Serologic Evidence of Severe Fever with Thrombocytopenia Syndrome Virus and Related Viruses in Pakistan

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We describe the seroprevalence of severe fever with thrombocytopenia syndrome virus (SFTSV) and the association of antibody occurrence with location, sex, and age among the human population in Pakistan. Our results indicate substantial activity of SFTSV and SFTSV-related viruses in this country.

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tickborne disease caused by the SFTS virus (SFTSV; genus *Banyangvirus*, family *Phenuiviridae*, order *Bunyavirales*). The disease is prevalent in East Asia countries. It was first detected in China in 2009 and later in Japan and South Korea (*1*) and is suspected to be widely spread across other parts of the world (*2*). The recent identification of SFTSV in Xinjiang, China (*3*), expanded our awareness of epidemic areas of SFTS and suggested the possibility of SFTSV spreading to bordering countries like Pakistan. However, the presence of SFTSV in Pakistan has been unclear. We investigated the seroprevalence of SFTSV in humans in Pakistan.

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The Study

For this study, we randomly collected human serum samples (n = 1,657) from 4 provinces in Pakistan during 2016–2017 (Figure). All participants were farmers of livestock (sheep, goats, cattle, buffaloes, and camels). We recorded and summarized testing results by sex, age, and geographic location (Table). The collection of human serum samples and subsequent tests were reviewed and approved by the Ethics Committees of Government College University, Faisalabad, Pakistan (approval number: GCUF/MICRO/18/1598). Adult participants and parents of participants <18 years of age provided written informed consent.

We used a 2-step approach to detect antibodies against SFTSV. First, we screened the samples for SFTSV IgG by using a SFTSV human commercial ELISA kit (NZK Bio-tech, https://hbnzk.com), which employs SFTSV nucleocapsid protein (NP) as the viral antigen. To set up negative and positive controls, we used serum samples from 3 healthy persons from Wuhan, China (*4*), and serum samples from 2 convalescent SFTS patients from Wuhan archived in the

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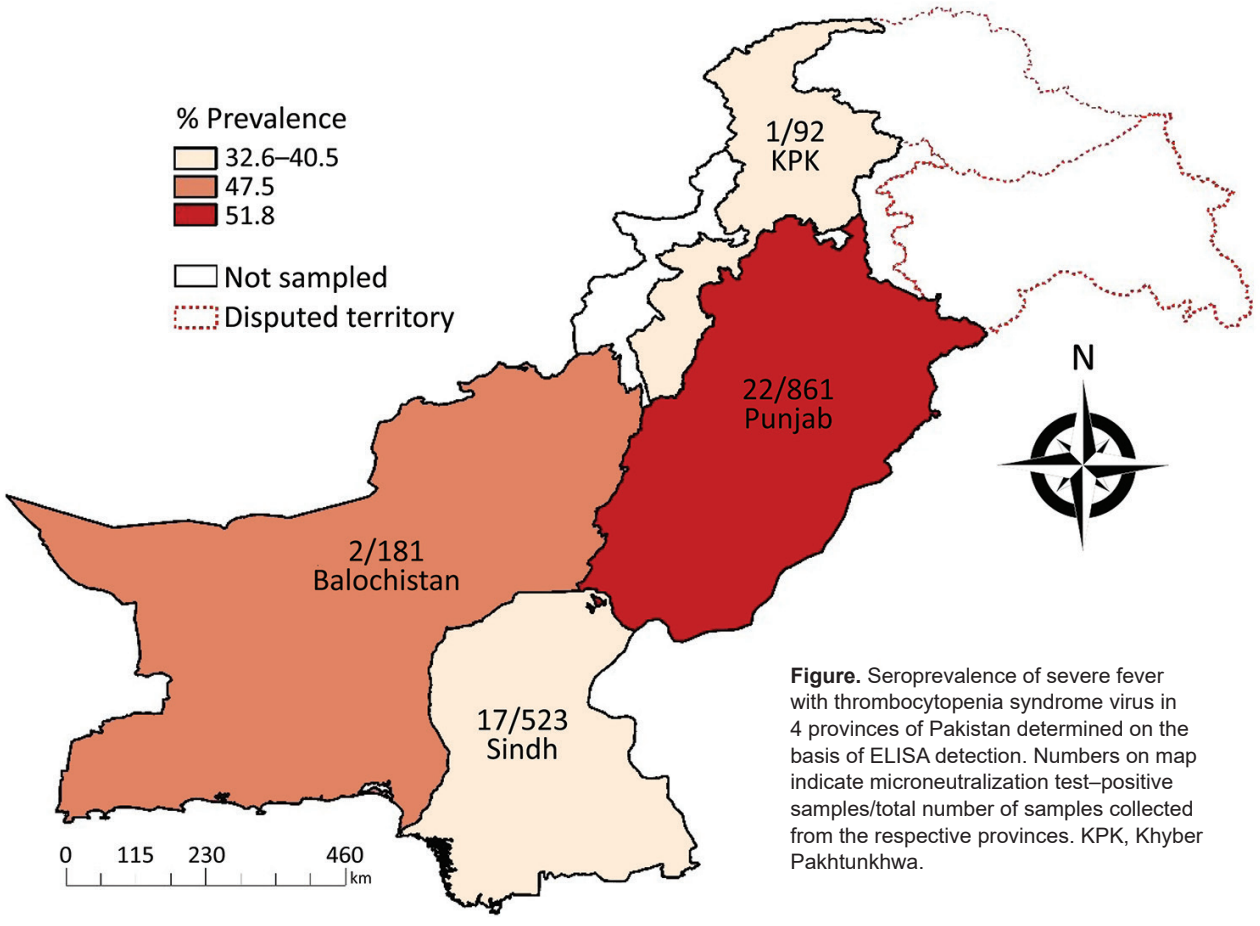

