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RESEARCH ARTICLE

Evaluation of Antiviral Efficacy of Recombinant Feline Interferon Lambda-1 Against Calicivirus

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A B S T R A C T

Feline calicivirus (FCV), causing respiratory disease in cats, contains a positive-sense single-stranded RNA genome of approximately 7.7 kb. Currently, there is no effective treatment for FCV infection. Interferon lambda (IFN-λ) is crucial for the innate immune response in epithelial cells. IFN-λ can exert antiviral activities by triggering specific sets of IFN-stimulated genes (ISGs). In this study, recombinant feline interferon lambda 1 (rFeIFN-λ1) was cloned and overexpressed in *Escherichia coli* and purified to demonstrate its antiviral activity against FCV in Crandell–Rees feline kidney cells. rFeIFN-λ1 was non-cytotoxic and displayed a dose-dependent induction of several antiviral protein genes, including ISG15, Mx1, PKR, and OAS1. rFeIFN-λ1 significantly suppressed FCV replication when the cells were treated 1 day prior to viral infection. Suppression of viral replication was confirmed using RT-qPCR, western blotting, plaque assays, and immunofluorescence assays. To our knowledge, this is the first *in vitro* study showing that rFeIFN-λ1 effectively inhibits FCV replication in feline cells. These results indicate that rFeIFN-λ1 may be clinically useful as a novel immunostimulatory antiviral agent for the treatment of FCV infection.

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INTRODUCTION

Feline calicivirus (FCV) is an important pathogen that affects cats. The virus commonly causes pathogenic effects in the oral cavity and upper respiratory tract, leading to oral ulcers, oculonasal discharge, and lethargy in cats (Weese and Evason, 2019). However, rare and highly virulent variants can also cause severe disease, including edema, icterus, skin necrosis, and even fatal outcomes (Radford *et al.*, 2021). FCV belongs to the Caliciviridae family and carries a positive-sense single-stranded RNA genome of approximately 7.7 kb. The genome consists of three open reading frames (ORFs). ORF1 encodes non-structural proteins such as 3C-like protease and 3D-like polymerase. ORF2 encodes the primary capsid protein (VP1), and ORF3 encodes the minor protein component (VP2) found within the virion (Pereira *et al.*, 2018). As with other RNA viruses, the FCV genome undergoes rapid mutations with minimal repair rates, contributing to increasing strain diversity over time (Johnson, 1992). FCV-infected cats can excrete the virus for an extended period (>30 days) where sometimes FCV can persist for several years post-recovery of the infected cats (Radford *et al.*, 2009), making FCV infection difficult to control. Several antiviral drugs have been used to treat cats infected with various viruses (Hartmann, 2015). Ribavirin is one of the few antiviral agents that can effectively inhibit FCV replication *in vitro*. However, its use in cats is limited due to its apparent toxicity and associated side effects (Povey, 1978). FCV vaccines have been used, but due to the antigenic diversity of FCV, these cannot completely prevent infection (Poulet *et al.*, 2008).

The innate immune response to viral infection is crucial for preventing initial viral infections (Kawai and Akira, 2006, Michael Carty et al., 2021). Type I and III interferons (IFNs) play a key role in inducing innate

immunity (Megan L. Stanifer et al. 2020), which helps in viral clearance during the early stages of infection. Type I IFNs are expressed by various cells, including immune- and tissue-specific cells, while type III IFNs are primarily expressed in epithelial cells. Both types of IFNs trigger the expression of similar sets of interferon-stimulated genes (ISGs) (Pervolaraki *et al.,* 2018, Franck J. Barrat *et al., 2019*). However, type I IFNs are more potent than type III IFNs and can lead to stronger side effects such as inflammation (Lazear *et al.,* 2019). Therefore, type III IFNs are generally considered safer antiviral agents compared to type I IFNs. While some studies have demonstrated the antiviral effects of type I IFN-λ in dogs, chickens, and pigs (Fan *et al.,* 2014; Li *et al.,* 2019; Reuter *et al.,* 2014), the potential of feline type III IFN-λ as an antiviral agent remains unexplored.

