

A serological study of severe fever with thrombocytopenia syndrome using a virus neutralization test and competitive enzyme-linked immunosorbent assay

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Severe fever with thrombocytopenia syndrome (SFTS) is caused by the SFTS virus (SFTSV). The SFTSV appears to have a wide host range, as SFTSV-positive ticks have been isolated from both farm animals and wild rodents. Therefore, it is important to monitor SFTSV-positive animals to prevent the transmission of SFTSV from animals to humans. Previously, we developed a competitive enzyme-linked immunosorbent assay (cELISA) to detect SFTSV-specific antibodies from field animals and compared the cELISA results to those from an indirect immunofluorescence assay (IFA). In this study, cELISA results were compared to and evaluated against the results from both an IFA and a virus neutralization (VN) test of 193 bovine serum samples (including two bovine positive control sera) and 70 horse serum samples. The consistency (98.9%) between cELISA and VN results was higher than that (97.4%) between cELISA and IFA for the bovine serum samples. Similarly, for the horse serum samples, the consistency (88.6%) between cELISA and VN results was higher than that (84.3%) between the cELISA and IFA. These findings indicate that our newly developed cELISA can be used for surveillance or epidemiological studies of SFTSV in animals.

Keywords: competitive enzyme-linked immunosorbent assay, severe fever with thrombocytopenia syndrome, virus neutralization test

Introduction

Severe fever with thrombocytopenia syndrome (SFTS) is a newly emerged tick-borne viral disease in humans. It has been reported in China since 2009 and in South Korea and Japan since 2012 and 2013, respectively; the fatality rate is 12%, 55%, and 61% in China, Japan, and Korea, each [8,17,19]. The name is derived from the clinical symptoms of the disease, *i.e.*, severe fever and thrombocytopenia; therefore, the pathogen is called the SFTS virus (SFTSV). The SFTSV belongs to the family *Bunyaviridae* and the genus *Phlebovirus* and has three single-strand negative RNA segments consisting of S, M, and L segments. The S segment encodes the nucleocapsid and non-structural proteins, the M segment encodes two glycoproteins for viral envelope components, and the L segment encodes an RNA-dependent RNA polymerase [10,11].

Domesticated animals from farms located in areas where human patients with SFTS have been reported had a high seroprevalence of SFTSV and shared genetic homology

[14,21]. These findings indicate that animals are an important reservoir for the SFTSV; however, little has been reported about the host species or transmission cycle because clinical signs of the illness or disease have not been observed in animals [14]. Therefore, serological diagnosis in multiple animal species is necessary to study how to prevent the transmission of the disease from animals to humans.

Enzyme-linked immunosorbent assays (ELISAs) have become the primary method for assessing a large number of samples with a simplified protocol and standardized results. However, previously developed, high-performance ELISA formats are not commercially available [6]. The competitive ELISA (cELISA), which we developed previously [10], is based on the competition between SFTSV-specific antibodies in the test serum and monoclonal antibodies (mAb) against the SFTSV nucleocapsid protein (NP). This cELISA was designed for the serodiagnosis of SFTSV in multiple animal species and was validated by using bovine serum samples. However, because we used an indirect immunofluorescence assay (IFA)

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as the reference test, it was difficult to predict the exact performance of the cELISA when using field samples with non-specific reactions. Therefore, we adopted a virus neutralization (VN) test as the reference to evaluate the developed cELISA. In this study, we collected bovine and horse serum samples and compared the results from the cELISA, VN test, and IFA.

Materials and Methods

Cell culture and virus

Vero E6 cells (CRL-1586; ATCC, USA) were used for virus amplification, neutralization, and the IFA. Vero cell culture was performed according to the maintenance protocol [1]. The SFTSV was obtained from the Korea Centers for Disease Control and Prevention and was isolated from a human patient in South Korea in 2013 [20].

