

# Chapter 2

## The Discovery Process of SFTS in China



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**Abstract** Heightened surveillance of acute febrile illness in China since 2004 led to the identification of a severe fever with thrombocytopenia syndrome (SFTS) with unknown etiology. Sporadic patients hospitalized with SFTS in 2009 and 2010 were identified and serum samples were collected. Sequence from a novel species of phlebovirus was identified by sequence independent single primer amplification (SISPA) from the serum of a patient with SFTS. The virus was isolated in Vero cell culture and its complete genome sequence was determined, only distantly related to other known phleboviruses. Electron microscopic analysis revealed a virion morphologically characteristic of phleboviruses. The virus was named as SFTS virus. The viral RNA and/or specific antibodies were detected from the blood of patients with SFTS. Serological assays demonstrated a virus-specific immune response in pairs of sera collected from patients at acute and convalescent phases. The pathogenic mechanisms of thrombocytopenia in human SFTS disease was resembled in a mouse model. The results had been collected to demonstrate that SFTS virus was etiologically associated with an acute and novel infectious disease, SFTS in humans.

**Keywords** SFTS virus · Phlebovirus · Emerging infectious diseases · Reduction of platelets · Fever

### 2.1 Introduction

Surveillance of infectious diseases in China has been significantly enhanced since 2004 after the outbreak of severe acute respiratory syndrome (SARS). Over the years sporadic cases of severe acute febrile illness with unidentified cause were noted. A unique group of hospitalized patients suffering from acute high fever accompanied with thrombocytopenia was identified. Although *Anaplasma phagocytophilum* found

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in some of these cases (Zhang et al. 2008), which was suggested as the cause of this disease, neither bacterial DNA nor antibodies against this bacterium could be detected in the majority of patients within this group. Other infectious causes including bunyaviruses including hantavirus, Rift Valley fever virus, Toscana virus, and Crimean-Congo hemorrhagic fever virus, flaviviruses including dengue virus, filovirus such as Ebola virus and Marburg virus, arenaviruses including Lassa virus and Junin virus, alphavirus, and rickettsia were also excluded by reverse transcription-polymerase chain reaction (RT-PCR) and/or specific antibody tests. All founded cases were from wooded and hilly upland areas and patients were mostly farmers who had ever worked in the field before the onset of disease.

In view of these unusual findings, an active surveillance was implemented in several provinces in China since May 2010 to identify patients with severe fever with thrombocytopenia syndrome (SFTS), which is characterized by acute high fever and thrombocytopenia. Collected sera were subjected to sequence independent single primer amplification (SISPA), which provides an opportunity for discovering novel microbial agents directly from clinical samples (Allander et al. 2001; Victoria et al. 2008; Ambrose and Clewley 2006; Jones et al. 2005). A novel phlebovirus was identified and subsequently isolated in cell culture. The complete genome of the novel virus was sequenced. Molecular and serological assays were performed to detect the virus in a larger cohort of patients with SFTS. The related data had been published (Yu et al. 2011). Here we described the process of the discovery of SFTS virus in China.

## 2.2 Case Surveillance

An active surveillance scheme was implemented in selected areas in Hubei, Henan provinces since May 2010 to identify hospitalized patients, who presented with an acute fever of  $\geq 38$  °C and thrombocytopenia with unknown causes (National 2010). Serum samples were collected preferably within 2 weeks after the onset of fever and during the convalescent phase from six provinces of Hubei, Henan, Shandong, Jiangsu, Anhui and Liaoning. The cases of SFTS occurring before May 2010 were also identified through retrospective review, and sera collected were requested from the hospitals. Cases fit the criteria but with clinical or laboratory confirmed diagnosis were excluded. The sera from 200 patient-matched healthy donors living in the same areas were also collected. The research protocol was approved by the human bio-ethics committee of the Chinese CDC, and all human participants gave written informed consent.