In this study, the recombinant feline interferon lambda 1 (rFeIFN-λ1) was cloned and overexpressed in *Escherichia coli* (*E. coli*) and purified to demonstrate its antiviral activity against FCV in Crandell–Rees feline kidney cells This is the first report showing the antiviral effects of feline IFN-λ1 *in vitro*. This study suggests that rFeIFN-λ1 could be developed into an effective antiviral agent for the treatment of several feline viral diseases.

MATERIALS AND METHODS

Cell culture and virus propagation: Feline IFN-λ1 antiviral activity was tested in Crandell–Rees Feline Kidney (CRFK) cells (American Type Cell Collection). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 8% heat-inactivated fetal bovine serum (FBS) and an antibiotic-antimycotic solution (penicillin and streptomycin). Feline calicivirus (FCV17D03-3P) was obtained from the Animal and Plant Quarantine Agency of Korea and propagated in CRFK cells at a multiplicity of infection (MOI) of 0.01.

Cloning and Expression of Recombinant Feline IFN-λ1 of feline IFN-λ1: The mRNA sequence for feline IFN-λ1 (GenBank accession No. XM_006941288) was cloned into the pET-21a (+) vector with a 6xHis tag using primers 5'-GTT GGA TGA ACT ACC CGC CAA TC-3' and 5'-CAT ATG CGG CTC TGA TGG CTT GAA ACT G-3'. The plasmid was transformed into *E. coli* BL21(DE3) cells. *E. coli* was cultured in Luria Bertani medium at 37°C until the optical density at 600 nm reached 0.6–0.7, then harvested, resuspended in xTractor Buffer (Takara, Japan; 20 mL per g of cells), and sonicated. The lysate was centrifuged, and the supernatant was used for protein purification.

Purification of rFeIFN-λ1: Recombinant IFN-λ1 was purified using a gravity-flow method with nickel-nitrilotriacetic acid (Ni-NTA) agarose. The lysate was mixed with Ni-NTA slurry, incubated, and then loaded onto a column. The lysate-Ni-NTA mixture was loaded onto the column. The bound protein was washed twice with wash buffer (Takara, Japan). The 6xHis-tagged rFeIFN-λ1 protein was eluted from the column using elution buffer (Takara, Japan). Following purification, the protein was dialyzed using 1 L of PBS and cellulose membrane tubing (MW 10,000) overnight at 4 °C. The protein concentration was measured using the BCA Protein Assay Kit (Thermo scientific, USA).

Recombinant feline interferon omega: Commercially available feline interferon omega (Virbagen Omega, France) was compared with rFeIFN-λ1 for antiviral activity.

Cell viability assay and Plaque assay: Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay (Sigma-Aldrich, USA), with rFeIFN-λ1 added at concentrations of 1, 10, 100, 1000, and 1000 ng/mL. For antiviral testing, cells were treated with rFeIFN-λ1 at 10 and 100 ng/mL. The cells were fixed with formalin and stained with crystal violet to examine the virus reduction value of IFN treatment compared with that observed in the positive control.

RT-Qpcr: Cellular mRNA was extracted from FCV-infected CRFK cells, treated or untreated with rFeIFN-λ1 or rFeIFN-ω at 1, 10, and 100 ng/mL, using the Xenopure PF-Total RNA Purification Kit (Xenohelix, Korea). Quantitative RT-qPCR was performed with the One Step TB Green PrimeScript RT-PCR Kit (Takara, Japan). Primers for ISGs and GAPDH (referenced in Table 1) were used, and data were analyzed using the delta-delta Ct method.

