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In-vitro inhibition of spring viremia of carp virus replication by RNA interference targeting the RNA-dependent RNA polymerase gene

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ABSTRACT

Spring viremia of carp, a fatal viral disease, is caused by the spring viremia of carp virus (SVCV) and can result in up to 70% mortalities in common carps and significant economic losses in several other cyprinid aquaculture. The present study aimed to investigate the possible control of SVCV replication in *Epithelioma papulosum cyprini* (EPC) cells using the RNA interference technology targeting the RNA-dependent RNA polymerase (L) gene of the SVCV that is essential for its replication. Three stealth small interfering RNA (siRNA) sequences were designed to target three different regions on the SVCV-L gene. The specific siRNAs designed were investigated individually or in combinations to inhibit the SVCV-L gene expression and the virus replication. Results showed that the most effective siRNA sequence was the siRNA-602 that specifically reduced the SVCV replication by two logs as indicated by the virus titration and quantitative real-time PCR. Results, also, showed that the minimum effective concentration of siRNA-602 was 20 nM when used to transfect the EPC cells before the virus inoculation. Results of this study clearly indicate that targeting the SVCV-L gene by RNAi can reduce the SVCV replication *in vitro*, that may lead to the control of SVCV in fish.

1. Introduction

Spring Viremia of Carp (SVC) is a notifiable, serious, contagious viral disease that affects common carp (*Cyprinus carpio*) and several other cyprinids and non-cyprinids fishes causing significant economic losses worldwide (Ahne et al., 2002). SVC causes significant morbidity and mortality in carp culture in Europe, Asia, North America and Middle East (Padhi and Verghese, 2008). In Egypt, SVCV was isolated from Nile tilapia (*Oreochromis niloticus*) the most important cultured fish species in Egypt, which has never been reported as a host for SVCV (Soliman et al., 2008). Externally, the major clinical signs of SVC are petechiae on the gills and skin with abdominal distension and inflammation of the vent, while internally, there are ascites, petechial hemorrhages of swim-bladder wall and skeletal muscle (Ahne, 1978).

The causative agent is the spring viremia of carp virus (SVCV), a negative sense single-stranded RNA virus that belongs to family *Rhabdoviridae* (Bachmann and Ahne, 1973). SVCV genome is about 11.019 kb in size that codes for five proteins which are nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA dependent RNA polymerase (L) respectively from 3' to 5' direction (Hoffmann et al., 2002). The RNA-dependent RNA polymerase, L-protein, is responsible for the synthesis of complementary RNA in infected cells and initiation of the virus replication (Bishop and Flamand, 1975). The virus is grown on cyprinid cell lines such as *Epithelioma papulosum cyprini* (EPC) and fathead minnow (FHM) at 20–25 °C where the cytopathic effects (CPE) appear as margination of nuclear chromatin followed by rounding up and lysis of cells (Fijan et al., 1983).

To our knowledge, no antiviral drugs are available to control the SVC; and the vaccines developed did not provoke an efficient protection against the virus (Emmenegger and Kurath, 2008; Kanellos et al., 2006). The current strategy to prevent and control SVCV relies primarily on detection and depopulation of the infected fish.

RNA interference (RNAi) is a post-transcriptional gene silencing strategy where double-stranded RNA (dsRNA) directs sequence-specific degradation of messenger RNA through cellular specific mechanism (Gitlin et al., 2002). RNAi can be triggered by 21–23 nucleotide duplexes of small interfering RNA (siRNA) that induce the degradation of mRNA, resulting in depletion of the encoded protein (Elbashir et al., 2001; Fire et al., 1998). The biological functions of RNAi are to regulate the gene expression and to protect the host cells from invading viruses,

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where it can inhibit the expression of crucial viral proteins by degrading targeted viral mRNA (Chen et al., 2004). RNAi-based technology was found to be useful protecting fish from diseases caused by viral and parasitic agents (Lima et al., 2013; Ruiz et al., 2009; Schyth et al., 2007; Tirasophon et al., 2007; Xu et al., 2007).

The present study aimed to investigate the possible control of SVCV replication in EPC cells by targeting the SVCV-L gene using siRNA as an *in-vitro* trial to control the SVCV infections; the minimum effective concentration and the best time for applying the siRNA were, also, determined.

2. Materials and methods

2.1. Propagation of the SVCV

The SVCV strain A-59/06 was kindly provided by the Austrian National Reference Laboratory of Fish Viruses, Clinical Division of Fish Medicine, University of Veterinary Medicine, Vienna, Austria. The EPC cells (Fijan et al., 1983) were grown in 25 cm^2 tissue culture flasks (Greiner Bio-One GmbH, Germany) with growth media of Eagle's minimal essential medium, MEM, with GlutaMax (Gibco, Life Technologies, UK) and supplemented with 10% fetal bovine serum, FBS (Gibco). Cells were incubated at 20 °C in a cooling incubator (Binder GmbH, Germany) till the formation of monolayer sheets.