Figure. Seroprevalence of severe fever with thrombocytopenia syndrome virus in 4 provinces of Pakistan determined on the basis of ELISA detection. Numbers on map indicate microneutralization test–positive samples/total number of samples collected from the respective provinces. KPK, Khyber Pakhtunkhwa.

National Virus Resource Center (accession nos. YB-17WIVS286, YB17WIVS294). Following manufacturer instructions (Appendix, https://wwwnc.cdc.gov/EID/article/26/7/19-0611-App1.pdf), we tested serum samples at 1:20 dilution; we considered samples IgG-positive when the absorbance was ≥2.1 times the mean absorbance of the negative control. Samples with optical density values ≥0.41 were considered SFTSV IgG–positive (Appendix Figure 1). We used an immunofluorescence assay modified from a previous study (*5*) to verify the validity of the commercial ELISA.

In the second step, we used a microneutralization test (MNT) assay to distinguish SFTSV-specific neutralizing antibodies from SFTSV-related viruses, as described elsewhere (*6*); we estimated SFTSV prevalence from ELISA and MNT results within 95% CIs. We performed statistical analysis of the data using the χ^2 test or Fisher exact test to explore the association of SFTSV with age, sex, and location. We performed the analysis in R version 3.5.1 with the Epicalc package version 2.15.1.0 (http://www.r-project.org).

The ELISA revealed a high seroprevalence (46.7%, 95% CI 44.3%–49.1%) of SFTSV in Pakistan (Table; Figure). Spatial distribution analysis indicated the highest prevalence (51.8%, 95% CI 48.4%–55.2%) in the Punjab province, followed by Balochistan (47.5%, 95% CI 40.1%–55.1%), Sindh (40.5%, 95% CI 36.3%–44.9%), and Khyber Pakhtunkhwa (32.6%, 95% CI 23.2%–43.2%). The prevalence was significantly higher (p = 0.004) in women (49. 9%, 95% CI 46.6%–53.2%) than in men (42.7%, 95% CI 39.1%–46.4%). The seroprevalence increased with age, but not uniformly; the highest seroprevalence (57.1%, 95% CI 28.9%–82.3%) was recorded in samples from persons ≥65 years of age. A technician unaware of the ELISA results and research details performed a single-blind test with 90 ELISA-negative and 252 ELISA-positive samples, randomly selected. This test confirmed results for 100% of the ELISA-negative samples and 212 (84.1%) of 252 of the ELISA-positive samples (Appendix Figure 2).

