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Original article

Expression of IFN- λ 1 from Congjiang pigs and its effect on anti-PRRSV proliferation

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Abstract

An eukaryotic expression system of Congjiang pigs IFN- λ 1 was constructed to obtain its expression in CHO-K1 cells and the inhibition effect of Congjiang pig IFN- λ 1 on PRRSV proliferation was verified. The eukaryotic expression plasmid pEGFP-PoIFN- λ 1 was constructed from the pig IFN- λ 1 gene fragment and transfected into CHO-K1 cells. Expression was detected by fluorescence microscopy and Western blotting. The influence on the proliferation of PRRSV was assessed. The results of the study showed that the recombinant plasmid pEGFP-PoIFN- λ 1 was constructed correctly. After transfection, green fluorescent signal was detected in CHO-K1 cells by fluorescence microscopy. Western blot analysis revealed that in cells at different time periods after transfection, porcine IFN- λ 1 was expressed, with the highest expression observed 36 h after transfection. The antiviral activity of the supernatant after 36 h of transfection was determined by the micro cytopathic inhibition method, and the biological activity was 2.1×10^3 U/mL. Quantitative PCR was used to detect the proliferation of PRRSV, and the results showed that Congjiang pigs IFN- λ 1 significantly inhibited the mRNA expression of PRRSV and viral proliferation in a dose- and time-dependent manner. This study established a Congjiang pig IFN- λ 1 eukaryotic expression system, and the quantitative PCR method showed that it has a significant inhibitory effect on the proliferation of PRRSV, which lays a foundation for the future production of antiviral drugs and clinical application.

Key words: porcine ifn- λ 1, porcine type III interferon, eukaryotic expression, anti-proliferation

Introduction

Interferon- λ (IFN- λ) is a new type III interferon discovered by Sheppard et al. in 2003. Its family members include IFN- λ 1, IFN- λ 2, IFN- λ 3, and IFN- λ 4 (Sheppard et al. 2003). In cells, IFN- λ 1 is expressed earlier than the other three types of IFN- λ due to differences in IFN- λ gene structures and transcriptional regulators. Therefore, IFN- λ 1 plays an important role in the early inhibition of viral proliferation (Xu et al. 2015). λ interferon is a kind of broad-spectrum antiviral glycoprotein produced after cells are invaded by viruses or stimulated by bacterial endotoxins and mitogens. It has a variety of antiviral and immunomodulatory effects (Hermant et al. 2014). With the development of science and technology, researchers have been studying interferon more deeply, starting from research on the signaling pathway of type I interferon to the clinical application of type II interferon, and then to the gradual exploration of the biological activity of type III interferon. This large family of cytokines has played an irreplaceable role in other factors in human history (Galani et al. 2017). From herpes simplex virus to hepatitis virus, from systemic lupus erythematosus virus to AIDS virus, and from acute diarrhea virus to influenza virus, the mechanism of action of λ interferon has played a certain role, and its protein activity has been shown to control virus spread. In the field of animal medicine, the study of λ interferon has been relatively slow, but in recent years there has been an increasing number of studies at home and abroad. Research on the cloning and expression of λ interferon genes and antiviral infections has expanded towards future clinical applications. Theoretical investigations have been carried out. Current studies have found that IFN- λ 1 has a good antiviral effect; in the continuous passage of a human lung epithelial cell line (Calu-3), increased expression of IFN- λ 1 can strongly inhibit the proliferation of H1N1 influenza virus (Ilyushina et al. 2017). Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences found that pig interferon IFN- λ 1 shows strong inhibitory effect on porcine epidemic diarrhea virus because it acts on the intestinal mucosa. In epithelial cells, the antiviral effect is more targeted than that of the previously known type I interferon, which provides new ideas for the further development of new antiviral drugs (Li et al. 2017).

The Congjiang pigs are a local miniature pigs breed in Guizhou Province, and because of its highly homozygous genes and high similarity to the human genome, it shows high research value in the medical field (Zhou et al. 2006, Shen et al. 2007). In this study, blood was collected from Congjiang pigs from the pig breeding farm of Guizhou University. Total RNA was extracted

from the isolated peripheral blood lymphocytes, and RT-PCR was used to amplify the IFN- λ 1 gene, which was then cloned and sequenced, followed by the construction of a eukaryotic expression vector. Evaluation of the biological function of its expression products and subsequent research on its antiviral activity against porcine reproductive and respiratory syndrome virus were performed.

