Original Article

Preparation of swine ISG15 polyclonal antibody and its application in classical swine fever virus

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Abstract

Interferon-stimulated gene 15 (ISG15) is known to participate in innate and adaptive immune responses against viral infections. Classical swine fever virus (CSFV) causes a global, economically important and highly contagious disease in pigs. To explore the interaction between ISG15 and CSFV, the *ISG15* gene was amplified, cloned and expressed in *E. coli* BL21 cells. The expressed ISG15 protein was purified by Ni-NTA agarose column and used to prepare a polyclonal mouse antibody. The anti-ISG15 antibody produced in mice could react with ISG15 expressed in the prokaryotic BL-21 cells as well as natural swine ISG15 expressed in PK-15 cells. This polyclonal antibody against swine ISG15 was used to perform immunofluorescence assay, western blot and flow cytometry for the detection of the ISG15 protein and to identify that ISG15 was up-regulated in PK-15 cells after infection with CSFV. This polyclonal antibody against ISG15 protein is important for further research on the interaction between CSFV and ISG15.

Keywords: Interferon stimulated gene 15 (ISG15), classical swine fever virus (CSFV), polyclonal antibody

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Received March 2, 2020.
Accepted August 18, 2020.

Introduction

Interferons (IFNs) play a key role in the innate immunity of vertebrates against viral infections by inducing hundreds of IFN-stimulated genes (ISGs), such as interferon-stimulated gene 15 (ISG15) (Álvarez-Torres *et al*., 2018). ISG15 contributes to antiviral defense in the innate and adaptive immune responses (Zhang and Zhang, 2011). ISG15 is stimulated by type I interferon (IFN-α and IFN-β) and virus infection (Hulo *et al*., 2011; Bazzigher *et al*., 1992; Der *et al*., 1998; Staehelia *et al*., 1986). The amino acid sequence encoded by ISG15 cDNA actually contains 165 amino acid residues in lengths of 17 kDa, which is the precursor of the ISG15 protein. When the COOH and 8 amino acids of NH2-terminal on the 17 kDa precursor form have been removed, it will quickly become 15 kDa mature form. Some studies have shown that the COOH terminal sequence may be a signal of intracellular lysosomal classification (Knight *et al*., 1988). ISG15 is the first reported member of the ubiquitin-like protein (UBL) superfamily (Haas *et al*., 1987; Huo *et al*., 2017) and as one of the ubiquitin-like modifiers, shares significant homology and functional characteristics with ubiquitin. Unlike ubiquitin and certain other ubiquitin-like modifiers that are more broadly expressed in species, ISG15 is only identified in vertebrates. Comparing the two ubiquitin-like domains of ISG15 with those of ubiquitin, the homology of the N-terminal domain is 29% (23/80 amino acids, aa); whereas the C-terminal domain is 31% (20/64 aa) including a highly conserved ending motif Leu Arg Leu Arg Gly Gly (LRLRGG) (Haas *et al*., 1987). The two ubiquitin-like domains of ISG15 play different roles in ISGylation, which is similar to ubiquitination. Protein ubiquitination or ISGylation requires the coordination of three modification enzymes: the activating (E1/UBE1L), the conjugating (E2/UBCH8) and the ligating (E3/HERC5) enzymes (Malakhov *et al*., 2002). Previous studies of the E3/HERC5 have demonstrated that more than 300 proteins were selected by HERC5 and underwent ISGylation in IFN-stimulated cells, ISGylation restrains the synthesis of new viral proteins in a cotranslational manner (Durfee *et al*., 2010).

ISG15 provides antiviral effect primarily in three ways (Chang *et al*., 2008; Sridharan *et al*., 2010; Yuan *et al*., 2002): as a cytokine directly inhibiting virus replication; as ubiquitin tagging foreign viral proteins for proteasomal degradation, such as in the case of the hepatitis C virus (Kim and Yoo, 2010; MacQuillan *et al*., 2003); competitively obstructing viral protein ubiquitination, thereby disrupting virus budding and release, such as occurs in HIV-1 (Okumura *et al*., 2006; Pincetic *et al*., 2010) and the Ebola virus (Malakhova and Zhang., 2008; Okumura *et al*., 2008).

Classical swine fever virus (CSFV) is the etiological agent of a highly contagious disease of swine around the world. CSFV glycoprotein E^{rns} is associated with double-stranded RNA (dsRNA) to disrupt the signaling pathway of Toll-like receptor 3 (TLR3) and repress the induction of type I interferon (Luo *et al*., 2009a; Luo *et al*., 2009b). CSFV N^{pro} protein influences type I interferon synthesis through at least three mechanisms: the inhibition of dsRNA mediated cell apoptosis; combining with interferon regulatory factor 3 (IRF3) in TLR3 dependent or independent signaling pathways and transferring the ubiquitination of IRF3 into proteasomes (Bauhofer *et al*., 2007; Seago *et al*., 2007); the interaction between Zn-binding domain of N^{pro} and IRF7 (Fiebach *et al*., 2011).

In our prior experiments, the gene expression profile in peripheral blood lymphocytes of pigs infected with CSFV showed that the mRNA levels of ISG15 were 2.63, 4.51, 4.43 and 2.18 folds up-regulated at 1, 3, 6 and 9 days post-infection (dpi), respectively (Li *et al*., 2010). To elucidate the effect of ISG15 on CSFV replication, in this study, a polyclonal antibody against swine ISG15 was generated. In the process of antibody preparation, we chose the complete CDS sequence of ISG15 to construct a eukaryotic expression vector. Notably, the precursor and mature form of ISG15 were both encoded by the same CDS region. Theoretically, our antibody could detect two forms including the precursor and mature form of ISG15 at the same time. However, we did not observe two bands in the lysate of PK-15 cells infected with CSFV, probably since only 2 kDa distance between the precursor and mature form of ISG15 was hard to distinguish in 12% separation gel of western blot. This antibody not only reacted well with ISG15 expressed in prokaryote but also with the natural swine ISG15 in PK-15 cells (a porcine kidney epithelial cell line). This anti-ISG15 antibody provides evidence that ISG15 protein and ISGylation are induced in PK-15 cells during CSFV infection *in vitro*.

Materials and Methods

Cell, virus and plasmids: PK-15 cells, a porcine kidney epithelial cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). The standard CSFV virulent Shimen strain was propagated in PK-15 cells. The cloning vector pMD18-T was purchased from Takara (Takara, Beijing). The prokaryotic expression plasmid pGEX-4T-1 and eukaryotic expression plasmid pcDNA3.0 were kindly provided by Professor Xiang-Tao Li (Guangxi University, China). The *E. coli* strains DH5α and BL21 (DE3) were purchased from Novagen (EMD Millipore, Massachusetts, USA).

