The Evolutionary History and Spatiotemporal Dynamics of the Fever, Thrombocytopenia and Leukocytopenia Syndrome Virus (FTLSV) in China

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Abstract

Background: In 2007, a novel bunyavirus was found in Henan Province, China and named fever, thrombocytopenia and leukocytopenia syndrome virus (FTLSV); since then, FTLSV has been found in ticks and animals in many Chinese provinces. Human-to-human transmission has been documented, indicating that FTLSV should be considered a potential public health threat. Determining the historical spread of FTLSV could help curtail its spread and prevent future movement of this virus.

Method/Principal Findings: To examine the pattern of FTLSV evolution and the origin of outbreak strains, as well to examine the rate of evolution, the genome of 12 FTLSV strains were sequenced and a phylogenetic and Bayesian phylogeographic analysis of all available FTLSV sequences in China were performed. Analysis based on the FTLSV L segment suggests that the virus likely originated somewhere in Huaiyangshan circa 1790 (95% highest probability density interval: 1756–1817) and began spreading around 1806 (95% highest probability density interval: 1773–1834). Analysis also indicates that when FTLSV arrived in Jiangsu province from Huaiyangshan, Jiangsu Province became another source for the spread of the disease. Bayesian factor test analysis identified three major transmission routes: Huaiyangshan to Jiangsu, Jiangsu to Liaoning, and Jiangsu to Shandong. The speed of FTLSV movement has increased in recent decades, likely facilitated by modern human activity and ecosystem changes. In addition, evidence of RNA segment reassortment was found in FTLSV; purifying selection appears to have been the dominant force in the evolution of this virus.

Conclusion: Results presented in the manuscript suggest the Huaiyangshan area is likely be the origin of FTLSV in China and identified probable viral migration routes. These results provide new insights into the origin and spread of FTLSV in China, and provide a foundation for future virological surveillance and control.

Introduction

In 2007, the fever, thrombocytopenia and leukopenia syndrome (FTLS; also called severe fever with thrombocytopenia syndrome, SFTS) was first reported in Henan province, China [1,2]. Since then, this life-threatening disease has been reported in many Chinese provinces, including Hubei, Anhui, Jiangsu, Liaoning and Shandong. The main clinical symptoms are sudden onset of fever (≥37.5°C), fatigue, marked anorexia, headache, myalgia, arthralgia, dizziness, lymphadenopathy, vomiting, and diarrhea. Laboratory tests show thrombocytopenia, leukopenia, increased urine protein, and elevated serum aspartate aminotransferase and alanine aminotransferase. Initially, this disease was diagnosed as suspended human granulocytic anaplasmosis (HGA) infection [3]. However, only 8% of patients were positive for HGA infection, indicating that other pathogens likely contribute to this syndrome. Samples collected in 2009 identified a novel virus in the Bunyaviridae family, genus Phlebovirus in FTLS cases; the virus has been named several times including severe fever with thrombocytopenia syndrome virus (SFSTV), Henan fever virus (HFV), fever, thrombocytopenia and leukopenia syndrome virus (FTLSV), and Huaiyangshan virus (HYSV) [1,2,4]. Serosurveillance has shown that 1 to3.8% of examined populations in hilly areas had FTLSV antibodies, suggesting that SFTSV has circulated widely in China but only a small proportion of infected individuals develop disease. The overall mortality of FTLSV...
FTLSV is novel bunyavirus belonging to genus Phlebovirus and was first found in Huaiyangshan area. The epidemiology and pathogenesis of FTLSV remain poorly understood. This lack of information underscores the importance of analyzing the movement and genetic history of FTLSV in China. Recent advances in Bayesian coalescent phylogenetic analyses have resulted in more sophisticated methods being available for determining the relative age of emerging pathogens. In this study, a phylogenetic and Bayesian phylogeographic analysis was performed for FTLSV whole genomic sequences isolated from China. Results identified the Huaiyangshan area as the most probable origin of FTLSV and suggested migrating routes of the virus. These results offer the first description of the movement and history of FTLSV in China. RNA segment reassortment was found in FTLSV, purifying selection appears to have been the dominant force driving the evolution of this virus. The results of this study could be used to facilitate the development of new strategies for controlling FTLSV.

Materials and Methods
Isolation and sequencing of viruses
Serum samples from FTLSV-infected patients (n = 12) were obtained by disease control and prevention workers in the Xinyang and Nanyang counties of Henan Province, China, during 2011–2012, and viral isolates were cultured from the sera [2]. All laboratory procedures involving viral cultures were performed under Biosafety Level-2 containment. All patients provided written informed consent for the research use of their samples. This research was approved by the Review Board of the Institute of Microbiology and Epidemiology [Beijing] and the Center for Disease Control and Prevention of Henan Province.

