TABLE 2 Primer sets used for cvPCR

Primer set	Forward	Reverse	Target region in the S segment (positions)
1	ATCGTCAAGGCATCAGGGAA	TTCAGCCACTTCACCCGRA	1045–1502
2	CATCATTGTCTTTGCCCTGA	AGAAGACAGAGTTCACAGCA	1076–1536

based on a Chinese strain, HB29, several candidate primer sets were designed using the DNADynamo sequence analysis software program (BlueTractorSoftware, Ltd., United Kingdom). Next, the primer specificity for the SFTSV genome and cross-reactivity within SFTSV strains were checked by Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sets 1 and 2, expected to be the most specific and broadly reactive primers to SFTSV strains, were selected. Moreover, to make sure they were compatible with both Chinese and Japanese SFTSV strains, a degenerate-base R (A or G) was substituted for a base in primer 1R. For the cvPCR assay, an aliquot of the extracted RNA solution was added to a reaction mixture of the SuperScript III one-step RT-PCR system with Platinum *Taq* DNA polymerase (Life Technologies), which contains 2× reaction mix, SuperScript III RT-Platinum Taq mix, H_2O, and 0.2 μM either specific primer set 1 (expected product length, 458 bp) or 2 (expected product length, 461 bp). After being denatured at 95°C for 2 min, a reverse transcription step was performed at 55°C for 30 min, followed by 45 cycles of amplification under the following conditions: 94°C for 30 s, 52°C for 30 s, and 68°C for 30 s in a PCR machine (Eppendorf, Hamburg, Germany). The positive-control plasmid, which provides 584 bp or 587 bp of PCR products by primer sets 1 or 2, respectively, was subjected to RT-PCR simultaneously with the specimens. A total of 8 μl of the PCR products was digested by 10 U/1iter μl of EcoRI with 1 μl of 10× H buffer as necessary. After electrophoresis on 1% agarose gels, the PCR products with the expected sizes were visualized by staining with GelRed (Biotium, Hayward, CA). For a definite diagnosis of SFTSV infection, the PCR products were purified by a MonoFas DNA purification kit I (GL Sciences, Tokyo, Japan) and sequenced using the ABI Prism 3100 genetic analyzer (Life Technologies), according to the manufacturer's protocol.

Quantitative one-step RT-PCR. Based on the nucleotide sequence of the Japanese strain, SFTSV YG1 (GenBank accession no. AB817995, AB817987, and AB817979), the specific quantitative one-step PCR (qPCR) primer and probe sets that were targeted to the RNA-dependent RNA polymerase (RdRp), glycoprotein (GPC), or nucleoprotein (NP) genes were designed using the DNADynamo sequence analysis software program (Table 1). The primer and probe specificities and cross-reactivities were checked by Primer-BLAST, as described above in the cvPCR primer selection procedure. For the qPCR assay, an aliquot of the extracted RNA solution was added to a reaction mixture for the QuantiTect probe RT-PCR kit (Qiagen, Hilden, Germany), which contains 2× QuantiTect probe RT-PCR master mix, QuantiTect RT mix, H_2O, and 10× primer-probe mix, which contains 4 μM each specific primer, 2 μM TaqMan probe(s), and 2 μM contamplicon probe. After PCR activation at 95°C for 15 min, the reverse transcription reaction was carried out at 50°C for 30 min, followed by 45 cycles of amplification under the following conditions: 94°C for 15 s and 60°C for 60 s in a LightCycler Nano (Roche).

The qPCR method was performed according to a previous report (6), with the PCR products amplified from an SFTSV strain, HB29, for the standards and using the QuantiTect multiplex RT-PCR NoROX (NR) kit (Qiagen) instead of synthetic viral RNA transcripts of HB29 and Ag-Path-ID with the one-step RT-PCR kit (Life Technologies).

Statistical analysis. One-way analysis of variance (ANOVA) with Bonferroni's multiple-comparison test was used to test the equality of the means of the viral RNA levels in the patient serum samples determined by either the reported qPCR method (6) or the qPCR method developed in this study, and to examine the relationship between the viral copy number level in the blood specimens as determined by qPCR and the prognoses of the patients by using the GraphPad Prism 5 software program (GraphPad software, La Jolla, CA). In the analysis, the copy number level was $\log_{10}$ transformed. If there were multiple blood specimens from a single patient, the specimen that contained the highest value was selected. A significant difference was considered to be present for any P value of <0.05.

Ethics statement. The clinical specimens for this study were used after obtaining informed consent from the patients themselves (for those who survived) or their responsible family members (for those who died). All of the protocols and procedures were approved by the research and ethics committees of the National Institute of Infectious Diseases (no. 489).

RESULTS

Development of the cvPCR assay and application for the diagnosis of SFTS. The cvPCR method, which adopted a one-step RT-PCR technique to facilitate the procedure, is targeted to the SFTSV NP gene in the S segment of the genome. As a positive control, a plasmid containing the target sequence, which was artificially modified by producing longer PCR products (the expected sizes for primer sets 1 and 2 are 584 bp and 587 bp, respectively) than those from the authentic viral genome (Fig. 1). In addition, the products were able to be digested by EcoRI (the expected sizes for primer set 1 are 347 bp and 237 bp, and those for primer set 2 are 316 bp and 271 bp) (Fig. 1). These characteristics of the method help to avoid false positives due to contamination with the positive control.

To determine the limit of detection of the cvPCR method, 10-fold serial dilutions of the SFTSV HB29, YG1, and SPL005 strains mixed with serum from a healthy donor were prepared. The purified viral RNA samples from each of the dilutions, which contained from 10^3 to 10^{-2} $TCID_{50}$s of the virus genome per reaction, were loaded in the cvPCR reaction mixture. The viral RNA of each strain was detected until the lowest 10^0 $TCID_{50}$/reaction with cvPCR using primer set 2, indicating that ≥125 $TCID_{50}$ of SFTSV in a 1-ml blood specimen can be detected (Fig. 1B and C). Clinical specimens collected from a total of 108 suspected SFTS patients were tested for the SFTSV genome by the cvPCR method. If positive, the amplified PCR products were confirmed by the determination of the SFTSV nucleotide sequences. The isolation of SFTSV from some of the PCR-positive patient sera was attempted. With the exception of the sera expected to contain small amounts of the virus based on the qPCR results, SFTSV was not successfully isolated from other samples (data not shown). As a result, 41 (17 patients who died, 24 who survived) out of these suspected patients were confirmed to have SFTSV infection by positive amplification of the SFTSV genome using the cvPCR method.

Establishment of the qPCR method. The amplification efficacy of qPCR targeted to the SFTSV NP, GPC, or RdRp gene was high, with PCR efficacy (E_{PCR}) values of >95% and coefficients of determination (r^2 values) of >0.997 (Fig. 2). These results indicated that the efficacies of all three qPCR strategies are close to the theoretical values (15).

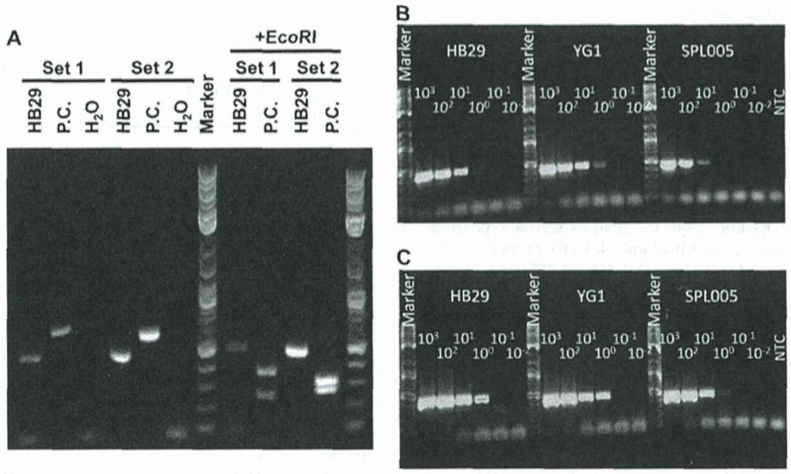

FIG 1 Establishment of the cvPCR method and the limits of detection. (A) Authentic SFTSV strain HB29 viral RNA (HB29), the plasmid pCR-SFTSV-posicon (P.C.), which contains the artificial sequence, and the nontemplate control (H_2O) were amplified by cvPCR using primer set 1 or 2. The PCR products were digested by *EcoRI* (+*EcoRI*). The sizes of the products were estimated by using a 2-log DNA ladder marker (NEB). (B and C) Isolated strains HB29, YG1, and SPL005, expanded in Vero cells, were diluted with serum from a healthy donor. The purified viral RNAs from the dilutions of virus-spiked serum were amplified. The resulting HB29, YG1, or SPL005 was amplified by primer set 1 (B) or 2 (C). The listed values are the dilutions of the viral strains. NTC, nontemplate control.

