Antiviral activity of porcine interferon omega 7 against foot-and-mouth disease virus in vitro

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Abstract
Foot-and-mouth disease (FMD) is a disease of worldwide economic importance, and vaccines play an important role in preventing FMDV outbreaks. However, new control strategies are still needed since FMDV outbreaks still occur in some disease-free countries. Currently, interferon (IFN)-based strategies have been demonstrated to be an efficient biotherapeutic option against FMDV; however, interferon omega (IFN-ω) has not yet been assessed in this capacity. Thus, this study evaluated the antiviral activity of porcine IFN omega 7 (PoIFN-ω7) against FMDV. After the PoIFN-ω7 was expressed and purified, cell proliferation assays and quantitative real-time reverse transcription-polymerase chain reaction were used to evaluate the effective anti-cytotoxic concentration of PoIFN-ω7 and its effectiveness pre- and post-infection with FMDV in swine kidney cells (IBRS-2). Results showed the rHis-PoIFN-ω7 fusion protein was considerably expressed using Escherichia coli BL21 (DE3) strain, and the recombinant protein exhibited significant in vitro protection against FMDV, including two strains belonging to type O and A FMDV, respectively. In addition, PoIFN-ω7 upregulated the transcription of Mx1, ISG15, OAS1, and PKR genes. These findings indicated that IFN-ω has the potential for serving as a useful therapeutic agent to prevent FMDV or other viral outbreaks in pigs.

KEYWORDS
antiviral activity, foot-and-mouth disease virus (FMDV), interferon stimulated genes (ISG), porcine IFN omega 7 (PoIFN-ω7), type I interferon

1 | INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious acute disease that affects cloven-hoofed domestic and wild animals, and the clinical symptoms include fever, lameness and vesicular lesions on the feet, tongue, snout, and teats.¹ Disease control includes sacrificing infected and susceptible animals, inhibiting animal movement, disinfecting contaminated premises, and vaccinating susceptible animals.² However, vaccination is not recommended in disease-free countries due to several reasons, including technical limitations in distinguishing vaccinated and infected animals, different antigenically variable strains of the virus, and trade restrictions.³ As a result, OIE has recognized that to be effective; control measures should include vaccination in combination with the use of antiviral agents and/or immunomodulatory molecules that could rapidly control the disease before an adaptive immune response is induced.⁴

Interferons (IFNs) are a group of cytokines that are divided into three types, including type I, type II and type III, and they constitute the first step in the immune response to pathogens.⁵ Type I IFNs, including IFN-α, β, δ, ε, ζ, η, ι, κ, τ are the largest group in the interferon family and have been well-characterized for their action against viral infections.⁶ IFN-ω genes were initially found in humans thirty years ago, and since then,
have been identified in other animals except canines and mice.\(^7\) Similar to other IFNs, IFN-\(\omega\) is produced by cells in response to viral infection and has identical antiviral, antiproliferative, and immunomodulatory activities by binding to the same receptors and activating similar pathways to the interferon-\(\alpha/\beta\) receptor.\(^8,9\) The feline IFN-\(\omega\) was the first licensed interferon compound, which is used in cats for the treatment of feline immunodeficiency virus and feline leukemia virus infections (Virbagen\(^\circledR\), Virbac) in Europe, Japan, Australia, New Zealand, and Mexico.\(^10\) Unfortunately, it has been not utilized for other viral infections. In fact, IFN-\(\omega\) also exerts a protective effect against several viruses, including bovine enterovirus, bovine viral diarrhea virus, infectious bovine rhinotrachitis virus, influenza virus, European bat lyssavirus, vesicular stomatitis virus, pseudorabies virus, canine and feline parvovirus, herpesvirus, calicivirus, coronavirus, and rotavirus.\(^7\) However, the antiviral effect of IFN-\(\omega\) has never been assessed against FMDV. Interestingly, different subtypes of IFN-\(\omega\) have distinct antiviral activity in vitro and in vivo. For example, among the four IFN-\(\omega\) pseudogenes and eight functional genes in the porcine IFN-\(\omega\) (PoIFN-\(\omega\)) family, porcine IFN omega 7 (PoIFN-\(\omega\)-7) has the highest antiviral activities, which are approximately 20 times than PoIFN-\(\omega\)-4, which has the lowest antiviral activity.\(^11\) Additionally, IFN-\(\omega\) has lower in vitro cytotoxicity, compared to other interferons, making it a therapeutic candidate for treating some viral diseases.\(^11\) In this study, PoIFN-\(\omega\)-7 was expressed in Escherichia coli, and its antiviral effects against FMDV was assessed in vitro.