For propagation of the SVCV, confluent EPC monolayers were washed with MEM and 1 mL of the SVCV suspension was then spread over the cells monolayer for 1 h at 15 °C. The viral suspension was then removed and 5 mL of MEM with 2% FBS (complete media) was added; the EPC monolayers inoculated were incubated at 15 °C and examined daily for the development of cytopathic effects (CPE). When CPE reached 70%, the supernatant containing the virus was harvested and centrifuged at 2000 xg for 5 min to remove any cell debris to be titrated. Tissue culture infective dose 50 ($TCID_{50}$) of the SVCV was calculated according to Reed and Muench (1938) and aliquoted before storage at −80 °C for future use. The virus identity was confirmed by amplifying a 369-bp segment according to Chimahara et al. (2016) using the One-Step RT-PCR Kit (Qiagen, Germany).

2.2. Design of the SVCV-L gene-specific siRNA

Various sequences of the SVCV-L gene obtained from the GenBank were aligned using the multiple sequence alignment software "Clustal W" to identify the conserved regions in the gene, which were subsequently used to design the stealth siRNAs by the Invitrogen Block-iT RNAi designer software. Out of ten stealth siRNAs designed, three were selected based on their target locations on the SVCV-L gene and synthesized by Invitrogen, USA as sense and antisense RNA strands (Table 1). The three specific siRNAs selected were named after the position of their target's 1st bp from the 5′ end of the L-gene as siRNA-602, siRNA-2605, and siRNA-5453. Moreover, three corresponding scrambled stealth siRNA sequences were designed (with base-pair mismatches to the same specific target selected above) to be used as controls (Table 1), and were named for their corresponding specific siRNA counterparts. Stealth siRNAs were suspended in 1% diethylpyrocarbonate-treated water and heated for 1 min at 90 °C for denaturation before incubation at 37 °C for 60 min for annealing to form siRNA duplexes, thereafter allowed to cool to room temperature and aliquoted for use.

2.3. SVCV-L gene-specific oligonucleotides and real-time PCR

GenBank deposited sequences of SVCV-L gene were aligned using "Clustal W" software to identify a conserved region that is suitable for designing of SVCV-L gene-specific primers and a TaqMan probe. Two primers designated as, SVC-L-qRT-forward (5'-AGG AAG TGG ATT TAT GCG AGG-3') and SVC-L-qRT-reverse (5'- GAT TGG TCA GAT ACT GTG CTC C-3') were designed to amplify a 134-bp fragment of the SVCV–L gene. To confirm the specificity of the amplified product, a TaqMan probe SVC-L-qRT-probe (5'-[FAM]TGT GAT GGG ATT ATA AGG GCC AGT GC[QSY]-3') labelled with 6-carboxyfluorescein [FAM] at the 5' end as a reporter fluorophore and with [QSY] at the 3`end as a quencher, was designed for inclusion in the PCR assay. These primers were used in a quantitative real-time PCR assay that was specifically developed to estimate the virus copy numbers throughout this study.

Table 1
Stealth small interfering RNA oligos designed to target the spring viremia of carp virus-L gene and the scrambled controls.

siRNA Name	Sequence (5' to 3')
Stealth- siRNA-602-sense	CGAUACUGUCCAGGAUGGAUCUUUA
Stealth- siRNA-602-antisense	UAAAGAUCCAUCCUGGACAGUAUCG
Stealth-siRNA- 602- scrambled - sense	CGAUGUCGACCGUAGCUAGUAUUUA
Stealth-siRNA-602- scrambled - antisense	UAAAUACUAGCUACGGUCGACAUCG
Stealth- siRNA-2605- sense	GACACUGUAUCUGGAUCCUUCAUUA
Stealth-siRNA- 2605- antisense	UAAUGAAGGAUCCAGAUACAGUGUC
Stealth- siRNA-2605- scrambled - sense	GACUGUCUAGGUCUAUUCACACUUA
Stealth-siRNA -2605- scrambled -antisense	UAAGUGUGAAUAGACCUAGACAGUC
Stealth-siRNA-5453- sense	CAGACUUCAGAAGUGUACUGUGUUA
Stealth-siRNA-5453- antisense	UAACACAGUACACUUCUGAAGUCUG
Stealth-siRNA-5453- scrambled- sense	CAGCUUAGAUGAAUGGUCGUACUUA
Stealth-siRNA-5453 - scrambled - antisense	UAAGUACGACCAUUCAUCUAAGCUG

Briefly, SVCV total RNA (1 µg) was reverse transcribed using Omniscript Reverse Transcription kit (QIAGEN) to obtain cDNA. A standard curve for the quantitative real-time PCR was generated using 10-fold serial dilution of 8.41×10^9 SVCV copies. The TaqMan real-time PCR assay was carried out in a final volume of 25 µL using QuantiTect Probe PCR kit, 2.5 µL of cDNA template10 µM of each of the SVC-L-qRT-forward and reverse primers, and 3 µM TaqMan probe. The cycling profile was set as initial activation step at 95 °C for 15 min and followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s in Mx3005 P real-time QPCR detection system (Stratagene, USA). The default settings of the program were used to define both the baseline and the threshold value and the fluorescent data were collected during the 60 °C step. Regression analysis, standard curve slopes, and amplification efficiencies were calculated using the MxPro-Mx3005 P software (v4.10 Build 389 Schema 85, Stratagene) supplied with the system. The standard curve generated from the cycle threshold (Ct) values obtained by amplification was used to estimate the SVCV copy numbers in the samples.

