REVIEW

A highly pathogenic new bunyavirus emerged in China

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Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease that was discovered in China in 2010. The causative agent has been identified as a new member of the *Phlebovirus* genus in the family *Bunyaviridae* and has been designated severe fever with thrombocytopenia virus (SFTSV). SFTSV infection can be transmitted person-to-person, and the average case fatality rate is approximately 10% in humans. There is a high seroprevalence of SFTSV infection in a wide range of domesticated animals, including sheep, goats, cattle, pigs, dogs and chickens. Ticks are suspected to be the vector that transmits the virus to humans. Currently, the SFTS endemic area is expanding. Therefore, SFTSV infection is an increasingly important public health threat.

INTRODUCTION

Since 2009, sporadic cases with clinical presentations of fever ($\geqslant 38\ ℃$), thrombocytopenia, leukocytopenia and gastrointestinal symptoms were frequently reported in rural areas of Hubei, Henan, Jiangsu, Anhui, Liaoning and Shandong provinces in central and northeastern China. Known pathogens that could cause similar symptoms were not detected in the majority of these patients, and initially the reported case fatality rate was 30%. In response to this emerging infectious disease with unknown causes, enhanced surveillance was implemented for severe fever with thrombocytopenia syndrome (SFTS) in 2010. More than 20 virus strains were isolated from SFTS patients in six provinces of China, and those isolates were identified as the causative pathogen of SFTS and therefore designated as SFTS virus (SFTSV).[1,2] According to the surveillance data,[3] SFTS cases have been identified in at least 13 provinces in China, and the average case fatality rate of SFTS is approximately 10%.[3,4] Moreover, a newly released report in 2012 revealed that two cases of new *phlebovirus* infections in Missouri in the United States had clinical presentations similar to those of SFTS, and a phylogenetic analysis showed that the novel virus identified in the United States is most closely related to SFTSV.[5]

MORPHOLOGY AND GENOME STRUCTURE

Viruses in the *bunyaviridae* family are generally spherical virions or sometimes are elongated, approximately 80–120 nm in diameter, with a lipid bilayer envelope that is 5–7 nm thick.[6] SFTSV virions have typical morphologic features of bunyaviruses. Negative-stain electron microscopy of purified SFTSV virions shows that the virus particles are enveloped spheres or ovals with diameters of 80–100 nm.[1] The surface projections could be observed on SFTSV virions, and the typical round and closely packed morphologic units on the virion surface further implicate SFTSV as a member of the *phlebovirus* genus.[7] The interior of the virions, as observed by thin section electron microscopy, has a filamentous or coiled bead appearance, which indicates the presence of a helical nucleocapsid structure comprised of the nucleocapsid protein and the viral genome segments.

Like other members of the family *Bunyaviridae*, SFTSV is an enveloped RNA virus with three single-stranded RNA genomes, consisting of the large (L), medium (M) and small (S) segments.[1] The L and M segments are of negative polarity. The L segment contains 6368 nucleotides with one open reading frame encoding the RNA-dependent RNA polymerase (RdRP), composed of 2084 amino acids. The M segment contains 3378 nucleotides with one open reading frame encoding 1073 amino acids of the precursor of glycoproteins Gn and Gc. The S segment of SFTSV contains 1744 nucleotides, which uses an ambisense strategy to encode the nucleoprotein (N) in antisense orientation and the nonstructural protein (NSs) in sense orientation, separated by a 54-bp intergenic region. The 5' and 3' termini of the L, M and S segments possess short noncoding sequences. The 5' noncoding regions of SFTSV strain HB29 are 16 nucleotide (nt) (L), 18 nt (M) and 42 nt (S), and the 3' noncoding regions are 100 nt (L), 141 nt (M) and 28 nt (S).