Western blot analysis: Cell lysates were prepared by centrifugation and mixed with Laemmli buffer (Sigma-Aldrich, USA), followed by SDS-PAGE. Proteins were transferred to a membrane, incubated with antibodies against FCV-1 (ab33990, Abcam, USA) and GAPDH (ab8245, Abcam, USA), and detected using a chemiluminescent substrate (Thermo Scientific, USA).

Immunofluorescence assay: CRFK cell fixation was performed for 10 min with 4% paraformaldehyde on samples treated with interferon (i) 24 h before infection, (ii) concurrently with infection, and (iii) 24 h after infection. Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei.

Statistical analysis: Data were analyzed using GraphPad Prism (version 8.0.2; GraphPad Software, USA). Differences were analyzed using paired t-tests and one-way ANOVA.A p-value<0.05 was considered statistically significant. The delta-delta Ct method was used to analyze qPCR data, with GAPDH as a normalization control. All *in vitro* experiments were repeated at least three times.

RESULTS

Expression and purification of rFeIFN-λ1: The plasmid encoding feline rFeIFN-λ1-His6 gene was transformed into *E. coli* BL21(DE3). The *E. coli* BL21(DE3) transformants expressing rFeIFN-λ1-His6 gene was confirmed by PCR (data not shown), and the selected clone was used for protein expression analysis. The size and identity of the purified recombinant protein was determined by SDS-PAGE and western blot analysis, respectively. As expected, the molecular weight of the purified rFeIFN-λ1-His6 was 17.8 kDa (Fig. 1).

Table 1: Primers used in the study for mRNA analysis of ISGs and FCV by RT-qPCR.

Target gene	Primer	Sequence (5' to 3')	Product size (bp)	Reference
ISG15	Forward	TCC TGG TGA GGA ACC ACA AGG G	125	X. Zhang et al, (2016)
	Reverse	TTC AGC CAG AAC AGG TCG GC		
OAS1	Forward	AAC GTT TGC AGT GCA GTT TG	166	designed in this study
	Reverse	TCT GGG GTC AGG TCT GTA GG		
PKR	Forward	GGA AGG CAG AGC GTG AAG TAA	66	X. Zhang et al, (2017)
	Reverse	TGT AGT AGT GAA CGA TAT TTG GGT GAT		
Mx1	Forward	TTC GGA GGT GGA GGA GGC AAT C	134	Y. Wang et al, (2022)
	Reverse	CAG GGA GGT CTA TCA GGG TCA GAT C		
GAPDH	Forward	GTC CCC GAG ACA CGA TGG T	60	Safi et al, (2017)
	Reverse	CCA TAA CCC GCG GAC C		
FCV	Forward	GTT GGA TGA ACT ACC CGC CAA TC	122	Kummrow et al, (2005)
	Reverse	CAT ATG CGG CTC TGA TGG CTT GAA ACT G		

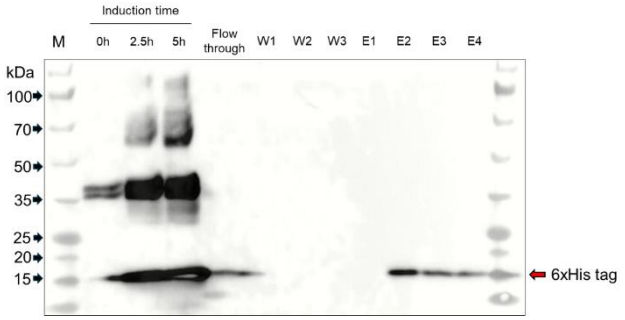

Fig. 1: Identification of recombinant rFeIFN-λ1 protein**.** The recombinant feline IFN-λ1 protein was confirmed through western blot using a 6x His tag antibody, which confirmed the expected size of 17.8 kDa. M: standard protein marker (7-240 kDa). Protein induction times of 0, 2.5, and 5 h. W1, W2, and W3: fraction of protein after 1st, 2nd, and 3rd washing, respectively. E1, E2, E3, and E4: elution 1, 2, 3, and 4, respectively.

