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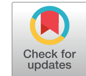
Designing and evaluation of MERS-CoV siRNAs in HEK-293 cell line



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ABSTRACT

Background: The MERS-CoV was identified for the first time from Jeddah, Saudi Arabia in 2012 from a hospitalized patient. This virus has now been spread to 27 countries with a total of 858 deaths and 2494 confirmed cases and has become a serious concern for the human population. Camels are well known for the transmission of the virus to the human population.
Methods: In this report, we have discussed the designing, prediction, and evaluation of potential siRNAs against the orf1ab gene of MERS-CoV. The online software was used to predict and design the siRNAs and finally, total twenty-one siRNA were filtered out from four hundred and sixty-two sIRNAs as per their scoring and specificity criteria. We have used only ten siRNAs to evaluate their cytotoxicity and efficacy by reverse transfection approach in HEK-293-T cell lines.
Results: Based on the results and data generated; no cytotoxicity was observed for any siRNAs at various concentrations in HEK-293-T cells. The ct value of real-time PCR showed the inhibition of viral replication in siRNA-1, 2, 4, 6, and 9. The data generated provided the preliminary information and encouraged us to evaluate the remaining siRNAs separately as well as in combination to analyses the replication of MERS-CoV inhibition in other cell lines.
Conclusion: Based on the results obtained; it is concluded that the prediction of siRNAs using online software resulted in the filtration of potential siRNAs with high accuracy and strength. This technology can be used to design and develop antiviral therapy not only for MERS-CoV but also against other viruses.
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Introduction

The MERS-CoV was emerged and identified for the first time in 2012 from Jeddah, Saudi Arabia, and has now become a serious concern for the human population [1]. Since September 2012, this virus has now spread to 27 countries with a total of 858 deaths and 2494 confirmed cases [2]. The camels are well reported for source virus transmission and infection to the human population [3–8]. The MERS-CoV-infected individuals develop a cluster of symptoms like fever, shortness of breath, and multi-organ failure in critically ill patients [9]. The MERS-CoV belongs to the family *Coronaviridae* and known as a large group of viruses infecting humans and animals continuously. Based on the genetic diversity and host range; coronaviruses have been divided into four main groups and designated as Alpha, Beta, Gamma, and Delta coronaviruses. The genome of Coronaviruses varies from 25.0 to 32.0 kb and is observed to have high genetic diversity which favors the high rate of recombination and emergence of new viral strains with novel characteristics and extended host range [10].

The regulatory role of RNA interference (RNAi) using short interfering RNAs (siRNAs), micro RNA (miRNAs) as significant antiviral therapy has been observed against many viral and deadly diseases. The RNAi is a valuable biological process to control the specific gene expression in eukaryotes by downregulating, silencing, and degrading the mRNAs of a specific gene [11,12]. The siRNAs are short sequences of RNA ranged from 21 to 23 base pairs associated with 5' phosphate group and a 3' hydroxyl group. These siRNAs bind to a specific protein and forms a complex known as RNA Induced Silencing Complex (RISC), which further binds to specific RNA sequences and finally degrades the RNA resulting in the silencing of mRNA expression [13]. Based on the current status of published information; the potential siRNAs have been used to

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evaluate the significant inhibition of viral replication in many cell lines against multiple viruses including HIV [14], Flock house virus (FHV) [15], Rous sarcoma virus [16], Dengue virus [17], Hepatitis C virus (HCV) [18], Influenza virus [19,20], Hepatitis B virus (HBV) [20,21], Human Papillomavirus (HPV) [22] and recently for SARS coronavirus (SARS-CoV) [23].

