Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola
ISSN 1516-635X Apr - Jun 2018 / v.20 / n.2 / 377-386

http://dx.doi.org/10.1590/1806-9061-2017-0662

Distribution of Ifitm3 in Yellow-Feathered Broilers and Inhibition of Avian Reovirus Multiplication by Ifitm3

■Author(s)

Wang Q[I,II]
Yuan X[I]
Li C[I]
Deng H[I]
Wang C[I]

[I] College of animal science, Fujian agriculture and forestry university, Fuzhou, 350002, China;
[II] Fujian key laboratory of traditional Chinese veterinary medicine and animal health (Fujian Agriculture and Forestry University) Fuzhou, Fujian 350002, China.

■Mail Address

Corresponding author e-mail address
Changkang Wang
Fujian Agriculture and Foresty University, Fuzhou, Fuzhou, Fujian, 350002, China.
Tel: 086-0591-83758852
Email: Wangchangkangcn@163.com

■Keywords

Yellow-feathered broiler, Interferon, Interferon-induced transmembrane protein 3, Gene cloning, Prokaryotic expression, Avian Reovirus.

Submitted: 10/October/2017
Approved: 08/January/2018

ABSTRACT

This study was carried to express the interferon-induced transmembrane protein 3 (*IFITM3*) in vitro and examine its function in inhibition of avian reovirus (ARV) replication. The recombinant prokaryotic vector expressing yellow-feathered broiler *IFITM3* was successfully constructed, and the recombinant protein was expressed in competent *Escherichia coli* BL21 cells. New Zealand white rabbits were immunized with the purified recombinant protein to prepare a polyclonal antibody, with a titer of 1:128,000. Immunohistochemistry, reverse transcription–PCR, and real-time fluorescence quantitative PCR showed that *IFITM3* was distributed in the yellow-feathered broiler immune organs, and the expression of *IFITM3* in bursa of Fabricius was more than in spleen and thymus. It was found that in the thymus, spleen and bursa of Fabricius the mRNA expression levels of IFNα and *IFITM3* were significantly induced after ARV infection. And it was also certified in the chicken embryo fibroblasts (CEFs) which infected with ARV. Then the *IFNα* was added into the cell culture medium before CEFs were infected with ARV. The results indicated that the mRNA of *IFITM3* expression was significantly increased and ARV multiplication was significantly inhibited. And when the expression of *IFITM3* was knocked down by siRNA-*IFITM3*, the expression of *IFITM3* was significantly reduced, but the ARV multiplication was significantly increased, which indicated that *IFITM3* protein could inhibit the ARV replication.

Abbreviations: avian reovirus, ARV; complementary deoxyribonucleic acid (cDNA); chicken embryonic fibroblasts, CEFs; double distilled water (ddH2O); Dulbecco's modified Eagle medium, DMEM; glyceraldehyde-3-phosphate dehydrogenase gene, GAPDH; interferon alpha, IFNα; interferon-induced transmembrane protein, IFITM; intravenous, iv; median tissue culture infective dose ,TCID50; multiplicity of infection, MOI; National Center of Biotechnology Information, NCBI; phosphate-buffered saline, PBS; polymerase chain reaction, PCR; ribonucleic acid, RNA; reverse transcription, RT; sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE; small interfering RNAs (siRNAs).

INTRODUCTION

The innate immune system can recognize pathogen infections and then initiates the mechanisms that inhibit pathogen replication (Amanda *et al.*, 2013). To defend against viral infection, the host will mobilize factors that challenge the virus. Interferons (IFNs) orchestrate a large component of the antiviral response (Brass *et al.*, 2009), triggering

antiviral pathways by inducing the expression of interferon-stimulated genes (ISGs) (Randall *et al.*, 2008). The interferon-inducible transmembrane (*IFITM*) proteins were first identified in IFN-treated neuroblastoma cells in 1984, and are encoded by a class of ISGs (Chen *et al.*, 1984). The IFITM gene family includes *IFITM1*, *IFITM2*, *IFITM3*, *IFITM5*, *IFITM6*, *IFITM7*, and *IFITM10* genes and some IFITM-like genes. These genes are induced by IFN and are widely expressed in tissues and organs, except *IFITM5*, which is only expressed in bone cells (Siegrist *et al.*, 2011). The biological functions of *IFITM*3 include the inhibition of both viral replication and cell entry. Brass *et al.* (2009) demonstrated that the IFITM proteins inhibit the early replication of flaviviruses, including *Dengue virus* and *West Nile virus*. Lu *et al.* (2009) confirmed that IFITM proteins inhibit HIV-1 replication, at least partly by interfering with viral entry.

Infectious diseases greatly influence poultry production, especially viral infections that induce immune suppression. Avian reovirus (ARV) infections affect the poultry industry throughout the world (Lorena *et al.*, 2009). ARV causes viral tenosynovitis, malabsorption, and respiratory diseases in chickens (Teng *et al.*, 2013), and ARV-induced immunosuppression is one of the most important pathogenic mechanisms of the virus (Chen *et al.*, 2015). However, how the innate immunity of chickens plays its roles during ARV infection requires further study. Therefore, in this study, the *IFITM3* gene of the yellow-feathered broiler was cloned, expressed, and confirmed, and the distribution of its expression in the chicken immune organs was investigated. And IFITM3 could inhibit ARV replication in vivo and in vitro that also was carried out.

MATERIALS AND METHODS

Materials

Virus

Avian reovirus strain S1133 was propagated in chicken embryonic fibroblasts (CEFs), which were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum, as previously described (Wang *et al.*, 2012). The median tissue culture infective dose ($TCID_{50}$) was $10^{-5.40}$ (multiplicity of infection [MOI] = 8/mL).

Cells

CEFs were grown in Dulbecco's modified Eagle medium (DMEM) containing streptomycin (50 µg/ml), penicillin G50 (50 µg/ml), and 5% foetal bovine serum, at 37 °C in 5% CO_2.

Vectors

The vectors used were the pGEM®-T Easy vector (Promega, Wisconsin, USA) and pET-32a(+) (Promega, Wisconsin, USA).