Cell cytotoxicity of recombinant protein using CRFK cells: The cytotoxicity of rFeIFN-λ1 was examined before infecting CRFK cells with calicivirus. The cells were treated with 10-fold diluted recombinant protein for 3 days and determined the level of cell cytotoxicity. The data showed that rFeIFN-λ1 did not induce cytotoxicity up to the highest dose tested of 1000 ng/mL (Fig. 2).

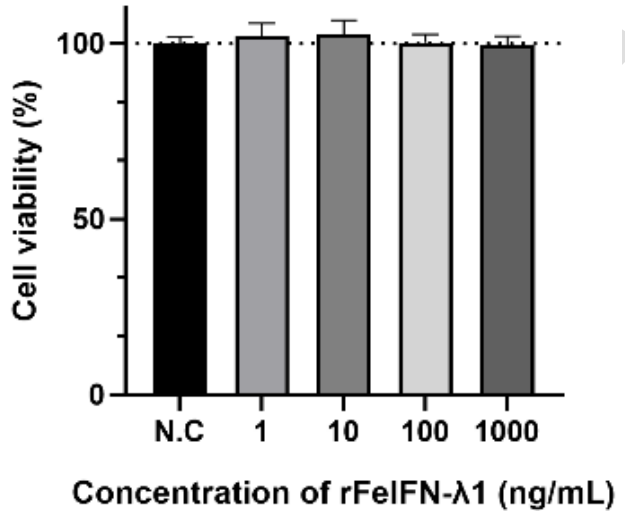

Fig. 2: Cytotoxicity analysis after rFeIFN-λ1 treatment.The viability of CRFK cells after treatment with different concentrations of feline IFN-λ1 was compared with that of untreated cells. Data are presented as mean ± standard deviation, derived from three independent experiments.

Reduction in viral replication as determined by plaque assay: To investigate the anti-viral activity of rFeIFN-λ1, plaque assay was performed. Treatment of calicivirus-infected CRFK cells with 10 ng/mL or 100 ng/mL rFeIFN-λ1, at -1, 0, and +1 dpi, showed significant reductions ($P<0.01$) in viral load (Fig. 3A and B). The cells treated with rFeIFN-λ1 at -1 dpi showed approximately 60% reduction in plaque numbers at both tested concentration of rFeIFN-λ1, compared to the positive control (Fig. 3B).

Reduction in viral replication as determined by immunofluorescent assay: The reduction in viral antigen levels in rFeIFN-λ1-treated cells was determined by immunofluorescence assay (IFA), after 36 h of viral infection. The most pronounced inhibitory effect was observed at -1 dpi. As expected, no fluorescence was observed in negative control, and the positive control cells displayed green fluorescent signals (Fig. 4). A dose-dependent decrease in fluorescent signals was observed in virus-infected cells treated with rFeIFN-λ1. These results suggest that rFeIFN-λ1 plays an important inhibitory role in FCV protein expression.

Comparison of antiviral activity between rFeIFN-λ1 and rFeIFN-ω by RT-qPCR and western blot: Since rFeIFN-ω is the only commercially available feline IFN, we compared its antiviral activity with that of rFeIFN-λ1. When calicivirus-infected cells were treated with 100 ng/mL of rFeIFN-λ1 and rFeIFN-ω at -1, 0, and +1 dpi, a significant reduction ($P<0.001$) in viral replication was observed by RT-PCR in the cells 1 day before viral infection (Fig. 5A). Similar results of viral reduction in cells treated with 100 ng/mL of rFeIFN-λ1 and rFeIFN-ω were identified by western blot assay at -1 dpi (Fig. 5B).