2 | MATERIALS AND METHODS

2.1 | Cells and viruses

IBRS-2 cells were maintained from our laboratory. E. coli DH5\(\alpha\) and BL21 (DE3) cells were purchased from Solarbio (Beijing, China). Two different serotypes of FMDV strains (O/MYA98/BY/2010 and A/GDMM/CHA/2013) were used to investigate the antiviral activity of PoIFN-\(\omega\)-7, and their TCID\(_{50}\) was measured with the Reed and Muench method.\(^12\)

2.2 | Plasmid construction

The DNA sequence of mature PoIFN-\(\omega\)-7 (Gene accession EU797621) was synthesized by Shanghai Sangon Biotechnology Co, Ltd (Shanghai, China). After digested by EcoRI and Hind III restriction sites from the vector, the target gene was ligated into the corresponding sites in the pET30a vector (Invitrogen, CA). Following ligation, the resulting pET30a-PoIFN-\(\omega\)-7 vector was transformed into chemically competent DH5\(\alpha\) E. coli cells. Single colonies were identified by colony polymerase chain reaction (PCR) using the forward primer of 5’-GGATCTCTAGGCTGACCTGT-3’ and the reverse primer of 5’-TCAAGGTCACCGGTGTCTACC-3’, and DNA sequencing by Shanghai Sangon Biotechnology Co, Ltd.

2.3 | Expression and Purification of PoIFN-\(\omega\)-7

E. coli BL21 (DE3) cells were used as the host strain to overexpress PoIFN-\(\omega\)-7 protein. Firstly, the positive recombinant pET30a-PoIFN-\(\omega\)-7 plasmid was transformed into induced E. coli BL21 (DE3) express stains. And then, a final concentration of 1 mM IPTG (Sigma) was added to induce protein expression in E. coli BL21 (DE3) cells at 37°C until the optical density reached at OD600\(_{\text{nm}}\) = 0.4-0.6. After which time, the bacteria were further cultured for 8 hours. After centrifugation at 11,000 rpm for 10 minutes at 4°C, the harvested cells were re-suspended in a final volume of 50 mL of Buffer A (0.1% v/v lysozyme, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.4% v/v Triton X-100, 20 mM Tris- HCl pH 8.0) and sonicated (at 200 W, 2 seconds on and 2 seconds off). Inclusion bodies (IB) were gained by centrifugation (11,000g, 30 minutes) and washed three times with Buffer A. And then, IB were re-suspended in a final volume of 20 mL of Buffer B (8 M urea, 2 mM beta-mercaptoethanol, 20 mM Tris- HCl pH 8.0), and incubated at 4°C overnight. The solution was centrifuged at 12,000 rpm for 30 minutes at 4°C, and the supernatant was collected. Subsequently, the supernatant was then loaded onto a 10 mL nickel Sepharose 6 Fast Flow column. To remove non-specifically bound materials, the column was washed with 100 mL Buffer B. Next, the column was washed with a linear gradient imidazole from 20 mM to 100 mM imidazole in Buffer B, using 100 mL for each step. Finally, the target protein was eluted with 500 mM imidazole in Buffer B. Fractions containing the rHis-PoIFN-\(\omega\)-7 protein were pooled and identified by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified PoIFN-\(\omega\)-7 protein was renatured with a reduced concentration of urea from 4 M to 0 M in Buffer C (100 mM Tris, 2 mM EDTA, 1% Glycine, 5% Glycerinum, pH 8.6). The final protein concentration was measured by Bradford Protein Assay Kit (Amresco) while Western blot analysis was used to confirm PoIFN-\(\omega\)-7. Endotoxin was analyzed by GenScript ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript) according to the instructions of the manufacturer.