Main reagents

The main reagents used included the TIANamp® Viral Total Nucleic Acid Purification Kit (TIANamp, Beijing, China); the GoScript™ Reverse Transcription System (Promega, Wisconsin, USA)); HilyMax™ Transfection Reagent (Dojindo, Shanghai, China); horseradish-peroxidase-labelled sheep anti-chick IgG antibody; *Escherichia coli* DH5α cells (Promega, Wisconsin, USA); *E. coli* BL21(Promega, Wisconsin, USA).

Total RNA extraction and cDNA amplification

Tissue samples (spleen, thymus, and bursa of Fabricius) were collected from healthy 20-day-old yellow-feathered broilers. Total RNA was extracted from the yellow-feathered broiler spleen with the TRIzol Plus RNA Purification Kit (TIANamp, Beijing, China). The first-strand cDNA was synthesized according to the manufacturer's instructions. The total volume of the reverse transcription reaction mixture was 20 µl. The reaction was performed at 45 °C for 15 min and 72 °C for 15 min.

Primers design

Oligonucleotide primers for the PCR reactions and real time-PCR were designed (Table1). *BstZ*1 restriction sites were incorporated at the 5¢ ends of the *IFITM3* gene primers.

Cloning the chicken *IFITM3* gene

The PCR product amplified with the specific primers was purified and cloned into the pGEM®-T Easy vector (Promega, Wisconsin, USA). This recombinant plasmid was designated pGEM-T–*IFITM3* and transformed into competent *E. coli* DH5α cells. Positive clones were selected with blue–white selection, and the positive plasmids were confirmed with PCR and sequencing.

The *IFITM3* sequence was compared using the National Center for Biotechnology Information (NCBI) online BLAST tool. The structure of the IFITM3 protein was predicted with the DNAMAN software. The palmitoylation sites were determined with the CSS-Palm2.0 software.

Construction of expression plasmids and expression of pEASY™-E1–*IFITM3*

The PCR product amplified with the specific primers was purified and cloned into pEASY™TM-E1

Table 1 – Primer sequences

Gene name	Primer sequences (5′-3′)	Amplification fragments(bp)
IFITM3-F IFITM3-R	5′ATGCAGAGCTACCCTCAGCA3′ 5′TCAGGGCCGCACAGTGTACAA3′	342
IFITM3 (*Bst*Z1)-F IFITM3 (*Bst*Z1)-R	5′ATTCGGCCGATGCAGAGCTACCCTCAGCA3′ 5′TAAGCCGGCTCAGGGCCGCACAGTGTACAA3′	360
IFITM3-F IFITM3-R	5′CAGAGCTACCCTCAGCACAC -3′ 5′CTGTCCTCCCATAGCTGCTG-3′	235
IFNα-F IFNα-R	5′ATGGCTGTGCCTGCAAGC -3′ 5′TTGCCTGTGAGGTTGTGGAT-3′	569
IFNα-F IFNα-R	5′GGACATGGCTCCCACACTAC-3′ 5′ATCCGGTTGAGGAGGCTTT-′	204
ARV- F ARV -R	5′GCTCCGCCATACGACGTTT-3′ 5′TAGATCGGCGTCAAATCGC-3′	441
ARV-F ARV-R	5′CCATACGACGTTTGAAGTGC-3′ 5′TCATAGATCGGCGTCAAAT-3′	250
β-actin-F β-actin-R	5′CCAAAGCCAACAGAGAGAAGATCA-3 ′5′TCACCAGAGTCCATCACAAT-3′	138

expression vector. The recombinant plasmid was designated PEASY™-E1–*IFITM3* and was transformed into competent *E. coli* DH5α cells. Positive clones were selected with blue–white selection and confirmed with PCR (using the T7 upstream primer and the target gene downstream primer), sequencing, and restriction enzyme digestion. And PEASY™-E1–*IFITM3* was transformed into Competent *E. coli* BL21(DE3) pLysS cells.

The pEASY™-E1–*IFITM3*-positive BL21(DE3) pLysS cells were cultured for 14 h at 37 °C with 1 mmoL/mL IPTG (Transgen Biotech, Beijing, China). The bacterial cells were collected and centrifuged at 12,000 g for 1 min. The pellet was then re-suspended in phosphate buffer (PBS) and broken with ultrasonography. The protein was denatured and identified with SDS-PAGE.

Purification of recombinant *IFITM3* (rIFITM3) and preparation of polyclonal antibody

The recombinant protein was purified with affinity chromatography on ProteinIso™ Ni-NTA Resin (Transgen Biotech, Beijing, China). rIFITM3 was identified with SDS-PAGE and Western blotting.

An anti-rIFITM3 polyclonal antibody was prepared as described previously (Zheng *et al.*, 2014). Three New Zealand white rabbits were inoculated by subcutaneous injection with 1 mL (1 mg/mL) of purified IFITM protein, which was emulsified with an equal amount of Freund's complete adjuvant. Immunization was boosted twice by inoculation with 1 mL of antigen mixed with an equal volume Freund's incomplete adjuvant at 2-weekly intervals. The fourth immunization was performed 7 days after the third immunization, with 1 mL (1 mg/mL) of purified IFITM protein by intravenous (iv) injection. The antibody titer was determined with the enzyme-linked immunosorbent assay.

Immunohistochemical localization of chicken IFITM3 in immune organs

The tissue samples including thymus, spleen and bursa of Fabricius, were dehydrated in a graded series of ethanol, embedded in paraffin, and sectioned at 4–6 μm with a histotome. After dewaxing and hydration, the sections were analysed with immunohistochemistry method. For immunohistochemical analysis, the sections were incubated with the rabbit anti-IFITM3 antibody at 37 °C for 60-120 min. After rinsed with PBS for 5 min three times, the sections were incubated with a biotin-conjugated goat anti-rabbit antibody (Keygen Biotech Co. Ltd, Nanjing, China) at 37 °C for 10-30 min. After the sections were washed, they were treated with streptavidin–peroxidase for 10 min and then with a diaminobenzidine solution. The sections were then counterstained with haematoxylin and mounted with neutral gum.