2.4. Optimizing the transfection protocol

Lipofectamine 3000 (Invitrogen, USA) was used for transfecting the siRNAs into EPC cells according to the manufacturer's protocol. To prepare the transfection complexes, each stealth siRNA duplex and the lipofectamine 3000 were separately diluted in Opti-MEM (Gibco), mixed together in equal amounts, incubated for 5 min at room temperature, and aliquoted in 50 u L volumes for use. Concentrations were adjusted to reach 1 u L of lipofectamine 3000 and 20 nM of stealth siRNA as final concentrations per 1 mL when 50 u L of the transfection complex was used to transfect the EPC monolayers. When combination treatments were used, the siRNA sequences were mixed in equal amounts and the final concentration of each siRNA used was adjusted to be 20 nM in the transfection complex.

2.5. SVCV-L gene knockdown

The three-specific stealth siRNA were used for the transfection experiments individually or in combinations. All experiments were

carried out in triplicate wells of 24-well plates with a final volume of 1 mL well^{-1}. At first, 50 µL of the siRNA-lipofectamine 3000 transfection complex were added to each well, and consequently, 3×10^5 EPC cells were seeded in each well with growth media; and the plates were then incubated at 20 °C for 18 h. The transfected, confluent monolayers were washed with MEM and inoculated with $2 \times 10^{6.83}$ mL^{-1} SVCV for 1 h at 15 °C as described above. The virus suspension was then removed and replaced with 1 mL of complete media, and plates were incubated at 15 °C and examined daily for CPE. For each specific stealth siRNA treatment (individual or combined), the following controls were included: (1) EPC cells transfected with the corresponding scrambled siRNA and inoculated with the SVCV, (2) EPC cells inoculated with the SVCV, (3) EPC cells transfected with that specific siRNA, (4) EPC cells transfected with the corresponding scrambled siRNA, and (5) EPC cells. Three days after incubation, 140 µL of the cell culture supernatant from each well of all treatments were sampled for virus titration as per Reed and Muench (1938). Virus copy numbers quantification by real-time PCR was used as a confirmatory procedure for the best three sequences (individual or combined) giving the highest inhibition of virus replication according to the titration results. Briefly, RNA was extracted from the cell culture supernatant (after centrifugation at 2000 xg for 5 min to remove any cell debris) using QIAamp Viral RNA Mini kit (Qiagen, Germany), and cDNA was then obtained by reverse transcription-polymerase chain reaction (RT-PCR) using OmniScript RT-PCR Kit. The cDNA obtained was used as template in the quantitative real-time PCR assay to estimate the virus copy numbers using the primers designed and TaqMan probe as described above.

2.6. Determining the effective concentration of the selected stealth RNAi-siRNA sequence

Various concentrations of the stealth siRNA-602 were tested to select the most effective concentrations that induce the highest inhibitory effects on the SVCV L-gene expression and virus replication. Three different concentrations (10 nM, 20 nM, and 40 nM mL^{-1}) were investigated with their corresponding scrambled and virus controls in triplicates in 24-well tissue culture plates using the same protocol described above. Three days after incubation, the cell culture supernatant was sampled for virus titration and virus copy number quantification by real-time PCR as described above.

2.7. Determining the best time to transfect the EPC cells in relation to the time of infection

A study was conducted to investigate the silencing efficacy of the stealth siRNA when applied either before, after, or simultaneously with the virus inoculation. All experiments were carried out in triplicates in 24-well plates with a final volume of 1 mLwell^{-1} using 1 u L of lipofectamine 3000 and 20 nM of the stealth siRNA-602 as final concentrations, where scrambled and virus controls were also used.

In case of transfecting the EPC monolayers with the stealth siRNA-602 before the virus inoculation, the experiment was done as previously described in the SVCV L-gene knockdown section. To investigate the silencing efficacy when stealth siRNA-602 transfects the EPC monolayers after the virus inoculation, the EPC monolayers were washed and inoculated with the SVCV at 15 °C for 1 h as mentioned above. Afterward, the SVCV suspension was removed and 50 u L of the stealth siRNA-transfection complex was added drop-wise to each well with 1 mL of complete MEM media. Plates were incubated at 15 °C and examined daily for CPE. To transfect the EPC monolayers with the stealth siRNA simultaneously with the virus inoculation, the EPC monolayers were washed and inoculated with the SVCV with the stealth siRNA-transfection complex at the same time and incubated at 15 °C for 1 h. Afterward, the SVCV suspension was removed and 1 mL of complete media was added to each well and incubated at 15 °C. Three days after incubation, the cell culture supernatant from each well of all treatments was sampled for virus titration and virus copy number quantification by real-time PCR as described above.

2.8. Statistical analysis

All experiments were repeated three times and the experimental design was completely randomized. One-way ANOVA was used to analyze the data of virus titers and copy numbers to measure the inhibition of virus replication for all treatments. When the overall model indicated significance at $P < 0.05$, Dunn's multiple comparison test was used for pairwise comparison of means at $P < 0.05$. All analyses were performed using programmed Graph Pads Prism® 5 Software (version 5.01).