To determine the limits of detection of the qPCR methods, the SFTSV HB29, YG1, and SPL005 strains were used as described above in the cvPCR section. Based on the RNA copy number in the culture supernatant containing 1 $TCID_{50}$ of SFTS, the theoretical values ranging from 10^3 to 10^{-2} $TCID_{50}$ were plotted (Fig. 3A to C). The qPCR method using either of the primer-probe sets detected the viral RNA until the lowest 1 $TCID_{50}$/reaction, which is equivalent to 17.8 (HB29), 20.0 (YG1), and 8.3 (SPL005) copies/reaction (average, 15.4 copies/reaction) at the theoretical value (Fig. 3A to C). The performance was equivalent to that of cvPCR (Fig. 1B and C). These results suggest that qPCR also possesses high efficacy for detecting Japanese and Chinese SFTSV strains.

Multiplex qPCR methods for NP and GPC with the contamplicon probe were also established. The limits of detection of multiplex qPCR (Fig. 3D to F), which was tested using the viral RNA samples, as described previously, were almost identical to those of the singleplex qPCR (Fig. 3A to C).

The cross-reactivities of the cvPCR and qPCR methods with the other phleboviruses were tested. Samples with 6×10^3 $TCID_{50}$/reaction of RVFV, 1×10^5 $TCID_{50}$/reaction of Forecariah virus, and 1×10^6 copies/reaction of synthetic RNA of the L, M, and S segments of Heartland virus were not amplified by either of the PCR methods (data not shown).

These results indicate that the application of the multiplex qPCR would be more convenient for the detection of viral genomes while maintaining its diagnostic efficacy.

Validation of qPCR using specimens from patients suspected to have SFTS. The efficacies of cvPCR and qPCR for the detection of the SFTSV genome were evaluated using specimens from suspected SFTS patients. The results determined by cvPCR and qPCR were well correlated (Fig. 4). Only four, four, and two of the specimens measured by the primer-probe set targeting NP, GPC, and RdRp, respectively, showed results that conflicted with those of cvPCR (i.e., were cvPCR positive but qPCR negative, or vice versa). All of these specimens showing opposite results between cvPCR and qPCR were either cerebrospinal fluid or urine samples from patients with SFTSV genome amplification-positive serum samples, except for one specimen, which was a peripheral blood specimen collected from an SFTS patient 7 days after onset. The viral RNA was not detected from five out of seven urine specimens and one out of two cerebrospinal fluid samples, or if it was detected, the RNA level was low (i.e., $<10^4$ copies/ml), although around 10^5 to 10^8 copies/ml of viral RNA were circulating in the blood of these patients during the acute phase (Table 3). On the contrary, the SFTSV genome was always detected in the throat swab specimens taken from patients who were positive for SFTSV viremia. The cvPCR and qPCR results were 100% identical if the acute-phase blood specimens were used for validation.

To compare the limits of detection of the cvPCR and qPCR methods developed in this study with that of the qPCR method reported previously (6), the viral copy numbers in some of the clinical specimens were measured by the method reported here (Table 4). All of the specimens determined to be negative by the developed cvPCR and qPCR methods were also determined to be negative by the previously reported qPCR method. However, the SFTSV genome was not amplified in the specimens (i.e., 064A and 097A) by using the S primer-probe set of the reported method. In addition, although the RNA copy numbers of HB29 determined by each of the methods as a control were not significantly different, those of 062A1, 067A, and 082A determined by the previously reported qPCR method were significantly lower than those determined by the new qPCR method. In particular, the viremia levels in 062A1 and 067A were >10 times higher than those determined by the previously reported qPCR method, especially for the detection of the Japanese strains.

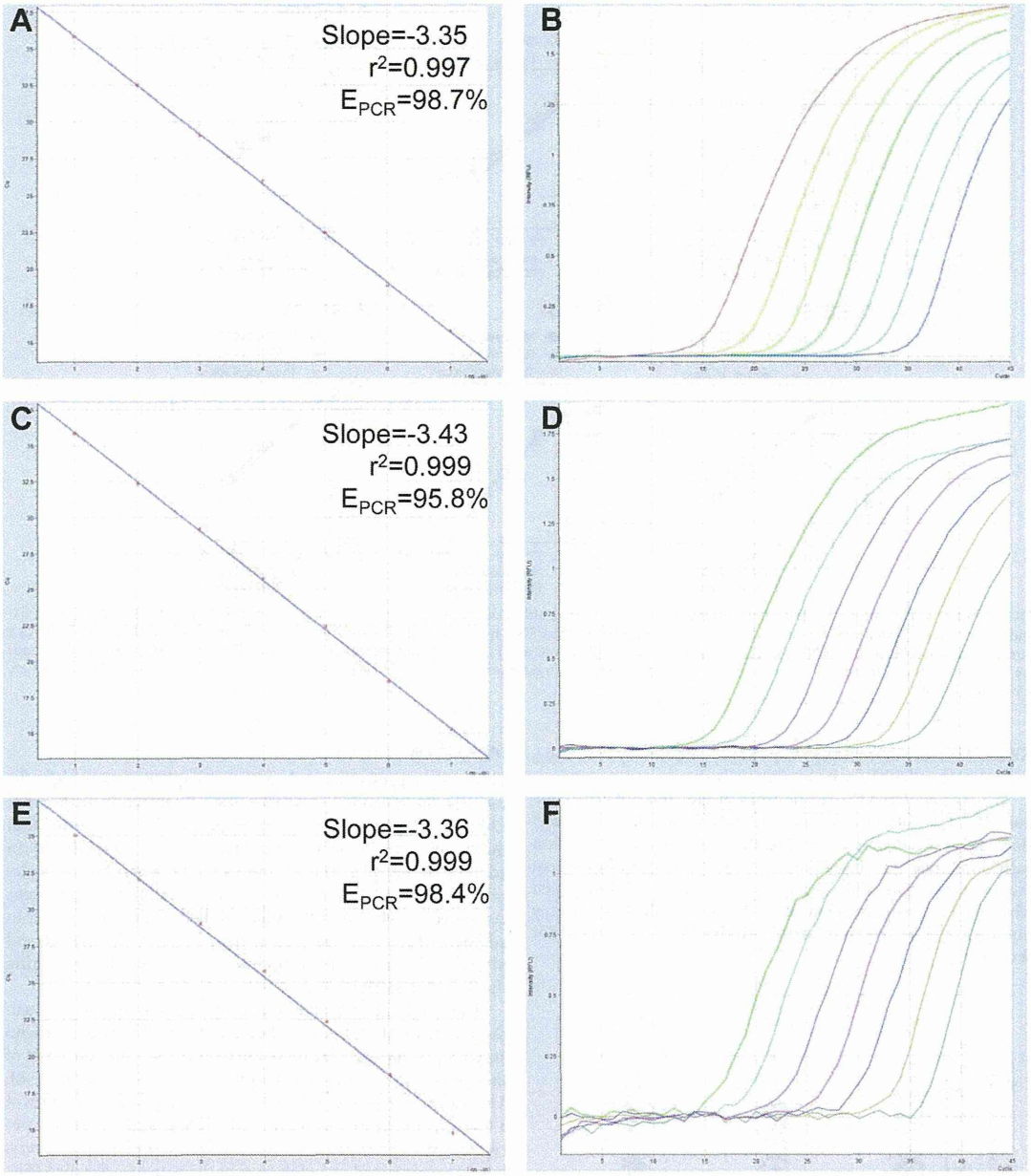

FIG 2 qPCR standard curves and amplification curves. The curves for NP (A and B), GPC (C and D), and RdRp (E and F) were derived from a dilution series of reference RNA. Values of the slope, correlation coefficient (r^2), and PCR efficacy (E_{PCR}) were calculated.

Viral copy number and patient outcome. To examine if the viremia level in the peripheral blood of SFTS patients is relevant to their survival outcome, the viral copy number in the blood specimens as determined by qPCR and the prognoses of the patients were analyzed. There were statistically significant differences in the copy numbers between the patients with fatal and nonfatal outcomes (Fig. 5A). The mean viral copy numbers in the patients who died and survived (6.4 versus 5.2 for NP, 6.7 versus 5.4 for

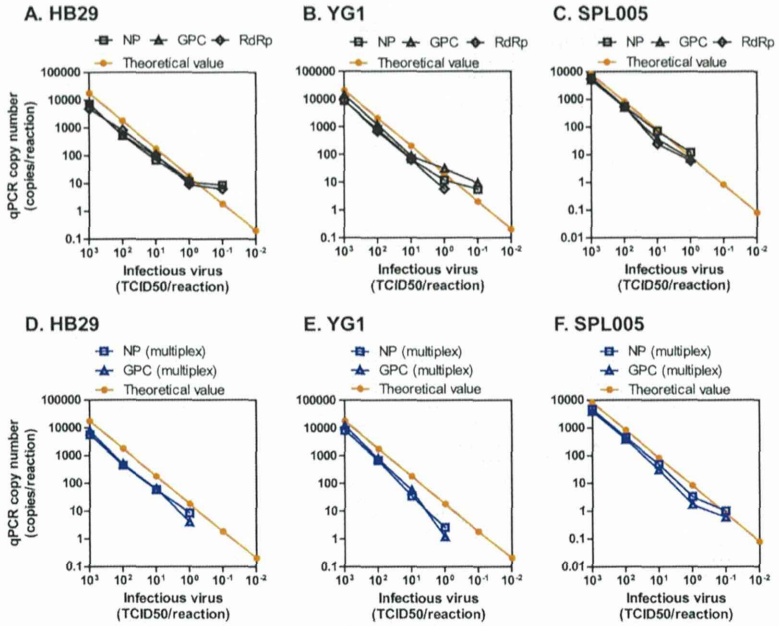

FIG 3 Limits of detection of the singleplex or multiplex qPCR to detect either the Chinese or the Japanese strain of SFTSV. Isolated strains HB29, YG1, and SPL005 were diluted with serum as described in Fig. 1, and purified RNA was amplified. The singleplex (A to C) or multiplex (D to F) qPCR copy numbers at each dilution of HB29 (A and D), YG1 (B and E), or SPL005 (C and F) that were detected by NP (square), GPC (triangle), or RdRp (diamond) are shown as dots and lines. The theoretical copy number of each strain estimated by undiluted viruses whose viral titers ($TCID_{50}$/ml) were known are shown in orange.

