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In vitro inhibition of feline coronavirus replication by small interfering RNAs

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ABSTRACT

Infection with virulent biotypes of feline coronavirus (FCoV) can result in the development of feline infectious peritonitis (FIP), a typically fatal immune mediated disease for which there is currently no effective antiviral treatment. In this study we demonstrate the ability of small interfering RNA (siRNA) mediated RNA interference (RNAi) to inhibit the replication of virulent FCoV strain FIPV WSU 79-1146 in an immortalised feline cell line. A panel of eight synthetic siRNAs targeting four different regions of the FCoV genome were tested for antiviral effects. Efficacy was determined by qRT-PCR of intracellular viral genomic and messenger RNA, TCID50 infectivity assay of extracellular virus, and direct IFA for viral protein expression. All siRNAs demonstrated an inhibitory effect on viral replication *in vitro*. The two most effective siRNAs, targeting the untranslated 5' leader sequence (L2) and the nucleocapsid gene (N1), resulted in a >95% reduction in extracellular viral titre. Further characterisation of these two siRNAs demonstrated their efficacy when used at low concentrations and in cells challenged with high viral loads. Taken together these findings provide important information for the potential therapeutic application of RNAi in treating FIP.

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1. Introduction

Feline infectious peritonitis, a significant cause of mortality in domestic cats worldwide, is caused by a virulent biotype of feline coronavirus (FCoV) often referred to as feline infectious peritonitis virus (FIPV) (Pedersen, 2009). As a member of the Family *Coronaviridae*, FCoV is an enveloped virus with a non-segmented linear single-stranded positive-sense RNA genome of approximately 30 kb (Fig. 1). The proximal two-thirds of the genome contain two large open reading frames that encode the non-structural replicase proteins while the four structural proteins; the spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins, and several accessory proteins of unknown function are encoded in the distal one third of the genome (Masters, 2006). There are two antigenically

distinct serotypes of FCoV: type I and type II. These serotypes differ primarily in their spike protein, with the type II viruses thought to have arisen from a recombination event with canine coronavirus while the type I viruses are considered wholly feline (Herrewegh et al., 1998). Type I FCoV are more commonly isolated from field cases, however most *in vitro* studies utilise type II strains due to the difficulty in propagating the former *in vitro* (Pedersen et al., 2009).

Infection with FCoV is extremely common in domestic cats as demonstrated by numerous seroprevalence studies (Addie and Jarrett, 1992; Bell et al., 2006; Cave et al., 2004; Holst et al., 2006). While most infections are asymptomatic, an unfortunate sequela of FCoV infection in a subset of infected cats is the development of FIP. FIP can occur following exposure to virulent biotypes of FCoV, either through the *de novo* mutation of a normally avirulent FCoV within an individual animal, the so called internal mutation theory (Chang et al., 2010; Pedersen et al., 2009; Poland et al., 1996; Vennema et al., 1998), or as more

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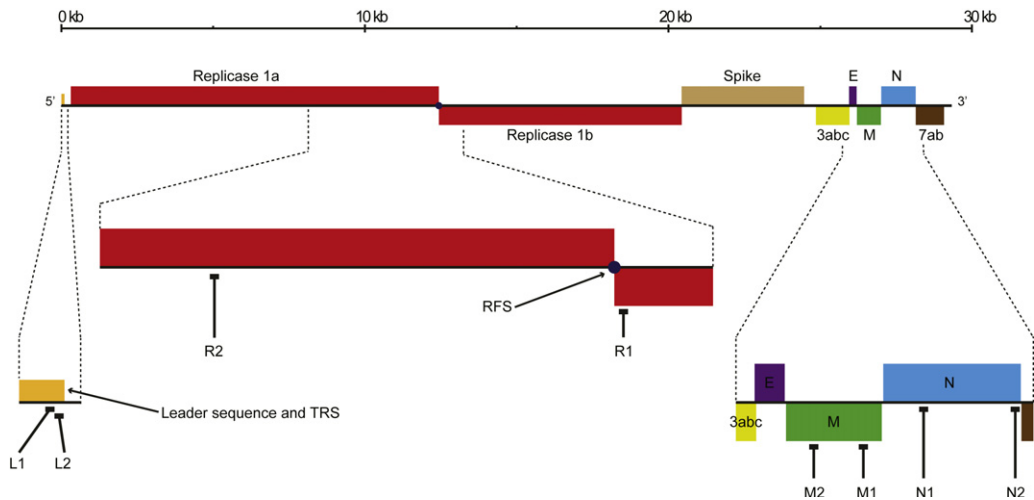


Fig. 1. Schematic of FCoV genome based on reported sequence of FIPV WSU 79-1146 (ascension number DQ010921). The FCoV genome is approximately 30 kb and encodes a large replicase polyprotein in two ORFs (1a and 1b) occupying the proximal 2/3rd of the 5' end. Polyprotein 1ab is produced following a ribosomal frameshift at the ribosomal frameshift element (RFS). The downstream region encodes the four structural proteins: spike (S), envelope (E), membrane (M) and nucleocapsid (N), in addition to several accessory proteins (3abc and 7ab). The unique discontinuous transcription of coronavirus mRNAs results in the production of a nested set of subgenomic mRNAs, each with an identical 5' leader sequence. The location of siRNAs targeting the leader sequence (L1, L2), replicase gene (R1, R2), membrane (M1, M2) gene, and the nucleocapsid (N1, N2) gene are indicated with small bars.

recently proposed, through exposure to circulating virulent strains (Brown et al., 2009). Regardless of their origin, the virulent FCoV biotypes demonstrate enhanced replication in cells of the monocyte/macrophage lineage which is thought to trigger the host immune dysregulation and massive systemic inflammatory response seen in FIP (Pedersen, 2009).