IFITM3 mRNA levels in chicken immune organs

IFITM3 mRNA levels in chicken immune organs were examined by RT-PCR and SYBR Green real-time PCR. RT-PCR was performed with 1 μL of cDNA, 0.5 μL of each primer, 1 μL of PCR mix, and 8 μl of double distilled water (ddH_2O), in a total volume of 20 μL. The cycling conditions for amplification were 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. The products were analysed with electrophoresis on 1.0% agarose gel and sequenced by Dalian TaKaRa Company (Dalian,China).

The *IFITM3* mRNA levels in the chicken immune organs were detected with SYBR Green real-time fluorescence quantitative PCR. Specific primers were designed (Table 1), and were synthesized by Invitrogen Company. Reaction volumes of 20 μL contained 10 μL of SYBR® Premix Ex Taq™ (2×) (Promega), 0.4 μL of FastStart Universal Probe Master, 0.4 μL of forward primer (10 μM), 0.4 μL of reverse primer (10 μM), 2 μL of cDNA, and 6.8 μL of RNase-free ddH_2O. The PCR cycling conditions were 45 cycles of initial denaturation at 95 °C for 30 s, 95 °C for 5 s, and 60 °C for 34 s. The data were analysed with the ΔCT method using the following formula: target gene expression = $2^{-\Delta Ct}$

($\Delta Ct = Ct_{target\ gene} - Ct_{reference\ gene}$). The glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) was used as the reference gene. Statistical differences were determined with *t* test in SPSS 17.0.

IFITM3 gene expression in ARV-infected chickens immune organs and CEF

Thirty 20-day-old yellow-feathered broilers were randomly divided into the control group (five and the experimental group) tweenty five. The experimental group chickens were infected with 0.2 mL ARV, andnd five control chickens were slaughtered at the first day of ARV iontreatment. Then five infection ducks were slaughtered ~~every~~ after every infection. The thymus, spleen, and bursa of Fabricius were collected from each broiler. The expression of *IFTIM3* and *IFNα* and the replication of ARV were detected using reverse transcription (RT)–PCR and SYBR real-time fluorescence quantitative PCR.

CEFs grew in six-well tissue-culture plates with MDEM cell medium for 37 °C 24 h. CEF cells were collected after inoculation with ARV 0 h, 12 h, 24 h, 48 h, 72 h respectively. The cells were t digested with 0.25% pancreatic enzyme. The total RNA was extracted and the mRNA expression of *IFN*, *IFTIM3* and ARV was determined with PCR and SYBR real-time fluorescence quantitative PCR.

Analysis of IFN sensitivity

CEFs grown in six-well tissue-culture plates were either mock-treated or treated with IFNα (100 IU/ml) for 0.5 h at 37 °C before viral inoculation. Then, these cells were challenged with 0.1mL of ARV for 24 h and digested with 0.25% pancreatic enzyme. The total RNA was extracted and the expression of *IFTIM3* was determined with PCR.

IFITM3-directed small interfering RNAs (siRNAs) were designed (F: 5¢-GAGCCUACGAAACCUUAAUdTdT-3¢ and R: 5¢-AUUAAGGUUUCGUAGGCUCdTdT-3¢) and transfected into CEF cells. The cells were inoculated with 0.1mL of ARV for 24 h and then digested with 0.25% pancreatic enzyme. The total RNA was extracted and the mRNA expressions of *IFNα*, *IFTIM3* and ARV were detected with PCR and SYBR real-time fluorescence quantitative PCR.

RESULTS

Cloning and analysis of the *IFITM3* gene

Total RNA was extracted from the spleens of chickens and reversed transcribed to cDNA. PCR with specific primers amplified the 342-bp target gene (Figure 1A). The PCR product was ligated to the linearized pGEM-T Easy cloning vector and competent *E. coli* DH5α cell were transfected with the recombinant plasmid. The recombinant plasmid was then identified with enzymatic digestion. The target gene was 342 bp on 1% agarose gel electrophoresis (Figure 1B), indicating that the cloning vector was successfully constructed. The sequence of *IFITM3* in the yellow-feathered broiler was determined and compared with homologous sequences using BLAST. The sequence of *IFITM3* in the yellow-feathered broiler was high ratio of homology to *Gallus IFITM3* (NCBI LOC422993).

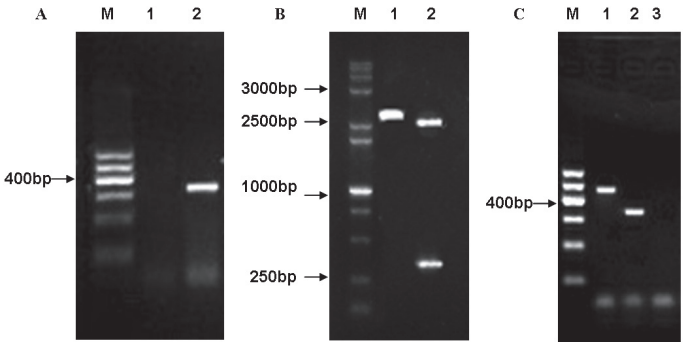

Figura 1 – Parte A: PCR with specific primers amplified the 342-bp target gene. Parte B: The target gene was 342 bp on 1% agarose gel electrophoresis, indicating that the cloning vector was successfully constructed. Parte C: The amplified fragment was consistent with the anticipated fragment (584 bp).

The *IFITM3* gene sequences of *Homo sapiens*, *Pan troglodytes*, *Macaca mulatta*, *Mus musculus*, *Sus scrofa*, *Bos taurus*, *Equus caballus*, *Felis catus*, and the white-throated sparrow (*Zonotrichia albicollis*) were downloaded from NCBI and their homology was analysed with the DNAMAN software (Figure 2, Table 2). The evolutionary distance between the yellow-feathered broiler and *Zonotrichia albicollis* sequences was smallest, whereas the yellow-feathered broiler sequence was distant from the mammal sequences, and most distant from the human sequence.