GPC, 6.4 versus 5.0 for RdRp, respectively) differed by >1.2 $\log_{10}$ copies/ml for all of the primer-probe sets.

We then analyzed the relationship of the viral NP gene copy number in the blood with the collection day (time after onset) (Fig. 5B). It was demonstrated that the average copy numbers on each blood collection day of the surviving patients were lower than those of the patients who died for ≥3 to 10 days after onset. Even when the data were analyzed using the GPC or RdRp gene copy numbers, the results indicated a similar tendency (data not shown).

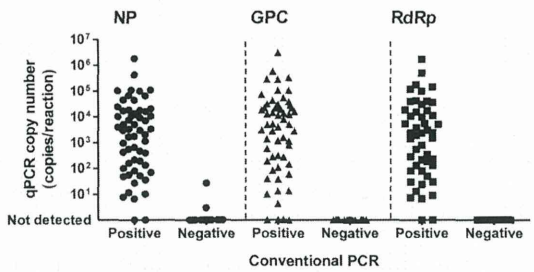

FIG 4 Agreement of cvPCR and qPCR results validated by clinical specimens from patients with suspected SFTS. The results (SFTSV positive or negative) determined by cvPCR (*x* axis) and the viral RNA copy number determined by qPCR (*y* axis) from each specimen are plotted as dots.

DISCUSSION

This study suggests that both cvPCR and qPCR possess high efficacy for the diagnosis of SFTS caused by infection with either Chinese or Japanese SFTSV strains, because it was reported that the viremia level in the SFTS patients was $>10^3$ copies/ml (6). In addition, serum/plasma is the most suitable matrix for the detection of the SFTSV genome, since the serum/plasma specimens contained a higher viral RNA load than the other specimens. In addition, there was 100% agreement between the cvPCR and qPCR results in the serum specimens that were collected during the first 10 days after onset.

There were significant differences in the viral copy numbers in the peripheral blood between the patients who died and those who survived, even when the number of days elapsed after the onset of the disease was not considered for the statistical analysis (Fig. 5A). The reason for this is that the viral copy number was maintained at a low level in patients who survived SFTS, at least during the period beginning 3 days after onset and lasting until their recovery, but was maintained at a high level in the patients who died (Fig. 5B). Although there were previously conflicting findings about the relevance of the viral copy number in patient blood samples with regard to their survival outcome (9, 10), our study confirmed that the SFTS viral RNA level, even the Japanese lineage, in patient blood samples is associated with their prognosis. The small number of cases may be a reason for the results reported by Gai et al. (10) that did not find any significant relationship between these

TABLE 3 Results of various specimens from patients confirmed to have had SFTS

Patient ID[a]	Outcome	Specimen source	Collection time (days after onset)	cvPCR result[c]	The $\log_{10}$ viral RNA copy no. (in 1 ml of specimen) of[b]:		
					NP	GPC	RdRp
010	Died	Serum	5	+	6.41	6.82	6.33
		Cerebrospinal fluid	5	+	–	–	3.05
067	Survived	Serum	6	+	6.75	7.13	6.72
		Urine	6	–	–	–	–
084	Died	Serum	5	+	8.35	8.57	8.32
		Throat swab	8	+	7.13	7.54	7.09
087	Survived	Serum	5	+	7.10	6.19	7.34
		Cerebrospinal fluid	11	+	2.99	–	2.92
		Throat swab	5	+	3.84	3.70	4.00
		Urine	5	+	–	–	–
089	Died	Serum	3	+	6.48	6.60	6.71
		Urine	3	–	2.57	–	–
097	Survived	Serum	6	+	5.08	5.69	4.32
		Throat swab	6	+	4.43	3.67	2.70
		Urine	6	–	–	–	–
		Serum	9	+	2.92	3.23	–
		Urine	9	–	–	–	–
104	Died	Serum	3	+	5.69	5.79	6.01
		Throat swab	3	+	3.16	3.21	3.29
		Urine	3	+	3.65	2.73	3.07
108	Died	Serum	3	+	6.93	6.96	7.15
		Urine	4	+	3.93	–	–

[a] ID, identification.
[b] NP, nucleoprotein; GPC, glycoprotein; RdRp, RNA-dependent RNA polymerase.
[c] +, detected; –, not detected.

two factors. Since several other significant prognostic indicators (e.g., cytokine levels, chemokine levels, platelet counts, lactate dehydrogenase [LDH] levels, and aspartate transaminase [AST] levels) have already been reported (9, 10, 16, 17), the combined use of the viral RNA level with these indicators may provide more substantial information that can be used to select the therapeutic strategy and predict patient prognosis.

Interestingly, all of the throat swabs collected from patients who were confirmed to have viral RNA in their serum contained a detectable amount of the viral RNA (Table 3). This suggests that it may be possible to develop a rapid and simple diagnostic system for SFTS based on immunochromatography, such as those widely used for the identification of influenza (18).

On the other hand, it was very hard to detect the viral RNA

TABLE 4 Comparison of the PCR methods

Specimen ID[a]	Reported qPCR method ($\log_{10}$ copies/ml of specimen) for segment:				cvPCR result for[b]:		qPCR result ($\log_{10}$ copies/ml of specimen):				
	S	M	L	Mean	Set 1	Set 2	NP	GPC	RdRp	Mean	*P* value[c]
057A	5.02	5.75	5.60	5.46	+	+	6.23	6.57	5.24	6.01	NS[d]
062A1	4.61	4.87	4.81	4.76	+	+	5.62	6.14	5.58	5.78	<0.01
064A	–	3.53	3.62	3.57	+	+	4.01	4.54	3.57	4.04	NS
067A	5.42	5.70	5.61	5.58	+	+	6.75	7.13	6.72	6.86	<0.001
078A3	3.67	4.15	4.11	3.98	+	+	4.69	5.23	4.46	4.79	NS
082A	4.76	4.89	4.89	4.85	+	+	5.81	5.99	5.47	5.76	<0.05
090A	5.28	5.57	5.51	5.45	+	+	6.06	6.46	6.14	6.22	NS
097A1	–	4.86	4.43	4.64	+	+	5.08	5.69	4.32	5.03	NS
076A	–	–	–		–	–	–	–	–		
079A	–	–	–		–	–	–	–	–		
081A	–	–	–		–	–	–	–	–		
092A	–	–	–		–	–	–	–	–		
093A	–	–	–		–	–	–	–	–		
094A1	–	–	–		–	–	–	–	–		
HB29 (2×10^5 $TCID_{50}$/ml)	6.01	6.13	6.02	6.05	+	+	6.46	6.48	6.38	6.44	NS

[a] ID, identification.
[b] +, detected; –, not detected.
[c] *P* value was computed using a one-way ANOVA with Bonferroni's multiple-comparison test.
[d] NS, not significant.

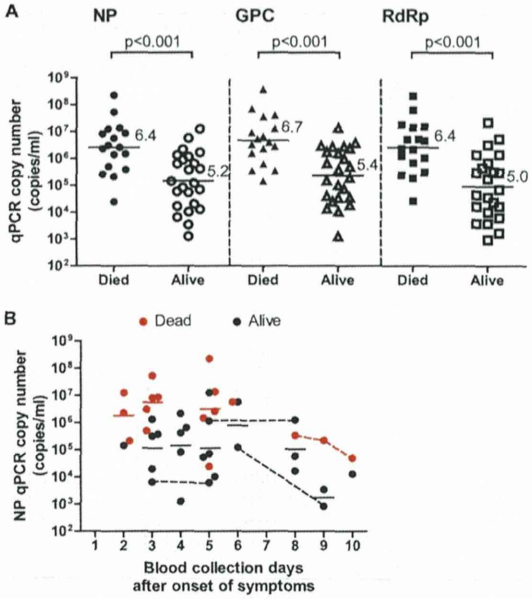

FIG 5 Relationship between the viral RNA level in the SFTS patient blood samples and the patient survival outcomes. (A) Viral RNA copy numbers in the blood samples from SFTS patients are plotted as dots and arranged based on the patients' survival outcomes. The mean of each group is indicated by a horizontal bar and the value. One-way ANOVA with Bonferroni's multiple-comparison test was used to determine the level of statistical significance. The calculated *P* values are shown above the groups that were compared. (B) Kinetics of the viral NP RNA copy number in the blood specimens after the onset of symptoms. The copy numbers in the blood specimens from SFTS patients who died (red) or survived (black) are plotted as dots for each of the collection days (days after the onset of symptoms). Horizontal solid lines for each day indicate the mean RNA copy number in the blood specimens collected from patients who died (red) or survived (black). The dots connected by dashed lines indicate specimens collected from the same patients.

from urine and cerebrospinal fluid samples (Table 3). It was previously reported that some patients exhibit neurological symptoms (1, 3, 4, 17). However, we tested cerebrospinal fluid samples from only two patients, so further studies are needed to determine whether the nervous tissue is a major site of virus multiplication. In addition, there was a possibility that blood contamination, especially in the cerebrospinal fluid samples, may have occurred during the collection.