3. Results

3.1. Establishing a quantitative real-time PCR assay

The standard curve of the TaqMan real-time PCR assay for the SVCV-L gene was established by using 10-fold serial dilutions of SVCV in triplicates. Amplification of SVCV cDNA at different concentrations showed linearity over a range of six orders of magnitude. The standard curve showed a linear correlation between the cDNA input and Ct values of SVCV copy numbers. The regression analysis yielded a correlation coefficient of 1.000 and a slope of -3.366 indicating amplification efficiency of 98.2%. The standard curve was highly reproducible with no significant differences in slopes between different runs of the same assay.

3.2. SVCV-L gene knockdown

Any specific siRNA sequence (individual or combined) was considered effective only when it results in significant reduction in the SVCV titers when compared to its both scrambled and virus controls. Additionally, any specific siRNA sequence (individual or combined) was considered specific in reducing the SVCV replication only when its corresponding scrambled control sequence results in insignificant differences in the SVCV titers when compared to the virus control.

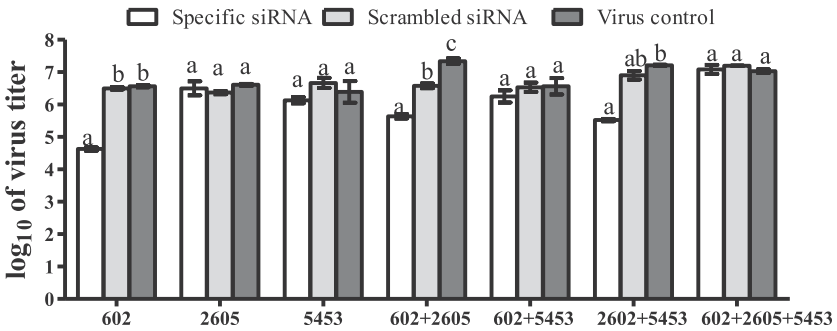

Fig. 1. Inhibition of the spring viremia of carp virus (SVCV) replication in *Epithelioma Papulosum Cyprini* cells transfected with 20 nM of siRNA sequences. Values are the mean of 3 experiments and represent $\log_{10}$ of SVCV titer ± standard deviation. Means of the same siRNA treatment with different letter designation are significantly different at $P < 0.05$.

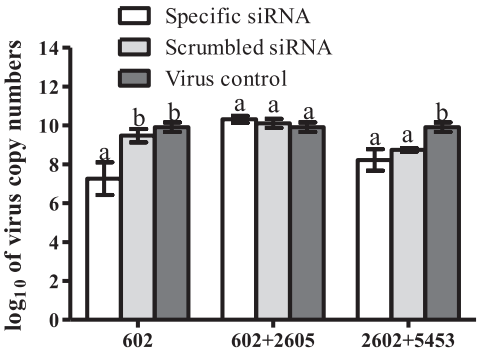

Fig. 2. Inhibition of the spring viremia of carp virus (SVCV) replication in *Epithelioma Papulosum Cyprini* cells transfected with 20 nM of siRNA sequences. Values are the mean of 3 experiments and represent $\log_{10}$ of SVCV copy numbers ± standard deviation quantified by real-time PCR. Means of the same siRNA treatment with different letter designation are significantly different at $P < 0.05$.

According to these criteria, only siRNA-602 has specifically interfered with the expression of the SVCV-L gene and consequently inhibited virus replication as indicated by the significant decrease in SVCV titer (Fig. 1) and copy numbers (Fig. 2). On the other hand, siRNA 602 + 2605 combination showed significant, but non-specific, reduction in the SVCV titration, as their corresponding scrambled sequences resulted in significant reduction in the virus titers when compared with the virus control. Furthermore, siRNA 2605 + 5453 combination showed high, but insignificant, reduction in the virus titers. Virus copy numbers quantification for treatments siRNA 602 + 2605 and siRNA 2605 + 5453 combinations are shown in Fig. 2. All other siRNA sequences used in this study either individually or in combination treatments were ineffective in silencing the L-gene, where they resulted in insignificant differences in the virus titers when compared to their controls (Fig. 1).

3.3. Determining the effective concentration of the siRNA-602

The most effective concentrations of siRNA-602 that significantly reduced the virus titers and copy numbers were the 20 nM and 40 nM (Figs. 3 and 4). The lower concentration, 10 nM, was ineffective in reducing both the virus titers and copy numbers (Figs. 3 and 4).

