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Preparation of A Polyclonal Antibody Against the Non-Structural Protein, NSS of SFTSV

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Keywords: PGEX-6P-NSs; Severe Fever with Thrombocytopenia Syndrome Virus (SFTSV); Polyclonal Antibody

Abstract

Severe fever with thrombocytopenia syndrome virus (SFTSV) is newly discovered virus, which is the member of the order Bunyavirales, family phenuiviridae, phlebovirus genus. Its genome is composed of 3 segments of negative-sense RNA L, M and S. NSs is a non-structure protein encoded by S segment which is important for viral replication and virulence. NSs protein of SFTSV is only involved in the regulation of host innate immune responses and suppression of IFN-promoter activities. So, the exact functions of this protein need to be studied deeply. To understand the exact role of NSs from SFTSV in viral replication and host immune response, a qualified antibody against this protein is required. In this study, NSs gene of SFTSV, was cloned into a bacterial expression vector (pGEX-6P-1) and the recombinant plasmid was transformed into Escherichia coli BL21 (DE3) cells. The SFTSV NSs fusion protein was purified using Ni-NTA agarose and utilized as an antigen to immunize rabbits and obtain an anti-SFTSV NSs polyclonal antibody. Proper expression of the fusion protein and polyclonal antibody specificity were confirmed by western blotting and immunofluorescence analyses. The polyclonal antibody recognized NSs from SFTSV specifically. This is the first report that NSs can form viroplasm-like structures not only in infected cells but also in transfected cells with NSs plasmids. This polyclonal antibody will be useful for future studies of NSs functions.

Abbreviations: DPRK: Democratic Peoples' Republic of Korea; SFTSV: Severe Fever With Thrombocytopenia Syndrome Virus

Introduction

Since 2011, Severe fever with thrombocytopenia syndrome virus (SFTSV) was reported in People's Republic of China (PR China) [1,2], South Korea [3], and Japan [4,5]. Severe fever with thrombocytopenia syndrome (SFTS) was caused by this virus. The major clinical features of SFTS are not specific. The symptoms are included high fever, gastrointestinal symptoms, fatigue, anorexia, myalgia, and regional lymphadenopathy, and thrombocytopenia. Liver enzyme level was found to be increased in some patients. Central nervous system manifestation was reposted in severe cases. SFTS is endemic to East Asia, PR China, South Korea, possibly including the Democratic Peoples' Republic of Korea (DPRK), and Japan. SFTSV is a novel phlebovirus of the family Bunyaviridae and is now classified in the genus Phlebovirus of the family Phenuiviridae [6]. SFTSV is a tick-borne virus, which is transmitted by tick bites [7] or contacting blood or body fluid of patients [8-12]. The genome of SFTSV is composed of three single-stranded negative sense RNA segments, including large (L), medium (M) and small (S) segments. Same as other members of the genus Phlebovirus, the L and M segments of SFTSV are of negative polarity, and the S segment is ambisense. RNA-dependent RNA polymerase (RdRp) is encoded by L segment.

The M segment encodes glycoprotein (Gn and Gc) precursor. The nonstructural protein (NSs) in the sense orientation and the nucleoprotein (NP) in the antisense orientation are encoded by the S seg-

ment contains [2]. Various studies have indicated that as seen with other bunyaviruses, SFTSV NSs is a potent IFN antagonist which can regulate host innate immune responses and inhibit IFN-promoter activities [13-16]. NSs protein can form viroplasm-like structures (VLSs) in SFTSV infected cells and interact or be associated with N protein and viral RNA in infected cells. Furthermore, NSs may play an important role in virus replication through formed VLSs colocalized with lipid droplets and inhibitors of fatty acid biosynthesis. Those studies suggest that NSs would potentially be a major determinant of virulence [17,18]. Characterizing the functions of the NSs protein in viral replication and virus pathogenesis will advance current understanding of the mechanisms of SFTSV replication. In the other way, NSs may interact with host cell factors to form VLSs during viral replication. To search that kind of factors is important for knowing the mechanism of viral replication. However, this research is hindered by the lack of a commercial antibody against NSs. Here, in this study, good quality anti-NSs antibody that may be applied in biological functions and provide insight into NSs protein-host cell interactions studies was prepared.

