



# Transcriptional responses of LSm14A after infection of blue eggshell layers with Newcastle disease viruses

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**ABSTRACT.** LSm14A is a key innate immunity component of processing body (P-body) that mediates interferon- $\beta$  (IFN- $\beta$ ) signaling by viral RNA. This is the first study to report chicken LSm14A (cLSm14A) cloning from *blue eggshell layer*, *high tibia* and *frizzle* chickens. The cLSm14A gene, encoding 461 amino acids, is highly homologous in the three types of chickens. The cLSm14A was extensively expressed in several tissues. The transcriptional level of cLSm14A was significantly increased in various stages of Newcastle disease virus (NDV) infection. In HEK293 cells, full length cLSm14A from *blue eggshell layer* was localized in the cytoplasm as dots. The results of this study indicated that cLSm14A is an important sensor that mediates innate immunity in chicken against NDV infections.

**KEY WORDS:** *blue eggshell layer*, Innate immunity, LSm14A, Newcastle disease viruses

LSm protein family is a class of highly conserved proteins that contain the Sm motif, also known as the Sm-like (Sm-Like, LSm) protein, and belongs to RNA-binding proteins that are widely distributed in eukaryotes, bacteria and plants [16, 18], and participate in many processes, including cell division, apoptosis [4], pre-nuclear precursor mRNA splicing, telomere maintenance, protein maturation, mRNA degradation, virus replication, etc [1, 7]. RNA-Associated Protein 55 (RAP55/LSm14A) is a member of the LSm family, and is a component of processing body (P-body) involved in mRNA decapping, translational repression, and P-body assembly. LSm14 was first identified in the oocytes of *Pleurodeles waltl* and *Xenopus laevis* as RAP55 (mRNA-associated protein of 55kDa) [10, 15]. RAP55 localizes to mRNA processing bodies and stress granules [19]. Two highly conserved paralogs, RAP55A (LSm14A) and RAP55B (LSm14B), exist in vertebrates [12]. Many studies have focused on LSm14A. Li *et al.* found that LSm14A is a sensor for viral nucleic acids and plays an important role in initiating IFN- $\beta$  induction in the early phase of viral infection [8]. Human, porcine and duck LSm14A could activate the IFN- $\beta$  signaling pathway to mediate innate immunity against viral infections [6, 8, 17]. Overexpression of LSm14A led to the formation of its related stress particles and also inhibited the replication of influenza virus [13]. In monkey embryonic kidney epithelial cells (Marc145), LSm14A inhibited porcine reproductive and respiratory syndrome virus (PRRSV) replication by activating the IFN- $\beta$  signaling pathway [9].

Although several LSm14A genes were identified in different animals, the function of chicken LSm14A (cLSm14A) gene remains unknown. In this study, we first cloned the cLSm14A (from *blue eggshell layer*, *high tibia* and *frizzle* chickens) and studied its mRNA expression levels in different tissues after infection with Newcastle disease viruses (NDV). We found that cLSm14A is significantly up-regulated at mRNA levels after viral infection against chickens, presumably playing an important role in chicken innate immunity.

## MATERIALS AND METHODS

### *Animals, tissues and viruses*

*Blue eggshell layer*, *high tibia* and *frizzle* chickens (7-week-old) were obtained from Guizhou province, China. The chickens were set free in an open ground under normal management conditions. Liver tissues from the three types of chickens were used for cloning the cLSm14A cDNA sequence. The tissues were collected, immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further use. NDV strains were stored at preventive veterinary laboratory of Guizhou University. The virus strains used in this study are shown in Table 1. F48E8 and IV strains (La Sota) were purchased from China Institute of Veterinary Drug Control. H2 (high virulent) and P3 (moderate virulent) strains were isolated from chickens in China.

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**Table 1.** Information of Newcastle disease virus (NDV) strains used in this study

NDV Strains	ICPI	MDT (hr)
F48E8	1.63	51.2
La Sota	0.00	109.0
P3	1.35	59.8
H2	1.69	59.4

ICPI, Interlocking Concrete Pavement Institute; MDT, mean death time.

