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Original article

Seroprevalence and genetic characterization of severe fever with thrombocytopenia syndrome virus in domestic goats in South Korea

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ABSTRACT

Severe fever with thrombocytopenia syndrome (SFTS) is a newly emerging tick-borne infectious disease caused by the SFTS virus (SFTSV). To investigate the prevalence of SFTSV in domestic goats in South Korea, we collected blood samples in commercial slaughterhouses in Chungbuk Province in 2017. Of the 207 samples tested, 4 (2%) were found to be positive for viral RNA by RT-PCR and 30 (14.4%) were positive for SFTSV antibody as detected by a nucleocapsid (NP) protein-based ELISA. Phylogenetic analysis of the non-structural protein (NS) sequences showed that all viruses belonged to the genotype B, although they were clustered into two different sublineages that showed the highest homology with the KR612076-JP01 and KY789441-CB3 human isolate from South Korea. Further, we confirmed the specificity of seropositive goat sera by $FRNT_{50}$ and western blotting analysis and found differential cross-reactivity of the sera with genotype A and B SFTSV strains. Collectively, this study suggests that relatively high numbers of goats are infected by antigenically different SFTSV strains, which might have a potential for zoonotic infection.

1. Introduction

Since the first detection of severe fever with thrombocytopenia syndrome (SFTS) in China in 2009 (Yu et al., 2011), cases have been identified in Japan (Takahashi et al., 2013) and South Korea in 2012 (Kim et al., 2013) with a case fatality rate of 6–30% in humans (KCDC, 2017; Zhan et al., 2017). The causative agent of this disease is the SFTS virus (SFTSV), a newly emerging tick-borne viral pathogen (Jin et al., 2012; Kim et al., 2013; Li, 2015; Takahashi et al., 2013). Although human-to-human transmission through contact with infected blood or body secretions has been reported (Tang et al., 2012), SFTSV infection in humans is believed to be predominantly transmitted by tick bites. Further, the prevalence of SFTS in both humans and animals is closely correlated with the seasonal distribution of ticks (Li, 2015). *Haemaphysalis longicornis* is a proven vector of SFTS virus. Other tick species, e.g. *Rhipicephalus microplus, Amblyomma testudinarium, Haemaphysalis flava* or *Ixodes nipponensis*, may be involved in the circulation of SFTSV, although this requires confirmation (Li et al., 2016; Luo et al., 2015; Meng et al., 2015; Zhang et al., 2012).

SFTSV infection is not limited to humans or ticks as it has also been found in domestic and wild animals (e.g., cattle, deer, goats, dogs, and cats) (Oh et al., 2016; Tian et al., 2017). Therefore, continuous surveillance of the prevalence of SFTSV in animals is necessary to identify the potential for animal-to-human transmission. Goats are at high risk for SFTSV infection through uptake of infected ticks or by being fed on by infected ticks during grazing in meadows (Jiao et al., 2015; Xing et al., 2017). Furthermore, as a domestic animal, goats have considerable contact with humans during which they could transmit the virus. However, the prevalence and genetic characterization of SFTSV in South Korean goats has not previously been investigated. Hence, in this study, we collected goat sera from two different commercial slaughterhouses in Chungbuk Province from March to October of 2017 and investigated the prevalence of SFTSV in goats by ELISA and RT-PCR.

2. Materials & methods

2.1. Sera collection

The 207 domestic goat blood samples were collected in commercial slaughterhouses during routine epidemiological surveillance in South

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Korea from March to October of 2017. The collected samples were centrifuged at 1660 × *g* for 20 min and sera were harvested and then stored at - 80 °C until use. Serum samples were heated at 56 °C for 30 min to inactivate complement before testing with SFTSV-specific ELISA.

2.2. RNA extraction and cDNA synthesis

RNA was extracted from goat serum using the TRIzol® Reagent (Thermo fishier, Massachusetts, USA) according to the manufacturer's instructions (Rio et al., 2010). Single strand cDNA was synthesized using viral RNA and primers specific for S segments and random hexamers using the cDNA synthesis kit (Omniscript Reverse Transcriptase, QIAGEN, Hilden, Germany). The extracted RNA, primers, and dNTP were incubated at 70 °C for 5 min and then immediately chilled on ice for at least 2 min. Then RT buffer and enzyme were added to the chilled mixture and reverse transcription initiated with the S gene primer: 5′-ACACAAAGAACCCCCTTCATTTGGAAA -3′ at 37 °C for 60 min.

2.3. qRT-PCR to detect SFTSV RNA

To quantitate viral RNA and viral copy number, quantitative real-time RT-PCR (qRT-PCR) was performed for the non-structural protein (NS) gene with the SYBR Green kit (iQ™ SYBR Green supermix kit, Bio-Rad, California, USA) as described elsewhere (Yoshikawa et al., 2014). Assays were performed on CFX96 Touch™ (Bio-rad, California, USA) and analyzed using CFX Maestro Software StepOne software (Bio-rad, California, USA). Real-time primers for the SFTSV NS gene were matched in PCR efficacy with NS forward: 5′- ACACAAAGAACCCCCTTCA TTTGGAAA-3′, NS reverse: 5′- AGTAGCACCTCATGTCCTTGTAGTA -3′.