Confirmation of ISGs mRNA levels in cells using RT-Qpcr: The mRNA expression levels of ISG15, OAS1, PKR, and Mx1 in cells treated with rFeIFN-λ1 and rFeIFN-ω were determined using RT-qPCR. The cells were treated with 1, 10, and 100 ng/mL of rFeIFN-λ1 or rFeIFN-ω and cell lysates were collected daily for 3 days. Time-dependent and dose-dependent increases in ISG15, OAS1, PKR, and Mx1 mRNA levels were observed in the cells treated with rFeIFN-λ1 (Fig. 6A). However, dose-dependent increases in mRNA expression of the four ISGs were observed in the cells treated with rFeIFN-ω (Fig. 6B).

DISCUSSION

Type I IFNs serve as the primary defense against microbial infections. Additionally, type III IFNs exhibit similar antimicrobial properties to type I IFNs, but with fewer side-effects, such as inflammation. Type III IFNs, also known as IL28, IL29, or IFN-λ, were previously

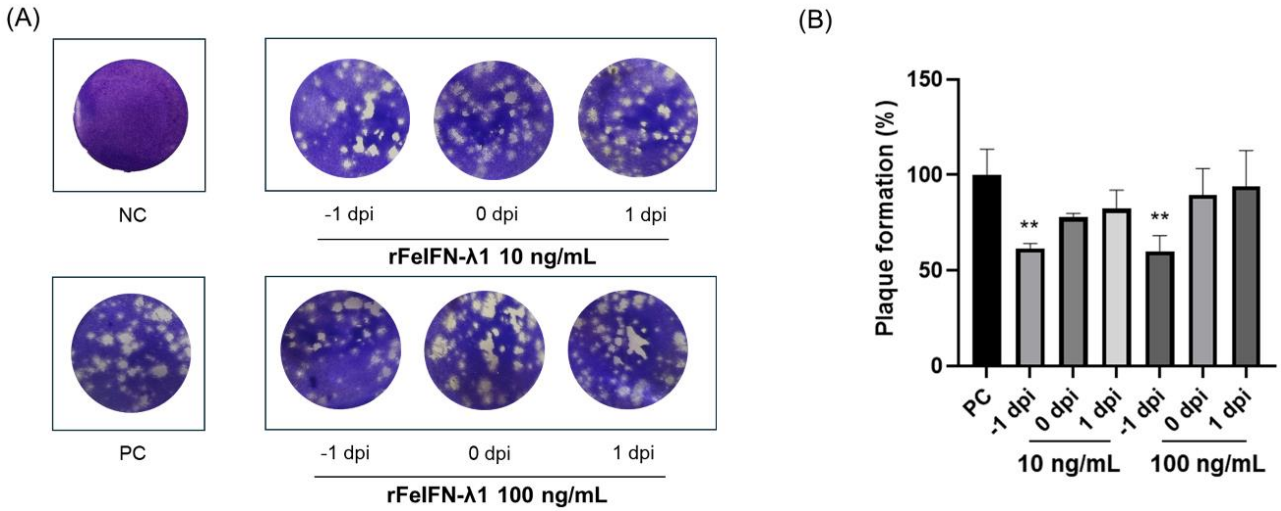

Fig. 3: Reduction in viral replication as determined by plaque assay. Plaque assay was performed to verify the anti-viral activity of rFeIFN-λ1. CRFK cell infected with calicivirus were treated with 10 ng/mL or 100 ng/mL at -1, 0, and +1 dpi. Viral replication was significantly reduced in the cells treated with rFeIFN-λ1 at -1 dpi (A and B). Compared to the positive control, the cells treated with rFeIFN-λ1 at -1 dpi showed approximately 60% reduction in plaque numbers at tested rFeIFN-λ1 concentrations (B). *P<0.05; **P<0.01; *** P<0.001