The present study included only one Chinese strain (HB29) for the evaluation. However, our PCR systems detected a Japanese SFTSV strain that clustered in the Chinese genotype (data not shown). In addition, the PCR primer specificity for the SFTSV genome and cross-reactivities within SFTSV strains were checked using the Primer-BLAST Web-based program.

In summary, we established cvPCR and qPCR methods that were able to detect SFTSV lineages distributed in both China and Japan. Both the cvPCR and qPCR methods were validated as being comparable in terms of their sensitivity and specificity for the detection of SFTSV. In addition, it was confirmed that the qPCR method developed in this study has a lower limit of detection than the qPCR method reported in previous studies, especially for the detection of Japanese strains. The cvPCR likely would be useful for phylogenetic analysis following a definite diagnosis and sequencing. In addition, cvPCR is much less expensive than qPCR. The qPCR method has the advantage that it is possible to know the kinetics of the SFTSV circulating in patients and to determine their prognosis during the acute phase.

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Development and validation of serological assays for viral hemorrhagic fevers and determination of the prevalence of Rift Valley fever in Borno State, Nigeria

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Background: Rift Valley fever (RVF) is endemic to the tropical regions of eastern and southern Africa. The seroprevalence of RVF was investigated among the human population in Borno State, Nigeria to determine the occurrence of the disease in the study area in comparison with that of Lassa fever and Crimean-Congo Hemorrhagic fever.

Methods: Recombinant nucleoprotein (rNP)-based IgG-ELISAs for the detection of serum antibodies against RVF virus (RVFV), Lassa fever virus (LASV), and Crimean-Congo hemorrhagic fever virus (CCHFV) were used to test human sera in Borno State, Nigeria. The presence of neutralizing antibody against the RVFV-glycoprotein-bearing vesicular stomatitis virus pseudotype (RVFVpv) was also determined in the human sera.

Results: Of the 297 serum samples tested, 42 (14.1%) were positive for the presence of RVFV-IgG and 22 (7.4%) and 7 (2.4%) of the serum samples were positive for antibodies against LASV and CCHFV, respectively. There was a positive correlation between the titers of neutralizing antibodies obtained using RVFVpv and those obtained using the conventional neutralization assay with the attenuated RVFV-MP12 strain.

Conclusions: The seroprevalence of RVF was significantly higher than that of LASV and CCHF in Borno State, Nigeria. The RVFVpv-based neutralization assay developed in this study has the potential to replace the traditional assays based on live viruses for the diagnosis and seroepidemiological studies of RVF.

Keywords: Nigeria, Rift Valley fever, Seroprevalence

Introduction

Rift Valley fever virus (RVFV) is a zoonotic mosquito-borne virus belonging to the genus *Phlebovirus* in the Family *Bunyaviridae*. It causes severe diseases in humans and livestock throughout Africa[1] and the Arabian Peninsula[2]. RVFV is also considered to be a potential bioterrorism agent. In the last few decades, Rift Valley fever (RVF) outbreaks have been reported in eastern and southern Africa (e.g. Kenya, Somalia, United Republic of Tanzania, Madagascar and South Africa).[3–7] In contrast, there have been very few reports on the recent occurrence of RVF in western and central Africa. Significant high- and low-prevalence clusters of RVF in sub-national areas on the African continent have been reported.[8] Since the spread of RVFV largely depends on the mosquito vectors and the translocation of animal hosts, an endemic situation usually occurs in the restricted geographical areas inhabited by their hosts and vectors. In Nigeria, RVFV antibodies have been found in sheep, goats, cattle, horses and camels in the northern states of Kaduna and Sokoto[9] and in the plateau area[10] suggesting that the virus may be enzootic in Nigeria. In addition, serological studies conducted on human sera have confirmed the existence of the disease in Nigeria.[11] The specific geographical location of Borno State in northeastern Nigeria, which shares international borders with three other African countries (Cameroun, Chad and Niger), makes it vulnerable to the transboundary spread of various diseases, including viral hemorrhagic fevers (VHFs). In addition, Borno State has been reported as the niche for Lassa fever virus (LASV) and possibly other VHFs. However, the epidemiology of RVF and other VHFs has not been extensively investigated in Borno State. A detailed and accurate investigation of the seroprevalence is necessary to ascertain the occurrence and spread of RVF in this area.

RVFV possesses a single-stranded tripartite RNA genome composed of three segments: S, M and L. The S segment encodes the nucleocapsid protein (NP) and non-structural (NS) protein, using an ambisense strategy. The M segment encodes the precursor for the glycoproteins Gn and Gc and two non-structural proteins of 78 kDa and 14 kDa. The L segment encodes the L protein.[12] The nucleotide sequence of the NP gene is highly conserved among various RVFV strains.[13] Serum antibodies against NP are readily detected early after infection and in convalescent individuals, providing a basis for the diagnosis of RVF.[14,15]

The traditional diagnostic assays for VHFs are based on immunoassays that use live viruses as the source of capture antigens. The use of highly attenuated RVFV (RVFV-MP12) does not require stringent biosafety measures and could readily be adopted in laboratories in developing countries where infrastructures for biosafety level 3 or 4 containments are lacking. The usefulness of recombinant viral nucleoprotein (rNP)-based serological assays, such as IgG-ELISAs and immunofluoresence assays (IFAs) for the detection of antibodies against VHFs such as Crimean-Congo hemorrhagic fever virus (CCHFV) and LASV have been reported.[16–18] Recombinant protein technology does not require high containment biosafety facilities and could readily meet the demand for a simple and reliable system not only for diagnosis of VHFs but also for comparative seroepidemiology of various VHFs in a cohort study.

In this study, the seroprevalence of RVFV infection in humans in Borno State, Nigeria, was determined using rNP-based IgG ELISAs, and the prevalence of RVFV antibody was compared with those of other hemorrhagic fever virus infections including LASV and CCHFV. In addition, we developed virus neutralization assays using vesicular stomatitis virus (VSV) pseudotype virus-bearing glycoproteins of RVFV, and the usefulness of the VSV pseudotype system was determined for a high throughput screening of neutralizing antibodies against RVFV.

Materials and methods

Serum samples

Two hundred and ninety-seven serum samples were collected between September 2011 and February 2012 from patients attending health facilities (government hospitals, private hospitals or clinics) in 10 out of the 27 local government areas (LGAs) in Borno State in northern Nigeria. A simple random sampling technique was used to obtain human sera from the selected LGAs, which consisted of at least three LGAs from each of the three senatorial districts (North, Central and South), and also from the town of Lassa.

Expression and purification of rNPs

Insect Tn5 cells[19] were infected with recombinant baculoviruses expressing rNPs of RVFV, LASV or CCHFV to produce recombinant His-tagged RVFV-rNP, LASV-rNP or CCHFV-rNP, respectively.[16,17,19] The rNPs were purified by Ni^{2+} column chromatography (QIAGEN GmbH, Hilden, Germany), as described previously.[17] The negative control antigen (ΔP) was prepared from a baculovirus (Ac-ΔP) that lacks polyhedrin expression using the same protocols as for the rNPs. Expression of the His-tagged rNPs and ΔP was analyzed by SDS-PAGE gels (12% polyacrylamide) stained with Coomassie blue (Bio-Rad Laboratories, Hercules, CA, USA) (Supplementary Figure 1).

IgG-ELISA

IgG-ELISA was performed as described previously.[16] Briefly, 96-well ELISA plates were coated with the predetermined optimal quantity of purified RVFV-rNP, LASV-rNP or CCHFV-rNP (approximately 100 ng/well) at 4°C overnight. Each well of the plates was then covered with 200 μl of PBS containing 5% skim milk and 0.05% Tween 20 (Sigma, St. Louis, MO, USA) (PBST-M), followed by incubation for 1 h at 37°C for blocking. The plates were washed three times with PBS containing 0.05% Tween 20 (PBST) and then inoculated with test serum (100 μl/well), which was diluted 1:400 and 1:1600 with PBST-M. After a 1 h incubation period, the plates were washed three times with PBST and then were inoculated with goat anti-human IgG antibody labeled with HRP (1:1000 dilution; Zymed Laboratories, Inc., South San Francisco, CA, USA). After a further 1 h incubation period, the plates were washed and 100 μl of ABTS solution (Roche Diagnostics, Mannheim, Germany) was added to each well. The plates were incubated for 30 min at room temperature, and the optical density at 405 nm (OD_{405}) was measured against a reference of 490 nm. The adjusted OD_{405} value was calculated by subtracting the OD_{405} value of the negative Ag-coated wells from that of the corresponding wells. The mean plus three standard deviations (mean+3SD) of the ELISA indices for the IgG-ELISAs was calculated using human serum samples that were confirmed to be negative for infection with the respective viruses by IFA (data not shown) and was used as the cut-off value for the IgG-ELISAs. In order to minimize false-positive results that could occur with single serum dilution, IgG response was considered to be positive if the sample showed adjusted OD_{405} values above the cut-off at both 1:400 and 1:1600 dilutions.