Gene cloning of swine ISG15: Total RNA was extracted from the PK-15 cells using Trizol (Invitrogen, Carlsbad, CA) following the manufacture's instructions. The first pair of primers, T1f: 5'-GAGCTTTTGCCCCACTATG-3'; T2r: 5'-TTACTAGCACTCGGTGAGGTG-3', was designed to amplify the 576 bp mRNA of the *ISG15* gene, which contains ISG15 CDS region. The second pair of primers, P1f: 5'-GCGGATCCGCCACCATGGG TAGGGAACTGAAG-3'; P2r: 5'-GCGAATTCTTAAT GGTGATGGTGGTGATGGCACTCGGTGAGGTGCT CCC-3', was designed to construct the fragment containing the 522 bp segment of ISG15 expression vector with restricted endonuclease sites (underlined), the 522 bp fragment was the CDS region of ISG15. The 6×His-tag sequence is indicated by double underlining and the Kozak motif is marked by continuous under points. Reverse transcription was performed with MLV reverse transcriptase (Takara, Beijing) and primer T2r at 42 °C for 1 h. The *ISG15* gene was amplified with

the primers T1f/T2r using ExTaq (Takara, Beijing) in a 50 µl reaction volume, 94 ℃ for 4 mins, followed by 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 56 ℃ for 30 s, and extension at 72 ℃ for 40 s; then final extension at 72 ℃ for 10 mins. The RT-PCR product was visualized by 1.5% agarose gel electrophoresis and purified by a Gel Extraction Kit (Watson, Japan) and sequenced by Sunbiotech Co. Ltd (Beijing).

Plasmid construction: The purified PCR product was inserted into the pMD18-T vector and then subcloned into pGEX-4T-1 to obtain a prokaryotic expression plasmid pGEX-ISG15 and subcloned into pcDNA3.0 to obtain a eukaryotic expression plasmid pcDNA-ISG15, respectively. Both recombinant plasmids pGEX-ISG15 and pcDNA-ISG15 were identified by BamH I/EcoR I and RT-PCR, respectively. The recombinant pGEX-ISG15 plasmid was transformed into the prokaryotic competent *E. coli* BL21 cells for producing recombinant ISG15 protein, whereas the pcDNA-ISG15 plasmid was transfected into the eukaryotic PK-15 cells for overexpression of ISG15.

Expression and purification of recombinant ISG15: After the pGEX-ISG15 was transformed into competent BL21 cells, a positive clone was selected to cultivate at 37 ℃ until the OD_{600} reached 0.6. The expression of ISG15 was induced by 1 mM IPTG at 37 ℃ for 3 h. The transformed BL21 cells were collected, suspended in 20 mM imidazole solution, ultrasonically disrupted at 300 W and then centrifuged at 12,000 rpm at 4 ℃ for 10 mins. The recombinant ISG15 (with 6×His-tag) in the supernatant (soluble form) or sediment (inclusion form) was determined by SDS-PAGE and purified by Ni-NTA agarose column (Qiagen) following the manufacture's instructions. The purified recombinant ISG15 was identified by SDS-PAGE and western blot, then was refolded and stored at -80 ℃ for preparation of polyclonal antibody in mice.

Preparation of ISG15 polyclonal antibody:
The purified recombinant ISG15 was administrated to Kunming mice for preparation of polyclonal antibody. The pre-immune serum of the mouse was collected before immunization. Each mouse was primarily-immunized by subcutaneous injection with a 100 µg mixture of recombinant ISG15 with an equal volume of Freund's complete adjuvant (Sigma) 3 weeks later, then subsequently boosted with 50 µg ISG15 mixture containing Freund's incomplete adjuvant (Sigma) three times at 2 week intervals. The antiserum was harvested at 1 week after the final immunization.

Screening of ISG15 stable expression cell line: The eukaryotic expression plasmid pcDNA-ISG15 was transfected into PK-15 cells for 24 h. Then the antibiotic neomycin G418 was added to the cell culture medium for 8 days (1200 µg/mL), so that the majority of cells without **the** antibiotic resistance gene would be killed. In further screening, a final concentration of 700 µg/mL of G418 was maintained in the cell culture medium for 15 days. Subsequently, the single cell cluster with ISG15 expression was picked for further culture.

SDS-PAGE and western blot analysis: SDS-PAGE was performed on 12% acrylamide gel in the Mini-protein Tetra system (Bio-Rad, USA). The recombinant ISG15 was visualized by Coomassie Blue R250 staining and its molecular weight was verified with protein molecular weight markers *(MBI* Fermentas, Germany). The recombinant ISG15 was separated and electroblotted on to a polyvinylidene difluoride membrane (PVDF, Millipore, USA) using a Semi-Dry Transfer System (Bio-Rad, USA). The non-specific proteins on the membrane were blocked by incubation in 5% defatted milk at room temperature for 1 h, and then the membrane was washed three times with TBS containing 0.05% Tween-20 (TBS-T). Primary antibodies containing monoclonal (anti-His tag, mouse) or polyclonal (anti-ISG15, mouse) antibodies were added to the membrane for 90 mins at 37 ℃. After washing 3 times with TBS-T, the membrane was subsequently incubated with secondary antibody (HRP-conjugated goat anti-mouse IgG) for 60 mins at room temperature. The final result was visualized using the HRP-DAB Kit (TIANGEN, Beijing) after washing five times with TBS.

Indirect immunofluorescence assay: The PK-15 cells were cultured on a 96-well cell culture plate and infected with CSFV Shimen strain. Both of the cells infected with CSFV and uninfected cells were washed with phosphate buffered saline (PBS) at 0, 3, 6, 12, 24, 48 and 60 hours post-inoculation (hpi) and fixed with a cold mixed liquor of formaldehyde and alcohol for 10 mins. Subsequently, both of the infected cells and uninfected cells were washed with PBS for 15 mins and incubated with anti-ISG15 polyclonal antibody at a 1:200 dilution for 1 h at 37 ℃. The cells were washed and incubated with FITC-conjugated goat anti-mouse IgG antibody (Sigma, USA) at 1:500 dilution for 1 h at 37 ℃. After incubation, the cells were washed again and analyzed using an Eclipse TE2000-U inverted fluorescence microscope (Nikon, Japan).

Flow cytometry analysis: PK-15 cells were infected with 1.0 m.o.i of CSFV Shimen strain; the uninfected cells were used as a control. The cells were collected at 0, 6, 12, 24 and 48 hpi for flow cytometry, of which, 0 h was used as uninfected cells. Both the infected cells and uninfected cells were washed with PBS three times, then incubated with anti-ISG15 polyclonal antibody and anti-CSFV-E2 polyclonal antibody, respectively, at a 1:200 dilution for 1 h at 37 ℃. The cells were washed and incubated with FITC-conjugated goat anti-mouse IgG antibody (Sigma, USA) at 1:500 dilution for 1 h at 37 ℃. After incubation, the cells were washed again and analyzed in a Guava Flow Cell System (Guava easyCyteHT, Millipore). The data was analyzed using guava Soft 2.6 software.