**Table 2.** Primers used for constructing clone plasmid vectors

Primer	Sequence (5'-3')
cLSm14A-F	5'-ATGAGCGGG GGGACGCCCTACAT-3'
cLSm14A-R	5'-CTATGCAGCAACTT TGTTGTCTTTCC-3'

### Sequence cloning of cLSm14A

Total RNA was extracted from the liver tissues of *blue eggshell* layer, *high tibia* and *frizzle* chickens with Trizol reagent kit (Sangon Biotech, Shanghai, China). Based on the manufacturer's instructions, the cDNA fragment was amplified by RT from total RNA using HiFiscript cDNA synthesis kit (CWBio, Beijing, China). According to the Gallus LSm14A coding sequence (GenBank accession number NM\_001012778), forward primer (cLSm14A-F) and reverse primer (cLSm14A-R) were designed to amplify the CDS sequence of cLSm14A (Table 2). The amplification conditions were as follows: initial denaturation at 94°C for 3 min, 35 cycles of 30 sec at 94°C, 30 sec at 55°C, 45 sec at 72°C and a final extension step at 72°C for 10 min. The DNA products were gel purified (Sangon Biotech) and then ligated into pMD-18T for sequencing.

### Sequence analysis of cLSm14A

The CDS (Coding sequence) sequences of cLSm14A from *blue eggshell* layer, *high tibia* and *frizzle* chickens were matched using DNASTAR software. The CDS sequences of cLSm14A have been uploaded to GenBank under the accession numbers MH331644, MH331645 and MH331646. Amino acid sequences of cLSm14A and other species were aligned using ClustalW2 of DNASTAR software. MEGA4 was used to construct a phylogenetic tree.

### Transcriptional responses of LSm14A in different tissues after NDV infection

This study was approved by the College of Animal Sciences, preventive veterinary laboratory (Approval number: GZU201608). All experiments were performed in accordance with the College's relevant guidelines and regulations. Forty-eight *blue eggshell* layers were randomly divided into six groups and inoculated with 200  $\mu$ l of  $10^6$  EID<sub>50</sub> of NDV strains (F48E8, La Sota, H2, P3) via ocular/nasal and muscular routes. The control chickens were similarly inoculated with PBS. The chickens were kept in separate rooms until the end of the experiment. At 12, 24, 48, 72 hr post infection (pi), the tissues were processed and stored at -80°C. At different stages of viral infection, the transcriptional levels of cLSm14A were tested with fluorescence quantitative PCR assays. Tissues, including heart, liver, spleen, lung, kidney, bursa, thymus, brain, intestine were surgically removed from each chicken and used for cLSm14A expression analysis. Total RNA was extracted using Trizol reagent kit (Sangon Biotech). The total RNA was reverse transcribed to cDNA using HiFiscript cDNA synthesis kit (CWbio). Then, cDNA was quantified by quantitative real-time PCR assay using the following conditions: an initial denaturation at 95°C for 15 min, 40 cycles of 95°C for 10 sec and 58°C for 20 sec, melting curve runs at 65–95°C with a heating rate of 0.5°C per second and a continuous fluorescence measurement. All samples were tested three times, and the level of chicken 18srRNA was used for normalization. Finally, the quantitative real-time PCR assay data were analyzed by the relative comparison *Ct* method ( $Qr=2^{-\Delta\Delta Ct}$ ), and the difference map was drawn by Graphpad Prism\_6.04 software (GraphPad Software, U.S.A.). The cLSm14A was amplified using the cLSm14A-q-F and cLSm14A-q-R, the 18srRNA was amplified using the 18srRNA-q-F and 18srRNA-q-R (Table 3).

### Expression plasmid construction

The entire open reading frame (ORF) of cLSm14A was cloned into pcDNA3.1-5'Flag (Table 4), resulting in the N-terminal flag tagged expression plasmid construct pcDNA3.1-5'Flag-cLSm14A encoding full length cLSm14A.

### Transfection and indirect immunofluorescence analysis

HEK293 cells were cultured on coverslips in 24-well dishes. Transient transfection was performed when the cells reached 90% confluence. The cells were transfected with 200 ng/well of expression plasmid pcDNA3.1-5'Flag-cLSm14A or an empty vector using Lipofectamine 2000™ reagent (Invitrogen, Carlsbad, CA, U.S.A.), as per the manufacturer's instructions. After 24 hr, the

**Table 3.** Primers used for q-PCR

Primer	Sequence (5'-3')
cLSm14A-q-F	5'-GACAATAGGGAACGTCGACCA-3'
cLSm14A-q-R	5'-CCTCTTCCACCTCGGAAACC-3'
18srRNA-q-F	5'-GTTTCAGCCACCCGAGATTGA-3'
18srRNA-q-R	5'-CCCATCACGAATGGGGTTCA-3'