The *In-silico* prediction and designing of siRNAs by using the computational biology approach provides the filtering of potential siRNAs candidates with high target specificity and reduced off-target effects. The basic rules and guidelines for siRNAs designing have been published in many reports. For designing of potential siRNAs, the basic criteria and other critical issues should be considered such as conserved genome sequences, off-target effects, siRNA folding, thermodynamic properties, target accessibility, stability, and immuno-stimulation of siRNAs [24–26]. In a recent study conducted using Vero cells, cytopathic effect was specifically inhibited resulting in less virus titer by siRNA targeting viral RNA polymerases of SARS-CoV [27]. Additionally, the function of the SARS-CoV-Spike protein gene was selectively silenced by DNA vector-driven siRNA in SARS-infected 293T cells. Another study showed that SARS-CoV infection and replication in fetal rhesus kidney cells was inhibited by siRNA duplexes targeting viral RNA polymerases, and spike protein gene. Currently, miRNA and siRNA-based candidate drugs are being evaluated in more than 20 clinical trials. Therapeutic siRNAs and miRNAs are found to be the most promising biopharmaceuticals in commercial space as oligonucleotide-based next-generation medicines [28]. Recently, we have elaborately reviewed the design and delivery of potential siRNAs for MERS-CoV [26]. For *In silico* designing and prediction of siRNAs against MERS-CoV, currently, two studies have reported using orf1ab replicase polyprotein [29,30]. The siRNAs were designed by using the integrative siRNA design including multiple bioinformatics tools and against the conserved regions (orf1ab) of the MERS-CoV genome. In this study, the *in silico* prediction, chemical synthesis, cytotoxic effect and antiviral activity of potential siRNAs against the MERS-CoV orf1ab region have been discussed. The designed siRNAs were synthesized commercially, and their cytotoxicity and efficacy have been validated using various dose-dependent experiments in HEK-293-T cells. The cells were transfected with selected siRNAs at various concentration and viral RNA was purified to determine the replication of virus inhibition by using real-time PCR assay. Based on the results generated from this work, we found that some siRNAs showed significant and potential antiviral activity and inhibited MERS-CoV replication at a low concentration (0.1 nM-50 nM) in HEK293-T cell lines.

Materials and methods

In silico prediction and screening of siRNAs

To predict and design the potential siRNAs, we isolated full-genome of MERS-CoV and performed multiple sequence alignment using BioEdit software. Based on the sequence identity, the MERS-CoV-orf1ab gene was selected as the target and the overall steps of siRNA designing and filtration have been presented in Fig. 1. We have used the online software as an integrated bioinformatics approach to predict, design, and filtration of potential siRNAs [31]. Based on the i-SCORE approach, a total of ten different siRNAs were selected by algorithm scores [32–35]. To filter the potential siRNAs, we used the consensus multi-score threshold filtration layer by defining threshold accepted scores. Based on the integrated bioinformatics approach and strict selection criteria with different scoring tools; finally, we selected the ten most probable potential siRNAs with the desired characteristics [29–31]. The detailed information about the selected siRNAs has been presented in Table 1.

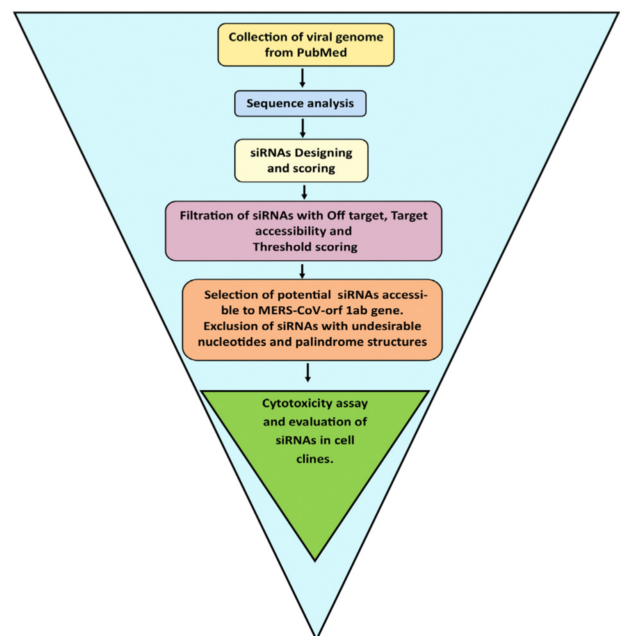

Fig. 1. Flow diagram of *In silico* prediction and design of siRNAs against MERS-CoV.