Materials and Methods

Main experimental material

The eukaryotic expression vector pEGFP-C1 was supplied by the Key Laboratory of Genetic Breeding and Reproduction of Plateau Mountain Animals of the Ministry of Education of Guizhou University, Chinese Hamster Ovary Epithelial Cells (CHO-K1) were purchased from Fenghui Biological Co., Ltd. *E. coli* DH5 α and IFN containing the target gene - λ plasmids were provided by the Institute of Animal Diseases of Guizhou University. Porcine Reproductive and Respiratory Syndrome virus Zhejiang isolate E11105 was donated by Associate Professor Wen Guilun of Guizhou University. Marc145 cells were preserved by Guizhou University Preventive Veterinary Laboratory.

Main reagent

Fetal bovine serum, double antibody solution (penicillin), 0.25% trypsin, high-glucose DMEM cell culture fluid were purchased from Gibco; liposome transfection kit LipofectAMINETM2000 was purchased from thermo; Xho I, EcoR I, T4 Ligase, RNA extraction kit, DL2000 Marker were purchased from Bao Bioengineering (Dalian) Co., Ltd.; PVDF membrane, DAB color development kit, sodium dodecyl sulfate (SDS), Tris-cl, glycine (Glycine), Coomassie brilliant blue R-250 and skimmed milk powder were purchased from Solibao Biotechnology Co., Ltd.; plasmid extraction kits were purchased from AxyGen; SDS-PAGE gel preparation kits were purchased from Biyuntian Biotechnology Company; GFP-labeled antibody primary antibody, secondary antibody goat anti-mouse IgG (H + L) HRP, Oligo (dT) 15 Primer, 2 \times Es Taq DNA Polymerase, and reverse transcription kit were purchased from Kangwei Century Biotechnology Co., Ltd.

Expression and activity determination of recombinant plasmid pEGFP-C1-IFN- λ 1

At 24, 36, and 48 hours after transfection, the expression of green fluorescent protein was observed with a fluorescent microscope and the growth status and cell

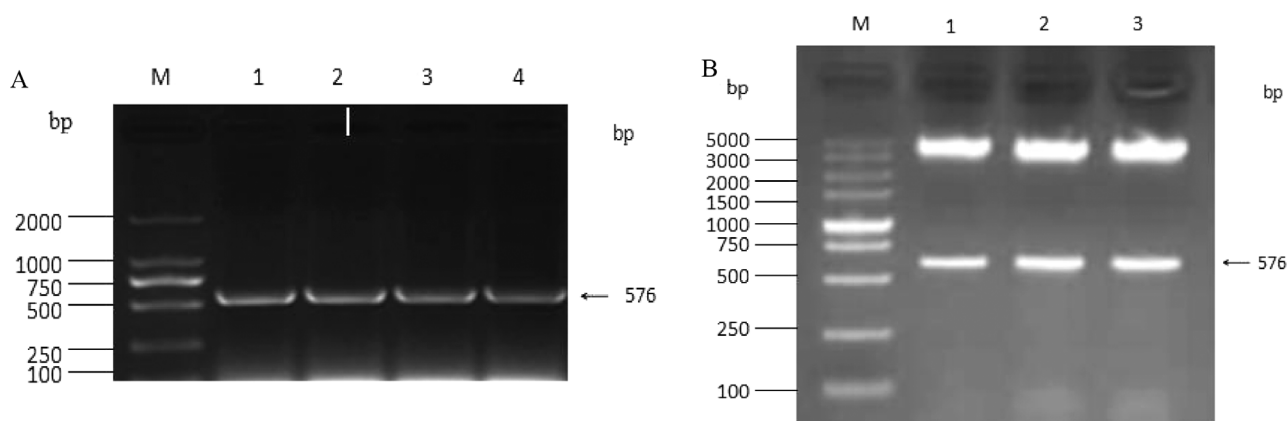


Fig. 1 A. Bacteria lysate PCR identification of recombinant plasmid, M: Marker; 1-4: Bacteria lysate PCR amplification product of recombinant plasmid. B: Analysis of eukaryotic expression vector pEGFP-C1-PoIFN- λ by enzyme digestion, M; DNA Ladder Marker; 1-3: Product from the recombinant plasmid pEGFP-C1-IFN- λ by enzyme digestion.