Table 2 – The evolutionary distance of IFITM3 gene in yellow-feathered broilers and other species

Species	Evolutionary distance
Homo sapiens	0.059
Pan troglodytes	0.061
Macaca mulatta	0.104
Mus musculus	0.183
Sus scrofa	0.102
Bos taurus	0.114
Equus caballus	0.114
Felis catus	0.120
Zonotrichia albicollis	0.209

Amino acid sequence prediction and analysis of the properties of *IFITM3*

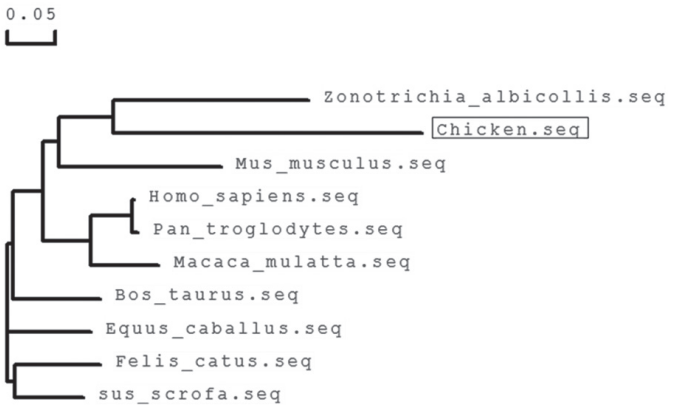

Figura 2 – The IFITM3 gene sequences of Homo sapiens, Pan troglodytes, Macaca mulatta, Mus musculus, Sus scrofa, Bos taurus, Equus caballus, Felis catus, and the white-throated sparrow.

The amino acid sequence of *IFITM3* was predicted and the protein properties were analyzed with DNAMAN. The isoelectric point (pI) of *IFITM3* is 8.8, and its secondary structure consists of helices, folds, and curls, but predominantly folds and curls (Figure 3A). *IFITM3* contains 113 amino acids, and has 20 fewer amino acids than human *IFITM3*. It contains a CD225 domain (Figure 2B), as does human *IFITM3*. There are two transmembrane domains, at amino acids 37–65 and amino acids 78–106 (Figure 3C), and three antigenic peptides. The *IFITM3* protein of the yellow-feathered broiler is hydrophobic (Figure 3 D).

The S-palmitoylation modification sites of *IFITM3* in the yellow-feathered broiler were analyzed with the CSS -Pal M 2.0 software. There are four potential palmitoylation sites at cysteines 45, 49, 53, and 89 (Table 3).

Table 3 – The IFITM3 protein of yellow-feathered broilers'S- palmitoylation modification site

Site	Locus	Possibility of palm acylating modified loci
49	NAFCLGL	0.65
89	FAFCVGL	0.61
53	LGLCALS	0.50
45	FVLCNAF	0.17

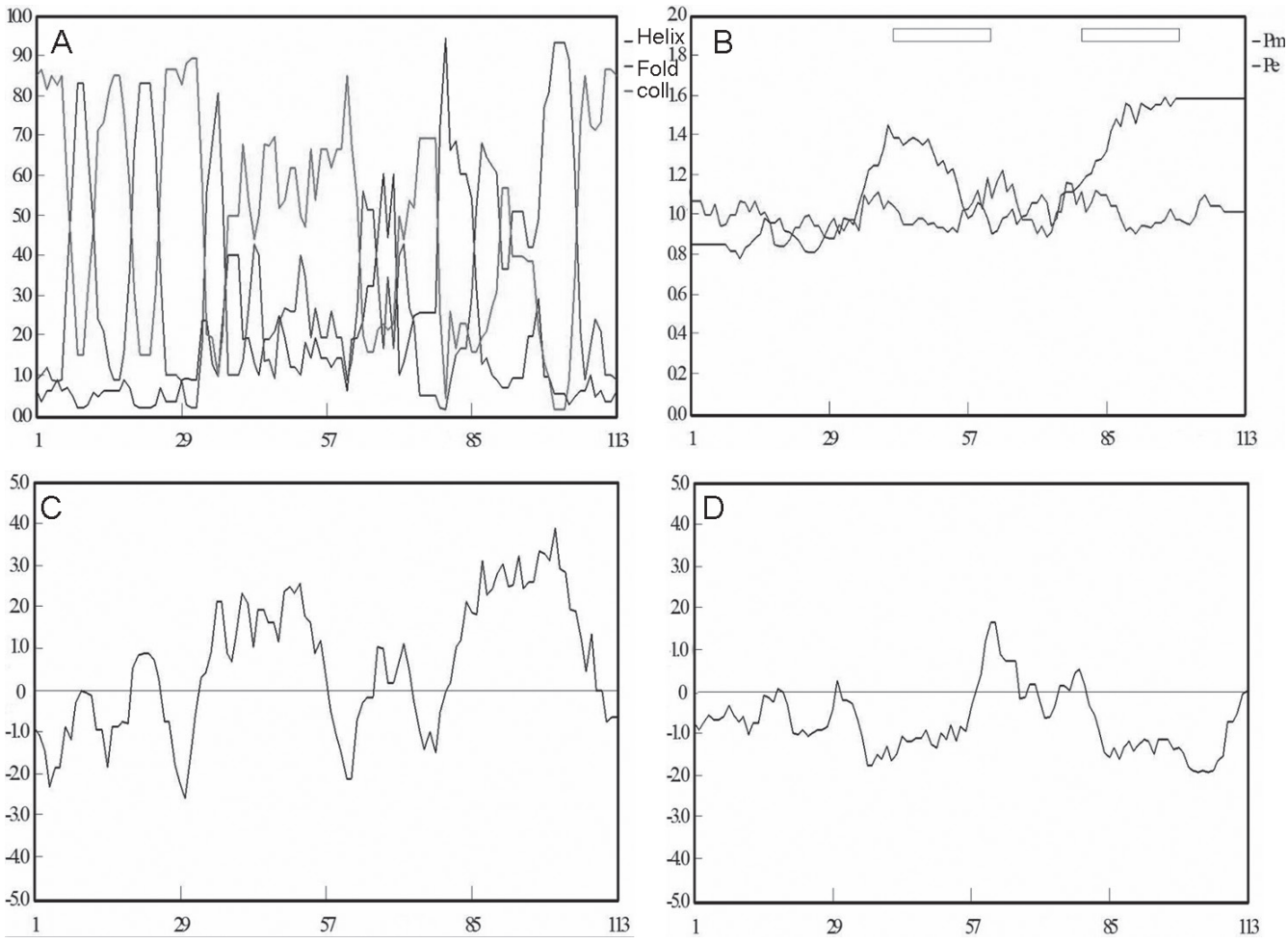

Figura 3 – The isoelectric point (pI) of IFITM3 is 8.8, and its secondary structure consists of helices, folds, and curls, but predominantly folds and curls. Parte A: The secondary structure of IFITM3 was mainly consisted of fold and curle . Parte B e C: IFITM3 contains CD225 domain structure and have two transmembrane proteins. Parte D: IFITM3 was a hydrophobic proteins.