Real-time quantitative PCR (RT-qPCR): PK-15 cells were infected with 1.0 m.o.i of CSFV Shimen strain; the uninfected cells were used as control. The cells were collected at 0, 6, 12, 24 and 48 hpi for RT-qPCR, which was performed using SYBR Green. Briefly, 1 µg of total RNA served as the template for the first-strand cDNA synthesis in a reaction using an oligo(dT) primer and Moloney murine leukaemia virus (MMLV) reverse

transcriptase under the conditions described by the manufacturer. A LightCycler 480 PCR detection system (Roche, Switzerland) was used for the quantitative assessment of CSFV genome RNA and *ISG15* mRNA under standard cycling conditions. GAPDH was served as the reference gene for all reactions. Relative fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. All primers used for RT-qPCR and associated sequences are listed in Table 1.

Table 1 Primers used for RT-qPCR

Genes	Primer names	Primers sequence (5′→3′)	Position	Fragment length (bp)
CSFV	CSFV-F	GCCATGCCCATAGTAGGACT	97	115
	CSFV-R	GCTTCTGCTCACGTCGAACT	212	
ISG15	ISG15-F	GCCTTCCAGCAGCGTCT	283	164
	ISG15-R	GCGTTGCTGCGACCCT	431	
GAPDH	GAPDH-F	TGGTGAAGGTCGGAGTGAAC	343	224
	GAPDH-R	GGAAGATGGTGATGGGATTTC	567	

Results

Histopathology results: Our group reported earlier, as

Construction of the recombinant expression plasmid: Using specific primers T1f/T2r, a 576 bp fragment of *ISG15* gene was amplified by RT-PCR (Figure 1A) and inserted into a cloning vector pMD18-T to obtain a pMD18-T-ISG15 recombinant vector, which was identified by the digestion of EcoR I/Pst I restriction enzyme (Figure 1B). Subsequently, the CDS of *ISG15* gene was amplified using primer P1f/P2r and subcloned into a prokaryotic expression vector pGEX-4T-1 and eukaryotic expression vector pcDNA3.0 by restriction endonuclease BamH I/EcoR I and the 6×His-tag sequence, respectively (Figure 1C and D).

Expression of recombinant ISG15: The recombinant pGEX-ISG15 was transformed into BL21 (DE3) and the ISG15 was induced by 1 mM IPTG at 37 °C and at 200 rpm rotation for 3 h. The expressed ISG15 fused with a 26 kD glutathione-S-transferase (GST) and was detected by SDS-PAGE (Figure 2A). The fused ISG15 was determined in a soluble expression form (Figure 2B). The purified ISG15 was harvested using histidine label with Ni-NTA resin affinity technique in non-denaturing conditions (Figure 3A). Then the purified ISG15 was further identified by western blot using an anti-6×His-tag monoclonal antibody (MAb), suggesting that the ISG15 was correctly expressed in prokaryotic BL21 (DE3) (Figure 3B).

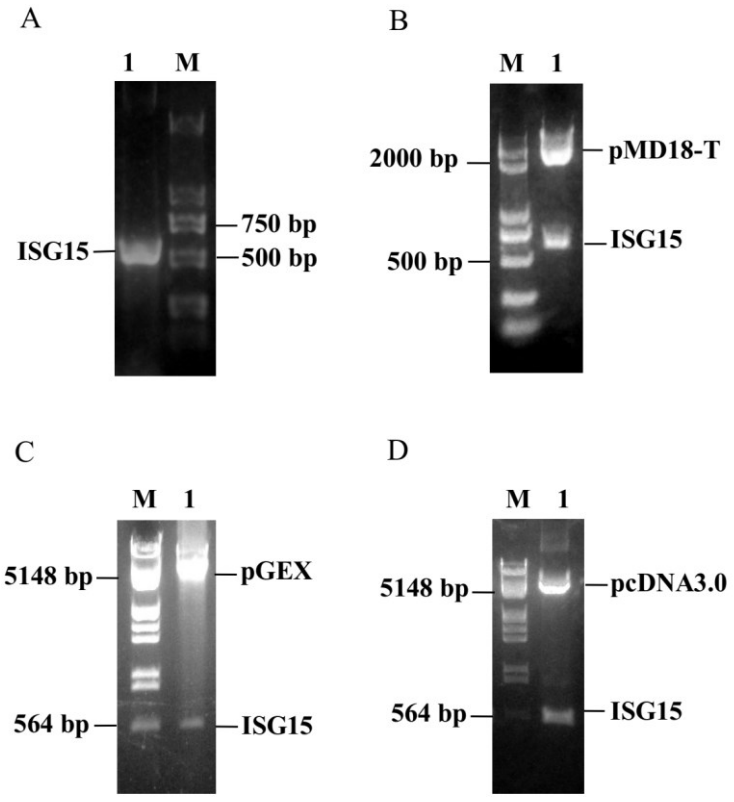

Figure 1 **Construction of the recombinant expression plasmid.**
(A) Amplification of ISG15 segment by PCR. Lane M, DL2000 DNA marker; lane 1, PCR products (576 bp). (B) Identification of the recombinant plasmid pMD18-T-ISG15. Lane M, DL2000 DNA marker; lane 1, pMD18-T-ISG15 was digested by EcoR I/Pst I. (C) Identification of the prokaryotic expression vector pGEX-ISG15. Lane M, λ DNA Hind III (MBI) DNA marker; lane 1, pGEX-ISG15 was digested by BamH I/EcoR I. (D) Identification of the eukaryotic expression vector pCDNA-ISG15. Lane M, λ DNA Hind III (MBI) DNA marker; lane 1, pCDNA-ISG15 was digested by BamH I/EcoR I.