morphology of CHO-K1 cells were also observed to further determine the success rate of transfection. Collect cells at 36 hours after transfection, reverse transcription after extracting cellular RNA, PCR amplification using specific primers for IFN- λ gene, and agarose gel electrophoresis to detect the transcription of the target gene, and Extract CHO-K1 cell RNA according to the kit method and perform reverse transcription verification. After observing the expression of the protein with a fluorescence microscope, a series of verifications are required, including SDS-PAGE electrophoresis and Western blot analysis. The renatured protein is removed, and a protein quantification kit is used. Protein quantification was performed, and its protein activity was measured in the MDCK-VSV system using the micro-cytopathic inhibition method, and its protein activity was directly determined by the Institute of Microbiology, Chinese Academy of Sciences.

Effect of recombinant plasmid pEGFP-C1-IFN- λ 1 on PRRSV proliferation

After treating Marc145 cells with PoIFN- λ 1 eukaryotic expression products at a dose of 0 U / mL, 50 U/mL, 100 U/mL, and 150 U/mL for 24 h, they were seeded with PRRSV and collected at 12 h, 24 h, and 36 h after inoculation, PRRSV-N gene RNA was extracted in order to optimize the reaction conditions. PRRSV-N gene real-time fluorescence quantitative RT-PCR was performed on the extracted RNA, at the same time a negative control was prepared, and the dilution factor was 10^{-3} . As a positive control, different amounts of PRRSV-N gene RNA were obtained by calculating different concentrations of IFN- λ 1, and the expression levels were analyzed.

Results

Verification of plasmid pEGFP-C1-IFN- λ 1

The plasmid containing the gene of IFN- λ 1 and the pEGFP-C1 plasmid were digested, gel-recovered, ligated and transformed. After the vector was constructed, PCR and enzyme digestion of the bacterial solution were performed for identification. The digested gene fragment was consistent with the predicted size (576 bp) (Fig. 1A and B). The results of sequencing performed by a commercial company were compared with the sequence in GenBank, which showed that the recombinant plasmid pEGFP-C1-IFN- λ 1 was constructed correctly and none of the bases had changed.

Transfection of recombinant plasmid pEGFP-C1-IFN- λ 1

The successfully constructed recombinant plasmid pEGFP-C1-IFN- λ 1 was transfected into CHO-K1 cells using Lipofectamine™ 2000 transfection reagent, and the cells were observed at 24 h, 36 h, and 48 h after transfection with a fluorescence microscope. A low level of fluorescence was observed at 24 hours, more scattered fluorescence could be seen at 36 hours, and the fluorescence intensity had decreased at 48 hours (Fig. 2). The experimental results showed that the pEGFP-C1-IFN- λ 1 recombinant plasmid was successfully transfected, the gene labeled with green fluorescent protein was expressed, and the construct could be used for further investigation of the expression of the Congjiang pig lambda interferon gene in CHO-K1 cells.

Observed with a bright-hold microscope, cell growth at 24 h after transfection of the recombinant plasmid pEGFP-C1-IFN- λ 1 was significantly better than that of cells at 36 and 48 h after transfection. After transfection with the empty plasmid pEGFP-C1, both