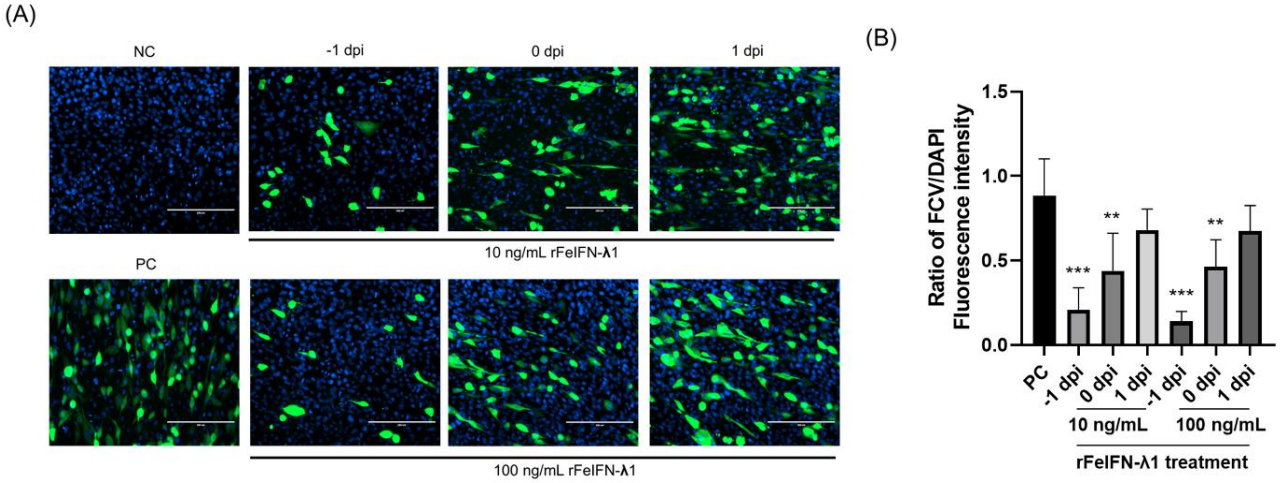

Fig. 4: Suppression of FCV replication by treatment with rFeIFN-λ1 as demonstrated by the IFA (A) CRFK cells were treated with 10 or 100 ng/mL of feline IFN-λ1 at -1, 0, and +1 dpi. The viral antigen was detected using the IFA method. (B) Virus reduction ratios. The data are presented as violin plots, showing the mean ± standard deviation from three independent experiments. *P<0.05; **P<0.01; *** P<0.001