Conventional neutralization assay

The conventional neutralization assay using infectious RVFV (RVFV-MP12 strain) was performed as described previously.[20] Briefly, heat-inactivated serum samples were diluted three-fold (from 1:40 to 1:1080) with Eagle's minimum essential medium (MEM, Sigma, St. Louis, MO, USA) containing 2% FBS (Invitrogen, Carlsbad, CA, USA). Each test sample (50 μl) was then mixed with the same volume of RVFV-MP12 at an infectious dose of 100 plaque forming units. The mixture was then incubated for 1 h at 37°C for neutralization. After incubation, the mixtures were tested for neutralization by the cytopathic effect inhibition assay using Vero E6 cells.[20] The neutralization antibody (NAb) titer was defined as the reciprocal of the highest dilution at which no cytopathic effect was observed.

Generation of VSV pseudotyped with RVFV-glycoprotein

The glycoprotein (GP) cDNA of RVFV-MP12 was cloned into the pKS336 vector[17] to construct an RVFV-GP expression plasmid, designated as pKS336-RVFV-GP. To generate the RVFV-GP-bearing VSV pseudotype (RVFVpv), a *G-VSVΔG/luc encoding luciferase gene (kindly provided by Dr. M. A. Whitt), instead of the VSVG gene, was inoculated into human kidney 293 T cells[21] transfected

with pKS336-RVFV-GP. After 24 h the culture supernatants were collected and used as a working seed for the RVFVpv.

Neutralization test using RVFVpv

The dilution of RVFVpv used was calculated to produce approximately 10^5 relative light units in control wells. Serum samples were mixed with RVFVpv at a dilution of 1:50 in MEM (Sigma) supplemented with 2% FBS (Invitrogen). Then, the mixture was incubated at 37°C for 1 h for neutralization. The serum-RVFVpv mixture was transferred to 96-well plates containing monolayers of Vero E6 cells.[20] After 24 h the cells were lysed, and the luciferase activities were measured using the Bright-Glo Luciferase Assay System (Promega, Madison, WI, USA) according to the protocol recommended by the manufacturer.

Statistical methods

The sensitivity, specificity and predictive values for positive and negative tests were calculated by standard methods. Spearman's rank correlation coefficient test, ROC curves and two-graph-ROC (TG-ROC) curves[22,23] were analyzed using the Stat Flex ver. 5 software program (Artech Co. Ltd., Osaka, Japan).

Results

rNP-based IgG-ELISA

In order to determine the seroprevalence of RVFV in humans in Borno State, Nigeria, sera were first subjected to an His-RVFV-rNP-based IgG-ELISA. An IgG response was considered to be positive if the sample had adjusted OD_{405} values higher than cut-off values at both 1:400 and 1:1600 dilutions. Of the 297 serum samples analyzed, 42 (14.1%) were positive for RVFV IgG (Table 1). Simultaneously, the serum samples were also tested for the presence of antibodies against LASV and CCHFV using the rNP-based IgG ELISAs, and a total of 22 (7.4%) and 7 (2.4%) of the samples were positive for antibodies against LASV and CCHFV, respectively (Table 1). The result indicated a high prevalence rate of RVF in the study area.

In order to confirm the efficacy of RVFV-rNP as an antigen and to determine the sensitivity and specificity of the RVFV-rNP based ELISA, the NAb assay was performed using RVFV-MP12. Of the 271 human serum samples examined, 34 (12.6%) were positive for NAb, with antibody titers ranging from 40 to 1080 (Table 1). Thirty-one out of 34 (sensitivity: 91.2%) NAb-positive samples were also positive in the IgG-ELISA, and 229 out of 237 (specificity: 96.6%) NAb-negative samples were also negative in the IgG-ELISA.

Neutralization assay with the VSV-based RVFV pseudotype

Virus neutralization assays using VSV pseudotype-bearing glycoproteins of viruses causing VHFs have been developed with high sensitivity and specificity.[24] In order to determine whether a VSV-based RVFV pseudotype can be applied for the screening of NAbs against RVFV infection, we produced RVFVpv and then determined whether the human sera collected from the study area could neutralize its infectivity. Single serum dilution (1:50) assay rather than titration by endpoint dilutions was performed in order to establish a high-throughput screening of NAbs. Since the infectivity of the RVFVpv, harboring the luciferase gene, could be ascertained by determining its luciferase activity, the neutralization activities of the sera were represented as the percent neutralization calculated from the reduction in the luciferase expression. Of the 278 serum samples tested, 43 (15.5%) showed more than 75% luciferase activity neutralization, and the remaining 235 (84.5%) showed less than 75% neutralization, compared with the non-serum control (Table 1). The sensitivity of the RVFVpv-based neutralization assay was determined by comparing the results with those obtained from the conventional neutralization assay using RVFV-MP12. All 34 serum samples that tested positive in the neutralization assay with RVFV-MP12 showed more than 50% neutralization of RVFVpv (Table 2). Furthermore, of the 211 serum samples that had less than 50% neutralization, none (0%) were positive for the neutralization assay with RVFV-MP12 (Table 2).

The ROC and TG-ROC analyses were performed in order to select cut-off points for the percent neutralization using RVFVpv (Figure 1). If the cut-off was defined as the intersection point of

Table 1. Summary of the results of the IgG ELISA (n=297), serum neutralization antibody (NAb) assay using VSV-RVFV-pV (n=278) and NAb assay using RVFV-MP12 (n=271) to determine seroprevalence of RVFV in humans in Borno State, Nigeria

	IgG-ELISA[a]			NAb assay	
	RVFV-rNP ELISA	LASV-rNP ELISA	CCHFV-rNP ELISA	RVFVpv NAb[b]	RVFV-MP12 NAb
No. positive (%)	42 (14.1)	22 (7.4)	7 (2.4)	43 (15.5)	34 (12.6)
No. negative (%)	255 (85.9)	275 (92.6)	290 (97.6)	235 (84.5)	237 (87.4)
Total	297 (100)	297 (100)	297 (100)	278 (100)	271 (100)

CCHFV-rNP: Crimean-Congo hemorrhagic fever virus recombinant nucleoprotein; LASV-rNP: Lassa fever virus recombinant nucleoprotein; NAb: serum neutralization antibody; RVFV: Rift Valley fever virus; RVFV-MP12: highly attenuated MP12 strain of RVFV; RVFV-rNP: RVFV recombinant nucleoprotein; RVFVpv: RVFV-glycoprotein-bearing vesicular stomatitis virus pseudotype.
[a] IgG response considered positive if the sample had a positive titer at both 1:400 and 1:1600 dilutions.
[b] >75% inhibition considered positive.

the sensitivity and specificity curves, the cut-off value was 75% neutralization, and the sensitivity and specificity were 94% and 95%, respectively. If the cut-off was defined as 50% neutralization, the sensitivity would increase to 100%, but specificity would decrease to 89% (Figure 1). There was a positive correlation between the titers of neutralizing antibodies obtained using RVFVpv and those obtained using the conventional neutralization assay with RVFV-MP12. Spearman's rank correlation coefficient (r_s) was calculated to be 0.77 (Figure 2). Thus, NAbs against RVFV can be screened by the RVFVpv-based neutralization assay using the single serum dilution format.

Table 2. The relationship between the results of the authentic virus (RVFV-MP12 strain)-based and RVFVpv-based neutralization assays (n=270)

NAb for RVFV-MP12	NAb for RVFVpv % Neutralization		
	>75	50–75	<50
Positive	32 (11.9%)	2 (0.7%)	0 (0%)
Negative	11 (4.1%)	14 (5.2%)	211 (78.1%)
Total	43 (15.9%)	16 (5.9%)	211 (78.1%)

NAb: serum neutralization antibody; RVFV-MP12: highly attenuated MP12 strain of Rift Valley fever virus; RVFVpv: RVFV-glycoprotein-bearing vesicular stomatitis virus pseudotype.