Construction of pEASY™-E1–c*IFITM3* and prokaryotic expression of the recombinant protein

The recombinant plasmid was amplified and identified with PCR using the T7 primer and target gene primer. The amplified fragment was consistent with the anticipated fragment (584 bp; Figure 1C.). The positive plasmid was sequenced by the Dalian TaKaKa Biotechnology Company, confirming the successful construction of the prokaryotic expression vector.

SDS-PAGE showed that the recombinant protein was successfully expressed in the BL21(DE3)pLysS cells, with the expected molecular weight of 11.5 kDa (Figure 4A). The recombinant protein was purified with affinity chromatography using ProteinIso™ Ni-NTA Resin. SDS-PAGE confirmed the successful purification of the recombinant protein (Figure 4B).

Specificity of the anti-*IFITM3* polyclonal antibody

After the immunization program, the anti-r*IFITM3* rabbit serum was collected and the antibody titer was determined with ELISA method. The antibody titer reached to 1:256000. The r*IFITM3* protein was detected with western blotting, demonstrating that the polyclonal antibody was specific for the r*IFITM3* protein (Fig. 4C).

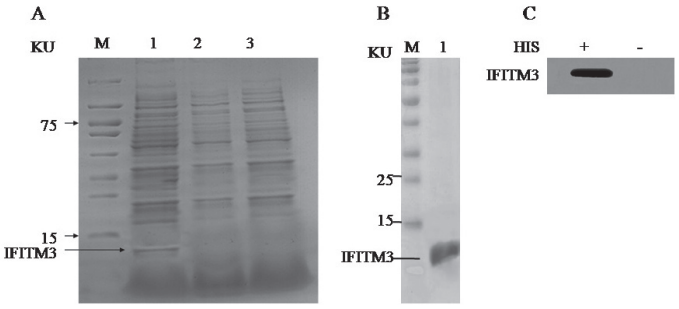

Figura 4 – Parte A: SDS-PAGE showed that the recombinant protein was successfully expressed in the BL21(DE3)pLysS cells, with the expected molecular weight of 11.5 kDa. Parte B: The recombinant protein was purified with affinity chromatography using ProteinIso™ Ni-NTA Resin. SDS-PAGE confirmed the successful purification of the recombinant protein. Parte C: The rIFITM3 protein was detected with western blotting, demonstrating that the polyclonal antibody was specific for the rIFITM3 protein.

Expression of *IFITM3* in immune tissues

The distribution of *IFITM3* in the chicken immune organs was examined with immunohistochemistry. *IFITM3* was distributed in the thymus (Figure 5A), spleen (Figure 5B), and bursa of Fabricius (Figure 5C). Positive cells were mainly distributed in the medulla of

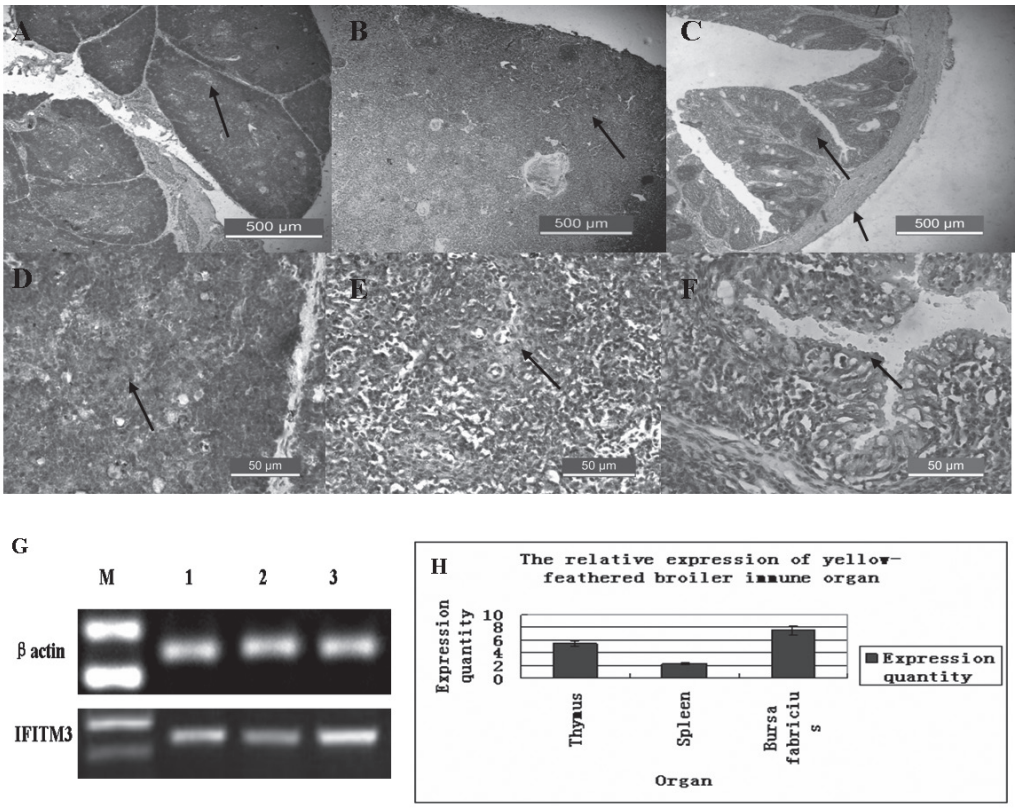

Figura 5 – The distribution of IFITM3 in the chicken immune organs was examined with immunohistochemistry. IFITM3 was distributed in the thymus (Fig. 5A), spleen (Fig. 5B), and bursa of Fabricius (Fig. 5C). Positive cells were mainly distributed in the medulla of the thymus (Fig. 5D), in the red pulp of the spleen (Fig. 5E), and in the epithelium of the bursa of Fabricius (Fig. 5F). The IFITM3 mRNA levels in 20-day-old yellow-feathered broilers were also investigated with RT–PCR and SYBR–PCR. IFITM3 mRNA was expressed in the three immune organs (Fig. 5G,H), with the highest expression in the bursa.