2.4 | Anti-FMDV activity of PoIFN-\(\omega\)-7 in IBRS-2 cells

2.4.1 | Pre-infection antiviral activity of PoIFN-\(\omega\)-7

Antiviral activity of the purified protein was tested using IBRS-2 cells and FMDV, as previously described.\(^13\) Briefly, IBRS-2 cells were grown in Dulbecco modified Eagle medium (DMEM; Gibco) supplemented 2% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 \(\mu\)g/mL), 3 x 10\(^4\) cells were seeded per well into a 96-well plate and incubated 24 hours at 37°C in a CO\(_2\) incubator. A 10-fold serial dilution of PoIFN-\(\omega\)-7 was added to the cells and was incubated for an additional 24 hours. After which time, the supernatant was removed and washed three times with DMEM; then, the cells were infected with 100 TCID\(_{50}\) of FMDV O/MYA98/BY/2010 for 1 h. The culture supernatants were replaced with DMEM containing 2% FBS, penicillin (100 U/mL) and streptomycin (100 \(\mu\)g/mL) at 37°C for 48 hours in a CO\(_2\) incubator. As a control, uninfected and non-treated cells (cell control), and infected with FMDV absence of IFN-treated cells (virus control) were maintained. Forty-eight hours post-infection, the viability of IBRS-2 cells was calculated using the MTS assay kit (Abcam, UK), according to the manufacturer’s instructions. Based on the OD readings at 490 nm,
TABLE 1 Primers sequences used in this study

| Genes  | Primer names       | Sequence (5'-3')                  | Sizes, bp | Reference |
|--------|-------------------|-----------------------------------|-----------|-----------|
| FMDV   | FMDV FMDVR        | CCCAGGGCCACCACTAG ACGTGTACCGGTTGTCG | 131       | 14        |
| Mx1    | Mx1F Mx1R         | GGGGTGGGAATCAGTCATG AGGAAGGTCTATGGGTCAGATCT | 81        | 15        |
| ISG15  | ISG15F ISG15R     | GCCCTCTCTAGTGGCGG AGGAGGCCCACCATAG | 110       | 15        |
| PKR    | PKRF PKRR         | AAAGCGGAAAGTGGAAAGG TCCACCTTACATGTCTTCTGA | 81        | 15        |
| OAS1   | OAS1F OAS1R       | GAGGTGAGGGAGGTCCCT TCCTGAGACAGGGATGA | 68        | 15        |
| β-actin| β-actinF β-actinR | GACCACCTCTAAGCTGATCA GTGTGGCGTAGGTCCTT | 63        | 16        |

the percent protection from cytopathic effect (CPE) was measured as previously described by Kawamoto et al.14 Total RNA of the cell lysates was extracted at 48 hours post-infection using TRIzol reagent (Invitrogen), and the relative RNA expression levels of FMDV was assessed by quantitative real-time PCR with gene-specific primer sets (Table 1) as described previously.13 The antiviral activity of PoIFN-ω7 against FMDV O/GDMM/CHA/2013 were tested using the similar methods as described above. Differently, IBRS-2 cells were challenged with 100 TCID50 of FMDV A/GDMM/CHA/2013.

2.4.2 | Post-infection antiviral activity of PoIFN-ω7

IBRS-2 cells were infected with FMDV by the method described above. The monolayers of IBRS-2 cells seeded in 96-well plates infected with 100 TCID50 of FMDV O/MYA98/BY/2010. After washed three times with DMEM, 10 ng/mL of PoIFN-ω7 were added to IBRS-2 cells at 0, 2, 4, 8, 16, and 24 hours after infection, respectively. As a control, cell control and virus control wells were maintained. After 24 hours post-infection, cell viability and viral mRNA expression levels were measured as described above.