Phylogenetic trees based on complete sequences of the L, M and S segments, from a panel of SFTSV isolates showed that the novel virus is classified in the genus of *Phlebovirus,* family *Bunyaviridae*. Further, phylogenetic analysis of the complete deduced amino acid sequences for the RdRp, glycoprotein (Gn and Gc), N and NSs proteins of six SFTSV strains, showed that all SFTSV isolates were clustered together in comparison with other known *Phleboviruses*, but were almost equally distant from the other two groups, including the sandfly fever group (Rift Valley fever, Punta Toro, Toscana, Massila, sandfly fever Sicilian viruses) and the Uukuniemi group.[1,2] The molecular and morphological data suggested SFTS virus to fit the prototype of a new member of the genus *Phlebovirus*. A comparison of amino acid sequence similarity provided further support of the separation of SFTSV from other *Phleboviruses*. Both the RdRp and glycoproteins (Gn and Gc) of SFTSV are slightly more closely related to counterparts in Uukuniemi group but only share a maximum similarity of 36%, while the most highly conserved protein of SFTSV, the N protein, only shared 41% similarity with Rift Valley fever virus (RVFV). With more diversity, the S segment-encoded NSs protein of SFTSV

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showed the lowest amino acid similarity to RVFV, with a value of only 11%–16%. The pairwise comparisons of SFTSV with the newly discovered Heartland virus in the United States showed that the amino acid sequences of the viral RdRp, glycoprotein (Gn and Gc), nucleoprotein, and non-structural protein shared homology of 73.4%, 62.6%, 62.8% and 63.5%, respectively.[1]

EPIDEMIOLOGY AND TRANSMISSION

During recent decades, phlebovirus infections have been mainly recorded in Africa and Europe. For major members of the *phlebovirus* genus, the phlebotomine sandfly-transmitted viruses (Toscana virus, sandfly fever Sicilian viruses, sandfly fever Naples virus), were reported around Mediterranean Europe; mosquito-transmitted RVFV was reported in Africa and the Arabian Peninsula, and the tick-transmitted Uukuniemi virus was mainly distributed in Europe.[6,8,9] There had been no reports of identification of phlebovirus in China until the discovery of SFTSV in 2009.

As a newly identified member in the *phlebovirus* genus, epidemiological findings revealed that most SFTSV cases occur in farmers living in wooded and hilly areas and working in the fields.[1,10,11] There is an obvious seasonal distribution of SFTS: the endemic starts around March, peaks between May and July, and ends around November.[1,12] The geographic and seasonal distributions, and the tick bite history of some SFTS cases, suggest that ticks were the insect vector to transmit the virus to humans. Studies showed that the tick species *Haemaphysalis longicornis* were dominant in the endemic regions, and SFTSV RNA was isolated from approximately 4.9% of the total *Haemaphysalis longicornis* pools collected.[13] The nucleic acid sequences of viruses isolated from ticks has 95% homology with the SFTSV isolated from patients.[1] These observations suggest that *Haemaphysalis longicornis* ticks are most likely the major vector of SFTSV.

Current knowledge suggests that many phleboviruses are maintained in their arthropod vectors by vertical (transovarial) transmission and that the vertebrate hosts play little or no role in the basic maintenance cycle of these agents; however, vertebrate hosts may serve as amplifying hosts. Current studies on domesticated animals reported that 47.7% of the animal sera were positive for SFTSV antibodies,[14] and 83% of goats in a county in Shandong Province were seropositive for SFTSV.[15] Our seroprevalence investigations of more than 3000 domestic animals, with five species in two epidemic regions, found that approximately 70% of sheep, 60% of cattle, 38% of dogs, 3% of pigs and 47% of chickens were SFTSV antibody positive. However, only a small proportion of studied animals (ranging from 1.7% to 5.3%) were found to carry low levels of viral RNA in sera. A genetic analysis of viral isolates from sheep, cattle, dog, and humans revealed a pairwise distance of the S segment of less than 4.6% (unpublished data). Therefore, it seems that there is an intense epizootic of SFTSV in endemic areas, and the domesticated animals might act as amplifying hosts for SFTSV. However, there is a lack of evidence to confirm vertebrate reservoir hosts and to clarify the possibility of vertical transmission in ticks.