**Table 4.** Primers used for constructing expression plasmid vectors

Primer	Sequence (5'-3')
cLSm14A-BamHI-F	5' <u>CGCGGATCC</u> AGCGGGGGGACGCCCTACATCG
cLSm14A-XbaI-TL-R	5' <u>CTAGTCTAGATGCAGCAACTTTGTTGTCTTTCC</u> T

Underline indicates the enzyme cutting site.

cells were fixed with 80% cold acetone for 30 min at  $-20^{\circ}\text{C}$ , washed three times with PBS (pH 7.2) and then incubated with anti-cLSm14A serum (1:50, anti-cLSm14A immune serum, produced by preventive veterinary laboratory, Guizhou University) followed by incubation with alexa fluor 555 labeled goat anti-rabbit IgG secondary antibody (Invitrogen). Subsequently, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) for 5 min at room temperature. Finally, after washing with PBS (pH 7.2) three times, the coverslips were mounted onto slides and imaged using fluorescence microscope IX81 (Olympus, Tokyo, Japan), Magnification,  $40\times$ .

## RESULTS

### *Molecular cloning and characterization of cLSm14A CDS*

A 1386 bp CDS sequence was amplified from the liver tissue of *blue eggshell* layer, *high tibia* and *frizzle* chickens. NCBI BLAST search revealed that the cLSm14A nucleotides were consistent and  $>99.1\%$  identical with Gallus (GenBank: NM\_001012778). The DNASTar software analysis predicted a protein translation of 461 amino acids. In comparison with the LSm14A amino acid sequences of representative animals of other species, cLSm14A from *blue eggshell* layer, *high tibia* and *frizzle* chickens demonstrated greater amino acid identity with *anas platyrhynchos* duck (96.8, 97.4, 97.6%) than pig (92.0, 92.6, 92.8%) and *Xenopus laevis* (80.7, 81.4, 81.6%) (Fig. 1A and 1B). Based on the amino acid sequences of cLSm14A and those of other organisms, a condensed phylogenetic tree was constructed (Fig. 1C). The phylogenetic tree revealed that cLSm14A of *blue eggshell* layer, *high tibia* and *frizzle* chickens showed the highest identity to avian LSm14A sequences, particularly to gallus and *anas platyrhynchos* duck, which was consistent with the sequence alignment results.

Sequence alignment and phylogenetic tree of amino acid sequences of 10 identified or predicted LSm14A from different species. The sequences were derived from Genbank entries with accession numbers NM001012778 Gallus (chicken), KJ466053.2 *Anas platyrhynchos*, NM001114093.1 *Homo sapiens* (human), NM001257954 *Macaca mulatta* (monkey), NM025948.2 *Mus musculus* (mouse), KC769582.1 *Sus scrofa* (pig), and *Xenopus tropicalis* NM213667.1.

### *Transcriptional responses of cLSm14A after NDV infection in different tissues*

The abundance of cLSm14A transcripts in tissues were determined by qPCR. As shown in Fig. 2, cLSm14A transcriptional levels were detected in all examined tissues indicating that cLSm14A was extensively expressed in chicken tissues. The thymus and bursa samples demonstrated the most abundant transcriptional levels of cLSm14A, while the liver showed the lowest level. At different stages of NDV infection, transcriptional levels of cLSm14A were significantly increased.

After infection with different NDV strains, the transcriptional levels of cLSm14A were increased significantly at different time points. After infection with different NDV strains, the transcriptional levels of cLSm14A frequently were fluctuated in most tissues of *blue eggshell* layers. The cLSm14A transcriptional levels were higher in all tissues 12 hr after infection with different NDV strains, excluding liver tissues infected with La Sota or P3.

### *Subcellular localization of cLSm14A*

Indirect immunofluorescence analysis revealed that cLSm14A was localized in the cytoplasm of HEK293 cells as dots, similar to the LSm14A localization in pigs and ducks (Fig. 3).

## DISCUSSION

LSm14A is a component of the P-bodies and a sensor of the IFN- $\beta$  signaling pathway, thereby mediating the innate immune response in humans [8], pigs [17] and ducks [6]. This was the first study on the molecular characterization of cLSm14A in Chinese *blue eggshell* layer, *high tibia* and *frizzle* chickens. The cLSm14A gene encodes a protein of 461 amino acids. The avian (duck, chicken), mammalian (human, porcine, mouse) and *Xenopus* LSm14A N-terminal 101 amino acid sequences, including the Sm-like domain, were highly conserved. Interestingly, unutilized clustalw showed that cLSm14A amino acids 150, 153, 156, 157, 169, 173, 187, 192, 223, 224, 231, 234, 242, 248, et, positions have distinct differences with other mammalian species. The biological significance of these differences among animal species needs further exploration.