SFTSV-positive control and field samples

All animal experiments followed the protocols of the Institutional Animal Care and Use Committee of the Animal and Plant Quarantine Agency of the Republic of Korea. SFTSV-positive control bovine serum samples were generated by immunizing laboratory animals with inactivated SFTSV [10]. Briefly, 60 mL of 10^8 TCID₅₀/mL SFTSV were inactivated with a 0.025% formalin solution and concentrated five-fold. Inactivated SFTSV (2 mL) was emulsified in adjuvant and injected into two 4-month-old cattle via intramuscular injection. The immunizing dose of virus particles was subcutaneously administered three times every other week, and a SFTSV-specific reverse-transcription polymerase chain reaction using white cells from the target animals was performed to confirm experimental safety.

Field bovine serum samples ($n = 191$) were collected from farms and slaughter houses in the southern part of South Korea (Gyeongbuk and Jeju provinces) from October 2013 to April 2015. Field horse serum samples ($n = 70$) were collected from farms in the southern part of South Korea (Jeonbuk province) from June 2013 to April 2015.

cELISA

To perform the cELISA, we followed the previously reported protocol [12]. Briefly, the recombinant NP was used as the coating antigen. Then, 100 ng/well of purified NP was coated onto Polysorp ELISA plates (Nunc, USA) for 16 h at 4°C. The non-specific protein interaction was blocked with 5% skim milk in 1× phosphate-buffered saline, and 50 µL of five-fold-diluted test sera and horseradish peroxidase-conjugated mAb (1:500) were added to the antigen-coated plate. The competitive reaction was performed for 90 min at 37°C. Then, the 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate (KPL, USA) was added as the horseradish peroxidase substrate.

Color development was terminated with TMB stop solution (KPL). Optical density (OD) was measured with a spectrophotometer (BioTek Instruments, USA) at both 450 and 630 nm. The OD at 450 nm minus the OD at 630 nm was used for background normalization. The percent inhibition (PI) values were calculated with the following formula: $PI = [1 - (OD \text{ test sample} / OD \text{ negative control})] \times 100$.

IFA

The IFAs were performed as previously reported [10]. Briefly, Vero E6 cells in 150 cm² culture vessels (Corning, USA) were sensitized with 2 mL of 1×10^3 TCID₅₀/mL SFTSV for 1 h at 37°C. The infected cells were detached with 0.25% trypsin-ethylenediaminetetraacetic acid (Gibco, USA) 6 days after infection. The floating cells were spotted onto reaction wells (Paul Marienfeld, Germany) and fixed with a methanol:acetone (1:1) solution. Serially diluted serum samples were incubated with fixed cells for 1 h at 37°C, and the fluorescence was detected using fluorescein isothiocyanate (FITC)-labeled secondary antibodies. The fluorescence was observed by using a Nikon TE-2000U fluorescence microscope (Nikon, Japan). The anti-bovine and anti-horse secondary antibodies (KPL) were diluted to 2.5 µg/mL prior to use.

Neutralization test

The VN test was performed as previously reported [3,5,19] with slight modifications. Briefly, heat-inactivated serum samples (30 min at 56°C) were serially diluted from a 1:2 stock. Then, 50 µL of the diluted serum was mixed with an equal volume of 200 TCID₅₀ SFTSV. The mixture was incubated for 1 h at 37°C. After incubation, the mixture and Vero cells (3×10^4 cells/100 µL) were cultured in 96-well culture plates for more than 3 days at 37°C. Virus back-titration was included in the test to measure the virus input. The presence of viral infection was determined by using NP-specific rabbit polyclonal antibodies [10] and FITC-labeled secondary antibodies after the cells were fixed. The highest serum dilution that completely protected the cells from virus infection was selected as the neutralizing antibody titer.

Statistical analysis

Efficacy of the cELISA was compared to the reference tests by using the field serum samples and the experimentally immunized serum samples. The Cohen's kappa coefficient was calculated to measure the agreement between the cELISA and reference test results [9]. The graphs and receiver-operating characteristic (ROC) curves [22] were drawn by using the graph function in Excel 2010 (Microsoft, USA).