Several studies have provided evidence to highlight the risk of person-to-person transmission of SFTSV through direct blood contact with the patient,[10,11,16,17] especially when the index patient has a high virus load in the blood,[10] with the virus titer in blood of index patients higher than 10^7 50% tissue culture infective dose/mL. These reports highlight the need to apply strict infection control for suspected clinical SFTS patients and to emphasize the appropriate use of personal protective equipment (PPE) when treating SFTS patients.

CLINICAL FEATURES

Most phlebovirus human infections are self-limited, resulting in a mild febrile illness.[6] Clinical presentations of SFTS patients are nonspecific; major symptoms include high fever, thrombocytopenia, leukocytopenia, gastrointestinal symptoms, and lymphadenopathy. Laboratory tests of SFTS patients commonly show elevated serum alanine aminotransferase, aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatine kinase (CK), creatine kinase MB fraction (CK-MB) levels, as well as a prolonged activated partial-thromboplastin time. These laboratory parameters are indicative of pathological lesions involved in the liver, kidney and coagulation systems of SFTS patients.[1,10,18,19] In severe SFTS patients, the clinical conditions may proceed quickly and end in multi-organ dysfunction syndrome, with clinical presentations of central nervous system (CNS) symptoms and hemorrhagic manifestations. Patients commonly die of disseminated intravascular coagulation and multi-organ failure.[1,10,18,19]

Dynamic monitoring of the disease course identified three major disease stages of SFTS patients:[18] the fever stage, the multi-organ dysfunction stage and the convalescent stage. During the first stage of the disease, day 1 to day 7 post-onset of illness, the initial serum viral load was high (average 10^5–10^6 copies/mL) and comparable in both fatal and non-fatal cases. Most SFTS cases in both groups had fever, marked thrombocytopenia and leukocytopenia in this stage. In addition, the alanine aminotransferase, AST, LDH, CK and CK-MB levels began to be elevated later during this stage. The second stage was between day 7 and day 13 post-disease onset. During this stage, the serum viral load decreased in non-fatal cases but still remained high in fatal cases (average 10^8 copies/mL). Coincident with the respective levels of viral load in non-fatal and fatal cases, the platelet counts reverted to a normal range in non-fatal cases but continued to decline in fatal cases. The levels of the serum enzymes AST, LDH, CK and CK-MB began to decline at approximately day 9 or day 11 in non-fatal cases but progressively increased in fatal cases. When the disease proceeded to the third stage after day 13, for most non-fatal cases, the patients could recover from the disease with most clinical parameters converting to normal. However, for fatal cases, the patients died of severe multi-organ dysfunction syndrome and disseminated intravascular coagulation during this stage, and the serum enzymes reached very high levels before death. The risk analysis revealed that high serum levels of AST (>400 U/L), LDH (>800 U/L), CK (>1000 U/L), and CK-MB (>50 U/L) were risk factors associated with death of SFTS patients.[18]

In the acute phase of SFTS human disease, the levels of a broad spectrum of serum cytokines, interleukin (IL)-1RA, IL-6, IL-10, granulocyte colony-stimulating factor (G-CSF), interferon-γ-induced protein 10, and monocyte chemotactic protein-1, were elevated in SFTS patients and were present at higher levels in fatal cases compared to non-fatal cases.[19,20] In contrast, levels of the cytokines platelet-derived growth factor-BB and regulated and normal T cell expressed and secreted decreased in SFTS patients. These eight cytokines reverted to normal during the convalescent phase of SFTSV infection. The cytokines IL-1β, IL-8, Macrophage inflammatory protein (MIP)-1α and MIP-1β showed a unique pattern of elevation in fatal cases but not in non-fatal cases. However, the levels of these cytokines increased in the convalescent phase of non-fatal SFTS cases. The regression analysis further revealed that the serum viral load correlated with the serum levels of these cytokines. Moreover, the levels of these cytokines correlated with various clinical parameters.[20] These findings suggest that the high viral load may lead to an overproduction of proinflammatory cytokines, which could further exacerbate disease