2.5 | Evaluation of ISG expression

The monolayers of IBRS-2 cells seeded in 12-well plates were treated 10 ng/mL of PoIFN-ω7, and untreated cells were maintained as the control group. 24 hours post-stimulation later, cells were scrapped from wells. Subsequently, total RNA was extracted using TRIzol reagent (Invitrogen) and treated with DNase I (TaKaRa, Dalian, China), according to the manufacturer’s protocols. The concentrations of the extracted RNA were measured using NanoDrop (Thermo Fisher Scientific). Quantitative real-time reverse transcription-PCR (qRT-PCR) was performed as described above. The list of primer sequences is shown in Table 1. Each reaction comprised 12.5 μL SYBR Green, 1 μL cDNA, 10 pmol of each primer and RNA-free water to a total volume of 25 μL. The q-PCR program started with a 30 seconds denaturation at 95°C, followed by 40 cycles of 5 seconds denaturation at 95°C, 30 seconds annealing at 56°C and 30 seconds elongation at 72°C for each specific primer, during which fluorescence was measured. All experiments contained a negative control, and all q-PCR reactions were performed in triplicate. DNA contamination in RNA samples was checked by real-time PCR using a “No-RT” control (reaction mix with no reverse transcriptase enzyme). The porcine β-actin was used an endogenous control to normalize the differences in the amount of total RNA of PoIFN-ω7-treated and non-treated cells. The relative quantities of mRNA accumulation were evaluated using the 2-ΔΔCt threshold cycle method.17

2.6 | Statistics analysis

Data were presented as means ± standards deviation (SD) for at least triplicate experiments. The statistical significance was analyzed by one-way ANOVA using the SPSS software package, followed by Tukey’s post-hoc multiple comparison test; while the graphical illustrations were produced by the GraphPad Prism software version Version 5.0. A value of P < 0.05 was considered as statistically significant.

3 | RESULTS

3.1 | Cloning and expression of PoIFN-ω7

According to the sequence of PoIFN-ω7, which was available in NCBI, the nucleotide sequences of PoIFN-ω7 were synthesized. pET-30a was used as the expression vector, which contained the sequence of one hexahistidine (his) tag fused to the N-terminus of PoIFN-ω7 (Figure 1). Recombinant pET30a-PoIFN-ω7 was identified by PCR, and DNA sequencing further confirmed that the PoIFN-ω7 gene was in accordance with the design and was 516 bp in length (data not shown). Select recombinant plasmids were transformed in E. coli BL21 (DE3) for protein expression. After induced by IPTG, PoIFN-ω7 protein was considerably expressed in E. coli BL21 (DE3) strain and the yields of the PoIFN-ω7 protein is approximately 200 mg/L from bacterial culture. As shown in Figure 2A, a mass of inclusion protein...
was detected. Using a Ni-NTA agarose (IMAC) column with a buffer containing imidazole, more than 90% purity of recombinant PoIFN-ω7 protein was obtained with a yield of 5 mg/L from bacterial culture (Figure 2A). Subsequently, the protein was further identified by Western blot analysis (Figure 2B). Endotoxin was undetectable in the purified recombinant PoIFN-ω7 protein (below the detection limit, <0.01 EU/mg).

3.2 | Pre-infection antiviral effect of PoIFN-ω7

To investigate the minimum protection antiviral concentration of purified protein for IBRS-2 cells, a serially diluted ten-fold from 100 ng/mL to 0.001 ng/mL of PoIFN-ω7 was added in five replicates to IBRS-2 cells. The lowest dilution of PoIFN-ω7 to offer protection to cells from CPE was 10 ng/mL, which displayed 100% protection. However, lower dilutions of PoIFN-ω7 used did not exert efficient protection from CPE caused by type O FMDV (Figure 3A). The concentration of 100 and 10 ng/mL decreased viral mRNA levels by 5.95-log and 6.278-log titers, respectively, compared to the VC group (Figure 3B). A considerable difference in CPE effect was observed between both the 100 ng/mL and 10 ng/mL groups, as well as the other concentration groups and the VC group (P < 0.05). In addition, PoIFN-ω7 could also exert its antiviral activity against type A FMDV (Figure 3C,D).