3.4. Determining the best time to transfect the EPC cells in relation to the time of infection

Applying the siRNA-602 before virus inoculation significantly

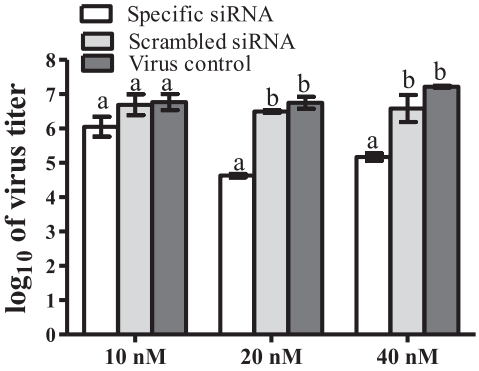

Fig. 3. Inhibition of the spring viremia of carp virus (SVCV) replication in *Epithelioma Papulosum Cyprini* cells transfected with various concentrations of siRNA-602 sequence. Values are the mean of 3 experiments and represent $\log_{10}$ of SVCV titer ± standard deviation. Means of the same siRNA concentration with different letter designation are significantly different at $P < 0.05$.

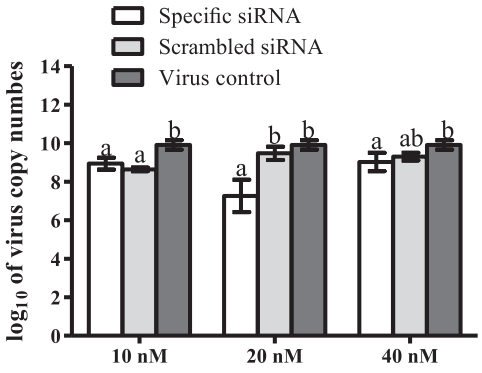

Fig. 4. Inhibition of the spring viremia of carp virus (SVCV) replication in *Epithelioma Papulosum Cyprini* cells transfected with various concentrations of siRNA-602. Values are the mean of 3 experiments and represent $\log_{10}$ of SVCV copy numbers ± standard deviation quantified by real-time PCR. Means of the same siRNA concentration with different letter designation are significantly different at $P < 0.05$.

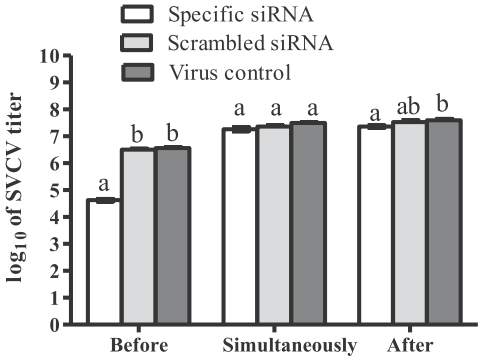

Fig. 5. Inhibition of the spring viremia of carp virus (SVCV) replication in *Epithelioma Papulosum Cyprini* cells transfected with 20 nM of siRNA sequences either before, after, or simultaneously with the virus inoculation. Values are the mean of 3 experiments and represent $\log_{10}$ of SVCV titer ± standard deviation. Means of the same siRNA application time with different letter designation are significantly different at $P < 0.05$.

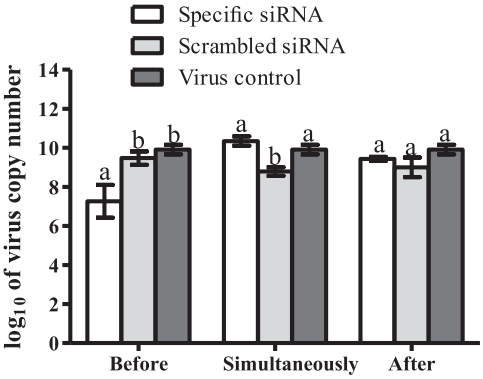

Fig. 6. Inhibition of the spring viremia of carp virus (SVCV) replication in *Epithelioma Papulosum Cyprini* cells transfected with 20 nM of siRNA either before, after, or simultaneously with the virus inoculation. Values are the mean of 3 experiments and represent $\log_{10}$ of SVCV copy number quantified by real-time PCR. Means of the same siRNA application time with different letter designation are significantly different at $P < 0.05$.

reduced the virus titer and copy numbers (Figs. 5 and 6). Conversely, the application of the siRNA-602 either with or after the virus inoculation did not significantly affect the virus titer nor the copy numbers (Figs. 5 and 6).

4. Discussion

The present study proved that the *in-vitro* replication of the SVCV in EPC cells can be controlled by targeting the RNA polymerase (L) gene using the RNAi technology. The RNAi became a promising antiviral strategy that may suppress the transcription of target viral genes (Yoshinouchi et al., 2003), or even blocks the viral replication (Hamasaki et al., 2003), and thus, is considered one of the interesting approaches to control viral infections of many viruses (Denli et al., 2004; Sui et al., 2009).

The L-gene was selected in the present study as a target for siRNA interference to reduce the virus replication, as it is necessary for the synthesis of complementary RNA in infected cells to initiate the virus replication (Bishop and Flamand, 1975). Thus, any interference with the expression of the L-gene was expected to result in a reduction in the virus replication in host cells. A previous study demonstrated that targeting of SVCV-N and SVCV-P gene expression by siRNA reduced SVCV replication in EPC cell line (Gotesman et al., 2014). The authors reported that the reduction in mRNA transcripts levels for N-gene was 25%, whereas it was 55% for the P-gene when targeted by specific siRNAs. Results of the present study, however, showed that targeting the L-gene can greatly reduce the viral replication in EPC cells by about two logs (~ 99%), and this may be because of its critical role in SVCV replication (Bishop and Flamand, 1975).