Discussion

In this study, the seroprevalence of RVF was investigated among human population in Borno State, Nigeria to determine the occurrence and spread of the disease in comparison with those of Lassa fever and CCHF. Since recombinant protein-based immunoassays with high sensitivity and specificity have been demonstrated to be useful for the diagnosis of VHFs in humans,[15–17,25,26] we have used rNP-based IgG-ELISAs for the detection of serum antibodies against RVFV, LASV, and CCHFV. Of the 297 serum samples tested for RVFV-IgG, a total of 42 (14.1%) showed positive results (Table 1). The antibody prevalence observed in this study is in agreement with the results of a surveillance study carried out in the 1980s, when 14.8% of the sera collected in more than 30 locations throughout Nigeria were found to be positive for a hemagglutination-inhibiting antibody against RVFV.[11] In this study a significant difference in the prevalence of antibodies was observed (RVFV rNP-ELISA [14.1%], LASV rNP-ELISA [7.4%] and CCHFV rNP-ELISA [2.4%]), and the highest prevalence was noted for RVFV rNP-ELISA antibody. In addition, more than 12% of the samples tested had NAb activity against RVFV. These results indicate that RVFV is more actively circulating in the study area compared with LASV and CCHFV. It is therefore important to undertake a risk assessment of RVFV infection in humans in Nigeria.

RVFV is transmitted through contact with body fluids from infected humans and animals or by mosquito bites and/or aerosols. The particular location of Borno State, with its shared geographical borders with three other African countries, indicates that a regional epidemiological study should be conducted not only in the LGAs in Nigeria, but also in the neighboring countries, to identify the possible risk factors for transboundary RVFV infection. The borders are porous and unrestricted human and animal

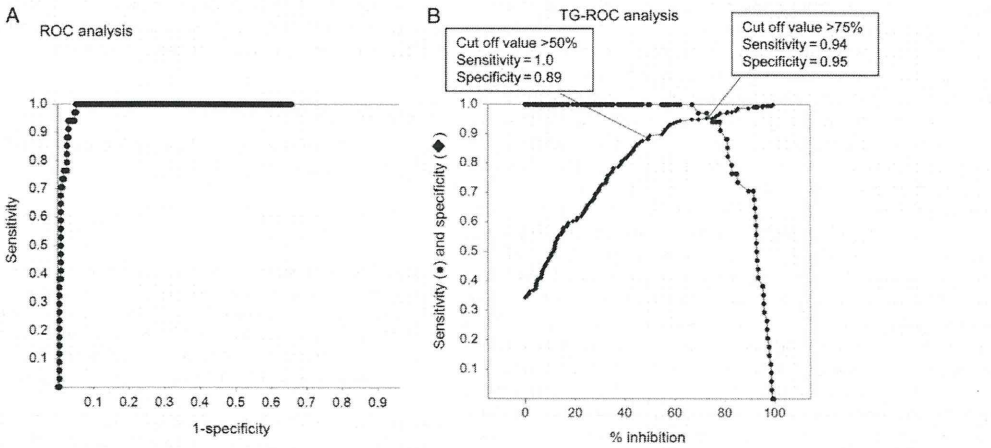

Figure 1. The results of the ROC and two-graph (TG)-ROC analyses of the vesicular stomatitis virus pseudotype virus-bearing glycoproteins of Rift Valley fever virus, (RVFVpv)-based neutralization assay. In (A) the ROC analysis graph, the specificity values are deducted from 1.0 in the x-axis, and the sensitivity vs 1-specificity data are plotted as dots. In (B) the TG-ROC analysis graph, sensitivity data are plotted as dots and specificity data are plotted as diamonds.

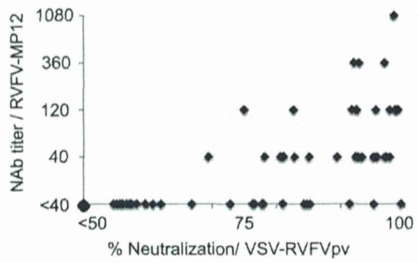

Figure 2. A comparison of the percent neutralization obtained from virus pseudotype virus-bearing glycoproteins of Rift Valley fever virus (RVFVpv)-based neutralization assay with the serum neutralization antibody (NAb) titers obtained from authentic virus (RVFV-MP12)-based neutralization assays. The data are plotted as diamonds on the scatterplot. Spearman's rank correlation coefficient (r_s) was calculated to be 0.77.

trafficking across borders is common throughout the year. In spite of the active circulation of RVFV observed in this study, no outbreak of the disease in humans or animals has been reported in the study area. This could be as a result of poor surveillance for the outbreak of the disease or a lack of expertise in disease recognition.[10,11] It is also possible that RVFV circulating in the region is a non-pathogenic strain. RVF in humans usually begins with a non-specific influenza-like acute fever, but can progress to serious hemorrhagic fever in some cases. VHFs, particularly Lassa fever, an acute viral hemorrhagic fever caused by LASV, has been reported to be endemic in Nigeria.[27,28] The symptoms of the disease also vary from a non-specific febrile illness to fatal viral hemorrhagic fever. Therefore, there is the possibility that patients with non-specific febrile illness caused by RVFV infection could be misdiagnosed with other endemic infectious diseases, such as malaria, typhoid fever or Lassa fever.

A VSV-based pseudotype system has been designed to enable the high-throughput screening of NAbs against viral infections.[29] We also developed a novel serum neutralization test using RVFVpv to detect serum antibody against RVFV. The reliability and usefulness of the assay were evaluated by comparing the results of the assay with those obtained from the widely used 'gold-standard' neutralization assay using infectious RVFV. Of the 43 serum samples that showed more than 75% neutralization by the RVFVpv-based assay, 11 were negative by the conventional RVFV-MP12 neutralization assay (Table 2). It is possible that the results of 11 negative samples were due to false-positive reactions in the VSV-RVFVpv-based assay. However, among these serum samples, one showed a higher OD_{405} value (0.443) than the cut-off OD_{405} value at 1:400 dilution, and another one was identified as a positive by the rNP-based ELISA, with OD_{405} values of 1.318 and 0.364 at the 1:400 and 1:1600 dilutions, respectively (Supplementary Table 1), indicating that the serum contained antibody to RVFV. Another possibility is that the VSV-RVFVpv-based assay was more sensitive than the standard neutralization assay using RVFV-MP12. This assumption is supported by the observation that the NAb titers measured using pseudotyped VSV bearing the GP of Nipah virus or SARS-coronavirus are higher than those obtained by using infectious viruses.[21,29,30]

The ROC and TG-ROC analyses indicated an appropriate cut-off value for the percent neutralization (75%) to demonstrate that the NAbs in the sera had high sensitivity and specificity. The conventional assay for measuring NAbs requires serial-dilutions of the test serum and takes several days for the virus to replicate to a level which results in plaque-forming or cytopathic effects in infected cells. However, the new assay based on RVFVpv uses a single serum dilution (1:50) and has a quantitative nature, where the luciferase activity can be determined one day after inoculation onto cells. Finally, pseudotyped VSVs do not produce infectious progeny viruses, thereby ensuring their safe use as diagnostic tools. Taken together, our results indicate that the RVFVpv-based assay for measuring NAb has safe, rapid and high-throughput diagnostic capabilities.

Our study has limitations, one of which is the relatively small sample size compared to the previous study, carried out in the 1980s, employing more than 3000 human sera from the different ecological zones in Nigeria.[11] In addition, we could not access detailed information on the subjects (age, profession, history of illness etc.). Although our data were obtained using the most recent serological procedures, the lack of demographic information on the study subjects makes it difficult to provide an advanced epidemiological understanding of VHFs in Nigeria. However, this study has important strengths: it provides information on RVFV infection with a high prevalence in human population in Borno State and demonstrates the usefulness of the VSV-based neutralization assay for the epidemiological investigations.

Conclusions

The results of rNP-based ELISA have shown that approximately 14% of the study population in Borno State, Nigeria, have a history of RVFV infection, and the seroprevalence of RVF was higher than those of other viral hemorrhagic fevers such as Lassa fever and CCHF. In addition, the RVFVpv-based NAb assay developed in this study has the potential to replace traditional assays based on live viruses for the diagnosis and seroepidemiological analysis of RVF in endemic and non-endemic countries.

Supplementary data

Supplementary data are available at Transactions Online (http://trstmh.oxfordjournals.org/).

Authors' contributions: DNB, FK and SSB conceived the study; SF and MS designed the study protocol; DNB, HT, TY, ST, KI and AF carried out the serological assays, and analysis and interpretation of the data. DNB and SF drafted the manuscript; MS, SM, MS and SSB critically revised the manuscript for intellectual content. All authors read and approved the final manuscript. SF and MS are guarantors of the paper.

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Original Article

Effects of Ribavirin on Severe Fever with Thrombocytopenia Syndrome Virus In Vitro

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SUMMARY: Severe fever with thrombocytopenia syndrome (SFTS) is a disease with a high case fatality rate that is caused by infection with the recently identified tick-borne SFTS virus (SFTSV), for which there are no specific countermeasures. We examined the effects of ribavirin and mizoribine, which are nucleoside analogue drugs with broad antiviral activities, on SFTSV proliferation in vitro. When 3 cell lines were treated with these drugs before and during infection with a Chinese SFTSV strain, the 99% effective concentrations (EC_{99}) of ribavirin were 19–64 μg/ml (78–262 μM); in contrast, the EC_{99} of mizoribine was >500 μg/ml (1,929 μM). Similar levels of inhibitory effects of ribavirin were observed with 4 Japanese SFTSV strains. However, when Vero cells were treated with ribavirin 3 days after inoculation, the inhibitory effect was dramatically decreased, indicating that ribavirin did not effectively reduce virus production in pre-infected cells. These results suggest that ribavirin could be used as post-exposure prophylaxis for the prevention of SFTS.