severity. Another study reported the elevation of IL-6, IL-10, G-CSF, interferon-γ, and acute phase proteins (phospholipase A, fibrinogen, hepcidin) in SFTS patients, and a higher production of IL-6, IL-8, IL-10, G-CSF, interferon-γ and phospholipase A in fatal SFTS cases than in non-fatal SFTS cases. The study also showed correlations between the viral load and host biomarkers indicative of fatal outcome, including liver enzymes AST, coagulation factors of activated partial-thromboplastin time, thrombin time and fibrinogen, as well as acute phase proteins phospholipase A and hepcidin.[19]

PATHOLOGY AND PATHOGENESIS IN ANIMAL MODELS

Currently, an infectious C57BL/6 mouse model with hallmark symptoms of thrombocytopenia and leukocytopenia has been established through intramuscular injection of SFTSV into adult mice. In this murine model, viral RNA was detected in the blood, spleen, liver, and kidney. The humoral immune responses of virus-specific IgM and IgG, neutralizing antibodies, and cellular responses were efficiently induced in the SFTSV-infected mice.[21]

Histopathological changes were identified in the spleen and bone marrow in the early phase of infection, and pathological lesions in the liver and kidney developed in a later stage. In the SFTSV infected spleen, the lymphocyte cellularity of the red pulp was visually decreased during the first week after inoculation and gradually recovered 14 days later. In addition, a marked increase of megakaryocytes was observed in the spleen. Similarly, megakaryocytes in the bone marrow also significantly increased in the early stage of infection. This increase of platelet progenitor cells in principle and secondary hematopoietic organs suggests extramedullary hematopoiesis may compensate for the depletion of circulating platelets. During the late phase of SFTSV infection, pathological changes were noted in the liver and kidney. The primary lesions in the liver consisted of ballooning degeneration of hepatocytes and scattered necrosis. The lesions in kidney typically included glomerular hypercellularity, mesangial thickening, and congestion in Bowman's space, while infiltration of inflammatory cells was absent. Pathological changes within the liver and kidney tissues were maximal on day 14 post-infection but had nearly recovered on day 28 post-infection. Therefore, the pathological features observed in the spleen and bone marrow during the early stage of infection were consistent with the hematological changes of thrombocytopenia and leukocytopenia.[21] The transient pathologic changes observed in kidney and liver at the later stage of infection were indicative of acute glomerular nephritis and acute hepatitis with self-limiting outcomes.

The viral RNA load and infectious titers increased in the spleen on day 3 post-infection, which suggests the spleen as the principle target organ for SFTSV replication.[21] Focusing on the spleen, we found that the number of macrophages and platelets dramatically increased in the infected spleen, and SFTSV colocalized with platelets in the cytoplasm of macrophages in the red pulp of the spleen. *In vitro* cellular assays further revealed that SFTSV adhered to platelets and facilitated the phagocytosis of platelets by macrophages, which, in combination with *in vivo* findings, suggests that the SFTSV-induced thrombocytopenia was caused by clearance of circulating virus-bound platelets promoted by splenic macrophages. Thus, the study elucidated pathology and pathogenic mechanisms of thrombocytopenia in a murine model that resembles human SFTS disease.[21]

The SFTSV infection in the established C57BL/6 mouse model could mimic major pathological changes of clinical infection in patients; however, lethal infection has only been induced in certain strains of newborn mice[22] but not in adult rodent models. It is suspected that the severe infection could only be induced in immune incompetent hosts, such as newborn mice, without a completely mature immune system, or certain strains of immune-deficient mice.