In the present study, the new-generation of siRNAs, stealth siRNA, that are 25 bp in length, chemically modified blunt-ended RNA duplexes were used to inhibit the SVCV replication in EPC cells. One of the main advantages of stealth siRNA over the regular siRNA is that it can minimize the immune responses or interferon induction (Schepers, 2006) as non-specific anti-viral effects (Bridge et al., 2003; Sledz et al., 2003). In addition, the modifications on the sense strand ensure that only the anti-sense strand is utilized by the RNA induced silencing complex (RISC) to degrade a target RNA. This decreases the potential degradation of off-target RNA sequences that could happen by partial homology of the sense strand with unselected targets (www.thermofischer.com).

The use of more than one potential siRNA sequence is an essential element of investigations deploying the RNAi technology (Lenz, 2005). It is appropriate to show that two or, preferably, three specific siRNAs can target different positions of the mRNA selected, each of which can specifically knock down the encoded protein expression (Whither, 2003). In the present study, more than ten siRNA sequences were designed, but only three siRNAs and their scrambled sequences of control were selected to study their interfering effects on the replication of SVCV virus.

The three siRNAs duplexes selected were used either as an individual treatment or in combinations with each other to investigate their interfering activities with the L-gene expression. When used as an individual treatment, the 602-siRNA duplex induced a specific degradation of the SVCV-L gene at the target location causing a reduction in the SVCV replication in EPC cells as indicated by the significant decrease in the virus titers and copy numbers when compared to the controls. The other two siRNA duplexes (siRNA-2605 and 5453) were excluded from any further investigations because of their ineffective and/or non-specific reduction in the virus replication when used either as an individual or in combination treatments.

The target site of the 602-siRNA sequence is near the 5′ end of the mRNA of the L-gene and degrading the RNA at this location may have a detrimental impact on the protein expression, while the target site for siRNA-2605 and 5453 are near the middle and 3′ end of the L gene respectively. The differences in the efficacy among the three siRNAs selected to silence the L gene may be attributed to the different position that each of them targets on the mRNA as was previously suggested by Holen et al. (2002). Furthermore, the differences in silencing efficacy of several siRNAs targeting different locations of the same gene mRNA is a well-known phenomenon (Bohula et al., 2003; Harborth et al., 2001; Vickers et al., 2003) and was described as “striking” by Holen et al. (2002). The local structure of the mRNA at the target position may affect the accessibility of the siRNA (Bohula et al., 2003).

Although all combined siRNA sequences, used in this study, target two or more loci on the L-gene mRNA, they resulted in insignificant or non-specific reductions in the virus replication. This may be explained by our finding that each siRNA sequence component of the combination treatment was ineffective or non-specific on its own. By contrast, other studies demonstrated that using combinations of different siRNAs enhances the knock-down of the target genes (Elbashir et al., 2001; Jingmin et al., 2003). Interestingly, the use of siRNA-602 in combination treatments resulted in either insignificant or non-specific reduction in the virus replication, despite the fact that it was effective as an individual treatment. This may be because of the sequence-independent reverse competitive effects that reduce the activity of the effective siRNA sequence when co-transfected with an ineffective siRNA into cells (Holen et al., 2002). Furthermore, in the combination treatments, double or even triple the optimum amount of the siRNA is used which may be ineffective.

Scrambled siRNAs sequences that contain nucleotides mismatch in their critical region to a specific mRNA target were used as negative controls for all RNAi experiments. Scrambled siRNA are incapable of specific binding to the complementary mRNA targets and, thus, cannot induce specific degradation of that target sequences (Cullen, 2006). A single mismatch can moderately reduce the effect of siRNA, while a double mismatch can cripple the mRNA degradation (Holen et al., 2002). On the other hand, the decrease in virus replication noticed with the application of some scrambled controls (particularly in combinations) may be due to considerable complementarity that the scrambled siRNA may have to other non-target mRNAs as was suggested by Birmingham et al. (2006) and Jackson et al. (2003) or due to tolerance of the RNAi machinery to base mismatch between the siRNA and its target mRNA by one or two bases (Holen et al., 2002).

It is important to transfect the minimum amount of the siRNA duplex that gives rise to a specific RNAi response (Cullen, 2006) to minimize or even totally avoid the off-target effects (Persengiev et al., 2004; Semizarov et al., 2003). Transfecting EPC cells with siRNA concentrations greater than 100 nM or lower than 10 nM frequently produce nonspecific off-target effects (Cullen, 2006). In the present study, the minimum concentration of the siRNA-602 that produced an effective reduction in the SVCV replication was 20 nM, as there were no significant differences in virus replication inhibition when 40 nM was used. On the other hand, the lower concentration of siRNA (10 nM) was ineffective as it might not be sufficient to reduce the gene expression.

Interestingly, a significant reduction in SVCV titers and copy numbers were only seen when siRNA-602 transfected the EPC cells before the SVCV inoculation; but was not seen when siRNA-602 transfected the EPC cells after or with the virus inoculation. This reduction in SVCV replication may be due to the formation of RISCs in the cytoplasm and being ready to degrade the L-gene RNA and control the virus replication when it starts. On the other hand, other studies concluded that the siRNA-derived inhibition of virus replication in cells pre-infected with viruses was more effective (Song et al., 2003), or even more long-lasting (Liu et al., 2005) than in cells transfected with the siRNA before virus inoculation.