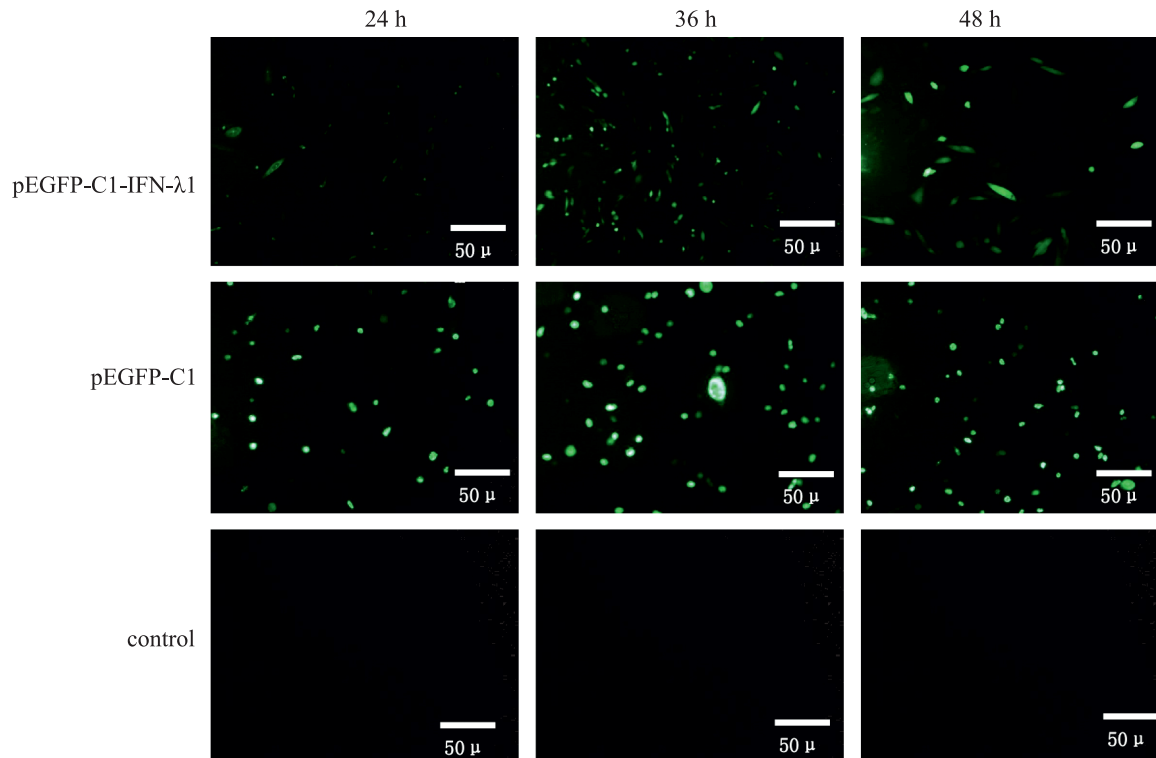


Fig. 2. Detection of CHO-K1 green fluorescence protein. Recombinant plasmid EGFP-C1-IFN- λ , pEGFP-C1 transfected CHO-K1 cells 24, 36 and 48 hours, respectively.

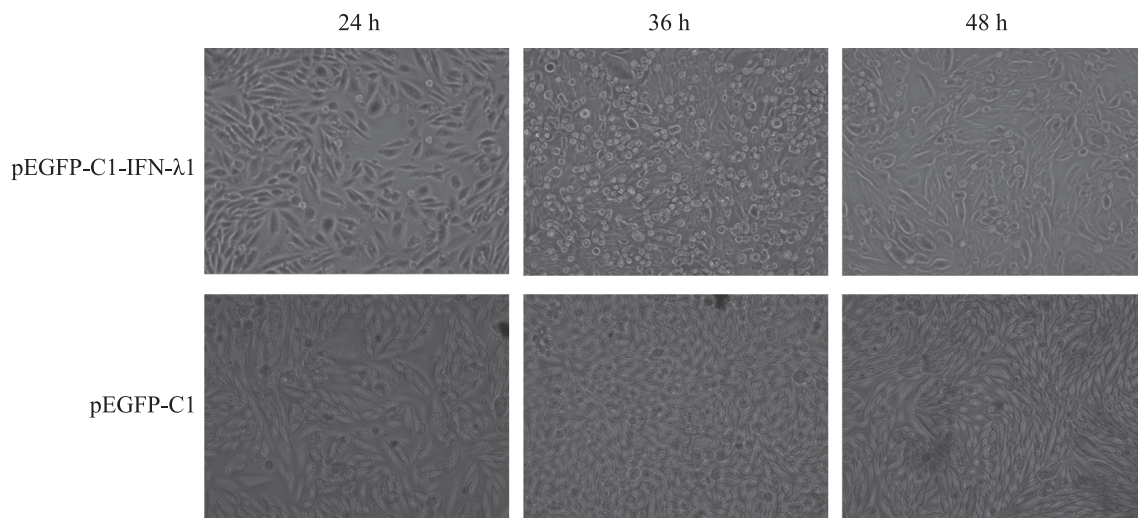


Fig. 3. Effect of recombinant plasmid pEGFP-C1-IFN- λ 1 transfection on CHO-K1 cells growth (10×10). Recombinant plasmid pEGFP-C1-IFN- λ 1, pEGFP-C1 transfected CHO-K1 cells in 24, 36 and 48 hours respectively.

the cell size and morphology indicated healthy cells, while it can be seen that the cell growth and morphology of pEGFP-C1-IFN- λ 1-transfected CHO-K1 cells changed (Fig. 3). The results indicate that the transfection of the recombinant plasmid pEGFP-C1-IFN- λ 1 affected the normal growth of CHO-K1 cells.