3.3 | Post-infection antiviral activity of PoIFN-ω7

To evaluate whether PoIFN-ω7 could protect cells from CPE following viral infection, the post-infection antiviral effects of PoIFN-ω7 was investigated. PoIFN-ω7 (10 ng/mL) were added to IBRS-2 cells at 0, 2, 4, 8, 16, and 24 hours post infection, respectively. The results showed that PoIFN-ω7 show over 86% protection against CPE at 0-8 hours post-infection (Figure 4A), and displayed a 2.13-log to 3.03-log decreases in FMDV mRNA levels at 0, 2, and 4 hours post-infection (Figure 4B) in comparison to the non-treated group (Non-tr). However, compared to the VC group, which displayed considerable CPE and its viral mRNA levels, no remarkable
FIGURE 3  The antiviral activity of PoIFN-ω7 against FMDV. A, CPE reduction efficacy of PoIFN-ω7 in IBRS-2 cells against type O FMDV. The percentage protection from CPE in treated group was compared to untreated virus and cell control. B, The reduction of FMDV viral mRNA expression levels by PoIFN-ω7 in IBRS-2 cells. IBRS-2 cells were pretreated with 10-fold dilutions of PoIFN-ω7 for 24 hours before infection with FMDV O/MYA98/BY/2010. VC (viral control) indicates the IBRS-2 cells infected with O/MYA98/BY/2010, and without PoIFN-ω7. C, CPE protection assay to evaluate the effectiveness of PoIFN-ω7 against type A/GDMM/CHA/2013. D, The reduction of FMDV viral mRNA expression following treatment with PoIFN-ω7 after infection with FMDV A/GDMM/CHA/2013. Non-tr indicates the IBRS-2 cells infected with O/MYA98/BY/2010, and without PoIFN-ω7. The graph represents three independent experiments with five replicates of wells in each experiment. (**P < 0.001 vs non-treatment group, analysis of variance). CPE, cytopathic effect; FMDV, foot-and-mouth disease virus

FIGURE 4 Post-FMDV infection antiviral activity of PoIFN-ω7 against FMDV O/MYA98/BY/2010. A, The protection from CPE in IBRS-2 cells after infection with FMDV O/MYA98/BY/2010. Protection was expressed as a percentage, compared to untreated virus and control cells. B, The reduction of viral mRNA expression following treatment with PoIFN-ω7 after infection with FMDV O/MYA98/BY/2010. IBRS-2 cells were infected with FMDV O/MYA98/BY/2010 and treated with PoIFN-ω7 at 0, 2, 4, 8, 16, and 24 hours post-infection. Untreated virus and cell controls were also maintained. Non-tr indicates IBRS-2 cells infected with O/MYA98/BY/2010, but lacking PoIFN-ω7 treatment. The graph represents three independent experiments with five replicates of wells in each experiment. (**P < 0.001 vs non-treatment group, analysis of variance). CPE, cytopathic effect; FMDV, foot-and-mouth disease virus
protection was found against CPE, and viral amounts were not downregulated at 16 hours and 24 hours post infection (Figure 4B).

3.4 | Evaluation of ISG expression

To better illustrate the mechanism of antiviral activity elicited by PoIFN-ω7 in IBRS-2 cells. The mRNA levels of ISGs, including Mx1, ISG15, PKR, and OAS1 in PoIFN-ω7-treated IBRS-2 cells and non-treated control groups were analyzed by q-PCR. As shown in Figure 5, compared to the non-treated control group, the mRNA expression for the antiviral genes Mx1, ISG15, PKR, and OAS1 mRNA in the cells treated with PoIFN-ω7 was 88.28-, 75.57-, 3.51-, and 70.16-fold higher, respectively (Figure 5).

4 | DISCUSSION

IFNs were reported 60 years ago by Isaacs and Lindenmann as antiviral proteins generated in response to viral infection. IFNs constitute the first step in the immune response against pathogens infection, and as a result, the induction of IFNs is a very powerful tool for the host to fight viral infections. In the case of FMDV, IFN-based strategies have been demonstrated to be an efficient biotherapeutic option against FMDV. Besides that, it has been demonstrated that IRF7/3 fusion protein could protect against FMDV infection by inducing type I IFN expression. However, the antiviral effect of IFN-ω against FMDV is still unknown. Herein, this study, for the first time, the anti-FMDV activity of IFN-ω was demonstrated in vitro.