5. Conclusion

In conclusion, the present study demonstrated that targeting of SVCV-L gene with 20 nM stealth siRNA-602 causes *in-vitro* reduction of SVCV replication in EPC cell line. The present study takes the RNAi-based antiviral strategy a step forward towards the control of SVCV and accordingly reduce the economic impact of this virus on the carp aquaculture.

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References

Ahne, W., 1978. Uptake and multiplication of spring viremia of carp virus in carp, Cyprinus carpio. L. J. Fish Dis. 1, 265–268.
Ahne, W., Bjorklund, H.V., Essbauer, S., Fijan, N., Kurath, G., Winton, J.R., 2002. Spring viremia of carp (SVC). Dis. Aquat. Org. 52, 261–272.
Bachmann, P.A., Ahne, W., 1973. Isolation and characterization of agent causing swim bladder inflammation in carp. Nature 244, 235–237.
Birmingham, A., Anderson, E.M., Reynolds, A., Ilsley-Tyree, D., Leake, D., Fedorov, Y., Baskerville, S., Maksimova, E., Robinson, K., Karpilow, J., Marshall, W.S., Khvorova, A., 2006. 3′ UTR seed matches, but not overall identity, are associated with RNAi off-targets. Nat. Methods 3.
Bishop, D.H.L., Flamand, A., 1975. Transcriptional process of animal RNA viruses. In: Burke, D.C., Russell, W.C. (Eds.), Society of General Microbiology, Symposium 25, Control Processes in Virus Multiplication. Cambridge University Press, Cambridge, pp. 95–152.
Bohula, E.A., Salisbury, A.J., Sohail, M., Playford, M.P., Riedemann, J., Southern, E.M., Macaulay, V.M., 2003. The efficacy of small interfering RNAs targeted to the type 1 insulin-like growth factor receptor (IGF1R) is influenced by secondary structure in the IGF1R transcript. J. Biol. Chem. 278, 15991–15997.
Bridge, A.J., Pebernard, S., Ducraux, A., Nicoulaz, A.L., Iggo, R., 2003. Induction of an interferon response by RNAi vectors in mammalian cells. Nat. Genet. 34, 263–264.
Chen, W., Yan, W., Du, Q., Fei, L., Liu, M., Ni, Z., Sheng, Z., Zheng, Z., 2004. RNA interference targeting VP1 inhibits foot-and-mouth disease virus replication in BHK-21 cells and suckling mice. J. Virol. 78, 6900–6907.
Chimahara, Y., Kurita, J., Nishioka, T., Kiryu, I., Yuasa, K., Sakai, T., Oseko, N., Sano, M., Dixon, P., 2016. Development of an improved RT-PCR for specific detection of spring viraemia of carp virus. J. Fish Dis. 39, 269–275.
Cullen, B.R., 2006. Enhancing and confirming the specificity of RNAi experiments. Nat. Methods 3, 677–681.
Denli, A.M., Tops, B.B., Plasterk, R.H., Ketting, R.F., Hannon, G.J., 2004. Processing of primary microRNAs by the microprocessor complex. Nature 432, 231–235.
Elbashir, S.M., Lendeckel, W., Tuschl, T., 2001. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. 15, 188–200.
Emmenegger, E.J., Kurath, G., 2008. DNA vaccine protects ornamental koi (Cyprinus carpio koi) against North American spring viremia of carp virus. Vaccine 26, 6415–6421.
Fijan, N., Sulimanovic, D., Bearzotti, M., Muzinic, D., Zwillenberg, L.O.Z., Chilmonczyk, S., Vautherot, J.F., Kinkelin, P., 1983. Some properties of the epithelioma papulosum cyprini (EPC) cell line from carp cyprinus carpio. Ann. Virol. (Inst. Pasteur) 134, 207–220.
Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double stranded RNA in Caenorhabditis elegans. Nature 39, 1806–1811.
Gitlin, L., Karelsky, S., Andino, R., 2002. Short interfering RNA confers intracellular antiviral immunity in human cells. Nature 418, 430–434.
Gotesman, M., Soliman, H., Besch, R., El-Matbouli, M., 2014. Inhibition of spring viraemia of carp virus replication in an Epithelioma papulosum cyprini cell line by RNAi. J. Fish Dis. 38, 197–207.
Hamasaki, K., Nakao, K., Matsumoto, K., Ichikawa, T., Ishikawa, H., Eguchi, K., 2003. Short interfering RNA-directed inhibition of hepatitis B virus replication. FEBS Lett. 543, 51–54.
Harborth, J., Elbashir, S.M., Bechert, K., Tuschl, T., Weber, K., 2001. Identification of essential genes in cultured mammalian cells using small interfering RNAs. J. Cell. Sci. 114, 4557–4565.
Hoffmann, B., Schutze, H., Mettenleiter, T.C., 2002. Determination of the complete genomic sequence and analysis of the gene products of the virus of Spring Viremia of Carp, a fish rhabdovirus. Virus Res. 84, 89–100.