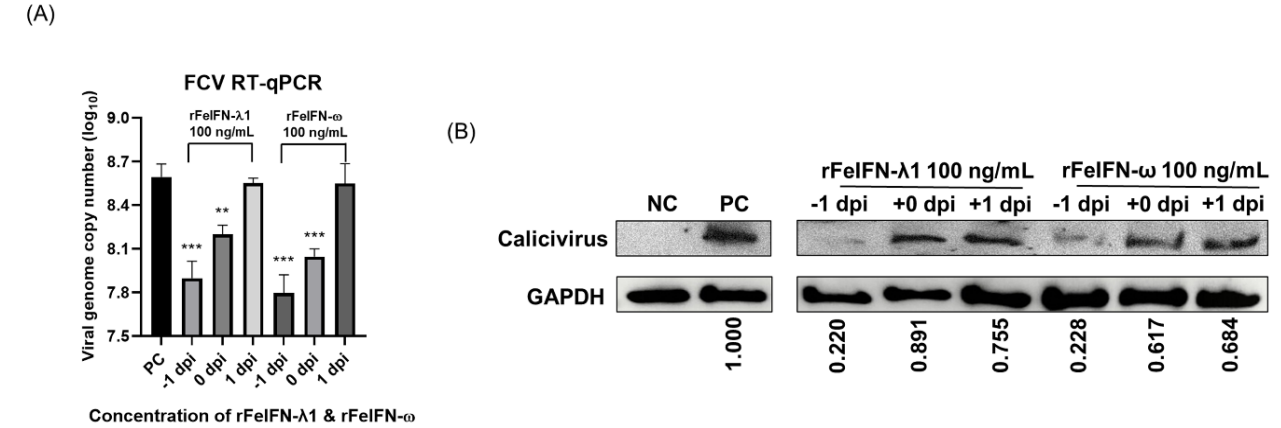

Fig. 5: Comparison of antiviral activity of rFeIFN-λ1 and rFeIFN-ω by RT-qPCR and western blot. Cells infected with calicivirus were treated with 100 ng/mL of rFeIFN-λ1 or rFeIFN-ω at -1, 0, and +1 dpi. In RT-PCR data, viral replication was significantly reduced at the cells which were treated with 100 ng/mL of rFeIFN-λ1 or rFeIFN-ω at -1 dpi (A). When determined by western blot assay, significant viral reduction in cells treated with 100 ng/mL of rFeIFN-λ1 or rFeIFN-ω were detected at -1 dpi (B). *P<0.05; **P<0.01; *** P<0.001

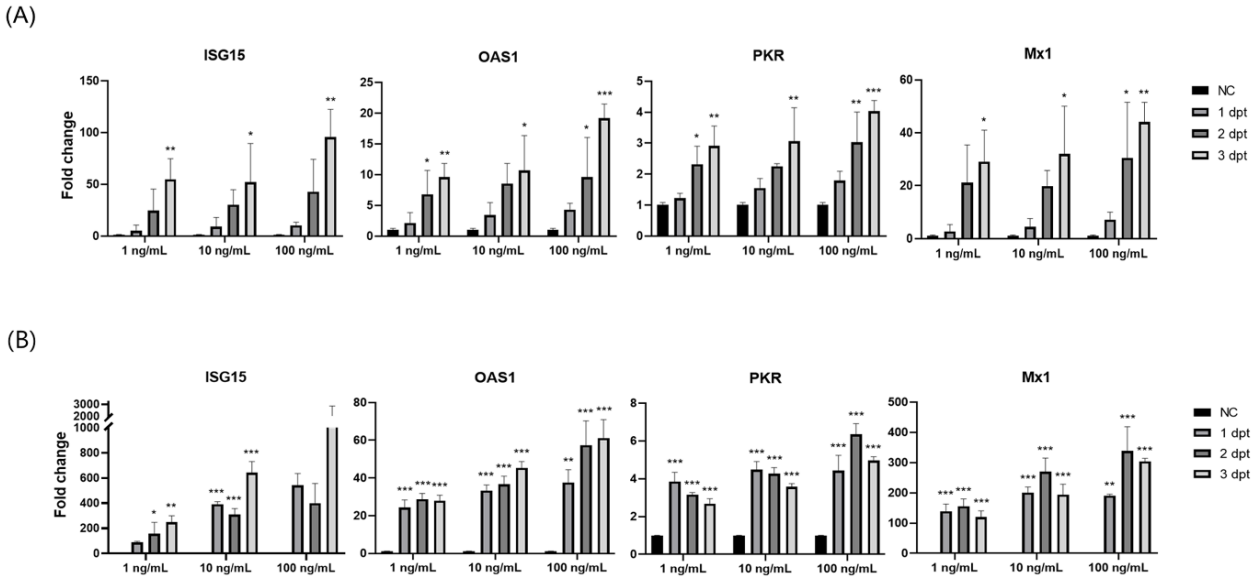

Fig. 6: Expression of interferon-stimulated gene (ISG) mRNA after rFeIFN-λ1 or rFeIFN-ω treatment**.** Expression levels of ISG mRNA determined by RT-qPCR in CRFK cells treated with 1, 10, and 100 ng/mL of feline IFN-λ1 or rFeIFN-ω. Furthermore, mRNA levels were measured as DPT (day post treatment). Cell lysates were prepared for mRNA extraction after 24, 48, and 72 h. (A) ISG15, (B) OAS1, (C) PKR, and (D) MX1 mRNA were analyzed by RT-qPCR using specific primers. The data are presented as violin plots, showing the mean ± standard deviation from three independent experiments. *P<0.05; ** P<0.01; *** P<0.001