Results

Generation of SFTSV-positive bovine sera for the cELISA and VN

In the absence of a reported SFTSV-positive control panel of sera, laboratory-generated SFTSV antibody-positive bovine sera were used to test the efficacy of the cELISA and to confirm the obtained titers with the VN test. Inactivated SFTSV was individually injected into two different cattle, and the antibody

titers were tested by using the cELISA and VN test before and after each interval of three antigen injections. The PI value gradually increased from 0% to 89.1% in cattle 1 and from 0% to 96.8% in cattle 2, and the neutralization antibody titer also significantly increased (Fig. 1).

Comparisons among the cELISA, IFA, and VN test for the field bovine sera

The cELISA results were compared to the results of the IFA

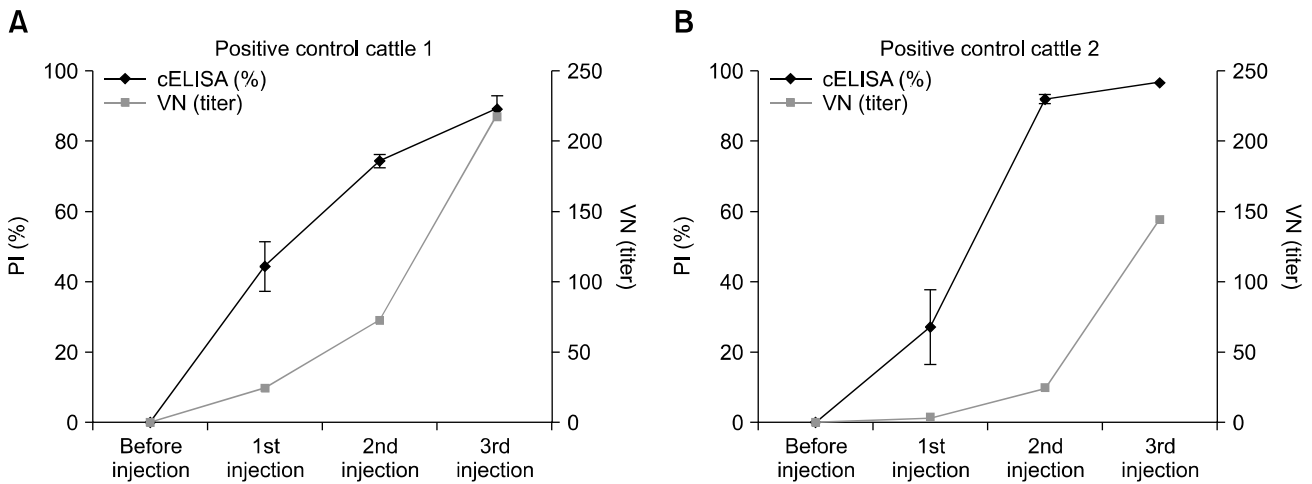


Fig. 1. The antibody titers against the severe fever with thrombocytopenia syndrome virus (SFTSV) in immunized cattle for the competitive enzyme-linked immunosorbent assay (cELISA) and the virus neutralization (VN) test. The serum titers were detected by using both the cELISA and the VN test. (A) and (B) represent the PI values for the cELISA and VN titers in two different cattle following immunization.