Materials and Methods

Cells and Viruses

Vero and human embryonic kidney 293T cells were obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA)supplemented with 5% fetal bovine serum (Gibco, Beijing, China), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco,Beijing, China). SFTSV named (SDYY007) used in this study was kindly provided by Institute of Microbiology, Chinese Academy of Sciences. Viruses were propagated in vero cells and the titer was determined by using Fluorescent Focus Assay (FFU), then the viruses were stocked in -80°C.

Plasmid Construction

Viral RAN was extracted from viruses by using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The cDNA synthesis was performed using the Prime Script First Reverse Transcriptase (Takara Bio, Dalian, China), according to the manufacturer's protocol. The cDNA template (10 μl) was used in a 50 μl reaction mixture and amplified using MMLV Taq (Takara Bio, Dalian, China). The primers are SFTSV-NSS F(EcoRI) 5'CGGAATTCATGTCGCTGAGCAAATGCTCCAACG3' and SFTSV-NSs R (XhoI) 5'CCGCTCGAGTTAGACCTCCTTCGGGAGGTCACCAATG3'.The amplification program included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 3 min. A final elongation step was then performed at 72°C for 7 min. The PCR products were detected on an agarose gel containing 0.5 μg/ml ethidium bromide, and amplicons of the gene encoding NSs were excised and extracted using the AxyPrep DNA Gel Extraction Kit (Axygen, Union City, CA, USA). The purified PCR product (878bp) was then cloned into the EcoRI and XhoI sites of PGEX-6P-1 (Invitrogen, Carlsbad, CA, USA) to generate the PGEX-6P- NSs plasmid. The NSs gene was also amplified with specific primers (the forward primer: 5'CGGAATTCCATGTCGCTGAGCAAATGCTCCAACG3', and the reverse primer: 5'CGGGATCCTTAGACCTCCTTCGGGAGGTCACCAATG3'). The PCR product was cloned into PCMV-Flag-3 (Sigma. Shanghai, China) through the sites of EcoRI and BamHI. The constructed plasmid named pCMV-3-NSs was confirmed by sequencing.

Expressed SFTSV Nonstructural Protein NSS in Escherichia Coli

The recombinant PGEX-6P-NSs plasmid was transformed into competent E. coli BL21 (DE3) cells, and the cells were subsequently cultured overnight in 5 ml of Luria-Bertani broth supplemented with ampicillin (100 μg/mL), until the optical density (OD600) reached 0.6–0.8. Expression of the PGEX-6P-NSs fusion protein was induced by treatment with isopropyl-β-d-thiogalactopyranoside, 0.1–1 mM (IPTG; Beyotime, Beijing, China) at 25–37°C on a shaker at 220 rpm/min. The expressed protein was then analyzed via SDS-PAGE.

Expression of SFTSV NSs Protein

The recombinant pGEX-6P-NSs plasmid was transformed into competent E. coli BL21 (DE3) cells, and induced by 1.0 mM IPTG (Beyotime, Beijing, China), which led to the production of GST-NSs fusion protein. In brief, colonies were inoculated into LB medium (100 μg/ml Amp; Solarbio and incubated in a high-speed shaker (220 rpm) at 37°C until the OD600 nm reached 0.6–0.8 (8 h). IPTG with the final concentration of 1.0 mM was added to the medium and incubated at 28°C for 12 h. The broth medium was centrifuged at 10,000 rpm, 10 min and the bacterial pellet was resuspended in lysis buffer for ultrasonication on ice. After centrifugation (10,000 rpm for 30 min), then ultrasonication on ice, the supernatant and the sediment were both resuspended in phosphate-buffered saline (PBS) and subjected to 12% SDS-PAGE. After electrophoresis of the proteins, the gel was stained in 50 ml Coomassie blue stain (0.25% Coomassie Brillian blue R-250, 10% glacial acetic acid, 25% methanol for 5 min at 95°C 30 min), followed by destaining in distilled water for more than 2 h until the protein bands were visible clearly. Balance Beyo Gold TMGST-tag Purification Resin (Beyotime), mix bacterial lysate with resin, followed by washing the mixture using elution buffer to get purification protein, then stained in Coomassie blue followed by electrophoresis on a 12% SDS-PAGE gel.