Removal of off-targets

To avoid any off-target binding of the designed siRNAs; we have used two different phases of selection. In the first phase of screening, we find out the near complimentary and similarity match with human mRNA sequences in PubMed by using the BLAST and Smith-Waterman algorithms as implemented in ParAlign and increase the validity percentage of the siRNAs and excluded those siRNAs with the most off-target effects. In the second phase of screening, we excluded the siRNAs which showed any seed-region similarity with human mRNA [36].

Final selection and chemical synthesis of siRNAs

The predicted, designed, filtered and probable potential siRNAs were selected based on the strict rules and parameters described in the integrated bioinformatics approach [24,34,35]. The selected siRNAs were chemically synthesized by Integrated DNA Technologies (IDT-USA) and used for further cytotoxicity and validation study.

Transfection of siRNAs to HEK 293-T cells

The reverse transfection method was used for the delivery of siRNAs in HEK 293-T cells grown in standard DMEM medium at 37 °C in 96-well plates with 60–80% confluency (1×10^4). All the experiments were performed in triplicates. The Lipofectamine 2000 (Invitrogen, USA) was used as a transfection reagent for making the siRNA-lipid complex as per the manufacturer's instructions. Briefly, various dilutions (0.1 to 50 nM) of siRNAs was were made from 50 μM stocks by adding the 100 μl Opti-MEM medium and Lipofectamine and incubated at room temperature for 30 min. Finally, the siRNA-lipid complex (1 μl) was mixed gently to the HEK 293-T cells and incubated for 24 h at 37 °C for further growth. We have used 3 different types of negative controls such as; HEK 293-T cells only, Opti-MEM with cells and, Lipofectamine with cells. The cytotoxicity was evaluated after 72 h post-transfection by using MTT assay as per manufacturer's instructions.

Table 1
List of predicted siRNAs from MERS-CoV orf1ab gene (KF958702).

S.No	Location of siRNAs in the viral genome (Start–End)	Target sequence	Predicted RNA oligo sequences (5′→3′)	Seed-duplex stability (Tm/°C) Guide/Passenger strand
1	791–813	AGCAATCTATTTTTACTATTAAT	UAAUAGUAAAAAUAGAUUGCU CAAUCUAUUUUUACUAUUAAU	6.3/6.6
2	1615–1637	ATGGATAATGCTATTAATGTTGG	AACAUUAAUAGCAUUAUCCAU GGAUAAUGCUAUUAAUGUUGG	6.9/8.7
3	1910–1932	GCGACTTTATGTCTACAATTATT	UAAUUGUAGACAUAAAGUCGC GACUUUAUGUCUACAAUUAUU	6.9/4.6
4	4018–4040	GACACTTTAGATGATATCTTACA	UAAGAUAUCAUCUAAAGUGUC CACUUUAGAUGAUAUCUUACA	6.6/9.8
5	5597–5619	ATGCTATTAGTTTGAGTTTTAAT	UAAAACUCAAACUAAUAGCAU GCUAUUAGUUUGAGUUUUAAU	13.3/2.8
6	5598–5620	TGCTATTAGTTTGAGTTTTAATA	UUAAAACUCAAACUAAUAGCA CUAUUAGUUUGAGUUUUAAUA	4.9/6.3
7	5819–5841	GAGCTAGTTTGCGTCAAATTTTT	AAAUUUGACGCAAACUAGCUC GCUAGUUUGCGUCAAAUUUUU	7.4/9.8
8	9495–9517	CTCTAATATCTTTGTTATTAACA	UUAAUAACAAAGAUAUUAGAG, CUAAUAUCUUUGUUAUUAACA	8.1/7.2
9	9533–9555	CTCTTAGAAACTCTTTAACTAAT	UAGUUAAAGAGUUUCUAAGAG, CUUAGAAACUCUUUAACUAAU	7.3/6.6
10	13605–13627	TGGTTTGATTTTGTTGAAAATCC	AUUUUCAACAAAAUCAAACCA, GUUUGAUUUUGUUGAAAAUCC	9.5/8.4