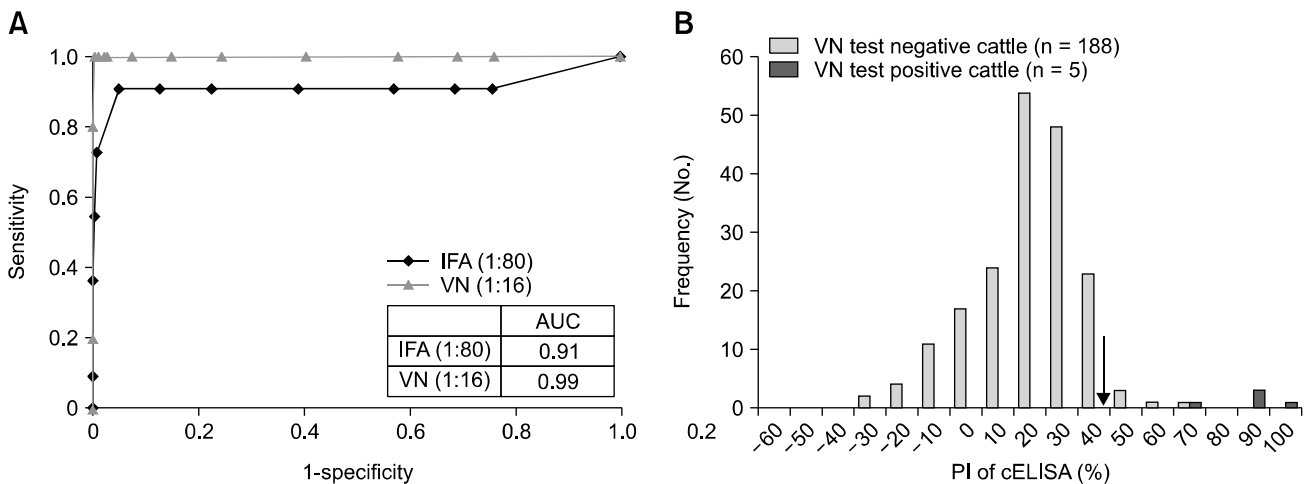


Fig. 2. Cattle receiver-operating characteristic (ROC) curves for the competitive enzyme-linked immunosorbent assay (cELISA) vs. the immunofluorescence assay (IFA) and the virus neutralization (VN) datasets (n = 193), and the frequency distribution of PI values in the cELISA from 193 bovine serum samples. (A) cELISA ROC curves comparing the correlations with the two different reference methods, i.e., the IFA and the VN test. The area under the curve (AUC) are provided in the inset table. (B) The frequency distributions of the percent inhibition (PI) values from 193 bovine serum samples that were determined to be the severe fever with thrombocytopenia syndrome virus-negative and -positive by the VN test (cut-off dilution rate = 1:16). The established cut-off PI value for the cELISA (48.0%) is indicated by an arrow.

and VN test by using field bovine serum samples. The ROC curves of each serum dilution for the IFAs and VN tests from 191 bovine serum samples and two positive control serum samples were used to determine the overall specificity and sensitivity and to define the optimal cut-off value for the developed cELISA. The ROC curves showed that the 1:80 dilution for the IFA and the 1:16 dilution for the VN test had the greatest correlation with the cELISA result (panel A in Fig. 2). The SFTSV-positive serum samples showed a specific staining pattern in the cytoplasm in SFTSV-infected Vero cells, and the 1:80 dilution was the last serial dilution that showed clearly discernible IFA results (Supplementary Fig. 1).

The cut-off dilution rate for the IFA was used as a positive predictive value; therefore, the cELISA result from the SFTSV-negative samples represents 14.5% of the mean PI value, with a standard deviation (SD) of 15.9%. We then determined the cELISA cut-off value to be 45.8% (mean + 2 SD), which indicated a 97.4% (188/193) consistency, 99.5% specificity, and 63.6% sensitivity (Table 1). Furthermore, the SFTSV-negative samples in the VN test represented a cELISA cut-off value of 48.0% (mean + 2 SD), which was determined from a 14.8% mean PI and a 16.6% SD. The total consistency between the cELISA and VN test was 98.9% (191/193), with 98.9% specificity and 100% sensitivity (Table 1). The frequency distribution of the cELISA PI values from the 191 field bovine samples and two positive samples that were primarily examined by using the VN test is shown in panel B in Fig. 2.