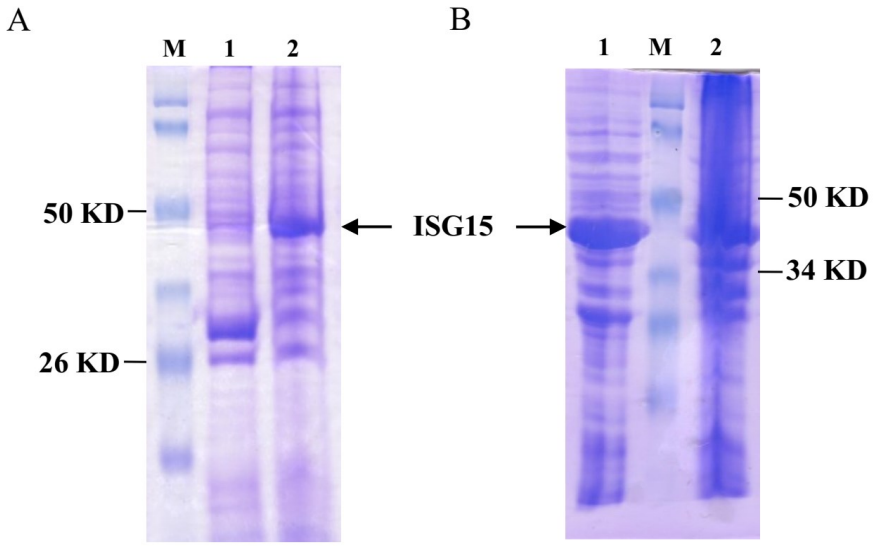

Figure 2 **Expression of recombinant protein ISG15 in *E. coli* BL21 (DE3) analyzed by SDS-PAGE.**
(A) The induced expression of pGEX-ISG15 recombinant bacteria. Lane M, protein molecular weight marker (MBI); lane 1, total cellular proteins of BL21 (DE3) harboring pGEX-4T-1 empty vector after induction for 3 h by IPTG in 1.0 mM concentration; lane 2, total cellular proteins of BL21 (DE3) harboring pGEX-ISG15 after induction for 3 h by IPTG in 1.0 mM concentration; (B) Determination of the recombinant protein ISG15 solubility. Lane M, protein molecular marker; lane 1, the recombinant ISG15 supernatant after re-suspending the lysate of the bacteria; lane 2, the recombinant ISG15 precipitate. The arrows indicate the position of the recombinant protein ISG15.

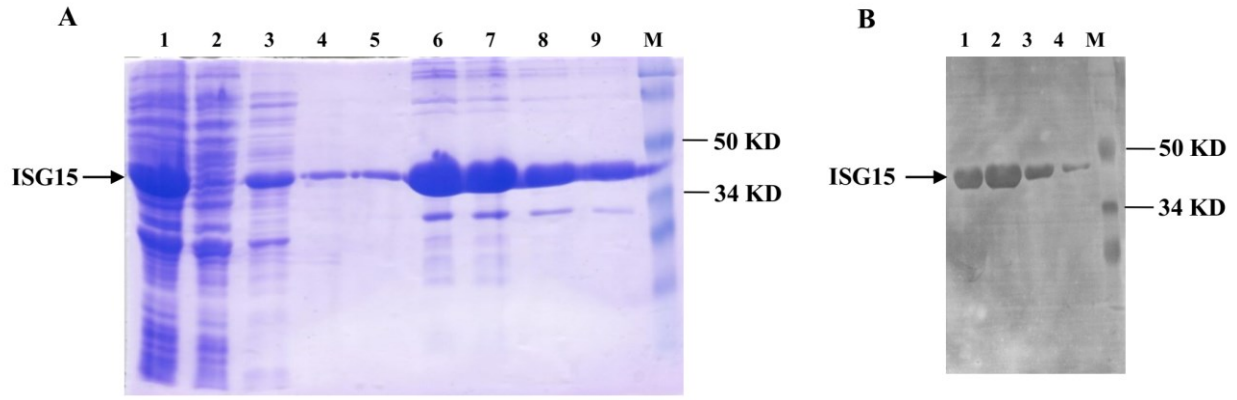

Figure 3 **Purification of recombinant 6×His-tagged protein ISG15 by SDS-PAGE and western blot analysis.**
(A) SDS-PAGE analysis of purified recombinant protein. Lane M, protein molecular weight marker (MBI); lane 1, supernatant of the lysis; lane 2, flow through; lane 3-4, eluted buffer with 70 mM Imidazole; lane 5-9, eluted buffer with 250 mM Imidazole. (B) Western blot analysis of recombinant protein ISG15 after purification, using mouse anti-6×His-tag monoclonal antibody. Lane M, protein molecular weight marker (MBI); lane 1-4, purified recombinant protein ISG15 by different batches of elution. Arrows indicate the position of the recombinant protein ISG15.

Characterization of polyclonal antibody against swine ISG15: We immunized the Kunming mice with the purified recombinant ISG15 protein and then obtained the polyclonal antibody. Western blot analysis was performed to determine the titer of anti-ISG15 polyclonal antibody in the immunized Kunming mice serum. The result showed that the 5 μg of the recombinant ISG15 protein could be well recognized by a 1:4000 dilution of the polyclonal antibody (Figure 4A). The specificity of the polyclonal antibody was further examined with the induced recombinant protein in BL21 cells (Figure 4B). The result revealed that the recombinant ISG15 protein was detectable at 1 h post induction and then stably expressed at 3 h after induction. Like the anti-6×His-tag monoclonal antibody, the anti-ISG15 polyclonal antibody appeared to have high specificity and strong reactivity with the recombinant protein in BL21 cells. In addition, the anti-ISG15 polyclonal antibody reacted well with natural ISG15 protein expressed in PK-15 cells induced by swine IFN-α2b, as determined by western blot (Figure 5A) and by IFA (Figure 5B).

The anti-ISG15 polyclonal antibody reacted with ISG15 stably expressed in PK-15 cells: Using cationic liposome mediated methodology, the eukaryotic expression vector pcDNA-ISG15 was transfected into an 80% monolayer of PK-15 cells for 24 h. The PK-15 cell-line with stable ISG15 expression was achieved after 15 d of antibiotic neomycin G418 screening. The ISG15 protein level in the pcDNA-ISG15 transfected cells was tested using our anti-ISG15 polyclonal antibody by IFA and by western blot (Figure 6A and B).

Identification of ISG15 induction in the CSFV infected PK-15 cells using anti-ISG15 polyclonal antibody: To further understand the role of ISG15 in CSFV infection, the expression level of the ISG15 protein was examined in PK-15 cells at 0, 3, 6, 12, 24, 48 and 60 hpi by IFA and western blot. As a result of IFA, the ISG15 protein was obviously detectable in the CSFV infected PK-15 cells after 12 hpi. The ISG15 in PK-15 cells infected with CSFV was increased compared with normal PK-15 cells (Figure 7A). Simultaneously, the outcome of western blot revealed a similar pattern (Figure 7B).