Therapeutic options for cats with FIP are extremely limited, and in most instances are at best palliative (reviewed by Hartmann and Ritz, 2008). Legendre and Bartges (2009) recently reported the use of a novel immunostimulant in the successful treatment of three cats with non-effusive FIP. This agent however was not effective in treating the more common effusive form of the disease. There are currently no specific antiviral chemotherapeutic agents that are effective in treating either effusive or non-effusive FIP, although a number of drugs have demonstrated *in vitro* efficacy against FCoV (Barlough and Scott, 1990; Barlough and Shacklett, 1994; Hsieh et al., 2010; Keyaerts et al., 2007). Given the central role of increased FCoV replication in the pathogenesis of FIP (Groot-Mijnes et al., 2005) it is likely that the development of an effective and safe antiviral agent will significantly advance our ability to treat this devastating condition.

RNA interference (RNAi) is a highly conserved post transcriptional gene silencing mechanism found in most eukaryotes that has recently attracted significant attention due to its potential therapeutic applications. RNAi can be induced in mammalian cells via the introduction of short interfering RNAs (siRNAs), ≈ 21 nucleotide RNA duplexes. Introduced siRNAs are incorporated into an RNA induced silencing complex (RISC) enabling it to bind to and target complementary sequences. Upon binding, an endonuclease within the RISC cleaves the targeted mRNA resulting in sequence specific gene silencing. The *in vitro* efficacy of

RNAi as an antiviral therapeutic has been demonstrated against a range of viral pathogens of medical and veterinary importance from diverse viral families, including HIV-1, influenza, SARS coronavirus, and foot and mouth disease virus (Ge et al., 2003; He et al., 2003; Kahana et al., 2004; Martinez et al., 2002). For some viruses these findings have been extended to *in vivo* animal models, where induction of a virus specific RNAi response results in a reduction in viral load and the associated pathology (Li et al., 2005a; Tompkins et al., 2004). The aim of this study was to evaluate the antiviral potential of a panel of eight siRNAs targeting four different regions of the FCoV genome using an *in vitro* assay. The most effective siRNAs were further characterised by examining the effect of multiplicity of infection and siRNA concentration on their inhibitory effect. The information gained from this pilot study is an essential first step to the development of an effective RNAi based treatment for FIP.

2. Materials and methods

2.1. Cells and virus

Crandall feline kidney (CRFK) cells were obtained from The Faculty of Veterinary Science, University of Melbourne. Cells were maintained in Dulbecco's modified eagles medium (DMEM) supplemented with 10% foetal bovine serum (FBS) (Sigma–Aldrich, Castle Hill, NSW, Australia). Cells were cultured in a humidified incubator at 37 °C in 5% CO₂ in air. Highly virulent type II FCoV strain FIPV WSU 79-1146 (ATCC: VR990) was purchased from The American Type Culture Collection (ATCC, Manassas, VA, USA). Working stocks of virus were produced in CRFK cells and quantified by plaque assay. Plaque assays were performed by inoculating

monolayers of CRFK cells in 6-well plates (Sarstedt, Numbrecht, Germany) with 250 μ l of ten-fold virus dilutions. After adsorption for 1 h at 37 °C the cells were overlaid with 2 ml of maintenance media containing 1% agarose (Amresco, Salon, OH, USA). After 48 h incubation, monolayers were stained with 500 μ l 0.05% neutral red (Sigma–Aldrich) for 3 h and plaques counted. Virus stocks were stored at –80 °C until use.

2.2. siRNA design

siRNAs were designed using Block-iT™ RNAi designer (Invitrogen, Mulgrave, VIC, Australia) using the consensus sequence of FIPV WSU 79-1146 (ascension number DQ010921). Four regions of the genome were targeted: the membrane and nucleocapsid structural genes, the replicase gene, and the common 5' leader sequence. The gene encoding the spike protein, the other main structural protein was not targeted in this study due to the genetic diversity of this region. Prospective siRNA sequences were aligned with other reported full- and partial-length FCoV sequences available on GenBank (Benson et al., 2010) using Geneious software (Biomatters Ltd., Auckland, NZ). Criteria for final siRNA selection were (1) high Block-iT™ RNAi designer ranking, (2) sequence homology to other reported FCoV strains, and (3) minimum homology to known feline sequences based on a BLAST search. Using these selection criteria the two highest ranking siRNAs targeting each of the four regions were selected (Table 1 and Fig. 1). A siRNA (NSC-GFP) was selected targeting green fluorescent protein mRNA to act as a non-silencing control. The 5' end of the sense strand of NSC-GFP was conjugated to

fluorescein isothiocyanate (FITC) to enable transfection efficacy to be monitored with fluorescent microscopy.

2.3. Transfection and FCoV infection

Low-passage sub-confluent CRFK cells were used for all experiments. Cells were dissociated with 0.25% trypsin/0.02% EDTA in HBSS (Sigma–Aldrich) and 6×10^4 cells/well were added to 12-well tissue culture plates (Corning Inc., Corning, NY, USA) 24 h prior to transfection. For immunofluorescence studies and for monitoring transfection efficacy cells were plated into wells containing sterile 13 mm glass coverslips (ProSciTech, Thuringowa, QLD, Australia). Transfection of the 40–50% confluent CRFK cells was performed using Lipofectamine 2000 (Invitrogen) as per the manufacturer's protocol. Cell monolayers were washed once with DMEM following a 6 h transfection period. The efficacy of transfection was assessed at this time using a BX60 epifluorescence microscope (Olympus, Melville, NY, USA) by assessing the percentage of cells containing intracytoplasmic FITC. Cultures were infected with FIPV WSU 79-1146 at a multiplicity of infection (moi) of 0.2, 2, or 20 or mock infected depending on experimental requirements. Virus was allowed to adsorb for 60 min at 37 °C, following which the viral inoculum was replaced by fresh maintenance media. Samples were harvested at various time points following infection depending experimental requirements. Tissue culture media was collected and stored at –80 °C prior to virus titration assays. For cellular viral RNA quantification cell monolayers were lysed with RLT buffer (QIAGEN, Doncaster, VIC, Australia) and the homogenised lysate stored at –80 °C prior to RNA extraction.