Detection of PoIFN- λ 1 Gene Transcription in CHO-K1 Cells

Cellular RNA was collected 48 h after transfection, and reverse transcription was performed. Specific primers for IFN- λ 1 gene were as follows: forward primer λ 1-IFN-F: 5'-CCCTCGAG CTATGGCTACAG CTTGGATCGTGGTGC-3' and reverse primer λ 1-IFN-R: 5'-CCGAATTC TCAGATGTGCAAGTCT CCACTGGTAACAC-3'. The gene was amplified by RT-PCR and after electrophoresis, the PCR product

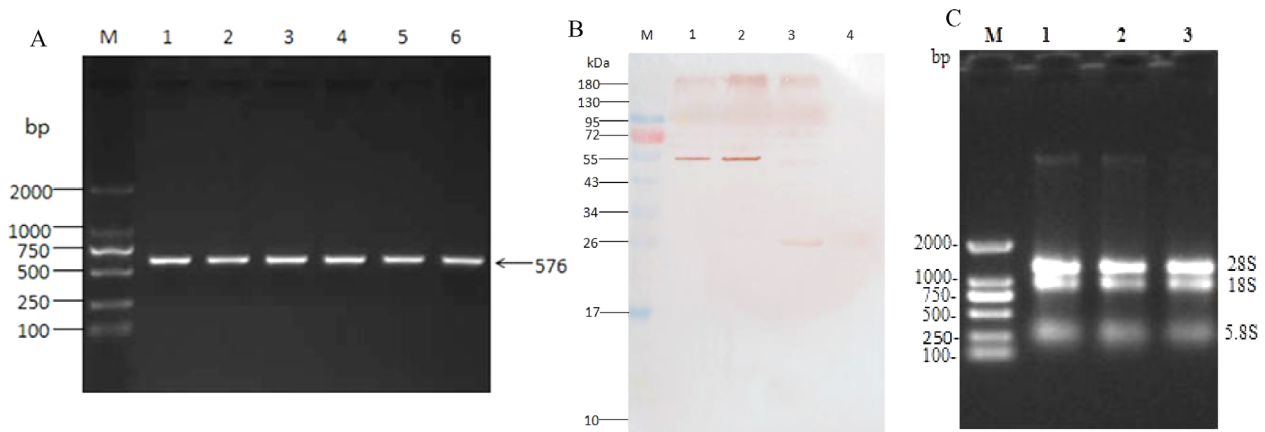


Fig. 4. A: Transcription of IFN- λ gene in CHO-K1, M: DNA Ladder Marker. 1-3: The amplified bands from IFN- λ 1 gene of the CHO-K1. B: Detection of IFN- λ protein by Western blot. M: Marker; 1: Fusion protein of pEGFP-C1-IFN- λ 1. 2: Fusion protein of pEGFP-C1-IFN- λ 1. 3: pEGFP-C1. 4: control. C: PRRSV total RNA M: Marker; 1-3 PRRSV total RNA sample.

Table 1. Parameter optimization of PCR amplification of PRRSV-N gene.

target gene	Annealing temperature °C	Primer concentration $\mu\text{mol} / \text{L}$	Probe concentration $\mu\text{mol} / \text{L}$
PRRSV-N	62	10	10

Table 2. Prrsv-n gene fluorescence quantitative PCR repeatability test results.

Gene name	Standard concentration (copies / μL)	Repeat within batch (C_T)			Repeat between batches (C_T)		
		average value	SD	CV (%)	average value	SD	CV (%)
PRRSV-N	1×10^7	17.36	0.13	0.74	17.73	0.23	1.30
	1×10^6	20.84	0.17	0.82	20.92	0.25	1.17
	1×10^5	24.06	0.31	1.29	24.58	0.40	1.61

of the target gene was detected. The electrophoresis results showed (Fig. 4A) that CHO-K1 cells transformed with the recombinant plasmid pEGFP-C1-IFN- λ 1 could be amplified the IFN- λ 1 gene fragment, which was 576 bp in length as expected, indicating that the recombinant plasmid pEGFP-C1-IFN- λ 1 interferon gene can be expressed in CHO-K1 cells.