Expression of ISG15 increased in PK-15 cells during CSFV infection: PK-15 cells were infected with 1.0 m.o.i of CSFV Shimen strain. The cells were collected at 0, 6, 12, 24 and 48 hpi. The PK-15 cells expressing-CSFV E2 and -ISG15 were counted by flow cytometry. With the increase of infection time, the proportion of cells infected with CSFV also increased; 68.31% of cells were infected with CSFV at 48 hpi. Similarly, the expression of ISG15 increased after infection with CSFV, and the proportion of cells expressing ISG15 reached 26.78% at 48 hpi (Figure 8A). After PK-15 cells were infected with CSFV, we detected the mRNA of *ISG15* gene and the genome RNA (gRNA) of CSFV. The result showed CSFV gRNA increased 160, 390 and 470 fold at 12, 24 and 48 hpi, respectively; and the *ISG15* mRNA of infected cells increased 68, 125 and 128 folds at 12, 24 and 48 hpi compared with uninfected cells, respectively (Figure 8B), corresponding with the trend of CSFV gRNA.

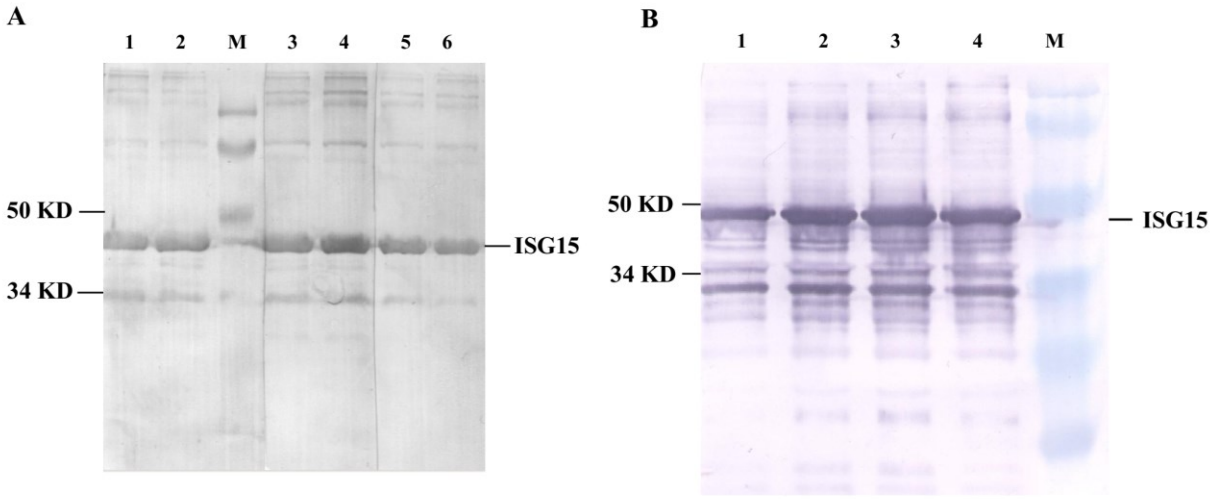

Figure 4 **Determining the titration and antigenicity of anti-ISG15 polyclonal antibody using western-blot analysis.**
(A) Determining the titration of anti-ISG15 polyclonal antibody. Lane M, pre-stained protein molecular weight marker; lane 1-2, 1/1000 dilution of positive serum; lane 3-4, 1/2000 dilution of positive serum; lane 5-6, 1/4000 dilution of positive serum. (B) Detect the antigenicity of anti-ISG15 polyclonal antibody with induced recombinant protein. 1, recombinant BL21 strain after induced 1 h; 2, induced 3 h; 3, induced 6 h; 4, induced 12 h. Arrow indicates the position of the recombinant protein ISG15.

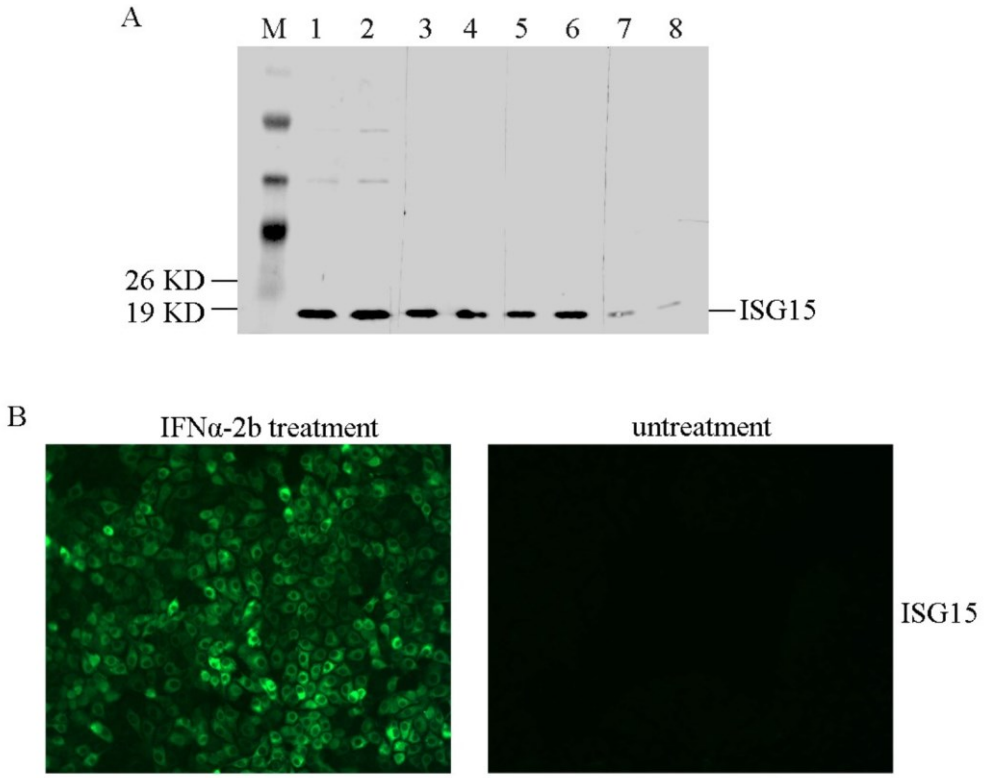

Figure 5 Western blot and indirect immunofluorescence analysis of ISG15 protein expression level in PK-15 cells using anti-ISG15 polyclonal antibody.
(A) Identification of ISG15 protein expression level in PK-15 cells by western blot. Lane M, protein molecular weight marker; 1-2, 1/1000 dilution of anti-ISG15 polyclonal antibody; 3-4, 1/2000 dilution of antibody; 5-6, 1/4000 dilution of antibody; 7-8, 1/8000 dilution of antibody. (B) Indirect immunofluorescence analysis for detection of ISG15 protein by the prepared ISG15 polyclonal antibody. left, PK-15 cells treated with IFNα-2b for 24 h; right, untreated PK-15 cells as a negative control.