Table 1

Sequence of siRNAs and their location within FCoV FIPV WSU 79-1146 genome (ascension number DQ010921).

siRNA	Sequence	Position in genome
Leader 1 (L1)		
Sense	5'-GCUAGAUUUGUCUUCGGAGcTdT-3'	64–82
Antisense	5'-GUCCGAAGACAAAUCUAGCdTdT-3'	
Leader 2 (L2)		
Sense	5'-GGACACCAACUCGAACUAdTdT-3'	79–97
Antisense	5'-UUAGUUCGAGUUGGUGUCcTdT-3'	
Replicase 1 (R1)		
Sense	5'-GCACGUAAGGAUCUUACAAdTdT-3'	12,479–12,497
Antisense	5'-UUGUAAGAUCUUACGUGCdTdT-3'	
Replicase 2 (R2)		
Sense	5'-GCUGAUUACCGCAUGGCUUdTdT-3'	8673–8691
Antisense	5'-AAGCCAUGC GGUAAUCAGCdTdT-3'	
Membrane 1 (M1)		
Sense	5'-CCUAGUAGAACCAUCGUUUdTdT-3'	26,611–26,629
Antisense	5'-AAACGAUGGUUCUACUAGGdTdT-3'	
Membrane 2 (M2)		
Sense	5'-GCUGGCUCGUUUUUGGCAUdTdT-3'	26,210–26,228
Antisense	5'-AUGCCAUAACGAGCCAGCdTdT-3'	
Nucleocapsid 1 (N1)		
Sense	5'-GGAGUCUUCUGGGUUGCAAdTdT-3'	27,112–27,130
Antisense	5'-UUGCAACCCAGAAGACUCCdTdT-3'	
Nucleocapsid 2 (N2)		
Sense	5'-GGCAUACACAGAUGUGUUUdTdT-3'	27,885–27,837
Antisense	5'-AAACACAUCUGUGUAUGCCdTdT-3'	
NSC-GFP		
Sense	5'-[FITC]CAAGCUGACCCUGAAGUUCdTdT-3'	
Antisense	5'-GAACUUCAGGGUCAGCUUGdTdT-3'	

2.4. Infectivity assay

Extracellular virus was quantified using a 50% tissue culture infective dose (TCID₅₀) assay in CRFK cells. Harvested tissue culture media was clarified by centrifugation at 1000 × g for 3 min and the supernatant serially diluted 10-fold with DMEM. Diluted virus suspension was added to subconfluent monolayers of CRFK cells in replicate wells of 96 well plates, six wells per dilution. Wells were scored for cytopathic effect 72 h post infection using an Olympus CKX41 inverted phase-contrast microscope (Olympus). TCID₅₀ endpoint values were calculated according to the method of Reed and Muench (1938).

2.5. Immunofluorescence assay

Cells grown on glass coverslips were rinsed with PBS, fixed in methanol-free 10% formalin (Polysciences, Warrington, PA, USA) in PBS, and permeabilised with 0.2% Triton X-100 (Sigma–Aldrich). Samples were blocked with 10% FBS in PBS and stained with FITC conjugated anti-feline infectious peritonitis virus antiserum (VMRD, Pullman, WA, USA). Cells were counterstained with DAPI (Invitrogen) and the coverslips mounted using Citifluor AF1 antifadent (Citifluor Ltd, London, UK). Images were acquired using a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Jena, Germany).

2.6. Quantitative real time reverse transcriptase PCR (qRT-PCR)

2.6.1. RNA extraction

Total RNA was extracted using RNeasy Mini spin columns (QIAGEN) as per manufacturer's protocol, including an on-column RNase-free DNase I (QIAGEN) treatment. RNA yield and purity was determined by spectrophotometry (OD₂₈₀/OD₂₆₀) and the integrity of RNA checked with 1.2% agarose/formaldehyde denaturing gel electrophoresis. Purified RNA was used immediately or stored at –80 °C prior to cDNA synthesis.

2.6.2. cDNA synthesis

cDNA was generated from purified RNA using Superscript[®] III (Invitrogen), a modified Moloney Murine Leukaemia Virus reverse transcriptase in a final reaction volume of 20 µl. The reactions consisted of 1 µg purified RNA, 100 ng random primers (Promega, Alexandria, NSW, Australia), 2.5 µmol of each dNTP (Promega), and RNase free water (Amresco) to a final volume of 14.5 µl. Samples were incubated at 65 °C for 5 min to denature RNA and then cooled on ice for 1 min. To this was added a mix consisting of 4.5 µl First Stand Buffer (Invitrogen), 20 units RNasin Plus RNase Inhibitor (Promega), and 100 units Superscript III. Tubes were incubated at 60 °C for 60 min followed by 15 min at 70 °C to inactivate the reverse transcriptase. cDNA was stored at –80 °C until use.

2.6.3. Quantitative real time PCR

Primers were designed using Primer3 software (Rozen and Skaletsky, 2000) using the published sequences of FIPV WSU 79-1146 and feline GAPDH mRNA (ascension number

Table 2

Primers used for quantitative real time reverse transcriptase PCR.

Gene	Primer sequence	Product size (bp)
GAPDH	Forward 5'-GCCGTGAACCACGAGAAGTATG-3'	236
	Reverse 5'-GCCAGTAGAAGCAGGGATGA-3'	
FCoV genome	Forward 5'-CGGACACCAACTCGAACTAAA-3'	171
	Reverse 5'-GAACCGCCGAGCTAATAC-3'	
Membrane mRNA	Forward 5'-CTTCGGACACCAACTCGAAC-3'	274
	Reverse 5'-GCCATAAACGAGCCAGCTAA-3'	
Nucleocapsid mRNA	Forward 5'-CTTCGGACACCAACTCGAAC-3'	202
	Reverse 5'-GGAACAAGGTCTCTCGGACAT-3'	

AB038241) and are shown in Table 2. The position of the identical forward primer for M and N genes within the common 5' leader sequence allows for the differentiation of sub-genomic mRNA from genomic RNA based on product length. Virus specific primers were designed targeting conserved regions of FCoV genome.