Cytotoxicity assay

To evaluate the cytotoxic effect of siRNAs on HEK 293-T cells; we used MTT assay commercial kit as per instruction (Invitrogen). The old DMEM media was changed with fresh media to the cells following the addition of 12 mM- MTT (10 μl) and further incubated for 4 h at 37 °C. The formazan crystals were thoroughly dissolved by adding 100 μL SDS-HCL solution following the further incubation for 4 h at 37 °C. After thoroughly mixing the cells; the absorbance was measured at 570 nm using SpectraMax i3x imaging cytometer and the mean OD value was used for cytotoxicity calculation using the standard formula.

Evaluation of MERS-CoV replication inhibition in HEK 293-T cells

Virus inoculation

We have used the previously standardized and published protocol for virus inoculation to the siRNAs transfected HEK 293-T cells (1×10^4) [8].The virus inoculation was performed after the siRNA transfection and cells were further grown and the cytopathic effect in HEK 293-T cells was observed daily for 3 days. After the full cytopathic effect, the cell supernatants and lysate were isolated for viral RNA isolation using QIAampViral RNA Mini Kit (Qiagen). The purified RNA was further used for real-time RT-PCR [8].

Total RNA extraction and real-time PCR

The viral RNA was purified from the cell supernatant and cell lysate by using the QIAamp Viral RNA Mini Kit (Qiagen), as per manufacturer's instructions. Finally, the real-time RT-PCR was performed by using the purified RNA for detection and quantification of virus inhibition using specific MERS-CoV open reading frame region orf1a and orf1b primers as described earlier [8].

Results

Prediction and selection of siRNAs

The whole-genome sequence of MERS-CoV was isolated and used to perform multiple sequence alignment and based on the alignment result, the orf1ab region was selected as target to design the siRNAs. The multiple sequence alignment results of the full-genome showed high similarity with all the analysed sequences of MERS-CoV. The online software was used for *In silico* designing and scoring of potential siRNAs targeting the targeted orf1ab gene of the MERS-CoV. By using the *In silico* prediction and selection criteria, the software generated many siRNAs to fulfil the criteria, but we selected only ten siRNAs based on the stringent filtration and screening strategy with functional, off-targets reduced siRNA. The predicted siRNAs were further evaluated for their cytotoxicity and inhibition of virus replication in the selected cell lines [24,34,35].

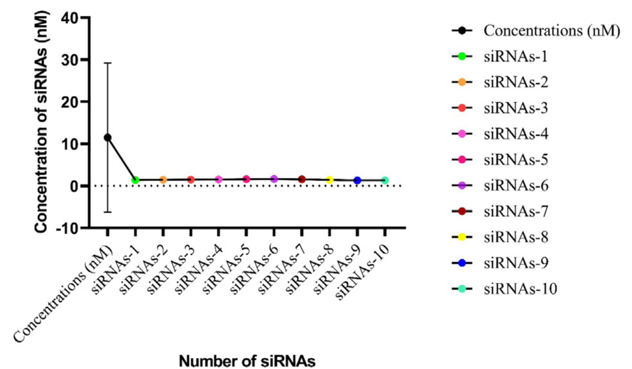

Fig. 2. Cytotoxicity of different siRNAs at various concentrations (0.1–50 nM).