We confirmed SFTSV infection using MNT, which revealed a low prevalence (2.5%, 95% CI 1.9%–3.4%) in Pakistan (Table; Appendix Figure 3). Women had a higher occurrence of anti-SFTSV neutralizing antibodies (2.7%, 95% CI 1.8%–4.0%) than men (2.3%, 95% CI 1.4%–3.7%), however, this difference was not

Table. Seroprevalence of severe fever with thrombocytopenia syndrome virus in Pakistan based on ELISA and MNT results

	ELISA			MNT		
Category	No. positive/no. tested	Prevalence, % (95% CI)	p value	No. positive/no. tested	Prevalence, % (95% CI)	p value
Province						
Punjab	446/861	51.8 (48.4–55.2)	<0.001	22/861	2.6 (1.6–3.8)	0.339
Balochistan	86/181	47.5 (40.1–55.1)		2/181	1.1 (0.1–3.9)	
Sindh	212/523	40.5 (36.3–44.9)		17/523	3.3 (1.9–5.2)	
KPK	30/92	32.6 (23.2–43.2)		1/92	1.1 (0–5.9)	
Sex						
M	313/733	42.7 (39.1–46.4)	0.004	17/733	2.3 (1.4–3.7)	0.619
F	461/924	49.9 (46.6–53.2)		25/924	2.7 (1.8–4)	
Age group, y						
15–24	196/413	47.5 (42.6–52.4)	0.919	8/413	1.9 (0.8–3.8)	0.120
25–34	310/669	46.3 (42.5–50.2)		21/669	3.1 (2–4.8)	
35–44	149/325	45.9 (40.3–51.4)		3/325	0.9 (0.2–2.7)	
45–54	89/184	48.4 (41–55.8)		8/184	4.4 (1.9–8.4)	
55–64	22/52	42.3 (28.7–56.8)		1/52	1.9 (0–10.3)	
≥65	8/14	57.1 (28.9–82.3)		1/14	7.1 (0.2–33.9)	
Total	774/1657	46.7 (44.3–49.1)		42/1657	2.5 (1.9–3.4)	

*KPK, Khyber Pakhtunkhwa; MNT, microneutralization test.

significant (p = 0.619). Neutralizing antibodies were detected in all age groups. Furthermore, we performed MNT for a novel virus, Guertu virus (GTV) (*4*), which is closely related to SFTSV, on 10 randomly selected serum samples that tested positive for SFTSV neutralization and 10 SFTSV IgG–positive samples that were negative for neutralization (Appendix Figure 3). All 10 samples that tested negative on the SFTSV MNT also tested negative on the GTV MNT. However, 3 of the 10 samples that tested positive on the SFTSV MNT also exhibited neutralization to GTV; the other 7 samples tested negative for neutralizing GTV.

Conclusions

This study highlights the activity of SFTSV and its substantial risks to the population in Pakistan. The observed high ELISA-based prevalence could be ascribed to the study population in this survey being livestock farmers, who could be more frequently exposed to tick vectors and livestock reservoirs. Higher estimates of SFTS prevalence in the Punjab province of Pakistan could be attributed to the high proximity of human and livestock populations in this region. Higher prevalence among women than among men was expected because livestock is mostly tended by female farmers.

In ELISA-based estimates of SFTSV in the human population reported from different areas of East Asian countries, seroprevalence has ranged from 0.23% to 9.17% in China (*7*), from 1.9% to 7.7% in Korea (*8*,*9*), and from 0.14% to 0.3% in Japan (*10*, *11*). In contrast to the findings from these reports (*7*–*11*), our study found a markedly high ELISA-based prevalence of SFTSV (46.7%) in Pakistan. The use of different SFTSV antibody detection methods may have led to the observed differences in results (*11*); nevertheless, results of the blind test using an immunofluorescence assay still suggested a high prevalence, such that 84.12% of the randomly selected ELISA-positive serum samples could react with the SFTSV antigen. Therefore, the low prevalence of neutralizing antibodies (2.5%) against SFTSV suggests the possibility of cocirculating antigenicity-related viruses that were not discernable in the indirect ELISA tests.