Quantitative real time PCR reactions were performed using a Rotor-Gene 3000 cyclor (Corbett Life Science, Mortlake, NSW, Australia). Each target was amplified in a separate 10 µl reaction in quadruplicate. The PCR reaction consisted of 1 µl of a 1:10 dilution of cDNA, 2 µl 5× GoTaq Colorless Reaction Buffer (Promega), 2.5 pmol forward and reverse primers, 2.5 µmol of each dNTP, 0.25 units GoTaq DNA polymerase (Promega), 1 µl 1:1000 SYBR Green (Invitrogen), and 25 µmol (GAPDH and FCoV genome targets) or 10 µmol (membrane and nucleocapsid mRNA targets) MgCl₂. Cycling conditions for GAPDH, FCoV genome, and nucleocapsid mRNA were as follows: initial denaturation of DNA at 95 °C for 2 min, 30 cycles of 30 s at 95 °C, 30 s at 62 °C, and 30 s at 72 °C, followed by a final extension at 75 °C for 30 s. Cycling conditions for amplifying membrane mRNA were identical with the exception of a 45 s annealing time during cycling. A post amplification melt curve analysis was performed from 74 °C to 96 °C at 10 s per degree to confirm product specificity for all reactions. Serial dilutions of purified PCR products (QIAquick PCR purification kit, QIAGEN) were amplified under identical conditions during each run to generate a standard curve from which copy number could be calculated.

2.7. Statistical analysis

All treatments were performed in triplicate and the results are expressed as geometric mean with 95% confidence interval from three independent experiments. For PCR data analysis, outliers within the PCR quadruplicates, defined as values >3 standard deviations from the mean of the remaining replicates, were excluded from further analysis. PCR viral genomic and mRNA copy number were normalised to the housekeeping gene GAPDH copy number. Relative viral copy number and titres were calculated by dividing individual treatment

values by the geometric mean of untreated samples. For statistical analysis, qRT-PCR and viral titre data was log transformed to normalise the distribution. Treatments were compared by one-way ANOVA followed by Dunnett's post hoc testing if indicated (GraphPad Prism V5.03 for Windows, GraphPad Software, San Diego, CA, USA). *p*-Values less than 0.05 were considered significant.

3. Results

3.1. Design of anti-FCoV siRNAs

The eight siRNAs designed in this study were selected to target conserved regions of the FCoV genome which were visually identified by aligning full- and partial-length FCoV sequences published on GenBank. Using this method we were able to design five siRNAs (L1, L2, R1, R2, N2) with 100% homology to all reported type I and type II strains. The remaining siRNAs showed complete homology at 18 of the 19 nucleotides (M1, N1) or 17 or 19 nucleotides (M2).

3.2. Effect of synthetic siRNA on FCoV infection in CRFK cells

An initial screening experiment was conducted with the eight siRNAs to identify duplexes with an inhibitory effect on FCoV replication *in vitro*. For the screening experiment

all siRNAs were tested at a final concentration of 100 nM. Efficacy of transfection of siRNAs into CRFK cells was greater than 99% under the stated conditions and was associated with minimal toxicity (data not shown). Cells were infected at a moi of 0.2. Untreated cells (infected and mock infected), cells treated with transfection reagents without siRNAs, and cells transfected with an irrelevant NSC siRNA were included as controls. The effect of siRNAs on viral replication was determined by qRT-PCR of intracellular virus (detecting viral genome and M and N mRNA), TCID50 infectivity assay of extracellular virus, and direct IFA staining for viral antigen, all conducted 24 hpi. Additionally, virus induced CPE was monitored in separate wells out to 50 hpi.

All siRNAs tested resulted in statistically significant inhibition of FCoV replication as measured by qRT-PCR and TCID50 infectivity assay however the most effective siRNAs were those targeted against L2 and N1. Treatment with Lipofectamine 2000 and transfection with the NSC siRNA had no significant effect on viral replication, confirming the sequence specificity of the siRNA mediated viral inhibition. Reduction of viral genomic RNA in FCoV specific siRNA treated cells ranged from 26.1% to 90.7% (Fig. 2a). Similar results were seen with viral mRNA, with M mRNA knockdown ranging from 27.7% to 92.3% (Fig. 2b) and N mRNA knockdown ranging from 23.1% to 93.8%