Holen, T., Amarzguioui, M., Wiiger, M.T., Babaie, E., Prydz, H., 2002. Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. Nucleic Acids Res. 30, 1757–1766.
Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Linsley, P.S., 2003. Expression profiling reveals off-target gene regulation by RNAi. Nat. Biotechnol. 21, 635–637.
Jingmin, J.I., Marion, W., Thomas, K., Peter, E., 2003. Enhanced gene silencing by the application of multiple speci¢c small interfering RNAs. FEBS Lett. 552, 247–252.
Kanellos, T., Sylvester, I.D., D'Mello, F., Howard, C.R., Mackie, A., Dixon, P.F., Chang, K.C., Ramstad, A., Midtlyng, P.J., Russell, P.H., 2006. DNA vaccination can protect *Cyprinus carpio* against spring viraemia of carp virus. Vaccine 24, 4927–4933.
Lenz, G., 2005. The RNA interference revolution. J. Med. Biol. Res. 38, 1749–1757.
Lima, P.C., Harris, J.O., Cook, M., 2013. Exploring RNAi as a therapeutic strategy for controlling disease in aquaculture. Fish Shellfish Immunol. 34, 729–743.
Liu, M., Chen, W., Ni, Z., Yan, W., Fei, L., Jiao, Y., Zhang, J., Du, Q., Wei, X., Chen, J., Liu, Y., Zheng, Z., 2005. Cross-inhibition to heterologous foot-and-mouth disease virus infection induced by RNA interference targeting the conserved regions of viral genome. Virology 336, 51–59.
Padhi, A., Verghese, B., 2008. Detecting positively selected codons in the glycoprotein of spring viraemia of carp virus (SVCV) isolates from the USA and China. J. Fish Dis. 31, 785–791.
Persengiev, S.P., Zhu, X., Green, M.R., 2004. Nonspecific concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). RNA 10, 12–18.
Reed, L.J., Muench, H., 1938. A simple Method of estimating fifty percent endpoints. Am. J. Epidemiol. 27, 493–497.
Ruiz, S., Schyth, B.D., Encinas, P., Tafalla, C., Estepa, A., Lorenzen, N., Coll, J.M., 2009. New tools to study RNA interference to fish viruses: fish cell lines permanently expressing siRNAs targeting the viral polymerase of viral hemorrhagic septicemia virus. Antiviral Res. 82, 148–156.
Schepers, U., 2006. RNA Interference in Practice: Principles, Basics, and Methods for Gene Silencing *C. elegans*, Drosophila, and Mammals. Wiley, pp. 101–135.
Schyth, B.D., Lorenzen, N., Pedersen, F.S., 2007. A high throughput in vivo model for testing delivery and antiviral effects of siRNAs in vertebrates. Mol. Ther. 15, 1366–1372.
Semizarov, D., Frost, L., Sarthy, A., Kroeger, P., Halbert, D.N., Fesik, S.W., 2003. Specificity of short interfering RNA determined through gene expression signatures. Proceedings of the National Academy of Sciences of the United States of America 100, 6347–6352.
Sledz, C.A., Holko, M., de Veer, M.J., Silverman, R.H., Williams, B.R.G., 2003. Activation of the interferon system by short-interfering RNAs. Nat. Cell Biol. 5, 834–839.
Soliman, M.K., Aboeisa, M.M., Mohamed, S.G., Saleh, W.D., 2008. First record of isolation and identification of spring viraemia of carp virus from *oreochromis niloticus* in Egypt. 8th International Symposium on Tilapia in Aquaculture. pp. 1287–1306.
Song, E.W., Lee, S.K., Wang, J., 2003. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat. Med. 9, 347–351.
Sui, H.Y., Zhao, G.Y., Huang, J.D., Jin, D.Y., Yuen, K.Y., Zheng, B.J., 2009. Small interfering RNA targeting M2 gene induces effective and long term inhibition of influenza a virus replication. PLoS One 4, e5671.
Tirasophon, W., Yodmuang, S., Chinnirunvong, W., Plongthongkum, N., Panyim, S., 2007. Therapeutic inhibition of yellow head virus multiplication in infected shrimps by YHV-protease dsRNA. Antiviral Res. 74, 150–155.
Vickers, T.A., Koo, S., Bennett, C.F., Crooke, S.T., Dean, N.M., Baker, B.F., 2003. Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. A comparative analysis. J. Biol. Chem. 278, 7108–7118.
Whither, 2003. RNAi? Nat. Cell Biol. 5, 489–490.
Xu, J., Han, F., Zhang, X., 2007. Silencing shrimp white spot syndrome virus (WSSV) genes by siRNA. Antiviral Res. 73, 126–131.
Yoshinouchi, M., Yamada, T., Kizaki, M., Fen, J., Koseki, T., Ikeda, Y., Nishihara, T., Yamato, K., 2003. Invitro and in vivo growth suppression of human Papillomavirus 6-positive cervical cancercells by E6 siRNA. Mol. Ther. 8, 762–768.