the thymus (Figure 5D), in the red pulp of the spleen (Figure 5E), and in the epithelium of the bursa of Fabricius (Figure 5F). The IFITM3 mRNA levels in 20-day-old yellow-feathered broilers were also investigated with RT–PCR and SYBR–PCR. *IFITM3* mRNA was expressed in the three immune organs (Figure 5G,H), with the highest expression in the bursa.

IFITM3 expression in ARV-infected CEF cells and yellow-feathered broilers

After ARV infection 12h and 24h, the mRNA expression levels of IFN-α and IFITM3 in CEF cells was higher than the control group (Figure 6 A,B).

To investigate the function of *IFITM3* after ARV infection, the mRNA expression levels of *IFITM3* and IFN-α in the thymus, spleen, and bursa were determined with RT–PCR and SYBR-qPCR. IFN-α expression was induced by ARV, and then stimulated the expression of *IFITM3* early in infection. *IFITM3* expression in the thymus (Figure 6 C, D) and bursa (Figure 6 E, F) peaked on day 3 after ARV infection, and then gradually decreased. In the spleen (Figure 6 G, H), *IFITM3* began to increase on day 1 after infection, and peaked on day 2. Therefore, ARV stimulated the secretion of *IFITM3* in the early stage of ARV infection.

IFN induces *IFITM3* expression to inhibit ARV replication

Cultured CEFs were infected with ARV. IFN-α (20 ng/mL) was added to the culture fluid of the experimental group and an equal amount of physiological saline was added to the control group. In CEFs with IFN activity, ARV expression peaked at 3 h, and then gradually decreased, whereas the expression of FITM3

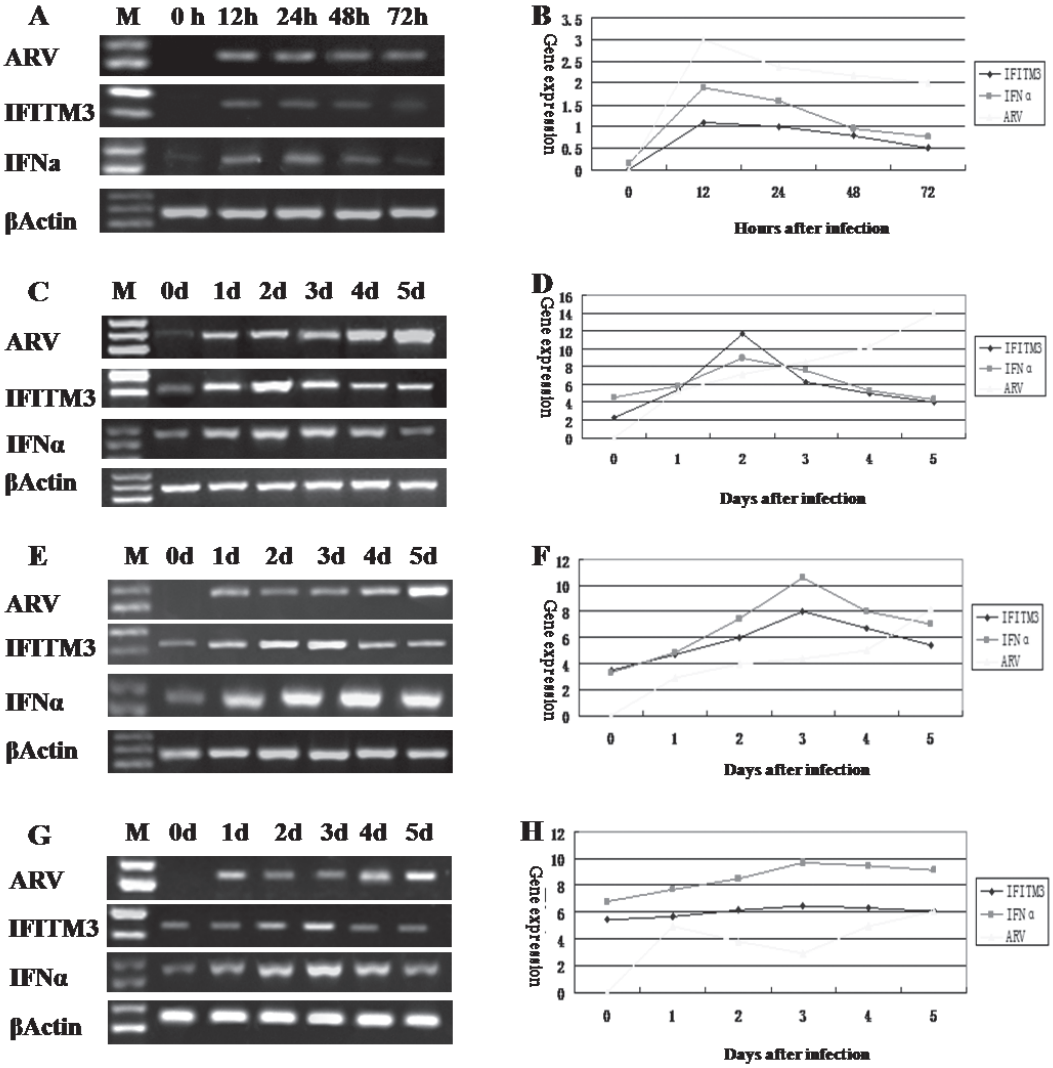

Figura 6 – Parte A e B: After ARV infection 12h and 24h, the mRNA expression levels of IFN-α and IFITM3 in CEF cells was higher than the control group. Parte C e D: IFITM3 expression in the thymus peaked on day 3 after ARV infection, and then gradually decreased. Parte E e F: IFITM3 expression in the bursa peaked on day 3 after ARV infection, and then gradually decreased. Parte G e H: In the spleen, IFITM3 began to increase on day 1 after infection, and peaked on day 2.

continued to increase gradually (Figure 7 A, B). But ARV expression gradually increased in IFN-null CEFs, and that the expression of *IFITM3* peaked at 3 h, gradually decreasing thereafter (Figure 7 C, D).