Purification of the NSs Fusion Protein

The PGEX-6P-NSs protein was purified from the inclusion body using Glutahione Sepharose 4B (GE Healthcare Biosciences, Little Chalfont, UK) following the manufacturer's instructions. Briefly, the bacteria were collected by centrifugation at an OD600 of 0.70 after induction with IPTG (1 mM) for 12 h at 28°C. The bacteria were then suspended in 20 ml of phosphate-buffered saline (PBS) and 200 μl of benzyl sulfonyl fluoride. Following ultrasonication on ice until the liquid started to clear, the sample was centrifuged at 6000 rpm/min for 30 min at 4°C.

Preparation of Antisera Against NSs of SFTSV

The antisera against the expressed NSs were prepared in three New Zealand rabbits, and intramuscular injections of 2 mg of purified protein were used. The protein (1 mg) was emulsified with an equal volume of Freund's complete adjuvant for the first injection and with Freund's incomplete adjuvant for subsequent injections (500 μg of the purified protein). For the final injection, the antigen (500 μg) was diluted in PBS. The rabbits were bled two weeks after the final injection. The serum fractions were collected and stored at −20°C. Animal work was approved by animal research ethics committee of Jinzhou Medical University. The approved number was 2018015. Rabbits were bred in the Laboratory animal facility of Jinzhou Medical University. Anesthesia of rabbit was done by giving xylazine (1-4mg/kg) intramuscular. Blood was taken by from blood. After that, Euthanasia of rabbits was done by intravenous administration of ketamine (29.1 mg/kg).

Immunoblotting

The cells were lysed in buffer consisting of 25 mM Tris–HCl pH7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS. After centrifugation, the soluble protein fractions were size fractionated using SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes (Millipore). Viral proteins were detected using Chemi-Lumi One Ultra (Bio-Rad Laboratories, Inc.) following incubation with antiserum-specific for NSs and Flag monoclonal antibody (Sigma, Shanghai, China) at a dilution of 1:2000 and HRP conjugated anti-rabbit or anti-mouse IgG secondary antibody (Sigma, Shanghai, China) at a dilution of 1:5000.

Immunofluorescence Assay (IFA)

Monolayers of vero cells were seeded onto 24-well plate and infected with the SFTSV viruses. After incubation of 24 h, cells were fixed with PBS containing 4% paraformaldehyde, washed with PBS, and incubated with NSs antiserum prepared above. After three washes with PBS, cells were incubated with CF488 Goat Anti-rabbit IgG second antibody (Invitrogen, Beijing, China) or Alexa Fluor 594 Goat Anti-Mouse IgG second antibody (Invitrogen, Beijing, China) at a dilution of 1:1000. The images were acquired with a fluorescent microscope (LEICA, DMI3000B).

Characterization of The Polyclonal Antibody

The reactivity and specificity of this polyclonal antibody against NSs were evaluated by western blotting and IFA. E. coli expressed NSs fusion protein samples, or the cell lyses from infected vero cells were subjected to 12% SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane for western blotting. Proteins were detected using Chemi-Lumi One Ultra (Bio-Rad Laboratories, Inc.) following incubation with antiserum-specific for NSs polyclonal antibody prepared in this study and HRP conjugated anti-mouse or anti-rabbit IgG secondary antibody (Sigma, Shanghai, China) at a dilution of 1:2000.

Result

Construction of Expression Vector of the NSs Gene of SFTSV

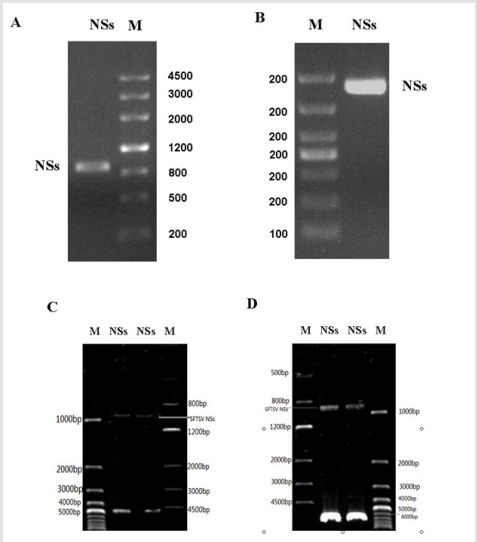

Figure 1:

A. Construction of the expression plasmids of pGEX-6p-NSs and pCMV-3-NSs (A) Amplified NSs gene for cloning into vector pGEX-6-p1. The lane M shows the DNA marker, lane NSs shows the SFTSV NSs gene.