The biological functions of LSm14 have been extensively studied. LSm14 was found to have adopted a divergent mode of binding DDX6 in order to support the formation of mRNA silencing complexes [2]. LSm14 also binds directly to tubulin and maintains mitotic spindle stability [14]. It plays an important role in innate antiviral responses by regulating MITA (an adaptor protein in the antiviral signaling pathways) level in DCs [11]. Kaycie *et al.* used RNAi technology to silence the LSm14A and EDC3 genes, and found that a slight loss of LSm14A or EDC3 could significantly increase Rift Valley fever virus infection [5]. LSm14A is known to be involved in antiviral signaling pathway in cell lines, but *in vivo* evidence is lacking. In this study, the transcriptional responses of LSm14A in *blue eggshell* layer tissues during different stages of NDV infection was investigated. The results showed that the LSm14A mRNA level of *blue eggshell* layer was dramatically up-regulated and peaked at 12 hr pi. This result is consistent with previous studies [8]. Interestingly, the LSm14A transcriptional levels of the spleen tissues remained high at 12, 24, 48 and 72 hr after La Sota infection, while other velogenic strains (F48E8 and H2) and moderate virulence strain (P3) had a decreasing trend at 48 hr pi. This is contrary to the results reported by Ecco *et al.*, who infected SPF chickens with different virulence NDVs and found that the expression of cytokines induced by strong strains was the strongest, while that induced by moderately virulent strains was relatively weak [3]. It is speculated that the stimulation of virulent strains is transient compared