The genus *Banyangvirus* currently includes the Bhanja and SFTS/Heartland groups. The 5 viruses of the SFTS/Heartland group have a wide geographic distribution. SFTSV is found mostly in China, Japan, and South Korea (*1*); GTV in northwestern China (XJUAR) (*4*), Heartland virus in the United States (*12*); Hunter Island group virus in Australia (*13*); and Malsoor virus in India (*14*).

Similar to findings in our study, a high seroprevalence (19.8%) of GTV was detected among the local residents of Guertu County in Xinjiang, China; however, only 3 (0.65%) of the 465 serum samples had neutralizing antibodies against GTV (*4*). Antigenic cross-reactivity between SFTSV and GTV was suspected because cross-neutralization was observed in mouse serum (*4*). However, serologic investigation of other bunyaviruses is limited, and serotypes of the 2 viruses, as well as other related viruses in the SFTS/Heartland group, remain unclear.

Our subsequent study found that a few serum samples exhibiting neutralization to SFTSV also exhibited cross-neutralization to GTV. All of these results indicate the presence in Pakistan of SFTSV and SFTSV-related viruses that might share antigenic similarity and could induce antibodies exhibiting cross-reactivity with each other. In addition, a recent study reported suspected

clinical SFTS cases in Pakistan; however, they were not confirmed using serologic or molecular tests (*15*).

Our findings suggest the potential risk for infection from SFTSV and SFTSV-related viruses in Pakistan. Further work on the discovery, identification, and ecology of these viruses in ticks, animal hosts, and human patients is needed because the viruses pose potential threats to public health.

