilum*-Apt$^+$ and VHSV in Japanese flounder. Values are cumulative mortality for each treatment during the 10-day experimental period

VHSV+*R. sulfidophilum*-Apt$^+$ F1 or F2 at 6 and 8 days post-infection and it was 0 % in fish treated with VHSV+*R. sulfidophilum*-Apt$^+$ C6 (Fig. 5b).

Discussion

The C6 aptamer was selected because it clustered with the dominant F1 and F2 aptamers (Fig. 1a). C8 was also in this cluster, but it was not selected because its predicted secondary structure is less stable (i.e., has a higher free energy) than that of C6 (Fig. 1b). The presence of two or more aptamers for a specific target molecule has been observed in a previous study on various RNA aptamers (Stoltenburg et al. 2007).

Each of the three RNA aptamer candidates (F1, F2, and C6) inhibited the growth of VHSV in HINAE cells as shown by the appearance of CPE (Fig. 4a) and by the plaque assay (Fig. 4b). The inhibition efficiency depended on the concentration of RNA aptamers, suggesting that the RNA aptamers prevented the replication of VHSV in HINAE cells. Some RNA aptamers have the same inhibitory effect in human viruses, such as Rous sarcoma virus (Pan et al. 1995) and influenza viruses (Gopinath et al. 2006). Thus, the three RNA aptamers may be useful in therapeutic and diagnostic studies of VHSV.

Aptamers bind to their target molecules through various combinations of structure compatibility, stacking of aromatic rings, electrostatic and van der Waals interactions, and hydrogen bonding (Hermann and Patel 2000). One possibility is that the aptamers in the present study bound to the antigenic receptors of VHSV and this in turn prevented the virus from binding to the host. This was the case of a DNA aptamer that was found to block the receptor-binding region of influenza hemagglutinin, preventing the virus from invading the host cell (Jeon et al. 2004). Similarly, an RNA aptamer with affinity for human influenza A virus subtype H3N2 was found to inhibit hemagglutinin-mediated membrane fusion (Gopinath et al. 2006). Further studies are needed to determine the target molecule on VHSV that the RNA aptamers bind to.

The present results show that the VHSV–RNA aptamers specifically bind to VHSV but not to other viruses such as KHV and VNN (Figs. 2 and 3b) and were shown to bind to another VHSV strain (strain Obama) from the same genotype group (genotype IV) (Nishizawa et al. 2006) (Fig. 3a).

A human influenza DNA aptamer was also found to inhibit different influenza strains (Jeon et al. 2004). In contrast, an influenza RNA aptamer was specific to its target strain (A/Panama/2007/1999 [H3N2]) and failed to recognize other human influenza viruses (Gopinath et al. 2006). It is unknown whether the VHSV–RNA aptamers could bind to VHSV from other genotype groups. One possibility is that they bind to a viral glycoprotein similar to the way RNA aptamers bind to the variant surface glycoprotein in African trypanosomes (Lorger et al. 2003). If this is the case, there is also a need to identify which VHSV genotype the VHSV–RNA aptamers bind to because, based on the sequence variation of the central region (aa 245–300) of the VHSV G gene, there are now four known VHSV genotypes (I, II, III, and IV) correlating with different geographical areas (continental Europe, Baltic Sea, British Isles, and North America, respectively) (Nishizawa et al. 2006; Kim and Faisal 2011).

Our results also demonstrate the utility of using the intact virus particle as a target molecule for application purposes. Pan et al. (1995) found at least three advantages of using intact virus rather than an isolated viral protein in selecting aptamers: (1) RNA selected by a viral protein may not interact with the protein complexed with a virion because of structural differences in the protein, (2) RNA selection by an intact virus does not require a full understanding of the usually very complex mechanism of viral infection, whereas selection by an isolated viral protein is limited to only a few cases in which the proteins responsible for the viral infection have been identified and isolated, and (3) RNA selection by an intact virus may lead to the identification of viral components that have not previously been known for their critical roles in viral infection. Another advantage of using

an intact virus is that intact viruses are easier and less costly to use than purified antigenic proteins.

Our results show that the three aptamers are promising candidates for inhibiting viral infection in vivo. By using the phototrophic marine bacterium *R. sulfidophilum*, which has the potential to secrete nucleic acids, it should be possible to produce VHSV–RNA aptamers on a large scale (Suzuki et al. 2010). Here we confirm that *R. sulfidophilum* can release RNA aptamers into the culture medium (Fig. 5a) and that the aptamers have the ability to inhibit viral infection in Japanese flounder (Fig. 5b). The extracellular RNA aptamers probably block viral infection by binding to viral particles in the sea water. Other advantages of *R. sulfidophilum* are that it does not produce any ribonucleases in the culture medium (Suzuki et al. 2010) and does not appear to have any toxic effects on Japanese flounder (data not shown).

In summary, this paper reported the identification of RNA aptamers that specifically bind to VHSV and showed their ability to prevent viral infection in vitro. This is the first report on the in vivo inhibitory ability of RNA aptamers produced by a microorganism. Our results show that RNA aptamers are promising candidates for antiviral agents for marine-cultured species.

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