siRNA transfection and cytotoxicity assay

The cytotoxicity of selected siRNAs was evaluated by applying the reverse transfection method using Lipofectamine 2000 for the delivery of siRNAs into HEK 293-Tcells. The cytotoxicity of selected siRNAs in HEK 293-T cells was found to be concentration-dependent. The CC50 was found to be variable for each siRNAs: siRNA-1:110.2, siRNA-2:205.1, siRNA-3:142.0, siRNA-4:118.1, siRNA-5:150.3, siRNA-6:131.2, siRNA-7:142.1, siRNA-8:132.5, siRNA-9:125.6, and siRNA-10:150.7. Based on the results obtained in this study, none of the tested siRNAs showed significant cytotoxicity to HEK 293-T cells tested up to 50 nM concentration. The cytotoxicity results are presented in Table 2 and Fig. 2.

Table 2
Mean OD value and cytotoxicity of different siRNAs at various concentrations (0.1-50 nM).

siRNAs Conc. (nM)	siRNA-1	siRNA-2	siRNA-3	siRNA-4	siRNA-5	siRNA-6	siRNA-7	siRNA-8	siRNA-9	siRNA-10
50	1.3	1.4	1.6	1.5	1.6	1.7	1.5	1.4	1.5	1.4
25	1.4	1.4	1.5	1.5	1.6	1.7	1.5	1.7	1.6	1.6
10	1.2	1.4	1.6	1.6	1.6	1.4	1.6	1.5	1.4	1.5
5.0	1.2	1.5	1.4	1.6	1.5	1.7	1.6	1.5	1.4	1.3
1.0	1.4	1.6	1.5	1.6	1.7	1.8	1.6	1.4	1.3	1.2
0.5	1.5	1.3	1.4	1.5	1.6	1.6	1.5	1.4	1.2	1.2
0.25	1.5	1.5	1.6	1.6	1.7	1.7	1.6	1.2	1.1	1.1
0.1	1.5	1.5	1.5	1.5	1.5	1.5	1.6	1.2	1.1	1.2
CC 50	110.2	205.1	142.0	118.1	150.3	131.2	142.1	132.5	125.6	150.7

Table 3
CT value of Real-time PCR results siRNAs at various concentrations in cell lysate/supernatant.

Conc. (nM)	SiRNA-1	SiRNA-2	SiRNA-3	SiRNA-4	SiRNA-5	SiRNA-6	SiRNA-7	SiRNA-8	SiRNA-9	SiRNA-10
50	12.83/14.48	11.56/11.31	12.99/12.38	13.75/13.50	13.52/12.21	13.13/12.98	12.78/14.67	14.41/13.99	14.75/13.91	14.21/13.97
25	15.21/15.98	14.65/14.21	15.41/15.12	15.82/15.71	14.43/13.94	14.65/13.98	15.56/16.55	16.12/15.78	16.34/15.89	15.75/15.67
10	19.99/19.67	16.57/18.31	14.54/14.21	16.61/18.26	15.99/14.90	19.21/18.19	15.97/17.67	17.46/17.50	19.89/19.71	17.51/17.23
5	18.95/18.87	19.95/19.87	14.81/14.38	19.84/19.39	16.75/16.10	16.12/13.99	16.78/16.98	17.95/16.93	20.91/19.98	17.96/17.81
1	13.11/13.20	13.21/12.95	13.19/12.99	13.65/13.91	13.15/12.97	13.78/19.99	13.34/15.84	14.61/13.86	14.31/13.89	16.78/16.34
0.5	12.91/13.0	12.36/12.10	13.85/13.25	13.21/12.98	13.45/13.90	13.72/13.58	13.80/14.69	14.32/13.75	14.64/14.31	14.91/14.74
0.25	12.78/13.02	12.01/12.59	12.78/12.19	13.91/13.29	13.25/13.11	13.10/13.0	12.56/14.53	14.87/14.21	14.83/14.39	14.68/14.51
0.1	15.81/17.75	17.95/16.71	12.21/12.35	18.49/18.98	16.11/15.98	18.64/18.28	13.35/15.87	15.94/15.79	19.23/18.87	16.95/16.90