In pre-infection concentration determination study, we found that the lowest concentration of PoIFN-ω7 was about 10 ng/mL, which provide 100% protection to IBRS-2 cells from CPE caused by O/MYA98/BY/2010. Interestingly, this concentration could also exert significant protection of cells from A/GDMM/CH/2013 infection (Figure 3C). In fact, unlike other vaccines, IFNs exert their antiviral activity against lots of viruses, no matter which the serotype or strain infected. This result was similar to the study from Usharani et al, in their study, they found that IFN-ω also exerted broad-spectrum antiviral effects against eight strains of FMDV. In post-infection study, PoIFN-ω7 (10 ng/mL) also provided cells considerable protection against CPE and displayed 1.29-log reduction in viral mRNA levels at 0-8 hours post-infection, compared to the VC group (Figure 4). Viral mRNA levels increased at 0 to 8 hours post-infection and raised considerably after 8 hours, while no considerable CPE was observed in this period, these results suggest that PoIFN-ω7 might serve as an useful adjunct treatment at the onset of the FMD outbreak as well as that within 8 hours of exposure to FMDV.

Generally, type I IFNs trigger the production of antiviral effectors by mediating the JAK/STAT pathway, these effectors include ISG, Mx1, OAS, and PKR. Our results showed that PoIFN-ω7 enhanced the mRNA levels of ISG15, Mx1, OAS1, and PKR. However, IFN-ω signaling was not verified by STAT1/STAT3 phosphorylation, and other effectors using western-blot in this study, further study is still needed, it will help to further elucidate porcine IFN antiviral innate immunity and address the question that why IFN-ω and IFN-α can exert different biological activities although both of them use type I signal pathway.

Several studies have revealed that combination interferon therapy has enhanced protection in comparison to single interferon therapy. For instance, results from a study by Moraes et al revealed that the combination of IFN-α and IFN-γ have a synergistically effect on suppressing FMDV replication and in vitro and in vivo. In future studies, it will be important to assess whether combining PoIFN-ω7 with type II IFN or IFN-λ leads to increasing efficacy against FMDV. Besides that, enhanced inhibition effects on FMDV were also observed by combinations of IFN-α and ribavirin in vivo and in vitro in 2012, therefore, whether the combination of PoIFN-ω7 and ribavirin has an enhanced antiviral activity should also be demonstrated in further study. Although the antiviral effect of IFN-ω was demonstrated against FMDV in vitro, this study did not confirm in vivo activities using animals, such as suckling mice (IFN-ω was not noted in mice). However, other target species, including bovine and porcine, are needed in the future to confirm the antiviral effects and evaluate the true potential of PoIFN-ω7 as an adjunct control measure for FMD outbreaks.

In recent years, there has been increasing interest in studying the consensus interferon (a type IFN that contain the most frequently occurring amino acids present among the non-allelic subtypes), which has been shown to have higher antiviral and anti-proliferative activities, natural killer cell activation and ISG-induction activities, compared to single IFN subtypes. Likewise, porcine IFN-ω is a multigenic family containing seven subtypes with different antiviral activities and different expression profiles. Therefore, a consensus IFN-ω could also be designed, and its efficacy against FMDV should be investigated in the future studies.

In conclusion, PoIFN-ω7 exhibited in vitro antiviral activities against FMDV and provides new insights for developing a novel
antiviral molecular that can be used in combination with vaccines. Such treatments would thus, protect animals before, or during the early stages of FMD viral infection, before the onset of the vaccine-induced immune response.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR’S CONTRIBUTIONS

HC and YZ conceived and designed the experiments. FS and MG performed the most experiments, FZ, JS, YL, and YX helped analyze the data. FS and MG wrote the paper. All the authors read and approved the final manuscript.

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