Comparisons among the cELISA, IFA, and VN test for the field horse sera

Serum samples from horses were tested to compare the cELISA results with the results from the reference methods (IFA and VN tests) to determine the developed cELISA usefulness in another animal species. According to the area under the curve (AUC) from the ROC curves from the reference tests, the IFA and VN test cut-off dilution rates that distinguished the positive and negative SFTSV samples were 1:32 and 1:16,

Table 1. Relative specificity and sensitivity of the competitive enzyme-linked immunosorbent assay (cELISA) compared to the immunofluorescence assay (IFA) and the virus neutralization (VN) test as determined by assessing 191 field bovine serum samples and two positive control bovine serum samples

	IFA*		VN†	
	Positive (≥ 80)	Negative (< 80)	Positive (≥ 16)	Negative (< 16)
cELISA‡ Positive	7	1	5	2
Negative	4	181	0	186
Sensitivity	63.6%		100%	
Specificity	99.5%		98.9%	

*The consistency between the IFA and cELISA was 97.4% (188/193) ($\kappa = 0.723$). †The consistency between the VN test and cELISA was 98.9% (191/193) ($\kappa = 0.828$). ‡The cELISA has a 45.8% cut-off percent inhibition (PI) for the IFA and a 48.0% cut-off PI for the VN test.

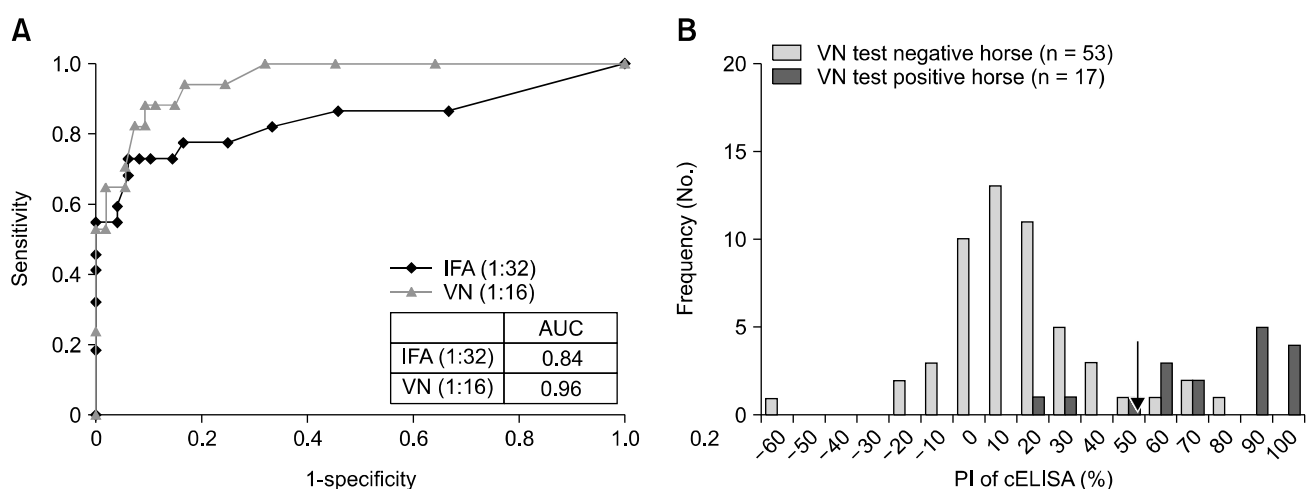


Fig. 3. Horse receiver-operating characteristic (ROC) curves for the competitive enzyme-linked immunosorbent assay (cELISA) vs. the immunofluorescence assay (IFA) and the virus neutralization (VN) datasets ($n = 70$), and the frequency distribution of the PI values in the cELISA from 70 horse serum samples. (A) The cELISA ROC curves comparing the correlation with the two different reference methods, *i.e.*, the IFA and the VN test. The area under the curve (AUC) are provided in the inset table. (B) The frequency distributions of the percent inhibition (PI) values from 70 horse serum samples that were determined to be the severe fever with thrombocytopenia syndrome virus-negative and -positive by the VN test (cut-off dilution rate = 1:16). The established cut-off PI value for the cELISA (57.4%) is indicated by an arrow.