Evaluation of virus replication inhibition

The inhibition of virus replication in HEK-293-T cells was evaluated by virus inoculation and analysing the ct value of real-time PCR against the selected siRNAs. All the experiments were performed in triplicates. The cell supernatant and lysate were used to purify the viral RNA after 72 h of virus inoculation. The transfection of all tested siRNAs showed the dose-dependent inhibition of MERS-CoV replication in HEK-293-T cells. The ct value of realtime RT-PCR generated variable slopes of virus inhibition for each siRNAs at various concentrations. Interestingly, the virus replication inhibition was observed better in siRNA 1, 2 4, 6 and 9 than others at 0.1 nM, 5.0 nM and 10 nM respectively. The positive sample was used as only cell lysate and the supernatant collected from infected cells. During analysis, we have observed that some siRNAs showed better inhibition at a 5−10 nM concentration in the only supernatant as compared to the cell lysate. The results of real-time PCR for tested siRNAs has been presented in Table 3 Fig. 3.

Discussion

The MERS-CoV was emerged in 2012 from Jeddah, Saudi Arabia. Currently, an enormous amount of research has been conducted and published. The published information contributed significantly to explore different types of strategies to control the virus spread and to design and develop disease management strategies. But still, many advanced studies are under progress towards the development of antivirals, therapeutic and preventive vaccines, and targeted drug discovery. The RNAi-based therapeutics have played a significant role and shown promising results against many viral diseases [37]. This technology is being used to significantly silence the expression of the desired gene against many diseases [28]. Currently, various types of siRNAs have been evaluated against HCV 5'-NTR, and found that HCV321, HCV353, HCV258 siRNA were the best and promising siRNAs for the inhibition of HCV replication. The MERS-S protein is responsible for the cell attachment and the replication of the virus is initiated by the binding of virus particles with cellular receptors. The orf1ab region includes two-thirds of the Coronavirus genome and encodes non-structural proteins [31].The proper use of RNAi technology was hampered due to many obstacles like; off-target binding, delivery, stability, and stimulation of immune responses. But due to continuous research effort, most of the obstacles have been successfully overcome and many siRNAs based therapeutics have reached to an advanced stage of clinical trials against multiple diseases [31,38–43].

In this study, we have predicted, designed, and evaluated the potential siRNAs in the HEK293-T cell line targeting the orf1ab gene of MERS-CoV using automated online software by filtering and excluding the off-target effects and considering various factors and stringent rules [24,34,35].The evaluated siRNAs were observed to be highly effective to inhibit MERS-CoV replication. As a preliminary test for the proof of concept, our primary objective was to predict and design the potential siRNAs against the orf1ab gene as a target region of MERS-CoV. The secondary objective was to evaluate the cytotoxicity study and experimentally evaluate the efficacy of predicted siRNAs into HEK 293-T cells by real-time PCR. Based on the extensive bioinformatics analysis, the outputs resulted in many potential siRNAs against the target orf1ab gene of MERS-CoV, but we selected only ten siRNAs with improved target accessibility and expected high antiviral potency with no off-target effect. The predicted siRNAs were chemically synthesized and evaluated in HEK293-T cells using Lipofectamine 2000 mediated delivery. The efficacy of predicted siRNAs was found to be effective against virus replication at variable doses. The ct value of Real-time PCR results for siRNAs 1, 2, 4, 6, and 9 was comparable and observed to be promising with other tested siRNAs. The replication of virus inhibition by the predicted siRNAs was found to be promising in the HEK293-T cells. Currently, very few siRNAs have been designed by using *In silico* online software but none of them have been evaluated in any cell lines [29,30]. Recently, some siRNAs against HCV have been evaluated and found to be very efficient to inhibit virus replication [31]. Based on the status and published information we have utilized the potential of RNAi technology to investigate the siRNAs and their potential as antiviral therapy against MERS-CoV. We have evaluated only ten siRNAs alone but as we expect that the combined use of multiple siRNAs may also provide better results as compared to alone as it has been observed for other viruses [31].