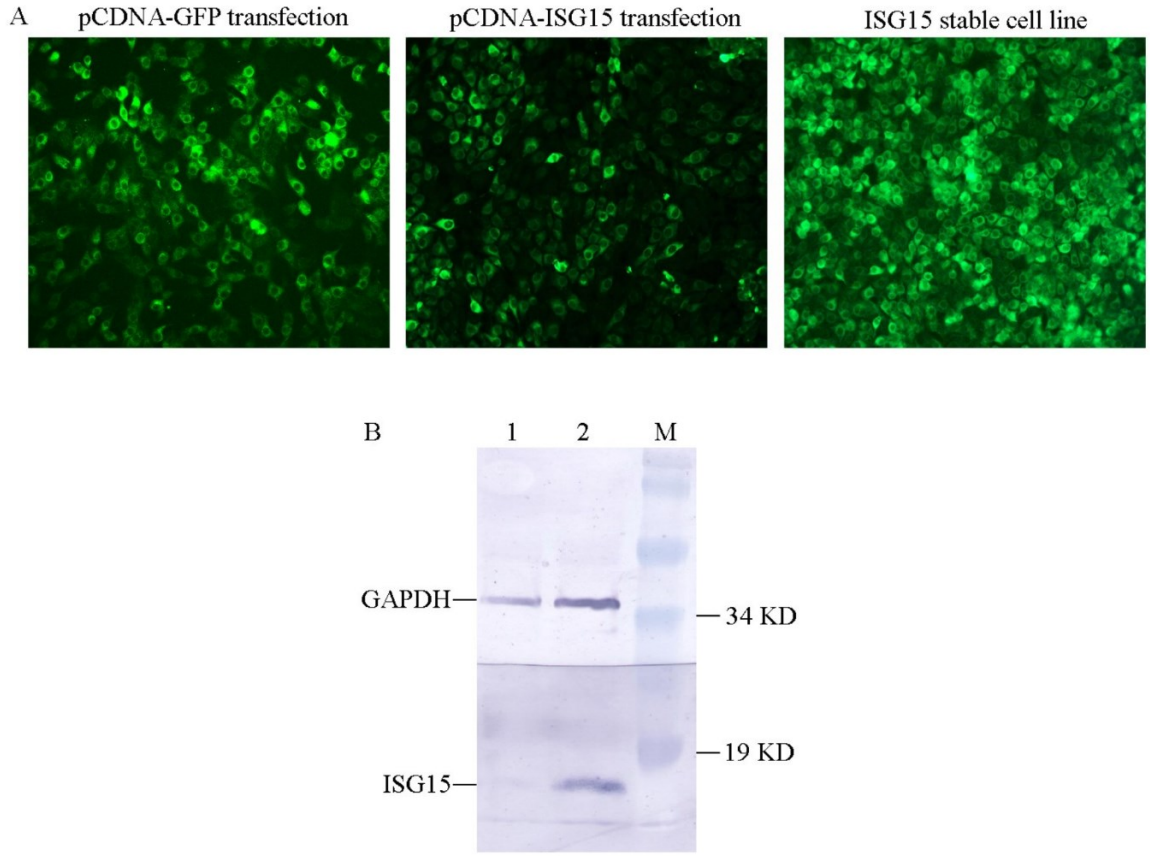

Figure 6 **Identification of ISG15 stable expression in PK-15 cell line by indirect immunofluorescence and western blot analysis.** (A) Detection of ISG15 expression in PK-15 cell by indirect immunofluorescence. left, pCDNA-ISG15 transfection PK-15 cells; middle, pCDNA-GFP empty vector transfection PK-15 cells; right, PK-15 cell lines with ISG15 stable expression were screened and obtained by G418. (B) Western blot analysis of ISG15 stable expression. Lane 1, normal PK-15 cells as negative control; lane 2, PK-15 cell line after transfected pCDNA-ISG15; M, protein molecular weight marker. Arrows indicate the position of ISG15 and GAPDH.

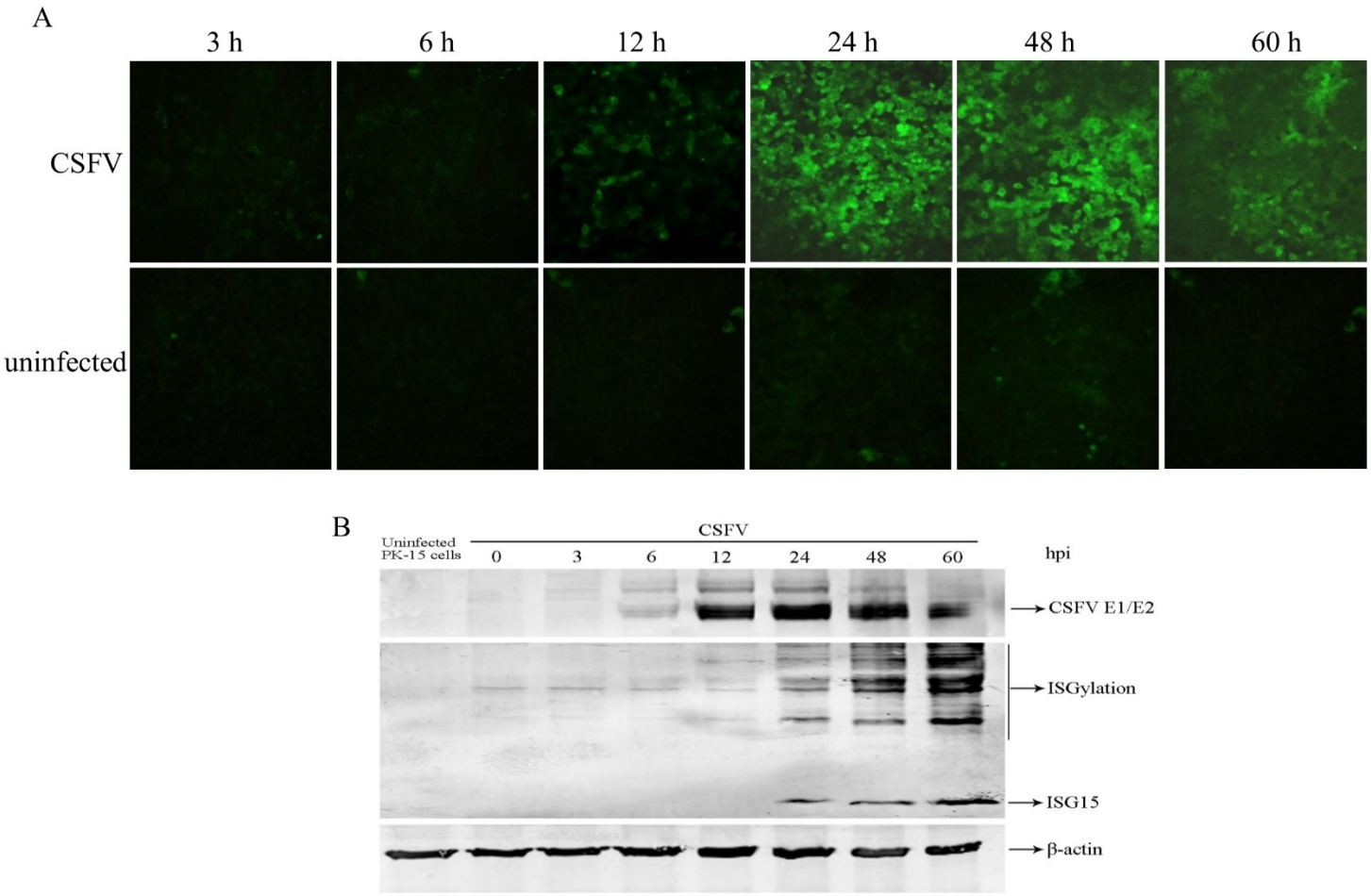

Figure 7 Detection of ISG15 expression levels in PK-15 cells infected or uninfected with CSFV by indirect immunofluorescence and western blot analysis.