### 2.3 Identification of the Viral Gene and Genetic Analysis

The amplification of microbial nucleic acids from serum was based on a modified version of the SISPA method (Allander et al. 2001; Ambrose and Clewley 2006; Jones et al. 2005). After filtration through a 0.2- $\mu$ m filter and digestion with turbo DNase (Ambion), benzonase (Novagen) and RNase One (Promega), RNA was extracted from serum (140  $\mu$ L) of patient HB29. RNA preparations were reverse transcribed into cDNA and second strand cDNA were synthesized. After purification, the DNA was ligated to a phosphorylated blunt adapter E19 (5'-AGCAATTCCGTTGCTGTCG-3'); and E12 (5'-P-GGCAACGACAGC-3'). The ligation product was PCR amplified and separated by agarose gel electrophoresis. Fragments of different size were isolated and cloned. A total of 576 cDNA clones were picked by SISPA from the serum of patient HB29 and sequenced. After trimming to remove sequences derived from the amplification primer, the data set was subjected to homology search with the GenBank databases of nucleic acids and proteins using BLASTN and BLASTX. Whereas the nucleotide sequence was essentially unrelated to other sequences in the existing GeneBank database (<http://blast.ncbi.nlm.gov>), the deduced amino acid sequence from 14 cDNA clones showed 20–30% homology to viral proteins, including RNA dependent RNA polymerase (RdRP), membrane polyprotein and nonstructural S protein, of known phleboviruses such as Rift Valley fever virus and Uukuniemi virus. Walking primers from both 5' and 3' directions were designed based on the sequence of gene fragments obtained from SISPA. The three genomic segments were assembled from a series of overlapping cDNA clones. The 5' and 3' termini of viral RNA segments were determined by rapid amplification of cDNA ends (RACE) using reagents purchased from Invitrogen. To eliminate the influence of cloning bias on genome sequencing, direct PCR re-sequencing of all three genomic segments were performed with newly designed primers. The partial sequences were obtained from the first isolated virus strain DBM, and the complete genomes of 11 isolates of SFTS virus were further sequenced (Yu et al. 2011). All isolates including strain DBM were closely related (Yu et al. 2011). Termini of three genomic segments of SFTS virus were found to be similar to counterparts in other phleboviruses.

Phylogenetic analyses were performed with the neighbor-joining method using the Poisson correction and complete deletion of gaps. Bootstrap values were estimated from 2000 replicates (95% confidence) with a random seed. Phylogenetic trees based on complete viral genomic sequence of L, M and S segments from strains (HB29, HN6, AN12, LN2, JS3 and SD4) from 6 provinces in China in comparison with other known phleboviruses showed that SFTS virus was related to prototypic viruses of Phlebovirus. Phylogenetic analysis using the deduced amino acid sequences of RdRp, glycoproteins (Gn and Gc), N and NSs proteins, the generated phylogenetic tree showed all SFTS virus isolates clustered together, but are almost equally distant from other two groups (Fisher et al. 2003), including the sandfly fever group (Rift Valley fever, Punta Toro, Toscana, Massila, sandfly fever

Sicilian viruses) and Uukuniemi group. The comparison of amino acid sequence similarity provided further support to the separation of SFTS virus from other phleboviruses. SFTS virus was assigned to be a novel species at the genus of *Phlebovirus* in *Bunyaviridae* in 2014, and was resigned to be a novel species at the genus of *Phlebovirus* in the family of *Phenuiviridae* by International Committee on Taxonomy of Viruses (ICTV) in 2016.

## 2.4 Isolation of the Novel Virus

In June, 2009, a blood sample in heparin anticoagulant was collected on day 7 after onset of illness from a patient from Xinyang City, Henan Province. Due to the lack of knowledge on the causes of the illness, the pathogen was isolated by inoculating multiple cell lines susceptible to both viral and rickettsial agents including DH82, L929, Vero, Vero E6, HL60 cells, and the tick cell line ISE6. Patients' white blood cells were inoculated to the cell monolayers. The cells were cultured at 37 °C in a 5% CO<sub>2</sub> atmosphere with medium changes twice a week. A month after inoculation of the cells with white blood cell (WBC) of the patient, cytopathic effect (CPE) was observed in the DH82 cells. The morphology of infected DH82 cells changed from round monocytes to a fibroblast-like shape, which had granular particles in the cytoplasm. In continuous culture, the CPE usually appeared on day 4 after inoculation of a fresh monolayer. But the pathogen was not characterized clearly. Subsequently, the virus was isolated by inoculation of Vero cells with serum or homogenized WBCs in 2010. After incubation for 10–14 days at 37 °C, culture fluid was passaged to new monolayers. Cells were monitored daily for presence of viral RNA in supernatant by real-time PCR. The novel phlebovirus was isolated in cultured Vero cells inoculated with acute-phase serum of patient HB29 from Shuizhou area, Hubei province. Virus isolation was also successful with sera from other 10 patients with SFTS from 6 provinces. Viral RNA was detected by real-time RT-PCR on day 7 after inoculation. Immunoreactivity of infected cells with convalescent-phase serum collected from RT-PCR confirmed patients was observed as punctate staining pattern distributed evenly throughout the infected cells at day 10 after inoculation. A plaque assay was performed on Vero cells to quantify the infectious particles in the supernatant. The plaques were visualized by staining with 3% neutral red solution in molten agarose. The virus titer was about  $6 \times 10^7$  pfu/mL in the culture supernatant at the 2–3 passage of the virus. Negative-stain electron microscopy revealed SFTS virus particles of 80–100 nm in diameter, with complex surface projections surrounding the periphery. Transmission electron microscopy (TEM) revealed viral particles in the DH82 cell cytoplasm. The viruses were observed inside vacuoles, presumably in the Golgi apparatus.