INTRODUCTION

Severe fever with thrombocytopenia syndrome (SFTS) is a recently-identified disease characterized by fever, gastrointestinal symptoms, thrombocytopenia, leukopenia, and elevated levels of liver enzymes in peripheral blood (1,2). Multiple organ failure and disseminated intravascular coagulation are often observed in severe cases (3). Its case fatality rate is approximately 12% (1). The causative agent of the disease is the SFTS virus (SFTSV) (family: *Bunyaviridae*, genus: *Phlebovirus*), the discovery of which was reported in 2011 (1,2). Although sporadic outbreaks of the disease have been recently found in Japan and South Korea, retrospective studies have indicated the emergence of SFTS in Japan in 2005 and in China in 2006 (4–6).

Epidemiology suggests that the transmission routes of SFTSV are tick bites and human-to-human transmission. The virus has been detected in several tick species including *Haemaphysalis longicornis* and *Rhipicephalus microplus*, which have been found in areas surrounding the places in which SFTS patients reside a substantial number of whom had a history of tick bites (1,2,7). Although not common, direct human-to-human transmission through close contact with virus-containing blood or excreta has also been reported (4,8–11).

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a guanosine analogue with broad antiviral activities, is effective in the treatment of Lassa fever (12) and hepatitis C when administered in combination with other drugs (13). Ribavirin is also used in the treatment of Crimean-Congo hemorrhagic fever (CCHF) (14); however, its efficacy in CCHF treatment remains to be proven. Ribavirin exerts its antiviral effects through various mechanisms, including the reduction of viral RNA-dependent RNA polymerase activity, mutagenesis in the viral genome, inhibition of RNA capping, reduction of cellular inosine monophosphate dehydrogenase (IMPDH) activity, and modulation of the host immune response (15). The drug has been used in the treatment of some SFTS patients, but at the time of use, its effect was unknown (4,16). Mizoribine (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate) is an imidazole nucleoside that has been used as an immunosuppressive agent in Japan. Although mizoribine shows inhibitory effects in vitro against herpes simplex virus (17), respiratory syncytial virus (18), influenza virus (19), and severe acute respiratory syndrome virus (20), there have been no reports on mizoribine usage or its effects with regard to SFTS or SFTSV.

In the present study, we examined the effects of ribavirin and mizoribine on SFTSV in vitro to clarify the potential of these drugs as countermeasures against SFTS. When ribavirin treatment was initiated before virus inoculation, SFTSV proliferation dramatically reduced, suggesting the prospective use of ribavirin as a

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post-exposure prophylactic.

MATERIALS AND METHODS

Cells and viruses: Monkey kidney-derived Vero, human hepatoma-derived Huh7, and human osteosarcoma-derived U2OS cells were cultured at 37°C in DMEM (Sigma, St. Louis, MO, USA) supplemented with 5% heat-inactivated fetal calf serum (FCS) and antibiotics. Cells inoculated with SFTSV or Rift Valley fever virus (RVFV) were maintained in DMEM supplemented with 2% FCS and antibiotics. The Chinese SFTSV strain HB29 was provided by Drs. Mi-Fang Liang and De-Xin Li (National Institute for Viral Disease Control and Prevention, China). The Japanese SFTSV strains SPL004, SPL010, SPL030, and YG1 have been previously described (5). Propagation of SFTSV and RVFV (MP-12) was performed with Vero cells.

Virus titration: SFTSV was serially diluted and inoculated onto Vero cells in quadruplicates. After 3 days of culture, cells were fixed with 10% formalin and permeabilized with 0.1% Triton-X100. Cells were subjected to incubation with rabbit anti-SFTSV nucleocapsid (N) protein (5) followed by further incubation with Alexa Fluor 488-labeled anti-rabbit IgG (H + L) (Life Technologies, Gaithersburg, MD, USA). Observation was performed under a fluorescence microscope (BZ-9000, Keyence, Osaka, Japan), and viral titers were calculated as median tissue culture infectious dose per ml ($TCID_{50}$/ml) using the Reed-Muench method (21). The same protocol was applied for titration of RVFV, with mouse anti-RVFV N protein (clone G2-36) and Alexa Fluor 488-labeled anti-mouse IgG (H + L) being used as primary and secondary antibodies, respectively.

Effects of drugs on virus/cell proliferation: Ribavirin and mizoribine (Kindly provided from Yamasa-Shouyu Co., Choshi, Japan) were dissolved in phosphate-buffered saline at a concentration of 100 mg/ml and, for treating cells, were further diluted with cell culture medium at indicated concentrations. A drug concentration of 0 μg/ml indicates the treatment of cells with cell culture medium containing no drugs. Cells were pretreated with ribavirin or mizoribine at indicated concentrations for 1 h. One hundred $TCID_{50}$ of SFTSV or RVFV was added to the culture without the removal of the drugs and then incubated for 3 days. Culture supernatants were harvested and subjected to virus titration.

To examine the cytotoxicity of the drugs, cells were cultured for 3 days in the presence or absence of the drugs without virus inoculation, and cell viability was measured using the cell proliferation reagent WST-1 (Roche, Penzberg, Germany) according to the manufacturer's protocol. Cell viability was calculated as follows: (absorbance of cells in the presence of the drug − absorbance of no cells in the presence of the drug)/(absorbance of cells in the absence of the drug − absorbance of no cells in the absence of the drug) × 100 (%).

Effects of ribavirin in SFTSV preinfected cells: Cells were inoculated with SFTSV (100 $TCID_{50}$) and cultured for 3 days, and then culture media were replaced with drug-containing media. After another 3 days of culture, supernatants were harvested and titrated.

Statistics: A paired t-test was used for statistical analysis of the drugs' effects.

RESULTS

Effects of ribavirin and mizoribine on SFTSV proliferation: Fig. 1 shows viral titers obtained from monkey Vero cells, which were inoculated with the Chinese SFTSV strain (HB29) in the presence of ribavirin or mizoribine (0, 4, 20, 100, and 500 μg/ml). Although statistically significant inhibitory effects ($P < 0.05$) of ribavirin and mizoribine were observed at 20–500 μg/ml and 100–500 μg/ml, respectively, ribavirin showed a greater reduction of viral titers than mizoribine (Fig. 1A, B). Ribavirin was also found to profoundly affect HB29 proliferation in 2 human cell lines, Huh7 and U2OS (Fig. 1C–F). Reduction curves were used to calculate 99% effective concentrations (EC_{99}), the drug concentrations at which viral titers were reduced by 2 logs (Fig. 1). The EC_{99} of ribavirin, was 64 μg/ml (263 μM), 20 μg/ml (83 μM), and 19 μg/ml (78 μM) in Vero, Huh7, and U2OS cells, respectively. In contrast, no 99% inhibition was observed with mizoribine, even at the highest concentration of 500 μg/ml (1,929 μM; Table 1).

Similar results were obtained with the Japanese SFTSV strain SPL030 (Table 1). The 3 additional Japanese strains (SPL004, SPL010, and YG1) were tested to further confirm the effects of ribavirin. Huh7 cells were inoculated with 100 $TCID_{50}$ of each strain in the presence of 20 μg/ml of ribavirin and cultured for 3 days. Titers of SPL004, SPL010, and YG1 strains were reduced by 1.83, 1.83, and 2.25 log(s), respectively.

Cytotoxicity was examined by measuring cell viability after cell culture in the presence of the drugs. As shown in Fig. 2, >60% viability was maintained in all 3 cell types, even at 500 μg/ml.

Effects of ribavirin on RVFV proliferation: To speculate on the potential effects of ribavirin on SFTS/SFTSV in vivo, the drug's inhibitory effects on RVFV proliferation were examined. RVFV is another member of the *Phlebovirus* genus, for which both the in vitro and in vivo effects of ribavirin have been reported (22–24). Ribavirin reduced RVFV proliferation in a dose-dependent manner (Fig. 1G), and the EC_{99} in Vero cells was 50 μg/ml (207 μM; Table 1).

Effects of ribavirin in SFTSV preinfected cells: We next examined the potential of ribavirin for reducing virus production from cells that had been previously infected with SFTSV. Vero cells were inoculated with SFTSV and cultured for 3 days (the culture period during which most inoculated cells became SFTSV-positive [data not shown]). Culture media were then replaced with ribavirin-containing media (0–500 μg/ml). After another 3 days of culture, supernatants were harvested and titrated. As shown in Fig. 3, ribavirin displayed statistically significant effects at 100 and 500 μg/ml; however, the reduction at 500 μg/ml was only about 1 log.