For sequencing, total RNA was extracted from 140 μl aliquots of cell culture supernatant obtained from each sample using a QIAamp viral RNA Mini Kit (Qiagen) according to the manufacturer’s instructions. cDNA was reverse transcribed from 6 μl of RNA at 45°C for 50 min in a 20 μl solution containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 100 ng of random hexamer primers, 200 units of Superscript II (Invitrogen), 25 units of RNasin (Promega) and 0.5 mM dNTPs. Overlapping PCR was performed, and products were subjected to Sanger sequencing [12]. Whole genome sequences were assembled using the SeqMan package in the DNASTAR 6.0 software.

Phylogenetic analysis
Phylogenetic trees for the three viral RNA segments were constructed using the maximum likelihood (ML) method, a feature of the software MEGA 5 [20], and the Bayesian MCMC method applied using MrBayes 3.2.1 [21]. The general time reversible (GTR) model with gamma distribution (GTR+G) was selected for all three segments. Preliminary analyses have also been shown [9,10,11], and phylogenetic analysis of partial L, M and S segments has indicated that there are five phylogenetic groups circulating in China [4]. The genome of FTLSV is similar to that of other Bunyaviridae; it is composed of three segments, designated L, M and S. The L segment contains 6391 nucleotides encoding an RNA-dependent RNA polymerase; the M segment contains 3366 nucleotides encoding glycoproteins Gn and Gc; and the S segment contains 1760 nucleotides encoding a nonstructural protein (NS) and a nucleocapsid protein (N) in opposite orientations. Partial sequencing of L and S segments from human cases and ticks confirmed that FTLSV is a novel Phlebovirus and is closely related to the non-pathogenic Uukuniemi virus (the pairwise nucleotide similarities for the L, M and S segments are 34%, 24% and 29%, respectively) [4]. All FTLSV sequences currently available in GenBank are similar regardless of their sampling locations (>90% sequence similarity), suggesting that they descended from a relatively recent common ancestor.

Phylogenetic trees for the three viral RNA segments were constructed using the maximum likelihood (ML) method, a feature of the software MEGA 5 [20], and the Bayesian MCMC method applied using MrBayes 3.2.1 [21]. The general time reversible (GTR) model with gamma distribution (GTR+G) was used for both methods. The reliability of the ML analysis was evaluated by a bootstrap test with 1000 replicates. The Bayesian MCMC method was run for 500,000 generations per segment.
all cases). Thus, the final Bayesian coalescent analyses used the GTR+Γ substitution model, a relaxed uncorrelated lognormal clock, and a constant population size. The S and L segment datasets were run for 50,000,000 generations, whereas the M segment dataset was run for 40,000,000 generations to ensure effective sample sizes (ESSs) of at least 200. Maximum clade credibility trees were summarized using TreeAnnotator [23] and depicted using FigTree [23]. FTLSV (SFTSV) sequences from Japan (AB817979–AB818002) were excluded from this analysis because the isolation time was not provided.

**Phylogeographic analysis based on the L segment**

To investigate the phylogeographic diffusion process along the posterior sets of the trees, relationships between locations were identified using the Bayesian stochastic variable search selection (BSSVS) applications in BEAST version 2.0.2 [22]. A Hasegawa-Kishino-Yano (HKY) substitution model, a constant population size tree prior, and a strict molecular clock were used. A standard discrete phylogeographic analysis using continuous-time Markov chains (CTMCs) was used to estimate the migration events between major geographic regions. For each taxon in the dataset, character states were assigned to the respective locations summarized in Table 1. FTLSV strains isolated from Japan (AB817979–AB818002) were excluded from this analysis because the isolation times were not provided.

**Evidence of RNA segment reassortment**

Two typical reassortment events appear to have occurred: 1) in the AHL/2011 strains, the L and M segments belonged to the D genotype, while the S segment fell into the B lineage (Figures 1, 2 and 3); and in the YSC3 strains isolated from Henan, the L and M segments belonged to the A lineage, while the S segment belonged to the E lineage.

**Selection pressure analyses**

Selection analyses were performed on the full dataset using the Datamonkey server implementation of HyPhy [30]. Single Likelihood Ancestor Counting (SLAC), Fixed Effects Likelihood (FEL), and Random-Effects Likelihood (REL) algorithms were used to assess the presence of selected sites in the L, M and S alignments. For the SLAC, FEL and IFEL results, a p-value cutoff of 0.05 was used; results of REL were considered significant if BF>100.0.

**Results**

**Phylogenetic relationships of FTLSV genotypes**

In this study, whole genome sequencing was performed on 12 FTLSV strains isolated in Henan Province, China. And the sequences were submitted into the Genebank (KF356517 to KF356552). Little variation was observed in the overall nucleotide lengths of the S, M, and L segments from viruses circulating in Henan from 2011 to 2012, and pairwise differences in the percent nucleotide identities were low, ranging from 0.1%–4.2%, 0.2%–4.7% and 0.1%–5.8% for the L, M, and S segments, respectively.