## 2.5 Detection of the Virus in the Patients with SFTS

Indirect immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA) and micro-neutralization test were used to detect the specific antibodies in the blood of patients with SFTS. For IFA, fixed Vero cells were incubated with diluted human serum and stained with fluorescein labeled anti-human IgG antibodies. ELISA methods were developed with purified recombinant nucleocapsid protein (NP) of the virus produced in *E. coli*. Specificity, sensitivity and cross reactivity of the methods were verified with serum samples collected from patients with SFTS confirmed by RT-PCR and sera collected from healthy donors from the areas without reported SFTS cases. For micro-neutralization test, sera were mixed with an equal volume of 100 50% tissue culture infectious dose (TCID<sub>50</sub>) of SFTS virus (isolate HB29) and incubated at 37 °C for 1.5 h. The mixture (100 µl) was then added to a 96-well plate in quadruplicates. The plates were incubated in 5% CO<sub>2</sub> at 37 °C for 12 days. Viral infection in wells was detected by specific IFA assays with a confirmed patient serum. The endpoint titer was expressed as the reciprocal of the highest dilution of serum. Viral RNA was detected from serum, whole blood or homogenized arthropods with one-step real-time RT-PCR assay using the primers and probes designed based on the 3 viral genome segments. The cutoff CT value for a positive sample was set at 35 cycles. Samples from which only one genomic segment was significantly amplified were verified by nested RT-PCR and sequencing.

SFTS viral RNA and/or specific antibodies were detected in 171 out of 241 hospitalized SFTS patients diagnosed according to the SFTS case definition (National 2010). These patients were found in central and north-eastern areas of China including of Henan (43 cases), Hubei (52 cases), Shandong (93 cases), Anhui (31 cases), Jiangsu (11 cases) and Liaoning Province (11 cases). But viral RNA and specific antibodies were not detected from the sera collected from 200 patient-matched healthy donors in the endemic areas, 180 healthy donors from non-endemic area, and 54 HFRS suspected cases. Viral RNA was not found in any of 5900 mosquitoes captured from the areas with SFTS patients living. On the other hand, 10 out of 186 ticks (5.4%) of the species *Haemaphysalis longicornis* collected from domestic animals in the same areas were positive for SFTS virus RNA.

To explore the seroconversion against SFTS virus in patients with SFTS, a cohort of 35 RT-PCR-confirmed patients was selected, sera at both acute- and convalescent-phase were collected. Specific antibodies were quantified using IFA, ELISA and micro-neutralization test. Four fold elevation of antibody titers or seroconversion from negative to positive reactivity in serological tests were found in all of the 35 patients. This indicated that high-titer neutralizing antibodies were generated during the convalescent phase of the disease. An antibody titer of more than 25600 in ELISA was found in sera from 15 patients at convalescent phase, indicating a robust humoral immune response against SFTS virus.

## 2.6 Conclusion

The discovery of the new SFTS virus was the result of the joint efforts of virologists, clinicians and epidemiologists, and also depends on the progress of laboratory testing technology. Robust epidemiological, clinical, serological and virological data had been collected to support that SFTS virus is the cause of the SFTS disease identified in central-eastern and northeastern parts of China. Of course, SFTS is not a disease with a single cause, in addition to non-infectious diseases, a variety of known microbial pathogens can cause SFTS, which needs to be differentiated from the other related pathogens.

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