(A) Indirect immunofluorescence analyzes ISG15 expression level. upper, ISG15 expression in PK-15 cells after 3, 6, 12, 24, 48 and 60 hours post CSFV infection; lower, ISG15 expression in PK-15 cells at 3, 6, 12, 24, 48 and 60 hours without CSFV infection as controls. (B) Western blot analyzes CSFV E2, ISG15 and ISGylation expression levels in PK-15 cells after 0, 3, 6, 12, 24, 48 and 60 hours post CSFV infection, the uninfected PK-15 cells were used as a control. Arrows indicate the position of CSFV E1/E2, ISG15, ISGylation and β-actin, respectively.

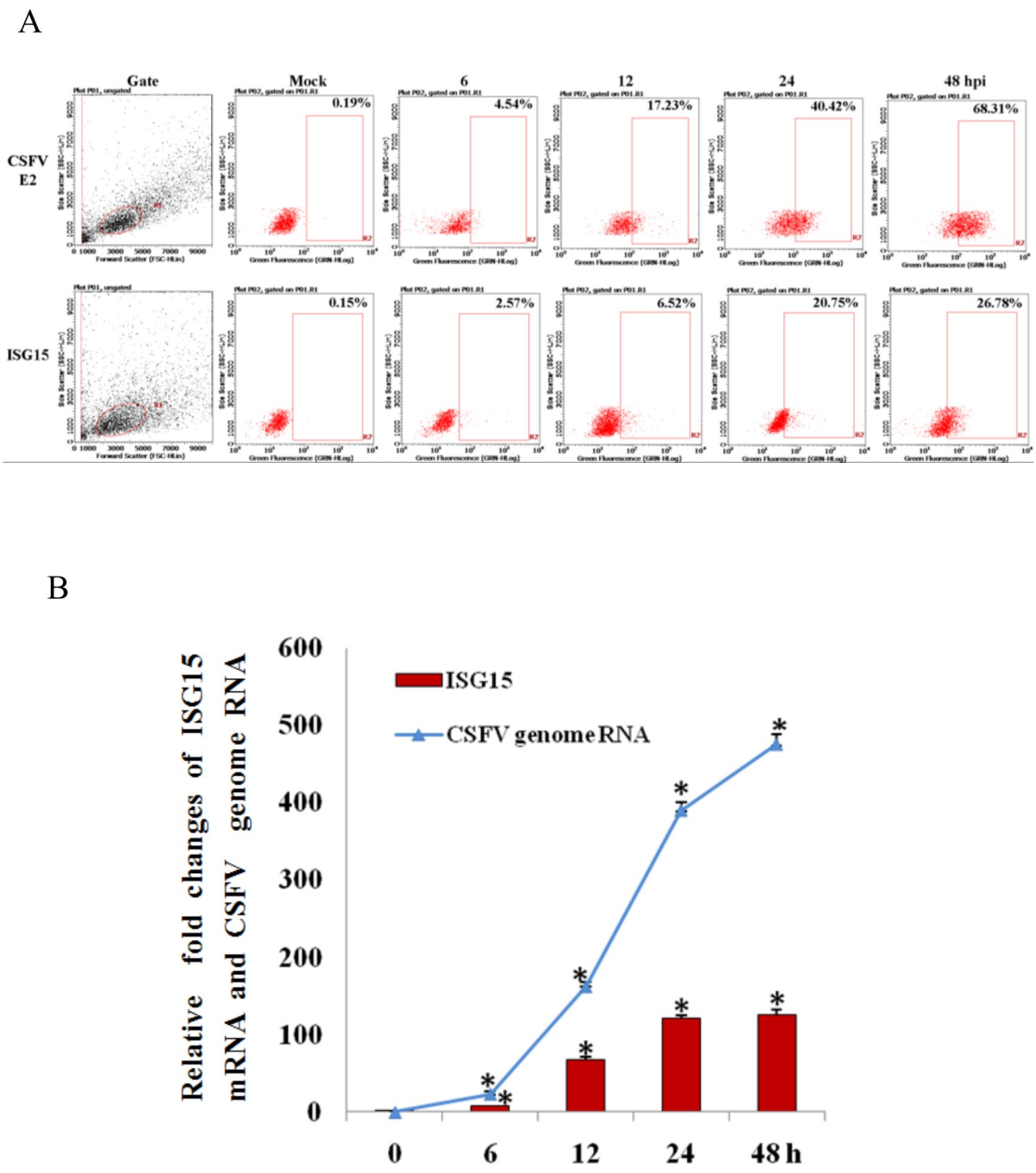

Figure 8 **The ISG15 expression level after CSFV infection.**
(A) Up-regulation of ISG15 after CSFV infection detected by flow cytometry. PK-15 cells were infected with 1.0 m.o.i of CSFV Shimen strain, the uninfected cells were used as a control. The cells were collected at 0, 6, 12, 24 and 48 hpi. The PK-15 cells expressing-CSFV E2 and -ISG15 were counted by flow cytometry, respectively. (B) Detection of CSFV gRNA and ISG15 mRNA by RT-qPCR. PK-15 cells were infected with 1.0 m.o.i of CSFV Shimen strain and the cells were collected for detection of CSFV gRNA and ISG15 mRNA at 0, 6, 12, 24 and 48 hpi by RT-qPCR, respectively.