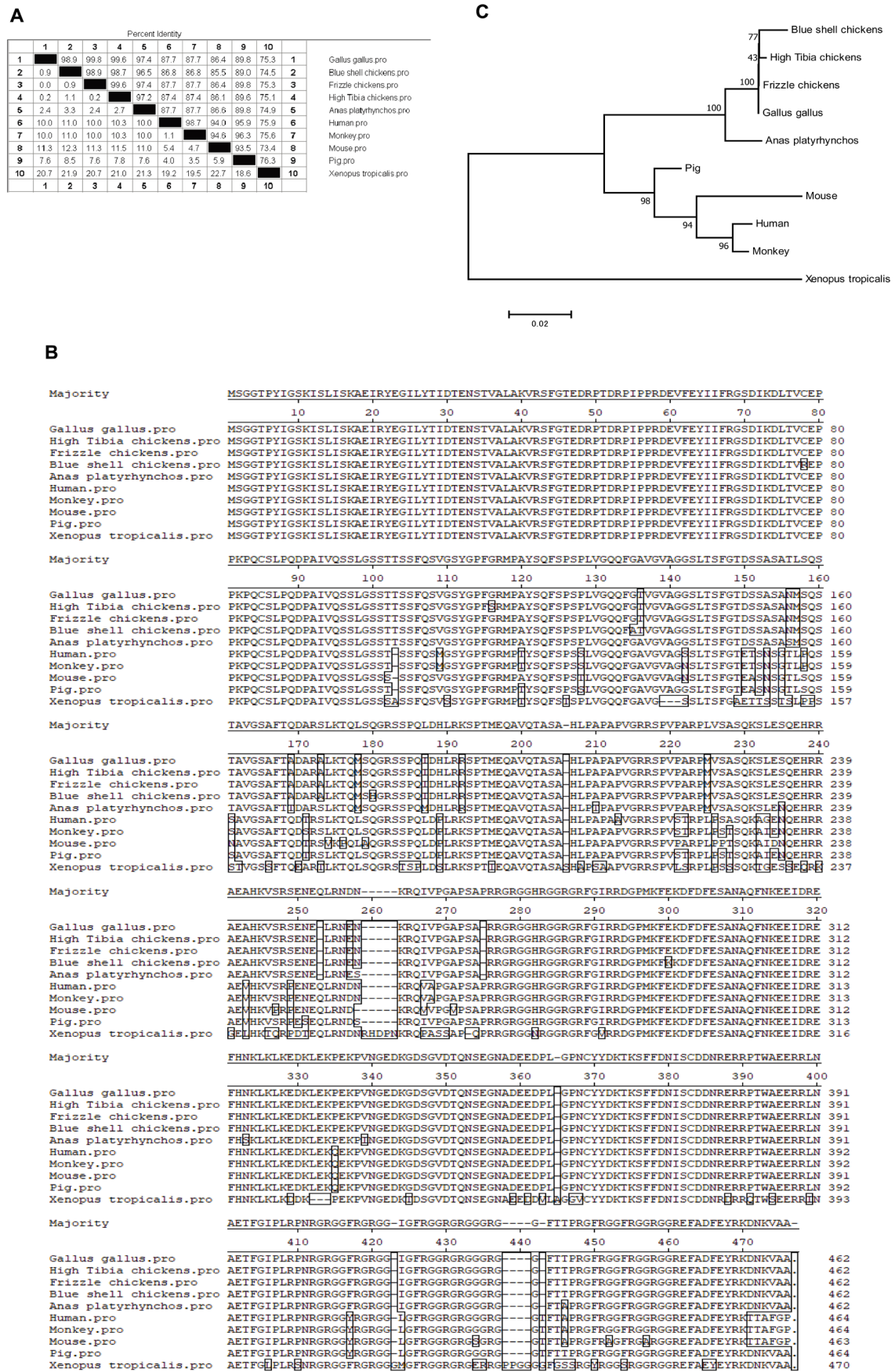
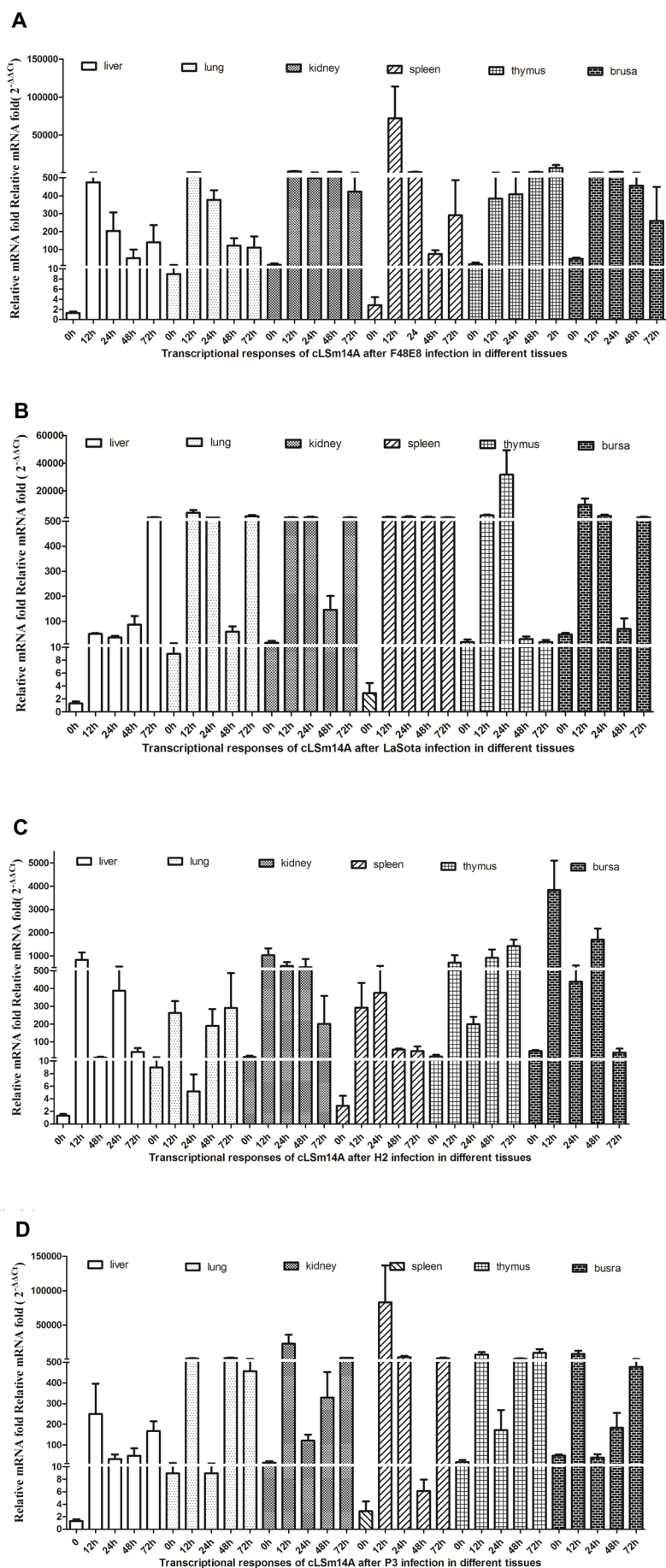
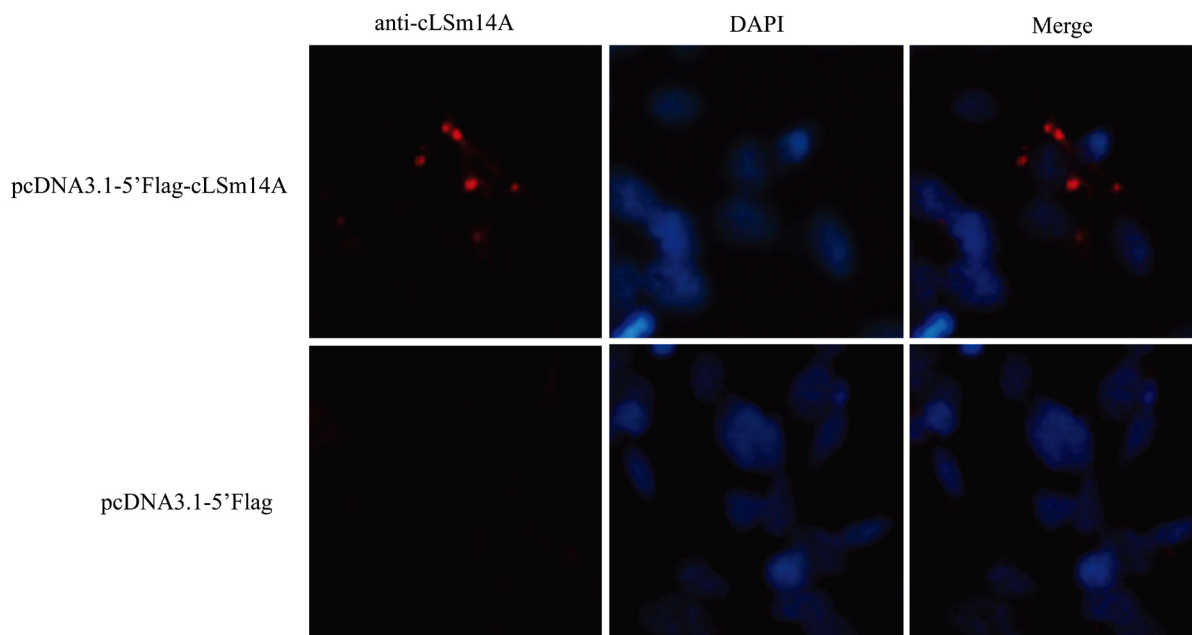