Table 2. Relative specificity and sensitivity of the competitive enzyme-linked immunosorbent assay (cELISA) compared to the immunofluorescence assay (IFA) and the virus neutralization (VN) test as determined by assessing 70 field horse serum samples

		IFA*		VN†	
		Positive (≥ 32)	Negative (< 32)	Positive (≥ 16)	Negative (< 16)
cELISA‡	Positive	13	2	12	3
	Negative	9	46	5	50
	Sensitivity	59.1%		70.6%	
	Specificity	95.8%		94.3%	

*The consistency between the IFA and cELISA was 84.3% (59/70) ($\kappa = 0.601$). †The consistency between the VN test and cELISA was 88.6% (62/70) ($\kappa = 0.676$). ‡The cELISA has a 54.8% cut-off PI (%) for the IFA and a 57.4% cut-off PI for the VN test.

respectively (panel A in Fig. 3). The IFA results for the serially diluted horse serum samples showed a significantly different staining pattern for the SFTSV-positive samples than for the SFTSV-negative samples at a 1:32 dilution (Supplementary Fig. 1).

The AUC values from the ROC curve indicated that the cELISA had an optimal threshold for detecting an SFTSV infection that was similar to that for the VN test but was dissimilar to the IFA threshold (panel A in Fig. 3). The SFTSV-negative samples from the IFA had a 54.8% cut-off value (mean + 2 SD) for the cELISA, with a 9.6% mean PI value and 22.6% SD. Compared to the IFA, the SFTSV-negative samples in the VN test were 57.4% (mean + 2 SD) of the cELISA cut-off value, with a 10.2% mean PI value and 23.6% SD. With this cut-off, the VN test supported the increased sensitivity of the cELISA from 59.1% to 70.6% (Table 2). The consistency between the cELISA and VN tests was 88.6% (62/70). The frequency distribution of the cELISA PI values from the 70 horse samples that were primarily examined by using the VN test is shown in panel B in Fig. 3.

Discussion

Serological studies of the SFTSV in animals are necessary to elucidate SFTS and to prevent the virus from being transmitted to humans. Previously, we developed a cELISA to diagnose SFTSV infection in multiple animal species and validated its performance with bovine sera by using an IFA as a reference method [10]. In the present study, we used the VN test as a reference to evaluate further the developed cELISA.

A known vector of the SFTSV is *Haemaphysalis longicornis*, which is one of the major species of ticks affecting and a principal parasite of cattle in Korea [4,7,15]. Therefore, serum samples from field cattle were primarily tested to detect

antibodies against the SFTSV. Moreover, the heartland virus (HRTV), which has a close genetic relationship with the SFTSV, has recently emerged in the United States [13]. As a 17.4% seroprevalence for the HRTV has been reported in horses in the US, we also checked serum samples from field horses in our country [2,16]. Therefore, in the present study, serum samples from both field cattle and horses were primarily tested to detect antibodies against the SFTSV.

The cut-off value for the cELISA was calculated by using SFTSV-negative samples, as determined by the IFA and VN test. Overall, the field serum samples demonstrated that the cELISA had a higher correlation with the VN test than it did with the IFA. Using the IFA as a reference test, the cELISA showed sensitivities of 63.6% and 59.1% for the bovine and horse serum samples, respectively. The low sensitivity is a result of the high number of false negative samples, which were positive in the IFA but negative in the cELISA. Four bovine and nine horse samples with positive signals in the IFA were determined to be false negatives, and the majority of those samples were confirmed to be negative by using the VN test. Consequently, by using the VN test as a reference, our cELISA showed a sensitivity of 100% for the bovine serum samples and a sensitivity of 70.6% for the horse serum samples.

Two bovine samples and three horse samples with PI values greater than the cut-off value displayed low VN titers (< 1:16), and five horse samples with high VN titers (≥ 1:16) had low PI values in the cELISA. This contradictory result between the VN test and cELISA was likely caused by differences in the target antigenic regions of the antibody. It was previously reported that antibodies that can neutralize the SFTSV target the glycoproteins Gn and Gc [5]. However, the developed cELISA detects antibodies against NP, which are not related to the virus-neutralizing reaction [18].

In conclusion, we confirmed the good performance of the newly developed cELISA, performance that is comparable to that of the VN test. We expect to use the developed cELISA to undertake surveillance and epidemiological studies of the SFTSV in animals.

Acknowledgments

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Conflict of Interest

The authors declare no conflicts of interest.

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