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About the Author

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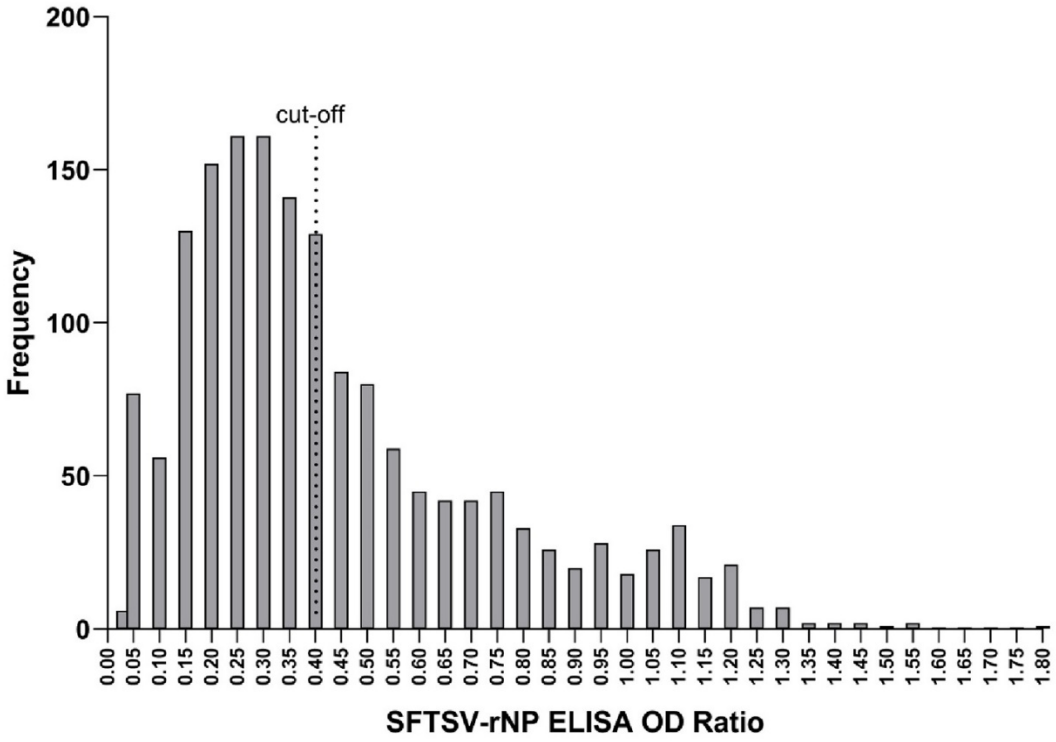
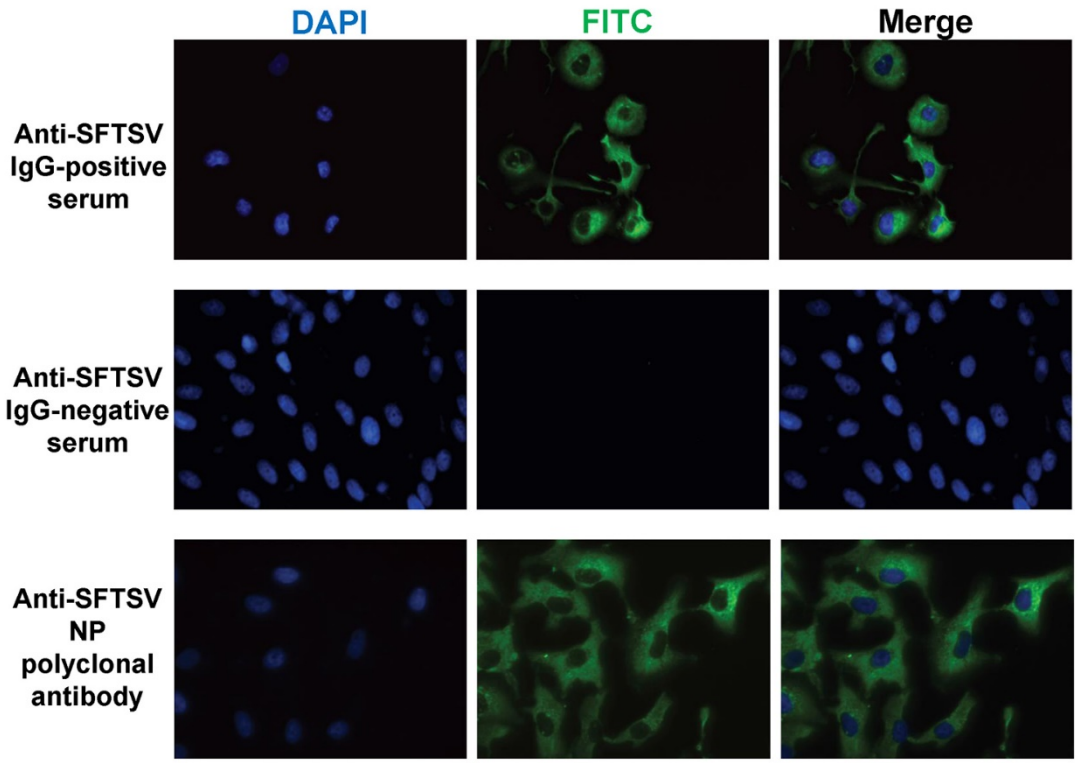
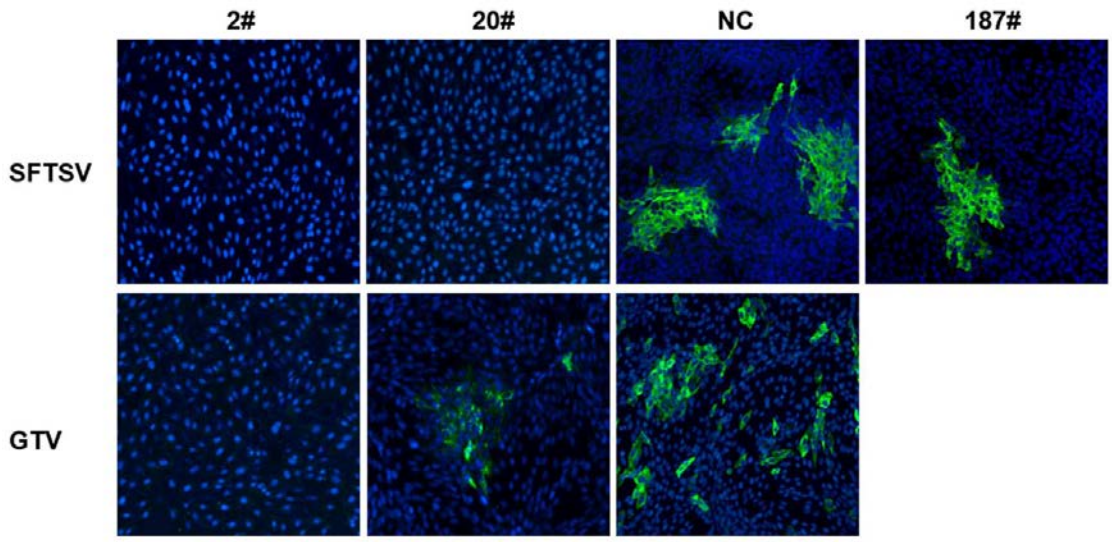
Appendix