believed to function similarly to both type I IFNs and the IL-10 families (Kotenko, 2011). However, their role in innate immunity differs from that of type I IFNs as they are crucial in epithelial cells without triggering widespread inflammatory responses (Wack *et al.*, 2015). Furthermore, type III IFNs are produced in response to specific viral infections, such as HBV and HCV, and exert antiviral effects *in vitro* (Broggi *et al.*, 2020; Robek *et al.*, 2005). Various viruses, such as calicivirus, can be fatal in cats (Addie *et al.*, 2000; Coyne *et al.*, 2006). However, only a few antiviral therapeutics are currently available for the treatment of FCV. Therefore, there is a need for the development of new antiviral drugs. In this study, the antiviral activity of rFeIFN-λ1, a representative type III IFN, was tested *in vitro*.

High concentrations of IFN-α2b have been shown to be cytotoxic (Liu *et al.*, 2020). In this study, the cytotoxicity of rFeIFN-λ1 was tested before further studies were conducted. No cytotoxicity was observed when cells were treated with rFeIFN-λ1 at concentrations of 1, 10, 100, and 1000 ng/mL. Next, a significant anti-calicivirus activity of rFeIFN-λ1 was confirmed through a plaque assay against, with the best efficacy observed when the interferon was pre-treated 1 day prior to viral infection. Further, through IFA, we validated the observed antiviral activity of the plaque assay. The IFA data were consistent with the plaque assay data. Further, we compared the antiviral activity of rFeIFN-λ1 with that of rFeIFN-ω, as rFeIFN-ω is known to display a comparable antiviral activity similar to type III IFN and it was commercially available. As expected, a decrease in FCV replication was observed when the viral-infected cells were treated with either rFeIFN-λ1 or rFeIFN-ω. The most effective antiviral effect was observed in cells treated with rFeIFN-λ1 and rFeIFN-ω 1 day before viral infections (Smith *et al.*, 2005).

Western blot analysis demonstrated a significant reduction in viral antigens in the virus-infected cells upon treatment with rFeIFN-λ1 or rFeIFN-ω. The gene expression analysis of various antiviral factors such as ISG15, PKR, Mx1, and OAS1 was studied in cells treated with IFNs (Sadler and Williams, 2008; Sooryanarain *et al.*, 2017), as specific antibodies to these feline antiviral factors were not available. The mRNA expression levels of these factors were upregulated in response to the treatment of rFeIFN-λ1 or rFeIFN-ω at different concentrations. This increase in expression was time-dependent. The data suggested that upregulated factors would induce reduction of virus replication. These results further strengthen our observations that the rFeIFN-λ1 used in this study has antiviral activity, through induction of ISGs that led to inhibition of viral gene expression and protein synthesis required for FCV replication *in vitro*.

Conclusions: Although several viral diseases, including FCV, cause serious pathogenic problems in cats, there is still no effective treatment available. In this study, we showed that rFeIFN-λ1 effectively inhibits FCV replication in cells. Therefore, rFeIFN-λ1 is a promising immunostimulatory antiviral candidate that could be developed into a novel antiviral agent against various viral diseases in cats.

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Conflict of interest statement: The authors declare no competing interests.

Authors contribution: KBL and ISC conceived, validated, and designed the study. DHK, JHK, HJG, SHH, and DYK conducted the experiments. DHL, JBL, SYP, CSS, SWL, and ISC supervised all experiments. KBL and ISC wrote the manuscript and prepared the figures. ISC contributed to funding acquisition. All authors interpreted

the data, critically revised the manuscript for important intellectual contents and approved the final version.

Data and materials availability: Data will be made available on request.

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