RNA interference downregulates the expression of *IFITM3* and affects ARV replication

CEF cells were transfected with siRNA of *IFITM3* for 0 or 24 h. The RNA was then extracted and reverse transcribed to cDNA, using β-actin as the internal control. RT–PCR was used to determine the effects of the different periods of *IFITM3* interference on ARV expression. When interference at 0 h was compared with no interference at 0 h, neither *IFITM3* nor ARV expression changed. After interference for 24 h, *IFITM3* expression decreased relative to that in the interference-free control, showing that a low level of RNA interference was achieved (Figure7 E, F). ARV expression increased when *IFITM3* expression was inhibited with *IFITM3* interference. With no interference, ARV expression decreased. These results indicate that *IFITM3* protein could inhibitreplication of ARV.

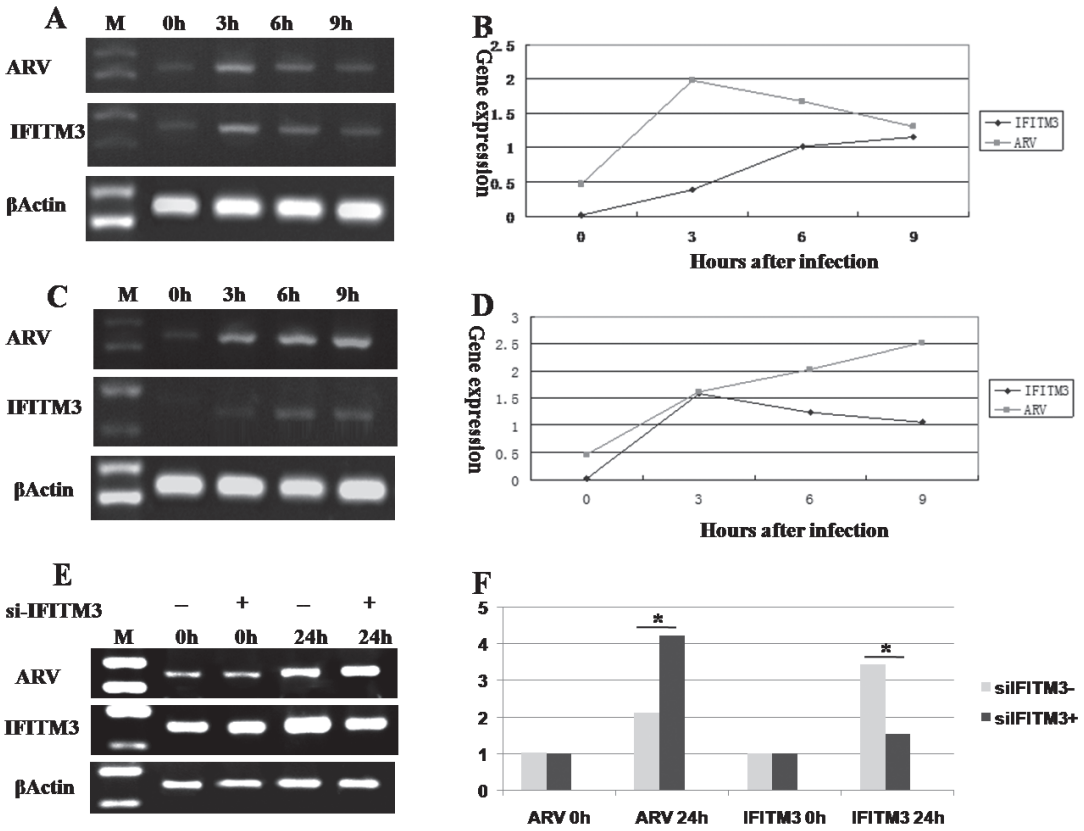

Figure 7 – Parte A e B: In CEFs with IFN activity, ARV expression peaked at 3 h, and then gradually decreased, whereas the expression of FITM3 continued to increase gradually. Parte C e D: ARV expression gradually increased in IFN-null CEFs, and that the expression of IFITM3 peaked at 3 h, gradually decreasing thereafter. Parte E e F: After interference for 24 h, IFITM3 expression decreased relative to that in the interference-free control, showing that a low level of RNA interference was achieved.

DISCUSSION

There are five members of the *IFITM* family in humans, *IFITM*1, *IFITM*2, *IFITM3*, *IFITM*5, and *IFITM*10, and these proteins are all induced by type I and type II IFNs (Laura *et al.*, 2014). They are involved in many physiological activities, including cell adhesion, cell differentiation, cell signal transduction, antitumor and antiviral activities, and immune surveillance (Perreira *et al.*, 2013). Research recently showed that IFITM was an important antiviral protein, resisting many kinds of viral infections. The replication of *influenza virus*, *West Nile virus*, and *Dengue virus* was significantly inhibited by *IFITM3* (Smith *et al.*, 2013). *IFITM*l is also an important factor in anti-hepatitis-C-virus infection mechanisms (Canoui *et al.*, 2016). Lu *et al.* found that the IFITM protein inhibited HIV-1 replication via IFN by uncoating the virus (Lu *et al.*, 2011). Yount *et al.* showed that the antiviral function of *IFITM3* was mediated by the posttranslational S-16 acylation of *IFITM3* (Yount *et al.*, 2010). However, the function of *IFITM*3 in poultry is still unclear.

The IFN-induced expression of the *IFITM* proteins is ubiquitous. Although *IFITM*5 is only expressed in bone cells, other *IFITM*-encoding genes are widely expressed in tissues and organs (Siegrist *et al.*, 2011). *IFITM*3 is mainly distributed on or in the cell membrane, and it inhibits viral entry into the cell (Bailey *et al.*, 2012). The immune organs (thymus, spleen, and bursa) of birds play important roles in resisting viral infection. Our experimental results found that the *IFITM3* gene was expressed in the immune organs of chickens, suggesting that *IFITM*3 was associated with the chicken's immune function.