B. Amplified NSs gene for cloning into vector CMV-3-Flag. The lane M shows the DNA marker, lane NSs shows the SFTSV NSs gene.

C. line NSs shows the verification of recombinant vector pGEX-6p-NSs which was cleaved by EcoRI and XhoI. The lane M is DNA marker.

D. Line NSs shows the verification of recombinant vector pCMV-3-flag NSs which was leaved by EcoR I and BamHI. The lane M is DNA marker.

The present study is aimed to express SFTSV NSs protein *in vitro* and produce its antibody. To this purpose, the SFTSV NSs gene was inserted into the pGEX-6P-1 and pCMV-Flag-3 between the restriction sites for EcoRI, XhoI and EcoRI , BamHI respevtively. The PCR product of NSs was checked by electrophoresis (Figure. 1A for cloning into pGEX-6P-1; Figure. 1B for cloning into pCMV-Flag-3). The recombinant plasmids (Figure 1C. pGEX-6P-NSs) and (Figure 1D. pCMV-3-NSs) were identified by double-enzyme digestion. The sequencing further confirmed that the NSs gene was in accordance with the design and was 878 bp in length. The constructed expression vector of pGEX-6P-NSs was introduced into E. coli BL21 for the purification of GST-NSs fusion protein. The recombinant plasmid, pCMV-3-NSs was transfected into 293T cells and further used in the verification of the NSs antibody.

Expression and Purification of NSs Fusion Protein

pGEX-6P-NSs was sequenced. Analysis showed that the NSs gene was inserted in a frame with GST. Competent E. coli BL21(DE3) harbored only small amount of PGEX-6P-NSs fusion protein in soluble fraction after 12 h induced by different concentration of IPTG at 28°C. After the sonication of bacteria induced by different concentration of IPTG after 12 h, the NSs protein as a fusion protein with GST-tag was expressed. The proteins were separated by SDS-PAGE and visualized by Coomassie blue staining (Figure. 2A). It was showed that NSs fusion protein mainly presented in an insoluble form. (Figure. 2B) The fusion protein can be induced to the highest amount at the concentration 1.0mmol/L of IPTG. (Figure. 2B. Line marked No. 6). On the contrary, the fusion protein was absent from un-induced cultures (both in Figure 2A and Figure2B. Line marked NO.1). According to the condition above, A large amount of this recombinant protein was collected and subjected to GST affinity column chromatography. The higher purity PGEX-6P-NSs recombinant protein was obtained. (Fig. 2C, Line marked 5-9). The most protein was eluated in Line Marked 6. (Figure 2C). SDS-PAGE revealed that the apparent molecular weight of the NSs protein was approximately 58 kDa (Figure 2B). This fusion protein is going to be used to immunize rabbit for making polyclonal antibody.