Detection of EGFP-PoIFN- λ 1 recombinant protein expression

After observing the expression of green fluorescent protein with a fluorescence microscope, it was confirmed that the recombinant plasmid pEGFP-C1-IFN- λ 1 was successfully expressed in CHO-K1 cells, and the cellular RNA was extracted by PCR amplification. Agarose gel electrophoresis verified that an amplification product was obtained, indicating that the gene was successfully transcribed and expressed in cells. After transfection, the CHO-K1 cells were subjected to SDS-PAGE electrophoresis, and the proteins were transferred to a nitrocellulose filter (NC membrane) and analyzed by Western blot technology. The results

showed that the product of transcription was mainly generated from cell pellets, and a specific band appeared at approximately 55 kDa in the recombinant plasmid pEGFP-C1-IFN- λ 1 group, but the band was absent in the empty plasmid group. No bands appeared in CHO-K1 cells alone and the blank control (Fig. 4B). This finding fully demonstrates that the eukaryotic expression vector of the lambda interferon gene from Congjiang pig was successfully constructed and successfully expressed in CHO-K1 cells, producing an approximately 55 kDa pEGFP-C1-IFN- λ 1 fusion protein.

Test results of total cell RNA

The total RNA of PRRSV was extracted and tested for concentration and purity. The OD260/OD280 of the RNA samples were between 1.8 and 2.0, and the concentrations were high. The quality of the extracted RNA was assessed, and subsequent experiments could be performed. The extracted RNA was subjected to agarose gel electrophoresis (Fig. 4C).