2.4. Sequencing and phylogenetic analysis

To analyze the nucleotide sequences of the S gene, partial genes were amplified using NS forward: 5′- ACACAAAGAACCCCCTTCATTT GGAAAC-3′ and NS reverse: 5′- ATTCGACAAAATTAGACCTCCTT-3′ primers. All PCR products were purified using a gel extraction kit (GeneAll, Lisbon, Portugal) followed by sequencing (Bionics, Seoul, Korea). NS genes were aligned using MegAlign version 5 (DNASTAR Inc, Wisconsin, USA) and compared for similarity to sequences deposited in BLAST (GenBank, Bethesda MD, USA). For phylogenetic analysis, sequence alignment and construction of the phylogenetic tree by the Maximum Likelihood (ML) method were performed using MEGA Version 7.0 software (MEGA, Pennsylvania, USA).

2.5. Detection of anti-SFTSV antibody by NP-ELISA

For the SFTSV enzyme-linked immunosorbent assay (ELISA), the nucleocapsid (NP) gene of CB1/2014 strain was cloned to an expression vector pGEX4T1. The recombinant NP protein (r-NP) was expressed in BL21(DE3) *E. coli* and purified using glutathione beads (Glutathione sepharose, GE healthcare, Little Chalfont, UK) according to the manufacturer's instructions. Purified proteins were electrophoresed in 0.8% SDS page gels to confirm correct sizes (data not shown).

Polysorp ELISA plates (Thermo Fisher, Massachusetts, USA) were coated overnight at 4 °C with 0.2 ug/well of r-NP protein. After incubation, the plates were blocked with 5% skim milk in phosphate buffered saline with tween® 20 (PBS-T) and incubated overnight at 4 °C. After washing, the plates were incubated with 100 ul of ten-fold diluted heat inactivated serum with 2% skim milk in 0.05% PBS-T for 2 h at room temperature. The plates were washed with 0.05% PBS-T three times, incubated with horse radish peroxidase (HRP) -anti-Goat IgG (KPL) diluted with 2% skim milk in 0.05% PBS-T for 1 h at room temperature, and washed five times. For detection of antibodies, the plates were overlaid with O-phenylenediamine dihydrochloride (Sigma, Missouri, USA) substrate and 1M H_2SO_4 was added to stop the reaction. Optical density (OD) was measured with a spectrometer (iMark™ Microplate Reader, Bio-Rad, California, USA) at 450 nm. The OD values for negative samples ranged from 0.1 to 0.2, and from 0.4 to 2.2 for positive samples.

2.6. Detection of anti-SFTSV antibody with fifty percent focus reduction neutralization titer ($FRNT_{50}$)

The $FRNT_{50}$ values of goat sera were determined using $FRNT_{50}$ as described elsewhere (Taniguchi et al., 2017). The CB1/2014 (genotype B) strain and CB2/2015 (genotype A) strain were used as a positive infection control in Vero-E6 cells. Briefly, 100 focus-forming units of the SFTSV CB1/2014 and CB2/2015 were mixed with serially-diluted sera and incubated for 1 h at 37 °C, then inoculated into confluent monolayers of Vero-E6 cells for an additional 1 h at 37 °C. The inoculums were removed and the media was changed to DMEM containing 1% fetal bovine serum (FBS) and 0.8% agarose. Seven days later, the inoculated cells were fixed with 10% formalin. Fixed plates were blocked with 3% bovine serum albumin (BSA) and treated with 10% Triton X-100. After washing with phosphate buffered saline (PBS), cells were incubated with mouse anti-NP antibodies and then HRP-mouse antibodies (The Jackson Laboratory, Maine, USA). The foci of infected cells were visualized using the 3,3′-diaminobenzidine (DAB) substrate kit (Vector Laboratories, California, USA). The $FRNT_{50}$ values were determined as a reciprocal of the highest dilution at which the number of the foci was < 50% of the number obtained without serum.

2.7. Detection of anti-SFTSV antibody with western blot

To identify the presence of viral antigen, whole inactivated CB1/2014 (genotype B) and CB2/2015 (genotype A) strains were ultracentrifuged (106750 × *g*) as described elsewhere (Lu et al., 1999). Total protein (0.5 ug) was separated by SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Darmstadt, Germany). After blocking with Tris-buffered saline containing 0.1% Tween20 and 5% skim milk, the membrane was probed with each goat serum followed by further incubation with a secondary antibody conjugated with HRP- anti-goat IgG (Sigma, Missouri, USA). Proteins were visualized using ECL Western blot detection reagents (Millipore, Darmstadt, Germany) and viewed under a Fujifilm LAS- 3000 Imaging System (R&D Systems, Minnesota, USA).

2.8. Ethical statement

All experiments in this study were approved by Chungbuk National University Animal Ethics Committee (Approval No.: CBNUA-1083-18-02). All blood samples taken from goats were processed with the consent of the responsible person within the slaughterhouse.

3. Results

3.1. SFTSV detection and phylogenetic tree analysis using viral RNA sequences

We collected sera from 207 goats in commercial slaughterhouses in Chungbuk Province during routine epidemiological surveillance from March to October of 2017. To detect SFTSV RNA, we conducted real-time RT-PCR on total RNA extracted from each serum sample. The results showed that 2% (n = 4) of samples were positive with viral RNA quantities ranging from 1.3 to 2.3 Log_{10} copies/ml. To isolate the viruses, we attempted to infect Vero-E6 cells with the positive sera; however, no virus was isolated from the cell culture. However, we were able to amplify the NS gene segment from the positive goat sera. Sequencing results (G93, G111, G190, and G192) revealed that all four viruses showed high homology with each other (95.9–99.8% nucleotide identity). Furthermore, G93, G111, and G190 showed the highest