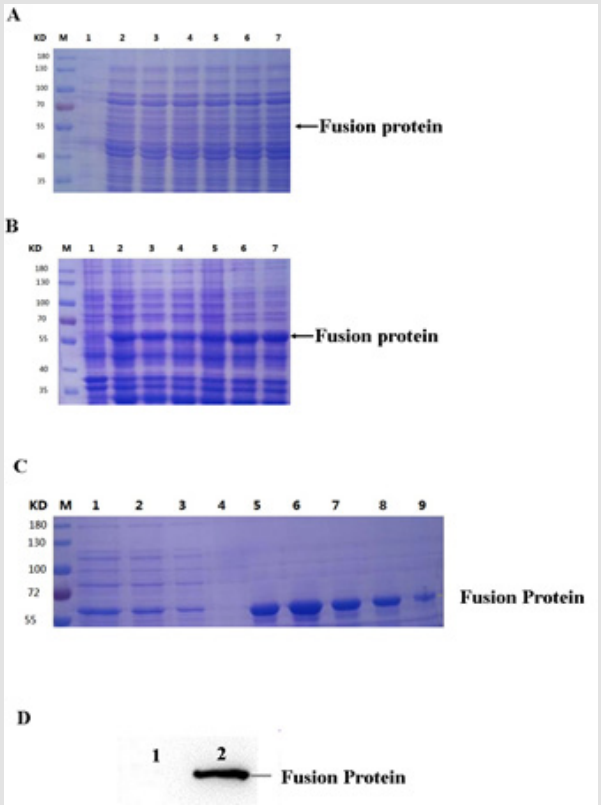

Figure 2: Electrophoretic analysis of expressed SFTSV nonstructural protein NSs in Escherichia coli. The BL21 (DE3) Escherichia coli cells that harbored Pgex-6P-NSs were induced using 0 mM–1.2 mM IPTG for 12 h at 28℃ at 220 rpm. The proteins were analyzed using 12% Tricine SDS-PAGE, and the gels were stained with coomassie brilliantblue G-250. IPTG concentrate was 0、0.2、0.4、0.6、0.8、1.0 and 1.2mmol/L, respectively. Lane 1-7, M: protein standard marker (Thermo Scientific, Waltham, MA, USA).

A. Purified recombinant Pgex-6P-NSs protein from supernatant after sonication.

B. Purified recombinant Pgex-6P-NSs protein from inclusion body. Line 1-4 eluted other proteins, Line 5-9 eluted target protein (NSs).

D. Western blot analysis of GST NSs fusion by using recombinant GST antibody. Line 1 is negative control from bacteria without plamid transfected. Line 2 is target protein (58kD).

Preparation and Characterization of The Polyclonal Antibody

The purified protein was used to immunize rabbits. After four times immunizations, the rabbit antisera were collected. IFA and western blotting were used to evaluate the reactivity and specificity of this polyclonal antibody against NSs protein. IFA results indicated that this antiserum could detect NSs both in SFTSV infected vero cells and 293T cells transfected with pCMV-3-NSs. VLSs were clearly observed both in SFTSV infected vero cells and 293T cells transfected with pCMV-3-NSs. No fluorescence signal was observed in Mock infection and no transfected cells. (Figure 3A) Western blot analysis results showed that this antibody was able to recognize the NSs protein from the SFTSV infected vero cells and 293T cells transfected with pCMV-3-NS. A strong band at a position that corresponds to 58 KDa was seen in infected cells and transfected cells. There were no bands observed in mock infection and non-transfected cells. (Figure 3B) These results suggested that this polyclonal antibody had good reactivity and specificity against NSs protein. This antibody would be used for study in future.

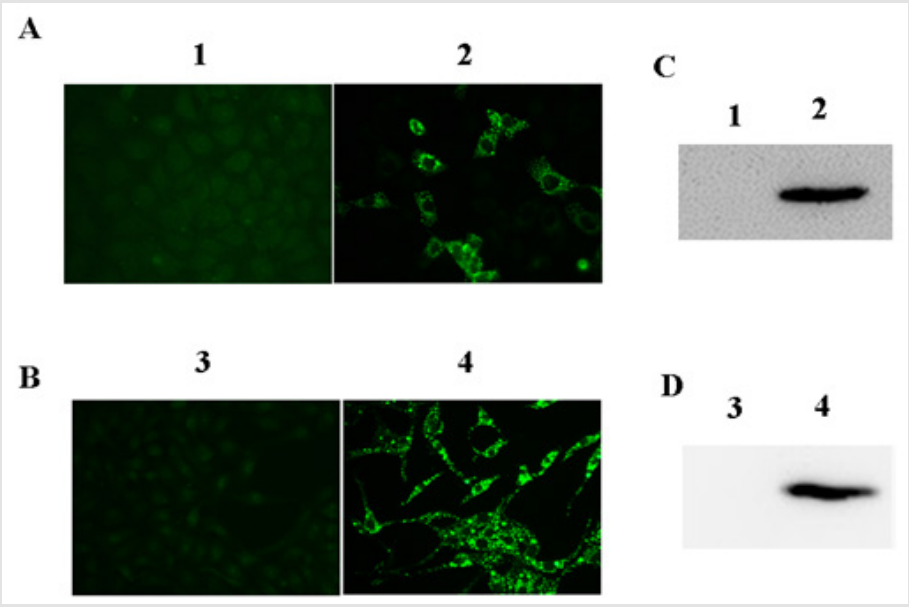

Figure 3: Verification of polyclonal antibody against NSs of SFTSV.