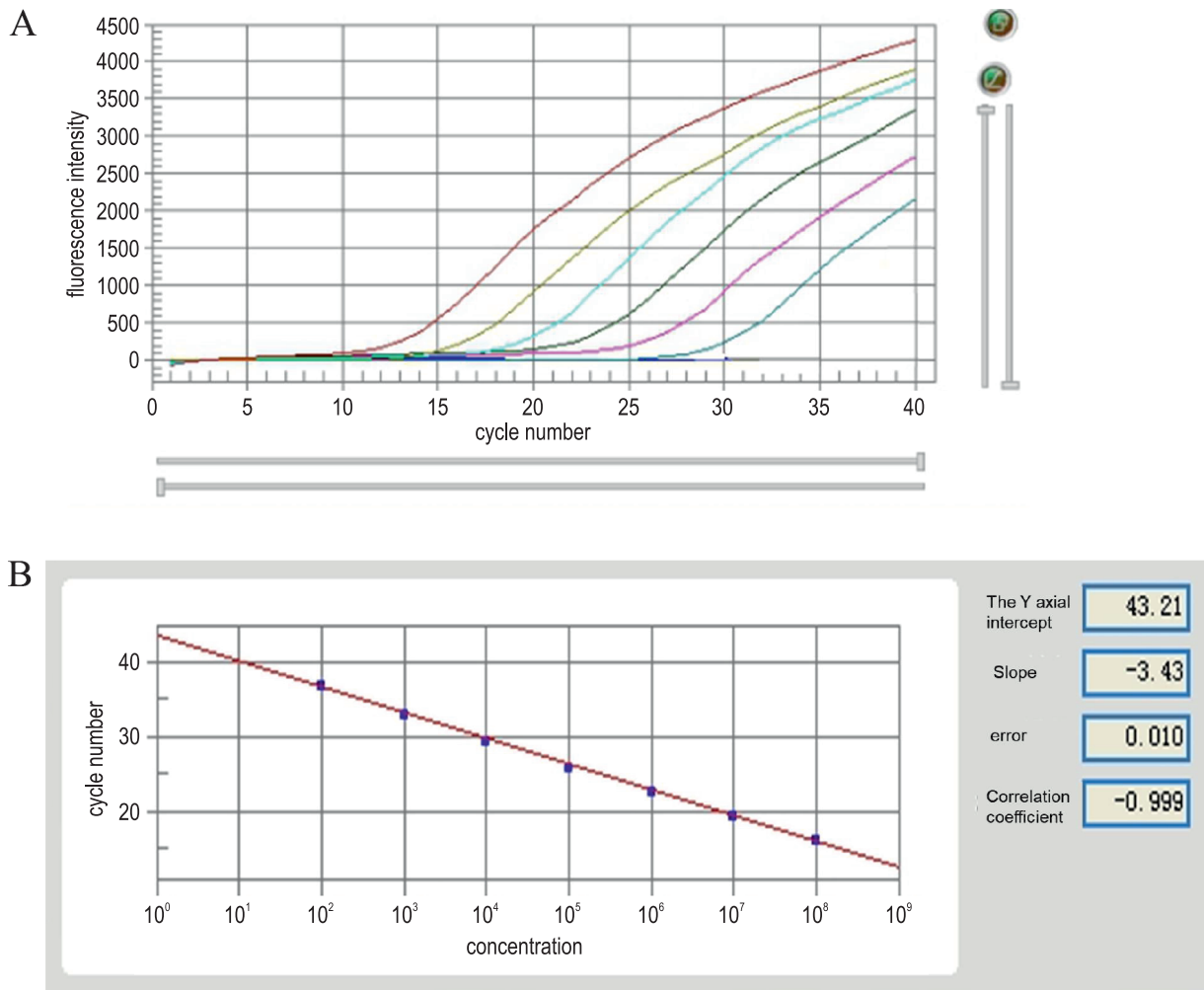


Fig. 5. PRRSV-N gene dynamic curve of fluorescent quantitative PCR and corresponding standard curve of the recombinant plasmid. A: the dynamic curves 1 to 5 represent 1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 , 1×10^3 , 1×10^2 copies/ μL of recombinant plasmid respectively; B: the standard curve and the 5snots represent the corresponding standard dilution.

The establishment of reaction system and optimization of conditions

The PCR amplification conditions of each PRRSV-N gene were 42°C for 5min; 95°C for 10s; 95°C for 5s, and annealing was performed for 30s (collecting fluorescence) for 40 cycles. The results of the reaction system optimization are shown in Table 1.

Establishment of the TaqMan fluorescent probe standard curve

A PRRSV-N gene standard with a copy number of 2.0×10^7 copies/ μL to 2.0×10^2 copies/ μL was used as a template, and a negative control was also set up to optimize the system and conditions for real-time quantitative PCR amplification. The established standard curve equation was $C_T = -3.43x + 43.21$, the correlation coefficient was 0.999, the C_T value increased as the template concentration decreased, and the tem-

plate concentration had a good linear relationship with the C_T value (Fig. 5). The repeatability test results showed that the established PRRSV-N gene mRNA expression standard curve had good repeatability within and between batches, and the CV% values were less than 2%, indicating that it had good repeatability and stability.

Effects of PoIFN- $\lambda 1$ on cell morphology and PRRSV proliferation

We further tested the cell morphology at different concentrations of IFN- $\lambda 1$ for 36 h ($100\times$) (Fig. 6A). The eukaryotic expression product of PoIFN- $\lambda 1$ was prepared at concentration of 0 U/mL, 50 U/mL, 100 U/mL, and 150 U/mL. After treating MARC-145 cells with 1 mL for 24 hours, PRRSV was inoculated, cell suspensions were collected at 12 h, 24 h, 36 h, and 48 h after inoculation, and PRRSV-N gene real-time fluorescence quantitative RT-PCR was performed