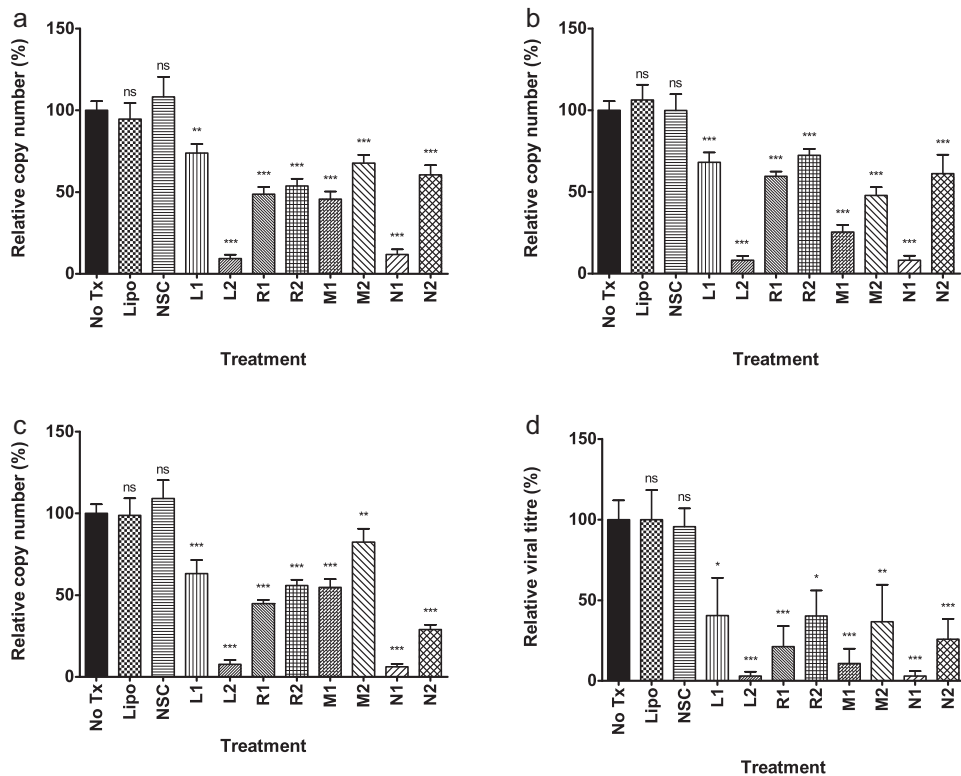


Fig. 2. Effect of siRNAs on replication of FIPV WSU 79-1146 in CRFK cells. Cells were infected at moi of 0.2 following transfection with 100 nM of each anti-FCoV siRNA (L1, L2, R1, R2, M1, M2, N1, N2). Untreated control (No Tx), transfection control (Lipo), and siRNA control (NSC) samples were included in each experiment. qRT-PCR for (a) FCoV genome, (b) FCoV membrane mRNA and (c) nucleocapsid mRNA was performed on total intracellular RNA 24 hpi. Extracellular virus titres (d) were determined 24 hpi by TCID50 end point assay. Values are expressed as geometric means \pm 95% confidence interval from three independent experiments. Asterisks indicate statistically significant differences between treated samples and the no treatment control. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, ns = not significant.

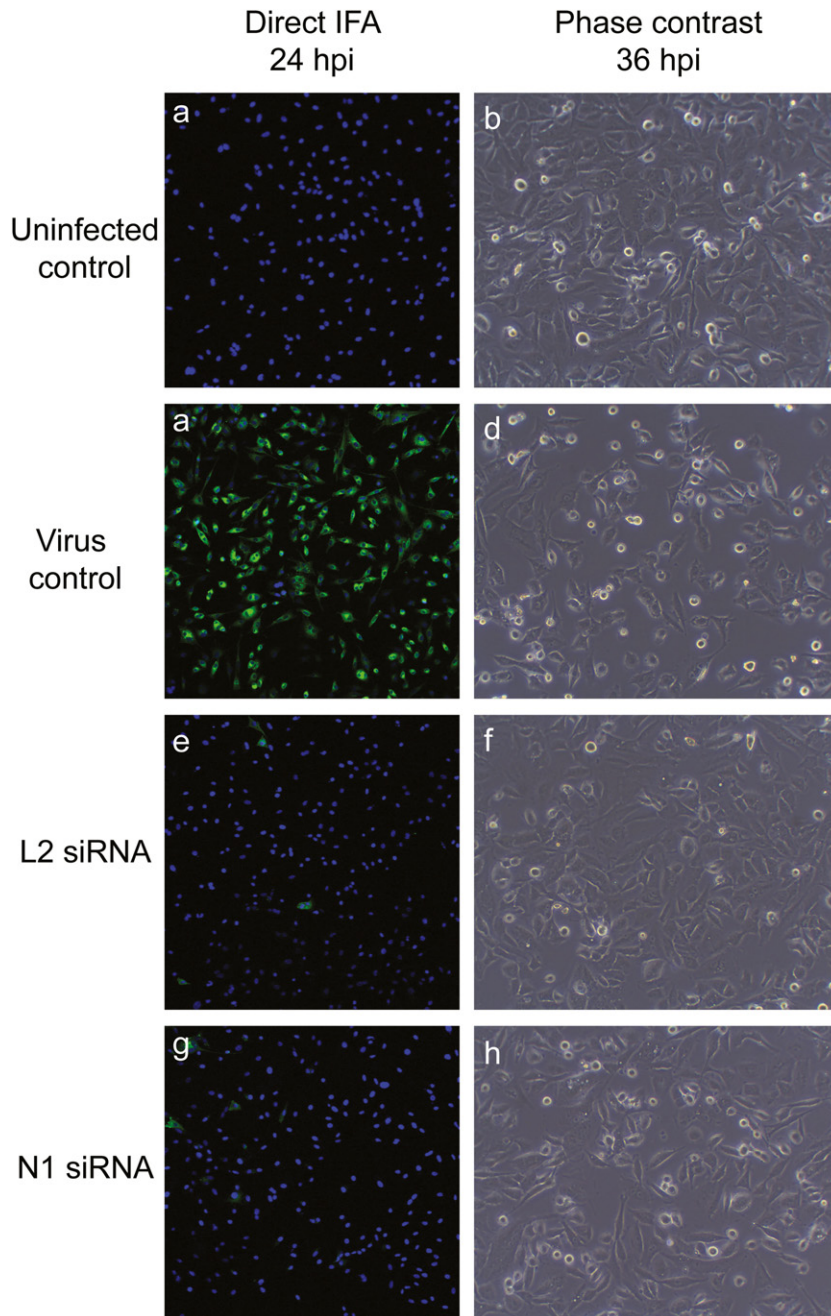


Fig. 3. Representative direct immunofluorescence and phase contrast micrographs from screening experiment showing results from healthy uninfected and untreated samples (uninfected control), infected cells with no prior siRNA treatment (virus control), and samples treated with siRNAs L2 and N1 prior to infection. Virus infected cells demonstrate diffuse and punctate green (FITC) cytoplasmic staining. Cell nuclei counterstained with DAPI appear blue.