LABORATORY DIAGNOSIS

In most phlebovirus infections, the diagnosis is based on serological tests.[8,9] In-house enzyme-linked immunosorbent assay (ELISA) methods are developed, commonly including IgM capture ELISA in acute phase serum or by IgG sandwich ELISA in paired sera from acute and convalescent phases.[23] Neutralization assays using early convalescent sera remain the reference method to specifically identify the viruses or to assess the antibody response specificity. Virologic diagnosis of RVFV infection is through detection of viremia in the acute phase of illness, initially using intracranial inoculation into suckling mice or infection of susceptible cells (Vero or mosquito cells) to grow the virus.[6] More recently, detection of serum or blood viral RNA copies by reverse transcription-PCR amplification has been developed and was found to be very useful for rapid presumptive diagnosis,[24] which could be followed by sequencing to further characterize the viral strains.

Currently, a variety of assays have been developed for laboratory diagnosis of infections with SFTSV. The early viral nucleic acid detection is performed by one-step Taqman real-time assays, using the highly conserved regions of all three genome segments L, M and S of SFTSV as targets.[25] Commonly, the viral nucleic acids can be detected in the acute phase serum of patients, within 2 weeks post-onset of the disease. The sensitivity and specificity of the assay is 98.6% and 99%, respectively.[25] The real-time PCR assay, based on the S segment, has been approved by the State Food and Drug Administration for application in clinical diagnosis in hospitals and disease surveillance by the local Center for Disease Control and Prevention. With respect to the detection of viral nucleic acids using simple instruments and saving time, a reverse transcription loop-mediated isothermal amplification assay to target eight distinct regions on the S segment of SFTSV has been developed. When combined with a fluorescent detection reagent, visible results could be determined by showing a color change within 30 min.[26] Another similar assay, which incorporates reverse transcription-cross-priming amplification with a vertical flow visualization strip based on the M segment of the SFTSV, could complete the entire procedure from specimen processing to result reporting within 2 h.[27] As for serodiagnosis, the in-house Mac-ELISA assay, indirect ELISA assay, and double antigen sandwich ELISA assay have been established and optimized to test virus specific IgM, IgG and total antibodies in serum samples of patients, respectively.[1,14] Indirect immunofluorescence assays were also established to test the virus specific antibodies using previously fixed SFTSV infected cells on slides. The serum neutralization test is generally regarded as the gold standard of various serological methods used for detection of virus-specific antibodies. However, this test is time consuming, expensive, and requires the manipulation of a live virus, so it is performed only in specialized reference laboratories housed in high-level bio-containment facilities. Two assays, the plaque reduction neutralization test and the microneutralization assay, are used for detection the neutralizing antibodies of SFTSV.[1]

To date, the criteria for laboratory diagnosis of SFTS case include: (i) viral RNA detected in serum or blood, (ii) virus-specific IgM positive in acute phase, (iii) a four-fold increase or conversion of virus-specific IgG in paired sera of acute and convalescent phases, or (iv) isolation of virus from patient samples. Satisfying any one of the above criteria will confirm an SFTSV infection of the patient.[1,10]

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PERSPECTIVES

Recent epidemics of SFTSV in China have emphasized the importance of understanding the transmission cycle and pathogenesis of this emerging virus. Although ticks appear to play an important role in viral transmission and propagation, and a variety of domesticated animals could be potential amplifying hosts, the reservoir hosts for the virus still needs clarification. SFTSV infection in animal models other than rodents, such as ferrets and monkeys, can also be tested. Because the endemic areas are expanding, there is an urgent need for the production of an efficient vaccine for high-risk populations, such as farmers in the endemic area and for the most susceptible animals, such as sheep, cattle and goats. Currently, the development of a vaccine strategy to use inactivated SFTSV is underway. Recent advances in reverse genetics, which can genetically manipulate the genomes of negative-stranded viruses and generate infectious viruses, will help to engineer viruses with modifications and facilitate the investigation of the pathogenesis of SFTSV and will hopefully open new perspectives to produce efficacious and safe SFTSV vaccines.

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