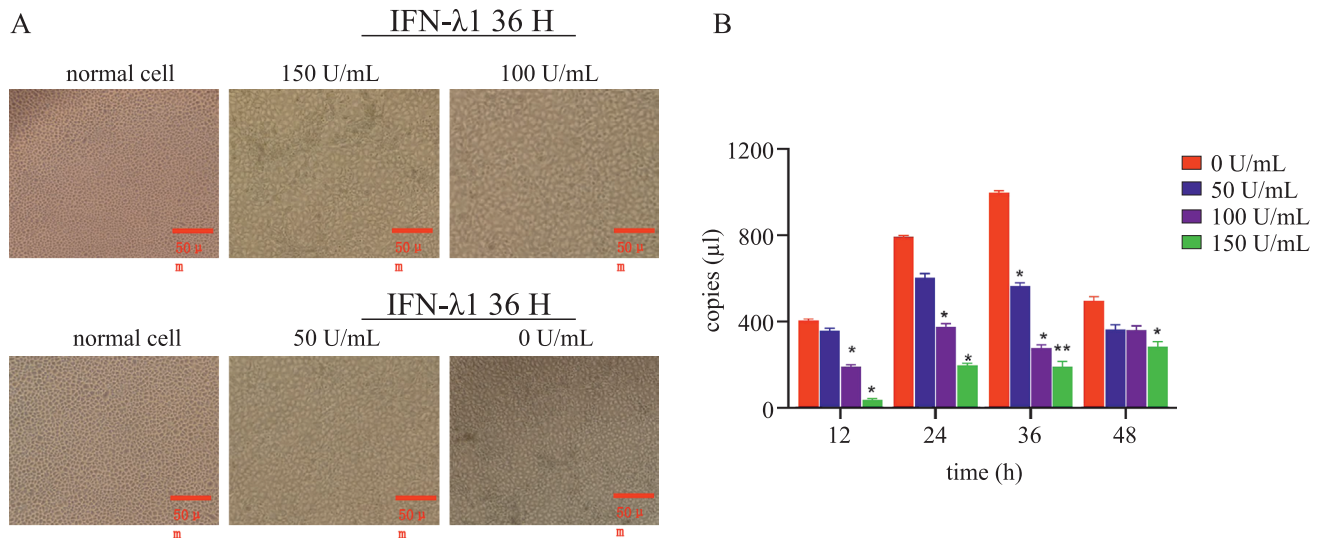


Fig. 6. Effects of PoIFN- λ 1 on cell morphology and PRRSV proliferation. A: cell morphology at different IFN- λ concentration in 36 h (100 \times). B: Fluorescent PCR Analysis of PRRSV mRNA Inhibition by PoIFN- λ 1.

on the extracted RNA. The eukaryotic expression product could inhibit the proliferation of the virus after inoculation in a time- and dose-dependent manner, and the inhibitory effect was the best at 36 hours after inoculation (Fig. 6B).

Discussion

In recent years, research on the biological activity of interferon has become a hot spot for researchers, especially research on the mechanism of interferon as an antiviral agent. The use of IFN- λ solves many bottlenecks in the field of medicine and plays different roles in different diseases. IFN- λ not only participates in inflammation but also inhibits the proliferation of tumor cells (Souza-Fonseca-Guimaraes et al. 2015). In one study, Wang Qiuli found that IFN- λ 2 is expressed in patients with allergic rhinitis and nasal polyps. IFN- λ can promote Th1 immune deviation by regulating the function of lung dendritic cells and reduce airway hyperresponsiveness (AHR) and cup metaplastic cells, thereby inhibiting airway allergic diseases (Wang 2016). Lasfar Ahmed et al. used animal models to show that IFN- λ shows a significant antitumor effect in a similar mild environment and verified their findings experimentally (Lasfar et al. 2016). Cheng Pengfei et al. used the immortalized human normal cervical cell line End1/E6E7 to establish an HSV-2-infected cell model and used this model to study the anti-HSV-2 mechanism of IFN- λ and found that human cervical cells express the IFN- λ body complex IL-28Ra/IL-10R β (Cheng et al. 2015). A study by Valadkhan Saba and others studies have shown that interferon can change the expression of a large number of long-chain non-

coding RNAs (lncRNAs) via the JAK/STAT pathway, making the virus unable to replicate and Further play a biological function against viruses (Valadkhan and Fortes 2018). Joana Rocha-Pereira et al. infected mice with this virus, which caused diarrhea in mice, and injected the animals with a substance that induced the expression of interferon lambda, which improved the symptoms of diarrhea in mice (Rocha-Pereira et al. 2018). This shows that IFN- λ can independently regulate and prevent the spread of many viruses, making it possible to protect elderly people and school-age children with reduced immune function.

In this study, total RNA extracted from peripheral blood lymphocytes of Congjiang pigs was used as a template to amplify the CDS region of the IFN- λ 1 gene and perform cloning and bioinformatic analysis. It was found that the IFN- λ 1 of Congjiang pigs have a complex secondary structure. Homology and phylogenetic tree analysis results showed that the Congjiang pig and wild boar IFN- λ 1 nucleotide homology was the highest (100%), which was the closest relationship on the basis of using PoIFN- λ 1 eukaryotic expression products. After pretreatment of MARC-145 cells for 24 h, the PRRSV strain was inoculated into MARC-145 cells, and it was found that the CPE of the cells decreased significantly with the increase of the dose of the expressed product, showing a dose and time dependence, with the highest inhibitory effect at 36 h. This shows that IFN- λ 1 can effectively inhibit the proliferation of PRRSV, which lays the foundation for the development and clinical research of recombinant IFN- λ 1 drugs.

Acknowledgements

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