(Fig. 2c). Reduction in extracellular virus titres paralleled the PCR data, ranging from 59.6% to 97%, confirming viral gene knockdown was associated with a significant reduction in the production of progeny virions (Fig. 2d). Qualitative assessment of viral protein expression using direct IFA demonstrated that treatment with FCoV specific siRNAs was associated with a reduction in the number of cells expressing FCoV antigen. This finding was most obvious for the highly effective siRNAs L2 and N1.

Additionally, phase contrast microscopy showed that the virus specific siRNAs provided some protection from virus induced CPE, in the case of L2 and N1 out to 50 h post infection. Representative micrographs are shown in Fig. 3.

3.3. Effect of siRNA concentration on FCoV replication

The two most effective siRNAs, targeted against L2 and N1, were used to examine the dose–response relationship

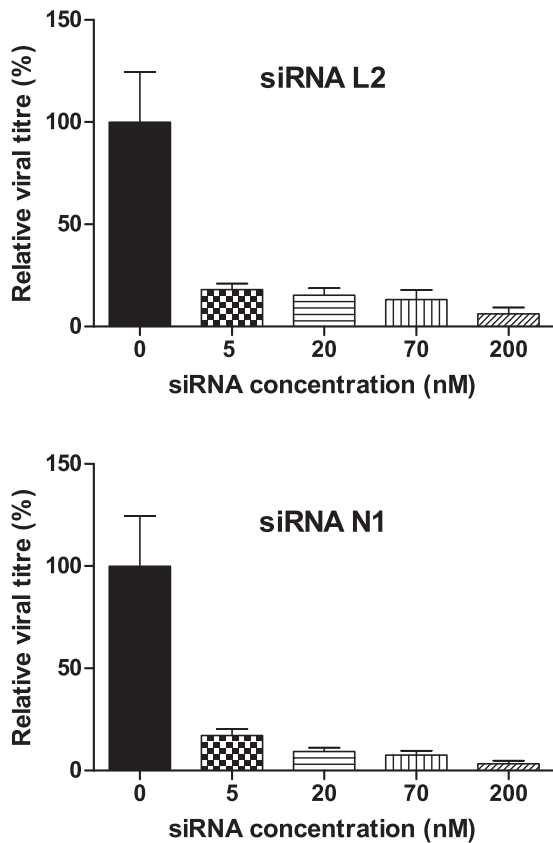


Fig. 4. Dose-dependent inhibition of FCoV replication. CRFK cells were transfected with varying concentrations of siRNA L2 and siRNA N1 and infected with FCoV. Extracellular viral titre was determined at 24 hpi by TCID50 infectivity assay. The value of the untreated control sample is defined as 100%. Values are expressed as geometric means \pm 95% confidence interval from three independent experiments.

of transfected siRNAs. The cell culture, transfection, and infection conditions were identical to those of the screening experiment, except for differing siRNA concentrations of 5 nM, 20 nM, 70 nM and 200 nM. Both tested siRNAs demonstrated concentration dependent inhibition of viral replication. Reductions in extracellular virus titres at 200 nM were 93.8% and 96.7% for L2 and N1 respectively, decreasing to 81.9% and 82.8% at 5 nM concentration (Fig. 4).

3.4. Effect of viral load on siRNA efficacy

Given the high viral loads demonstrated in cats with FIP, we sought to determine if siRNAs remained effective at inhibiting FCoV replication in the face of increased viral loads. To this end, the inhibitory effect of L2 and N1 siRNAs on FCoV replication was tested in CRFK cells using transfection and infection conditions identical to those of the screening experiment except cells were infected at a moi of 0.2, 2, or 20. siRNA efficacy was monitored by measuring extracellular virus titre and measurement of intracellular viral genome copies. Infection of CRFK cells with FIPV WSU 79-1146 at high multiplicity of infection was associated with significant cytopathic effect and cell

loss by 24 hpi (data not shown). To allow harvesting of sufficient cells for determination of intracellular viral loads, samples were harvested at 18 hpi, prior to the development of significant CPE. Although extracellular virus was tested 18 hpi, it has been demonstrated that there is only a slight increase extracellular FCoV titres when grown in CRFK cells between 12 and 24 hpi (Dewerchin et al., 2005). As shown in Fig. 5, both siRNAs remained effective at all tested moi, with >75% inhibition under all tested infection conditions. Results of qRT-PCR of viral genome showed a less pronounced reduction at higher moi compared to the viral titre results. This difference was greatest for siRNA N1, which demonstrated a reduction in viral genome copy number of only 35% at a moi of 20.

4. Discussion

RNAi has been demonstrated *in vitro* and in animal models to be a potent antiviral strategy for a diverse range of viral pathogens (Ge et al., 2003; He et al., 2003; Kahana et al., 2004; Martinez et al., 2002). This current study has demonstrated the effectiveness of specific siRNA mediated RNAi in substantially inhibiting FCoV replication *in vitro*, without cytopathic effects in a feline cell line. This provides important preliminary data on the potential effectiveness and safety of these antiviral agents, leading the way for extension into *in vivo* animal models and as a potential therapeutic agent for the deadly disease FIP, for which there is currently no effective treatment. This approach may also have a potential application as an antiviral treatment of asymptomatic chronic carriers and shedders of FCoV that serve as a continual source of infection for other cats in the environment, although the likely high cost of RNAi based therapeutics may preclude such a widespread application.