Fig. 1. Sequence alignment and phylogenetic tree of cLSm14A (A: The identity of cLSm14A with other species, B: The sequence alignment results of cLSm14A, C: The phylogenetic tree of cLSm14A).



**Fig. 2.** Transcriptional levels of cLSm14A after Newcastle disease virus (NDV) infection in different tissues at different time points. Relative expression levels of cLSm14A in blue eggshell layer tissues infected with different NDV strains were measured by quantitative real-time PCR. Results represent the mean relative expression levels of two independent experiments normalized to 18srRNA (A. Transcriptional levels of cLSm14A after F48E8 infection, B. Transcriptional levels of cLSm14A after La Sota infection, C. Transcriptional levels of cLSm14A after H2 infection, D. Transcriptional levels of cLSm14A after P3 infection).



**Fig. 3.** cLSm14A expression in HEK293 cells. The red indicate cytoplasmic localization of the cLSm14A, and blue is nucleus, 200 $\times$ .

with the sustained stimulation of attenuated strains, so the transcription level of LSm14A decreases at 48 hr under the action of virulent strains (F48E8 and H2) and moderately virulent strains (P3). The underlying reason needs to be further explored.

### Conclusion

This is the first study to isolate and characterize the cLSm14A ORF of *blue eggshell layer*, *high tibia* and *frizzle* chickens. The transcriptional level of cLSm14A was high in the thymus and bursa. Furthermore, the transcriptional level of cLSm14A increased in all the tissues after infection with different strains of NDV. The cLSm14A from *blue eggshell layer* was localized in the cytoplasm of HEK293 cells.

**CONFLICT OF INTEREST.** The authors declare that they have no conflict of interest.

**INFORMED CONSENT.** Informed consent is not required because no human participants were involved in this article.

**RESEARCH INVOLVING HUMAN PARTICIPANTS AND/OR ANIMALS.** This article does not contain any studies with human participants performed by any of the authors. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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