Conclusions

Based on the results obtained data analysis, it is concluded that the *In silico* predicted and designed siRNAs against MERS-CoV

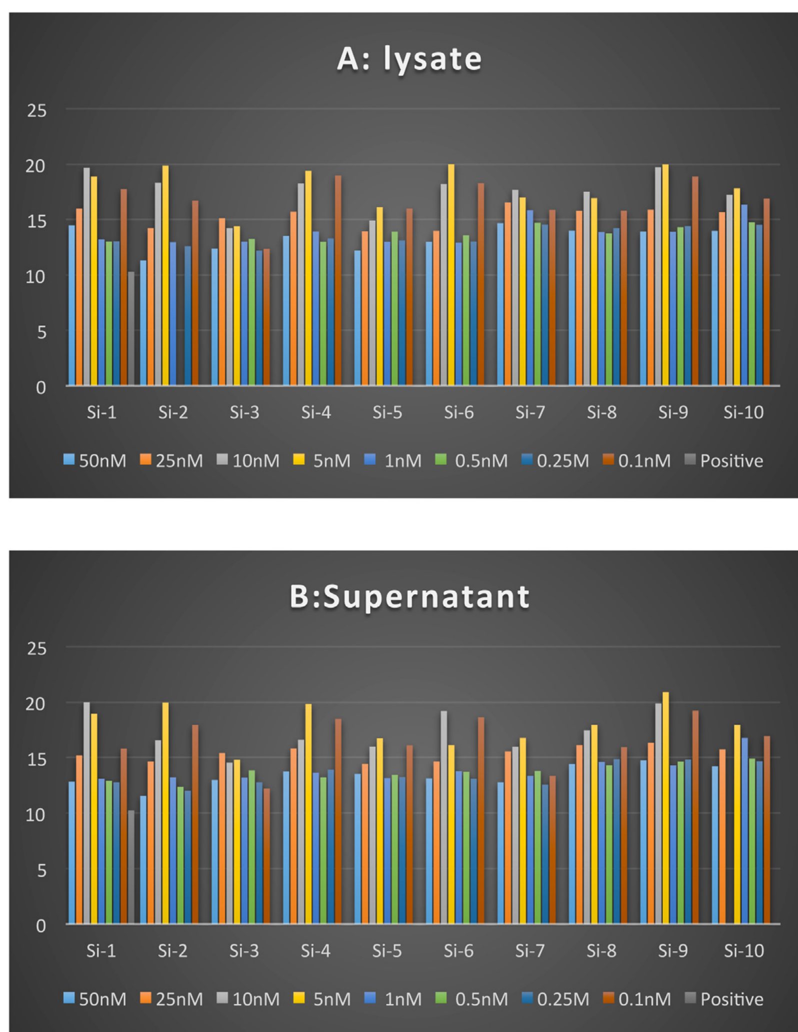

Fig. 3. Graphical representation of Ct value of RT-PCR result. A: Lysate B: Supernatant.

can be used as oligonucleotide-based antiviral therapeutics. The online software provided many potential siRNAs without off-target effects, minimal cytotoxicity, high efficiency, and improved specificity. The siRNA-1, 2, 4, 6 and 9 were found to be better than others to inhibit the replication of MERS-CoV at various concentrations. By using this technology, the potential siRNAs can be designed, evaluated, and used to develop antiviral therapeutics against viral diseases.

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Competing interests

None declared.

Ethical approval

Not required.

Author contributions

SSS, SAE designed and executed the experiments, ZM, SSS performed bioinformatics study. SSS, SAE, ZM wrote the manuscript. EIA: Contributed in designing and execution of experiments and critically reviewed the manuscript. All authors provided critical feedback and analysis of manuscript. All authors reviewed the MS and approved.

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