An initial screening experiment was conducted to identify siRNAs that could effectively inhibit FCoV replication. The eight siRNAs tested targeted different regions of the FCoV genome, including structural genes (M1, M2, N1, N2), non-structural genes (R1, R2), and untranslated regions (L1, L2). Selection of appropriate siRNA targets is a critical first step in using RNAi as an antiviral therapeutic. As a Baltimore group IV virus, the lifecycle of FCoV theoretically enables the simultaneous degradation of both viral genomic and messenger RNA via RNAi. Additionally, the unique discontinuous transcription mechanism of coronaviruses, resulting in a nested set of subgenomic mRNAs (Masters, 2006), enables targeting of multiple viral mRNAs and viral genomic RNA with a single siRNA. A common leader sequence, which plays a critical role in coronavirus gene expression, is present on the 5' end of each viral mRNA (Masters, 2006). siRNAs directed against the untranslated leader sequence can target all viral mRNA species and has been reported as an effective method for inhibiting coronavirus replication using an *in vitro* SARS-CoV model (Li et al., 2005b). Similarly, a number of papers have reported success in inhibiting the replication of SARS-CoV by targeting regions encoding the replicase, membrane, and nucleocapsid proteins (He et al., 2003, 2006).

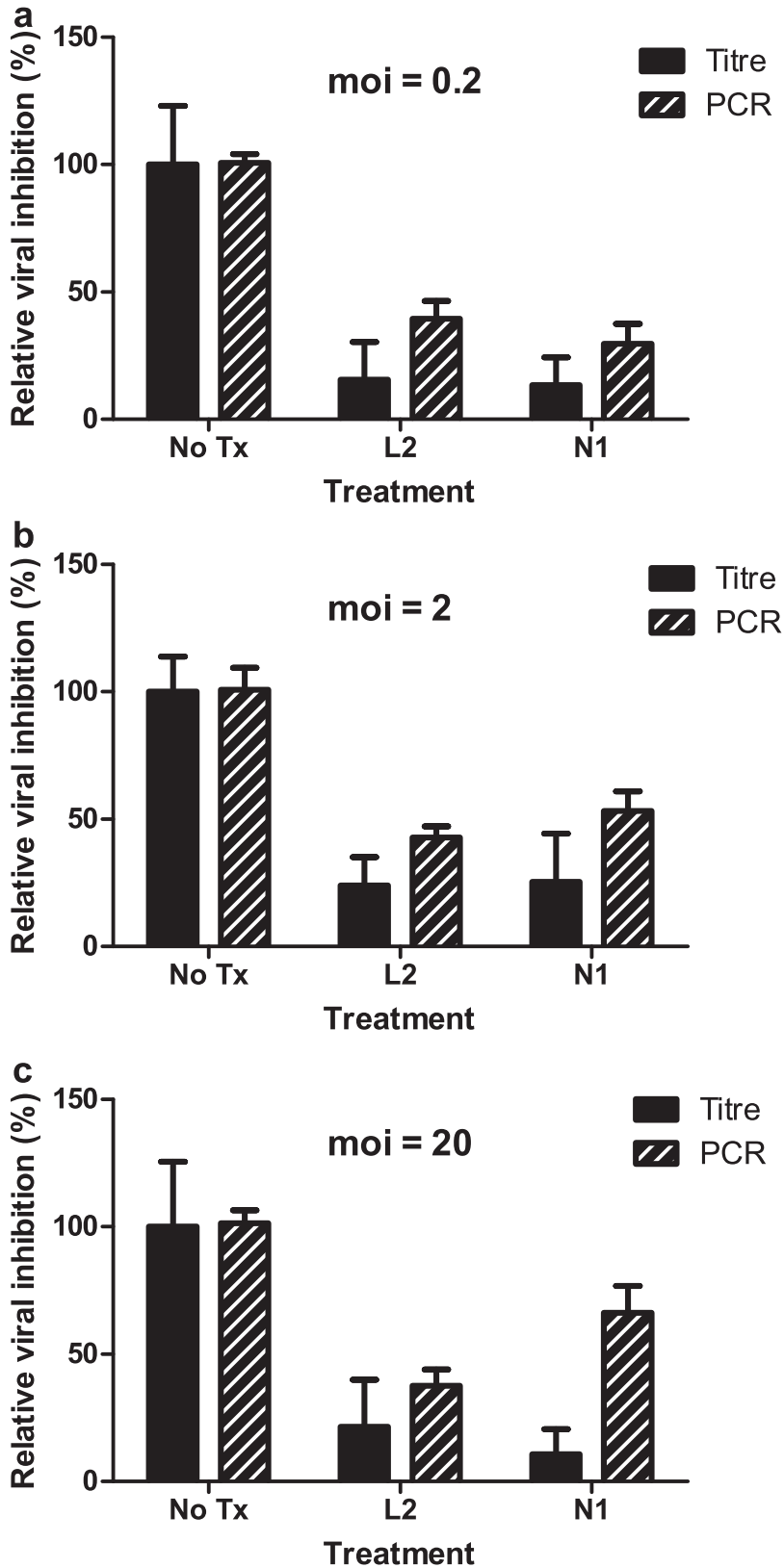


Fig. 5. Effect of increased multiplicity of infection on siRNA efficacy. CRFK cells transfected with 100 nM siRNA L2 or N1, or untreated were infected with FIPV WSU 79-1146 at a moi of (a) 0.2, (b) 2, or (c) 20. Extracellular viral titre was determined by TCID50 infectivity assay and intracellular viral genome copy number determined by qRT-PCR at 18 hpi. The value of the untreated control sample is defined as 100%. Values are expressed as geometric means \pm 95% confidence interval from three independent experiments.

An additional complicating factor in designing siRNAs for use against FCoV is the genetic heterogeneity inherent in the quasispecies nature of RNA viruses. Ideally an antiviral siRNA therapeutic should be effective against all field isolates, and thus conserved regions of the viral genome should be targeted. Such a strategy may also help to limit the development of escape mutants during treatment as highly conserved regions are likely to be structurally or functionally constrained. Our simple approach to siRNA design was able to identify five siRNAs with 100% homology to all reported FCoV strains. A more advanced approach for identifying appropriate siRNA targets in highly divergent viruses, such as that applied by Naito et al. (2006), would likely identify other suitably conserved targets.