DISCUSSION

In the presence of ribavirin, SFTSV proliferation was moderately reduced, and the inhibitory effects in in vitro experiments were not strain-specific (Fig. 1; Table

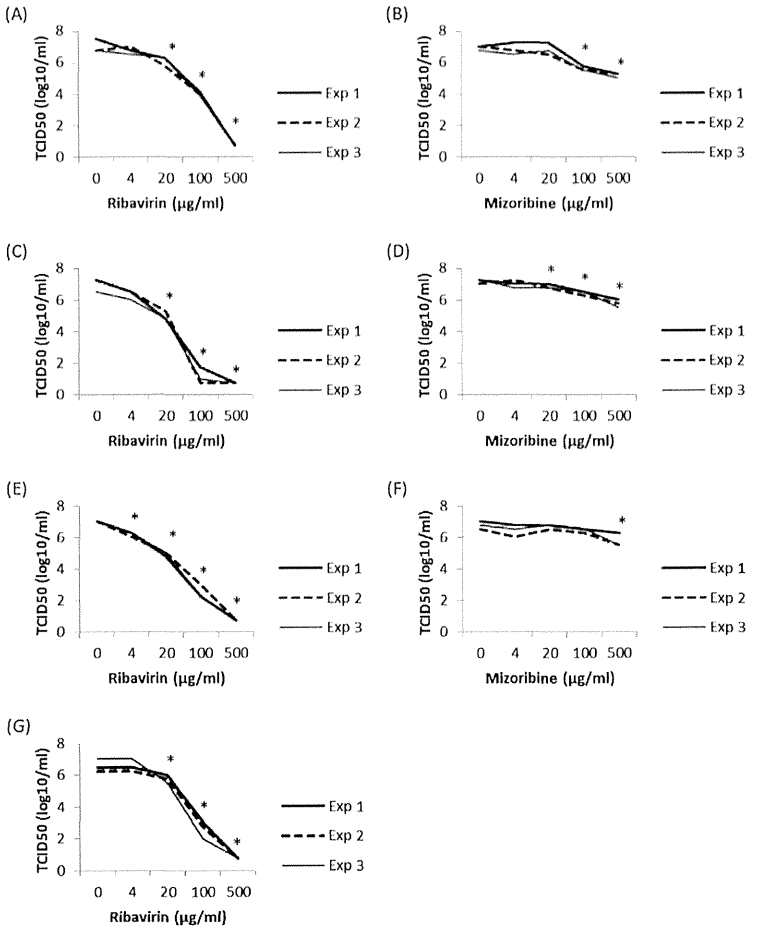

Fig. 1. Effects of ribavirin and mizoribine on SFTSV/RVFV proliferation. Vero cells (A, B), Huh7 cells (C, D), and U2OS cells (E, F) were inoculated with SFTSV HB29 strain in the presence of indicated concentrations of ribavirin (A, C, E) or mizoribine (B, D, F) and titers of supernatants 3 days after inoculation are shown. Vero cells were inoculated with RVFV in the presence of indicated concentrations of ribavirin. Titers of supernatants at 3 days after inoculation are shown (G). Results were obtained from 3 independent experiments. *, $P < 0.05$.

1; data not shown). Diversity among the nucleotide sequences of SFTSV strains reported from China, Japan, and South Korea is less than 5% (5,6), which suggests that ribavirin would likely show similar inhibitory effects against all SFTSV strains, regardless of areas from where the viruses have been isolated. Although both ribavirin and mizoribine have been shown to inhibit several viruses including herpes simplex virus, respiratory syncytial virus, influenza virus, and severe acute respiratory syndrome virus (17–20), mizoribine did not show an efficient inhibitory effect on SFTSV proliferation. The finding that effective doses of ribavirin against SFTSV were higher in Vero cells than in the other 2 cell lines (Table 1) is consistent with previous reports by Huffman et al. (25) and Peters et al. (23), in which the impact of ribavirin on several DNA/RNA viruses was compared in several cell lines. Given that the known functions of mizoribine are the inhibition of inosine monophosphate synthetase and guanosine monophosphate synthetase (26), other functions of ribavirin, such as viral polymerase inhibition, mutagenesis, RNA capping inhibition, and/or reduction of cellular IMPDH, might be critical in its effects on SFTSV proliferation.

To speculate on the potential effects of ribavirin on SFTS/SFTSV in vivo, the drug's inhibitory effects on RVFV proliferation (another member of the *Phlebovirus* genus) were examined in our assays and compared with those on SFTSV. Peters et al. reported that ribavirin at 23 μg/ml showed 2-log inhibition against RVFV in Vero cells (23). In animal models, ribavirin treatment (75–100 mg/kg/day) led to a 60%–75% survival rate in RVFV-inoculated mice (22–24), while it suppressed viremia in RVFV-infected monkeys (30 mg/kg/day, more than 2-log reduction of viral titers) (23). In the present study, the EC_{99} of ribavirin in Vero cells against SFTSV

Table 1. Effects of ribavirin and mizoribine on SFTSV/RVFV proliferation

Virus	Cell (origin)	Ribavirin, EC_{99}, μg/ml (μM)[1)]	Mizoribine, EC_{99}, μg/ml (μM)[1)]
SFTSV HB29	Vero (monkey)	64 ± 17 (263 ± 68)	>500 (1,929)
	Huh7 (human)	20 ± 5 (82 ± 20)	>500 (1,929)
	U2OS (human)	19 ± 2 (78 ± 6)	>500 (1,929)
SFTSV SPL030	Vero (monkey)	104 ± 22 (424 ± 88)	>500 (1,929)
	Huh7 (human)	15 ± 2 (63 ± 7)	>500 (1,929)
	U2OS (human)	19 ± 4 (73 ± 15)	>500 (1,929)
RVFV MP-12	Vero (monkey)	50 ± 12 (207 ± 68)	ND[2)]

[1)]: Data are mean ± SD from 3 independent experiments.
[2)]: Not done.

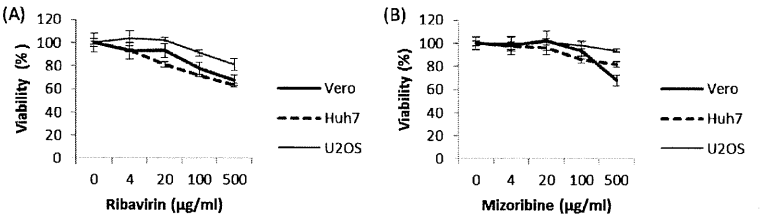

Fig. 2. Cytotoxicity of ribavirin and mizoribine. Viability of Vero cells cultured in the presence of ribavirin (A) or mizoribine (B) was measured. Cell viability was calculated as follows: (absorbance of cells in the presence of the drug − absorbance of no cells in the presence of the drug)/(absorbance of cells in the absence of the drug − absorbance of no cells in the absence of the drug) × 100 (%). Experiments were performed in triplicate and means ± standard deviation are shown.

(64–104 μg/ml, 2-log inhibition) suggests that SFTSV may be slightly less sensitive to ribavirin than RVFV (Table 1). However, because the impact of ribavirin in human cell lines occurred at 15–20 μg/ml (Table 1) and a single dose of ribavirin at 600–2,400 mg reached peak serum concentrations of 47–161 μM (equivalent to approximately 11–39 μg/ml) (27,28), the effects of ribavirin in vivo could be substantial. Recently, an animal model using interferon α/β receptor-knockout mice was reported in which SFTSV infection was fatal (29). The model might be useful for understanding the pathogenesis of SFTS and for evaluating the in vivo effects of antiviral drugs, including ribavirin, against SFTS/SFTSV.

In the present study, ribavirin added before virus inoculation inhibited SFTSV proliferation. In some SFTS cases, exposure to SFTSV can be recognized immediately (4,8–11). Thus, suggesting that ribavirin could be an effective post-exposure prophylaxis that will minimize the severity of SFTS in cases where individuals are deemed to be at a high risk of SFTSV infection, such as in cases in which contaminated sharp instruments penetrate skin or in which an individual comes into close contact with the patient's blood or excreta.

Ribavirin did not show an effective reduction of virus production in pre-infected cells, when added 3 days after inoculation (Fig. 3). This suggests that ribavirin has no effect on fully-infected cells or in patients in whom SFTSV has already expanded systemically. In SFTS patients, serum viral loads are very high on hospital admission, particularly in fatal cases (30,31). It is therefore unlikely that ribavirin would be effective in the treatment of patients who are already showing symptoms of SFTS. This corresponds with a report by Liu et al., which noted that neither fatality ratios nor

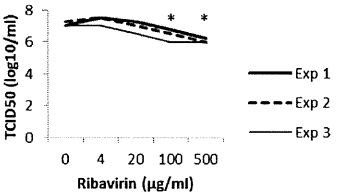

Fig. 3 Effects of ribavirin on SFTSV production from SFTSV-pre-infected cells. Vero cells were inoculated with SFTSV and cultured for 3 days. Culture media were then replaced with ribavirin-containing media. After a further 3 days culture, supernatants were harvested and viral titers were determined. Results were obtained from 3 independent experiments. *, $P < 0.05$.

blood laboratory test results of SFTS patients improved with ribavirin therapy (32). However, in hepatitis C, combination therapies that include ribavirin (along with interferon and/or specific antiviral drugs) have been shown to be very effective, while the usage of ribavirin alone is less profound (13,33). As such, the possibility exists that ribavirin could be effective as a part of combination therapy in the treatment of SFTS patients.

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Conflict of interest None to declare.

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