Does *IFITM*3 suppress the proliferation of chicken viruses? Smith *et al.* found that the *IFITM*3 protein of chickens inhibited the replication of ARV (Smith *et al.*, 2013). ARV is one of the most important viruses in poultry production, and understanding the effect of *IFITM*3 on ARV infection is important for the discovery of new strategies to impede ARV transmission. In this study, we found that ARV activated the expression of IFN-α and *IFITM*3 in the immune organs (thymus, spleen, and bursa of Fabricius) in 20-day-old yellow-feathered broiler chickens artificially infected with ARV. The expression of *IFITM*3 was also induced in ARV-infected CEFs. When *IFITM*3 was strongly expressed after its induction by IFN, the replication of ARV was inhibited, but when the *IFITM3* gene was knocked down with shRNA, the replication of ARV was enhanced. Therefore, we consider that the FITM3 protein could inhibit the replication of ARV.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

This work was supported by the Chicken Industry System Program of Fujian China (NO. K83139297) and the National Spark key Program of China (2015GA720001).

COMPLIANCE WITH ETHICAL STANDARDS

All chicks used in the experiments were treated in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of China. The animal protocol used in this study was approved by the Research Ethics Committee of the College of Animal Science, Fujian Agriculture and Forestry University, Fujian, China.

REFERENCES

Anafu AA, Bowen CH, Chin CR, Brass AL, Holm GH. Interferon-inducible transmembrane protein 3 (IFITM3) restricts reovirus cell entry. *The journal of biological chemistry* 2013;288(24): 7261–17271.

Bailey CC, Huang IC, Kam C, Farzan M. IFITM3 limits the severity of acute influenza in mice. *PLoS Pathog* 2012;8:e90991002.

Brass AL, Huang IC, Benita Y, John SP, Krishnan MN, Feeley EM, Ryan BJ, Weyer JL, van der Weyden L, Fikrig E, Adams DJ, Xavier RJ, Farzan M, Elledge SJ. IFITM proteins mediate the innate immune response to influenza A H1N1 virus, West Nile virus and Dengue virus. *Cell* 2009;139(7): 1243–1254.

Canoui E, Noël N, Lécuroux C, Boufassa F, Sáez-Cirión A, Bourgeois C, Lambotte O. IFITM1expression in Cd4 T cells in HIV controllers is correlated with immune activation. *Jaids-Journal of Acquired Immune Deficiency Syndromes* 2016; Aug 19. [Epub ahead of print] PMID: 27552157.

Chen YS, Shen PC, Su BS, Liu T., Lin CC, Lee LH. Avian reovirus replication in mononuclear phagocytes in chicken footpad and spleen after footpad inoculation. *Canada Journal Veterinary Research* 2015;79(2): 87–94.

Chen YX, Welte K, Gebhard DH. Induction of T cell aggregation by antibody to a 16kd human leukocyte surface antigen *Immunology* 1984;133: 2496–2501.

Lu J, Pan Q, Rong L, He W, Liu SL, Liang C. The IFITM proteins inhibit HIV- 1 infection. *Journal of virology* 2011; 85(5) : 2126-2137.

Lorena V, Irene L, José M, Javier B. Avian reovirus sigmaA localizes to the nucleolus and enters the nucleus by a nonclassical energy-and carrier-independent pathway. *Journal of virology* 2009; 83(19): 10163–10175.

Miller LC, Jiang Z, Sang Y, Harhay GP, Lager KM. Evolutionary characterization of pig interferon-inducible transmembrane gene family and member expression dynamics in tracheobronchial lymph nodes of pigs infected with swine respiratory disease viruses. *Veterinary Immunology and Immunopathology* 2014;159(3-4):180-191.

Perreira JM, Chin CR, Feeley EM, Brass A.L. IFITMs restrict the replication of multiple pathogenic viruses. *Journal of Molecular Biology* 2013;425(24):4937-55.

Randall RE, Goodbourn S. Interferons and viruses: an interplay between induction, signaling, antiviral responses and virus countermeasures. *Journal of Gene Virology* 2008;89:1–47.

Smith SE, Gibson MS, Wash RS, Ferrara F, Wright E, Temperton N, Kellam P, Fife M. Chicken interferon-inducible transmembrane protein 3 restricts influenza viruses and lyssaviruses in vitro. *Journal of Virology* 2013; 87(23):12956-12966.

Siegrist F., Ebeling M, Certa U. The small interferon-induced transmembrane genes and proteins. *Interferon Cytokine Reseach* 2011; 31:183-197.

Teng LQ, Xie ZX, Xie LJ, Liu JB, Pang YS, Deng XW, Xie ZQ, Fan Q, Luo SS. Complete genome sequences of an avian orthoreovirus isolated from Guangxi, China. *Genome Announc* 2013;1(4): e00495-13.

Wang QX, Wu YJ, Cai YL, Zhuang YB, Xu LH, Wu BC, Zhang YD. Spleen transcriptome profile of Muscovy ducklings in response to infection with Muscovy duck reovirus. *Avian Disease* 2015;59(2):282-290.

Yount JS, Moltedo B,Yang YY. Palmitoylome profiling reveals S-palmitoylation-dependent antiviral activity of IFITM3. *Nature Chemestry Biology* 2010; 6:610-614.

Zheng HL, Li H, Sun YS, Yang ZY and Yu Q. Parathyroid hormone-related peptide (PTHrP): prokaryotic expression, purification, and preparation of a polyclonal antibody. *Genetics and Molecular Research* 2014;13 (3): 6448-6454.

Erratum

In the article Distribution of Ifitm3 in Yellow-Feathered Broilers and Inhibition of Avian Reovirus Multiplication by Ifitm3 published in the Revista Brasileira de Ciência Avícolas/Brazilian Journal of Poultry Science, v20 (2):305-316, in page 380, 381, 382 and 383 where it was written:

Figura

the correct form is

Figure

http://dx.doi.org/10.1590/1806-9061-2017-0662