The effectiveness of the siRNAs was assessed at the genetic level, measuring viral genomic and messenger RNA, and downstream, by a qualitative assessment of viral protein synthesis and quantification of the production of progeny virions. All of the siRNAs tested demonstrated an inhibitory effect on FCoV replication; however the magnitude of inhibition varied significantly. The highly effective siRNAs L2 and N1 were able to reduce extracellular virus titres by >95%, while less effective siRNAs such as L1 resulted in a reduction of approximately 60%. Such variability in potency between different siRNAs is frequently encountered in RNAi studies, even when they target closely associated regions. This is demonstrated in the current study where the L2 siRNA resulted in a greater than 10-fold inhibition in extracellular viral titre compared to the L1 siRNA, despite the two targeting contiguous regions of the genome.

For each treatment, the magnitude of inhibition determined by measurement of virus titre was greater than the inhibition seen in viral genome copy number. Although these two assays are looking at different endpoints, the consistent discrepancy may be in part explained by considering that the qRT-PCR for viral genome is able to detect cleaved, and therefore non-infectious viral genomes, that have not yet been degraded, and thus likely underestimates the potency of the siRNAs. Viral mRNA was also assayed by qRT-PCR. For siRNAs targeting structural genes, the knockdown of the respective mRNA was greater than the knockdown of viral genome. This finding may be due to the viral genome being at least partially protected from the effects of these siRNAs, perhaps due to their association with viral proteins. A critical step in virus assembly is genome encapsidation by the N protein to form a helical nucleocapsid (Masters, 2006). The tight genome–N protein interaction may serve to partially protect progeny genomes from RNAi mediated degradation through steric hindrance of RISC access to the target site, as has been suggested for other viruses (Bitko and Barik, 2001; Hu et al., 2002). Differences in RNA secondary structure between the smaller viral mRNA and the full length genomic copies may also limit target site accessibility (Shao et al., 2007). Alternatively, the difference may be attributed to the smaller mRNAs being degraded more rapidly following RISC mediated cleavage than the larger full length genome copies.

In this study we demonstrated that the two most effective siRNAs, L2 and N1, inhibit FCoV replication in a dose dependent manner. Most importantly, both of these highly effective siRNA were still able to significantly inhibit FCoV replication at the lowest tested concentration. This is important from a potential therapeutic point of view, as off-target effects associated with RNAi are in part concentration dependent (Behlke, 2006), and thus can be minimised by using low concentrations of highly potent siRNA.

In the screening and titration experiment the cells were infected at a relatively low moi (0.2). To assess the usefulness of the siRNAs in a therapeutic setting siRNAs L2 and N1 were tested at higher moi – 0.2, 2, and 20. The development of FIP is associated with high viral loads, and it was considered that the higher moi would more accurately mimic the findings in a natural infection (Kipar et al., 2006). Both siRNAs remained effective, showing greater than 75% inhibition of extracellular virus compared to the control samples even when challenged with a 100-fold increase in virus. As with the earlier experiments the reduction demonstrated in viral genome copies is less than the reduction in extracellular virus production. Again, a likely explanation is that qRT-PCR will detect non-infectious cleaved genomic RNA prior to its degradation. This hypothesis would explain the significantly increased N1 qRT-PCR results compared with L2 results, as the target site and the location of the PCR primers in the former are separated by over 25 kb.

We utilised the type II FCoV FIPV WSU 79-1146 in this study. Although the incidence of type II FCoV varies worldwide, in all reported studies infection with type I viruses is more common than type II viruses (Pedersen, 2009). It is unlikely that the higher prevalence of type I infections would invalidate any therapeutic application of the siRNAs tested in this study, as the primary difference between type I and II FCoVs is the spike protein, a region not targeted by any of the tested siRNAs. Additionally, siRNAs were selected in part based on homology to reported FCoV strains, including both type I and type II FCoVs.

The therapeutic application of RNAi technology faces a number of important challenges. Translation of *in vitro* results into an animal model requires the development of an appropriate delivery system. Delivery options for RNAi include chemical modifications to the siRNA duplex and lipid- and polymer-based delivery systems (Akhtar and Benter, 2007). Direct conjugation strategies which allow for targeted delivery of siRNAs to specific tissues and cell types will enhance the activity of siRNAs *in vivo* and reduce unwanted effects (Akhtar and Benter, 2007). Additional complications associated with RNAi *in vivo* are off-target effects. siRNA sequences used in this study were subjected to a BLAST search to identify and eliminate any anti-FCoV siRNAs with significant homology to feline genes. The current light coverage of the feline genome (1.9-fold) means that homologous feline sequences may be missed during BLAST search, resulting in unexpected and potentially adverse off-target effects. More in depth coverage of the feline genome will help to reduce the chances of off-target effects, however as many of the off-target effects are

mediated by the micro RNA pathway, and can be triggered by limited sequence homology in the seed region of the siRNA (Jackson and Linsley, 2010), it is more appropriate to talk of minimising off-target effects rather than avoiding them (Behlke, 2006).

Taken together, the results of this study suggest that RNAi may be a useful therapeutic option for the treatment of FIP. It is however unlikely that antiviral siRNA therapeutics, or any antiviral therapeutic for that matter, will be effective as a monotherapy in treating FIP. While increased viral replication is a triggering and perpetuating event in the disease pathogenesis, the characteristic pathological lesions of widespread serositis, vasculitis, and pyogranuloma formation, and the attendant clinical signs are immunopathological in nature. Thus effective treatment of FIP will likely require immunomodulatory therapy in addition to antiviral therapy. The results of this current study demonstrate that siRNA mediated RNAi may be an appropriate choice in fulfilling the latter requirement.

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