Appendix Figure 1. A plot showing the frequency of optical density (OD) values from all 1657 human serum samples. The cutoff value at which samples were considered anti-SFTSV IgG-positive was set at an OD value ≥0.41, according to the ELISA kit instructions.



Appendix Figure 2. Images of indirect immunofluorescence assay (IFA) that was used to verify the results from ELISA. Representative images are shown, including an ELISA-positive serum sample that was also detected anti-SFTSV IgG-positive by IFA (upper panel); an ELISA-negative serum sample which was detected anti-SFTSV IgG-negative (middle panel); and an anti-SFTSV nucleoprotein polyclonal antibody (*4*) used as a positive control (bottom panel).



Appendix Figure 3. Images of microneutralization (MNT) assay showing the representative results of SFTSV or GTV infection in cells after incubating with human serum samples. Neither SFTSV nor GTV infection could be observed if serum sample could neutralize both viruses. Of the 10 serum samples MNT-positive to SFTSV, 3 samples were detected having cross-neutralization to GTV. Images from sample 2# were shown. The other 7 serum samples could neutralize SFTSV but could not react with GTV. Images from sample 20# show that SFTSV infection was prevented and GTV infection was observed. SFTSV and GTV infection were both observed in the negative control (NC) when viruses (100 $TCID_{50}$) were mock incubated. SFTSV infection could be observed in cells when viruses were incubated with anti-SFTSV IgG-positive samples, but they had no neutralization to the virus (187#).

Manufacturer Instructions

The following pages show the manufacturer instructions for the ELISA used in this study.

Cat. NZK-E17003

For Research Use Only

NZK biotech

Severe fever with thrombocytopenia syndrome virus (SFTSV) Human IgG ELISA kit

Product Manual

URL: https://hbnzk.com/products/materials

I. Intended Use

The Severe Fever with Thrombocytopenia Syndrome Virus (SFTSV) Human IgG ELISA Kit is an in vitro enzyme immunoassay for the specific determinations of human anti-SFTSV IgG in serum.

II. Principle

The Severe Fever with Thrombocytopenia Syndrome Virus (SFTSV) Human IgG ELISA Kit is a solid phase assay based on an indirect method that utilizes purified recombinant SFTSV nucleoprotein (NP) to detect antibodies in human serum samples. The protein is coated on the microtiter plate to create the solid phase. Non-specific binding is blocked by a blocking buffer. Serum samples are diluted with dilution buffer and incubated in wells. Then, the wells are washed, and incubated with the second antibody, anti-human IgG antibody conjugated with horseradish peroxidase (HRP). During the incubation, anti-SFTSV NP antibodies in human serum were captured, then the specific IgG was recognized with the second antibody. Reaction between HRP and substrates results in color development with intensities proportional to the amount of anti-SFTSV NP antibody present in serum samples. The results can be measured by the absorbance with a microplate reader. Serum samples positive for anti-SFTSV NP IgG can be determined by comparing the absorbances with those of the negative control.

III. Reagents and Material

Each kit includes reagents sufficient for 96 wells. The expiration date for the complete kit is stated on the outer box label and the recommended storage temperature is 2-8 °C.