Discussion

ISG15 plays an important role in antiviral defense. In vivo, ISG15 is an antiviral factor providing resistance to DNA or RNA viruses (Lai *et al*., 2009). The previous in vitro experiments involved with either overexpression or knockdown of mouse or human ISG15 in cultured cells showed that ISG15 inhibited the growth of many viruses. Singh *et al*. (2019) used RT-qPCR and immunoblot analysis to assess the expression of innate inflammatory and antiviral genes, supplementation of recombinant ISG15 (rISG15) and gene silencing approaches confirmed the role of ISG15 in corneal antiviral defense. Hsiao *et al*. (2010) found that the overexpression of ISG15 in human medulloblastoma cells significantly inhibited the Japanese encephalitis virus (JEV) replication. In addition, ISG15 knockout mice (ISG15$^{-/-}$) were more susceptible to the influenza virus, herpes simplex virus (HSV) and Sindbis virus. The C-terminal motif (LRLRGG) of ISG15 and the arginine residue at position 151 (Arg151) were essential for antiviral activity to the Sindbis virus (Giannakopoulos *et al*.,

2009; Lenschow *et al*., 2005). Certain viral structural or nonstructural proteins were targeted for ISGylation. The major binding site of ISG15 to nonstructural protein 1 (NS1) of influenza A was a key lysine residue (K41) in the N-terminal RNA-binding domain (RBD). The modification of ISG15 K41 blocked the association of the NS1 RBD domain with importin-α, which mediated nuclear importation of the NS1 protein. The modification of ISG15 K41 inhibited the replication of influenza A virus (Zhao *et al*., 2009).

Many viruses have evolved strategies to combat the antiviral actions of ISG15 and its conjugating enzymes. HSV-1 induces the synthesis of IFN antagonism proteins to inhibit the phosphorylation of STAT1, interferes with the signaling pathway of IFN, disrupts the expression of ISG15 protein and stimulates the replication and spread of HSV-1 (Johnson and Knipe, 2010; Nicholl *et al*., 2000). Our previous study revealed that the transcription level of *ISG15* gene in lymphocytes of peripheral blood in pigs during CSFV infection increased to the maximum at 6 dpi and then decreased at 9 dpi, as evidenced by microarray (Li *et al*., 2010). However, the role of ISG15 in CSFV replication remains unclear. There has been a lack of a suitable anti-ISG15 antibody. To further clarify the action of ISG15 during CSFV infection, we determined to prepare an anti-swine ISG15 antibody.

In the present study, two pairs of primer T1f/T2r and P1f/P2r were designed according to the ISG15 sequence on GenBank (accession: EU584557.1) for constructing the ISG15 recombinant plasmids. Using the cDNA of PK-15 cells as a template, the first pair of primer T1f/T2r was used to amplify the ISG15 sequence of 576 bp, which contained ISG15 CDS region. The T1f/T2r PCR product was ligated to the pMD18-T vector to obtain the recombinant plasmid of pMD18-T-ISG15. The plasmid pMD18-T-ISG15 was used as a template, and the second pair of primers P1f/P2r was used to amplify the 522 bp CDS region of ISG15, the coding sequence of swine ISG15 gene encoding a peptide of 168 amino acids was amplified. This two primer pairing used to construct the plasmid was easy to amplify the ISG15 fragment and confirm the specific fragment. The amplified ISG15 coding sequence inserted 6×His-tag was fused with a fragment of GST peptide and was subcloned into the prokaryotic expression plasmid pGEX-4T-1. The experimental results revealed that the larger fused protein containing ISG15, 6×His-tag and GST peptide was well expressed in *E. coli* BL21 cells and demonstrated excellent antigenicity. Using this larger fused protein ISG15/6×His-tag/GST, a high quality polyclonal antibody with high specificity and reactivity was prepared for administration to Kunming mice. The immunized mice serum at 1:4000 dilution reacted well with 5 μg of purified ISG15/6×His-tag/GST through western blot. The anti-ISG15 polyclonal antibody not only strongly reacted with the recombinant protein in BL21 cells, but also well with natural ISG15 protein in PK-15 cells induced by swine IFN-α2b, as demonstrated by western blot, IFA and flow cytometry. Firstly, the ISG15 polyclonal antibody could not detect any band in the lanes of normal cell samples but the antibody could detect one specific band at 15 KDa in the lane of PK-15 cell line after transfected pCDNA-ISG15 samples (Figure 6B). Furthermore, Figure 7A indicated that the ISG15 polyclonal antibody could detect obviously specific fluorescence after 24 hpi with CSFV (Figure 7A upper), but could not detect fluorescence without CSFV infection at any time (Figure 7A lower); and Figure 7B indicated that ISGylation of ISG15 protein increased with the time of CSFV infection and the ISG15 content increased with CSFV infection. From all of these, the ISG15 antibody is specific and could not cross-react with other proteins in the cells following CSFV replication.

Our study demonstrated that the expression of ISG15 was increased in PK-15 cells during CSFV infection, as assessed by IFA (Figure 7A), western blot (Figure 7B) and flow cytometry (Figure 8A). Theoretically, after CSFV infection, there would not only be a 15 kDa mature form but also a 17 kDa precursor of ISG15 be detected, but we did not observe two bands, probably since 2 kDa difference between precursor and mature forms of ISG15 was hard to distinguish in 12% separation gel of western blot. Precursor would be cleaved then turn into the mature form, as well as the remaining and newly transcript premature forms being readily stimulated in the cycle of activation. According to the increase of ISGylation after CSFV infection (Li *et al*., 2020), the expression level of the mature form of ISG15 must be raised (Figure 7B). To further explore the interaction between ISG15 and CSFV, the preparation of a suitable anti-swine ISG15 polyclonal antibody is a crucial step. With this tool now available, we will aim, in further efforts, to clarify how ISG15 and ISGylation interact with CSFV. Our previous study demonstrated that both porcine IRF1 and ISG15 were shown to be up-regulated in PK-15 cells following stimulation with dsRNA or CSFV infection (Li *et al*., 2018). PK-15 cells were infected with the CSFV Shimen strain (1.0 m.o.i), the cell lysates were collected and the total RNA was extracted at 0, 3, 6, 9, 12, 24, 36 and 48 hpi, IRF1 and ISG15 mRNA levels were detected by RT-qPCR. The IRF1, ISG15 and CSFV E1/E2 proteins were detected by western blot analysis simultaneously. In vitro infection with CSFV confirmed that ISG15 expression could be up-regulated in PK-15 cells compared with uninfected cells and the result of this study is consistent with the previous conclusion (Li *et al*., 2018). In the other experiments, we had revealed that once ISG15 expression was inhibited by ISG15 siRNA, the expression of CSFV E1/E2 significantly increased compared to the control (data not shown), demonstrating that ISG15 inhibited the replication of CSFV. Therefore, the porcine ISG15 could inhibit CSFV replication in cells, and the ISG15 polyclonal antibody prepared in this study could be powerfully used to detect the ISG15 expression level after CSFV or other virus infection.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31660722), the Project in Natural Science Foundation of Guangxi Province (2017GXNSFAA198137), National key research and the development project of China (2018YFD0500104).

Declaration: *All authors declare that they have no competing interests.*

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