A. 293T cells were transfected with pCMV-3-NSs, after 24 hours, cells were applied for IFA by using polyclonal antibody prepared in this study, followed by secondary antibody, goat anti-rabbit conjugated with FITC. The fluorescence was observed under the microscope. (NO. 2) Cells transfected with empty vector pCMV-3 were used as negative control. (NO. 1)

B. Vero cells were infected with SFTSV after 48, cells were applied for IFA by using the polyclonal antibody prepared in this study, followed by secondary antibody goat anti-rabbit conjugated with FITC. The fluorescence signal was observed under the microscope. (NO. 4) Uninfected cells were used as negative control (NO. 3).

C. Western blotting confirmed the expression of NSs in 293T cells transfected with pCMV-3-NSs, by using polyclonal antibody prepared in this study, followed by secondary antibody HRP anti-rabbit (NO. 2). Cells transfected with empty vector were used as negative control (NO. 1).

D. Western blotting confirmed the expression of NSs protein in infected cells by using the polyclonal antibody prepared in this study followed by secondary antibody HRP anti-rabbit (No. 4). Uninfected cells were used as negative control (No. 3).

Discussion

NSs protein is major virulence factor which takes a role in the disruption of the innate immune response to infection by affecting the IFN antiviral response pathway [19]. NSs acts as a virally encoded IFN antagonist and is dispensable for virus replication [20]. The concentration of IFN-α as well as other cytokines (IFN-γ, G-CSF, MIP-1α, IL-6, and IP-10) has been reported to be related with the severity of SFTS. It suggests that type I interferon may not be significant in resistance SFTSV infection in humans and it may play an import role in cytokine storm [21]. Also, VLSs formed by NSs protein are regarded as viral replication factory which is important for virus replication. It is necessary to study the function of NSs deeply and screen the interaction protein of host cell proteins that is essential for formation of VLSs. For those purpose, a good quality antibody against NSs is indispensable. At present, no commercial antibodies that target NSs protein were available. E. coli is the most widely used prokaryotic expression system for the production of heterologous protein [22]. E. coli expression system has many advantages, such as well-known genetic background, high production capacity, inexpensive culture media and relatively easy protein purification methods.

The present study addressed the possibility of the use of expressed NSs-GST protein to produce specific antiserum against NSs protein of SFTSV and possible application for NSs function research. The expression of the entire NSs as a fusion protein was achieved high mount successfully by induction with 1 mM IPTG at 28°C. The expressed protein mainly presented in an insoluble fraction. (Figure 2B) Purified protein from insoluble fraction was used to immunize rabbits. The antiserum against NSs was successfully obtained. The produced polyclonal antibody could recognize NSs protein both in infected cells and cells transfected with pCMV-3-NS plasmid which were confirmed both by western blotting and IFA assay (Figure 3). VLSs were clearly observed under the microbiology in IFA assay both in SFTSV infected cells and plasmid transfected cells. (Figure 3) This is the first time to show that NSs can VLSs in the cells transfected with NSs plasmid. This polyclonal antibody could be used for further study in future.

So far as we know, this is the first report on the preparation of polyclonal antiserum against the recombinant non-structural protein NSs of SFTSV. Purified protein from *E coli* has many advantageous compared with other antigen preparation methods, such as virions purification from infected cells, because it is not necessary to do virus purification and propagation of live virus. Also, expression of protein from bacterial (*E. coli*) is simple and fast. High amounts of purified protein can be obtained at low cost by this way. Protein with GST tag can facilitate purification process. In this study, NSs is over-expressed and purified in a sufficient yield for antiserum production. The resulting polyclonal antiserum against NSs can be used to efficiently detect SFTSV infections and for the research on function of NSs.

Disclosures/Conflict of interest

The authors declare that there is no conflict of interest.

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