A. Materials Provided

(1) SFTSV NP coated microtiter plate, 1 plate (96 wells: 8 wells × 12 strips)

(2) HRP-conjugated anti-human IgG antibody, 12 mL

(3) Sample dilution buffer, 12 mL

(4) Washing buffer (20×), 50 mL

(5) Substrate solutions

Solution A, 5 mL

Solution B, 5 mL

(6) Stop buffer, 5 mL

(7) One zip-lock bag

B. Equipment and Materials Required but not Provided

(1) Pipette, micropipette, and tips

(2) Microplate reader

(3) Incubator (37±1°C)

(4) Absorbent paper

IV. Precautions

- Do not mix reagents from different kit lots.

- Do not use reagents beyond expiration date on label.

- In order to avoid reagent contamination, use disposable pipette tips and/or pipettes.

- Do not expose Substate Solution to strong light during storage or incubation.

- Avoid contact of Substrate and Stop Solution with skin or mucous membranes. If these reagents come into contact with skin, wash thoroughly with water. Do not pipette by mouth. Do not smoke, eat, or drink in area where specimens or kit reagents are handled. All blood fluids should be considered as potentially infectious.

- Avoid contact of Substrate Solution and Stop Solution with any metal surface. Disposable glassware or test tubes are recommended for handling the Substrate Solution. If non-disposable glassware is used, it must be acid

washed and thoroughly rinsed with distilled, deionized water.

- Do not use the Substrates if its color is changed to thick blue.

V. Specimen Collection and Handling

This kit is only for use of detection of anti-SFTSV IgG antibody in human serum. Remove the serum from the clot or red cells, respectively, soon after clotting and separation. Samples containing a visible precipitate must be clarified prior to use in the assay, and should not be contaminated with microorganisms. Fresh samples could be stored at 2-8°C for one week. If the length of time between sample collection and assay is to exceed 24 hours, samples are suggested to be stored frozen under-20℃ or lower temperature. Excessive freeze-thaw cycles should be avoided. Do not use grossly hemolyzed or lipemic specimens. Prior to assay, frozen samples should be brought to room temperature slowly, and gently mixed by hand. Do not thaw samples in a hot bath. Do not vortex or sharply agitate.

VI. Procedure

Notes: The microtiter plates should be brought to room temperature 30 min before use. For thorough mixing, the microtiter plate can be gently agitated on a plate mixer or by mixing the plate sporadically by hand. Dilute the Washing Buffer (20×) in ddH_2O to working concentration (1×), which should be prepared directly before the assay.

A. The serum samples of positive control and negative control, and blank should be set up. Pipette 100μL of each into one well. Samples must be diluted in the Dilution Buffer (v/v=1:1 for the first test), 100μL per well.

B. Mix, seal the microtiter plate in the zip-lock bag, and then incubate for 30min at 37°C.

C. Remove sample solution and control and wash the wells 6 times with

200 μL of Washing Buffer (1×). Let the plate stand for 15-30sec for each time. Between the separate washing steps, empty out the microtiter plate and vigorously tap onto paper towel, especially after the last washing.

D. Pipette 100 μl of HRP labelled anti-human IgG antibody into one well. Mix, seal the microtiter plate in bag and incubate for 30 min at 37℃.

E. Remove the solution and wash the wells 6 times as described above (**C**) (It is especially important after this step to thoroughly empty out the remaining fluid before adding the substrate).

F. Add 50 μl of Solution A and 50 μl of Solution B into each well. Seal in bag and incubate at 37℃for 6-8 min.

G. Add 100 μl of Stop Solution into each well in the same order as for substrate. Tap plate gently to mix.

H. Measure the absorbance at 450 nm with a microplate reader. The absorbance should be read within 15 minutes after the completion of the assay. It may be read up to 1 hour after addition of Stop Solution if wells are protected from light at room temperature.

VII. Results

Record the absorbance at 450 nm for each sample well.

Cut-off value equals to 2.1 folds by the mean absorbance of negative controls (N) (Cut-off value =2.1×N). If mean value (N) was less than 0.05, the cut-off value equals to 2.1 by 0.05 (0.105).

The absorbance of Negative control should be no more than 0.2, or else the assay must be repeated. Samples are considered negative if absorbance < Cut-off value, and are considered positive if absorbance ≥ Cut-off value.

VIII. Contact Us

URL: https://hbnzk.com/products/materials

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