For the three viral RNA segments, Bayesian analysis yielded highly supported tree topologies that were consistent with the ML results (Figures 1, 2 and 3). Phylogenetic analysis confirmed the presence of the five genotypes previously identified in China (designated A–E) [4,31] and clustered the Japanese FTLSV strains, into a novel genotypic group. The Chinese genotypes were representative of the locations in China with the greatest burden of FTLS (Jiangsu, Henan, Liaoning, Shandong, Anhui, and Hubei Provinces and the Huaiyangshan region). Strains isolated from animals mainly clustered into the A lineage based on L and M analysis; however, the analysis of the S segments indicated that animal-isolated strains also included B and C genotypes. Strains isolated as part of this study were classified into A, B, D and E genotypes (Figures 1, 2 and 3).

**Selection pressure on FTLSV**

Analysis with the SLAC algorithm showed that the global ratio of nonsynonymous to synonymous nucleotide substitutions (dN/dS) in the L segment was 0.0480, suggesting the presence of predominantly purifying selection. The SLAC algorithm further identified 72 sites in the L segment that appeared to be under negative selection, while the FEL, IFEL and REL analyses detected one site (positions D691E/G) that appeared to be under positive selection in this segment. For the M segment, the SLAC algorithm identified 72 sites under negative selection, while the FEL, IFEL and REL analyses identified 72 sites in the L segment that appeared to be under negative selection.
| Isolated strain          | Year of isolation | Location   | Host    | NCBI Access number (L segment) | NCBI Access number (M segment) | NCBI Access number (S segment) |
|-------------------------|-------------------|------------|---------|-------------------------------|-------------------------------|-------------------------------|
| Phlebovirus_AH12_2010   | 2010              | Anhui      | Human   | HQ116417                      | HQ141590                      | HQ141591                      |
| Phlebovirus_AH15_2010   | 2010              | Anhui      | Human   | HQ141592                      | HQ141593                      | HQ141594                      |
| Phlebovirus_HB29_2010   | 2010              | Hubei      | Human   | HM745930                      | HM745931                      | HM745932                      |
| Phlebovirus_HN13_2010   | 2010              | Henan      | Human   | HQ141598                      | HQ141599                      | HQ141600                      |
| Phlebovirus_HN6_2010    | 2010              | Henan      | Human   | HQ141595                      | HQ141596                      | HQ141597                      |
| Phlebovirus_J52007_01   | 2007              | Jiangsu    | Human   | JF837593                      | JF837594                      | JF837595                      |
| Phlebovirus_J524_2010   | 2010              | Jiangsu    | Human   | HQ830163                      | HM802201                      | HQ830165                      |
| Phlebovirus_J56_2010    | 2010              | Jiangsu    | Human   | HQ830169                      | HQ830170                      | HQ830171                      |
| Phlebovirus_JSD1_2011   | 2011              | Jiangsu    | Dog     | JF267783                      | JF267784                      | JF267784                      |
| Phlebovirus_J526_2010   | 2010              | Jiangsu    | Human   | HQ830166                      | HQ830167                      | HQ830168                      |
| Phlebovirus_SD24_2010   | 2010              | Shandong   | Human   | HM802200                      | HM802201                      | HM802205                      |
| Phlebovirus_SD4_2010    | 2010              | Shandong   | Human   | HM802202                      | HM802203                      | HM802204                      |
| 2010_FQM_2010           | 2010              | Huaiyangshan | Human | HQ419227                     | HQ419236                     | HQ419240                     |
| 2010_T112_2010          | 2010              | Huaiyangshan | Human | HQ419228                     |                               |                               |
| 2010_WSQ_2010           | 2010              | Huaiyangshan | Human | HQ419226                     |                               |                               |
| 2012_1_L_2012           | 2012              | Henan      | Human   | KF356550                      | KF356539                      | KF356527                      |
| 2012_2_L_2012           | 2012              | Henan      | Human   | KF356551                      | KF356540                      | KF356528                      |
| AHL_China_2011          | 2011              | Anhui      | Human   | JQ670934                      | JQ670930                      | JQ670932                      |
| AHZ_China_2011          | 2011              | Anhui      | Human   | JQ670929                      | JQ670931                      | JQ670933                      |
| HB154_China_2011        | 2011              | Hubei      | Human   | JQ733561                      | JQ733560                      | JQ733562                      |
| HB155_China_2011        | 2011              | Hubei      | Human   | JQ733564                      | JQ733563                      | JQ733565                      |
| HB156_China_2011        | 2011              | Hubei      | Human   | JQ733567                      | JQ733566                      | JQ733568                      |
| Henan_CHN_01_2010       | 2010              | Henan      | Human   | HQ642766                      | HQ642767                      | HQ642768                      |
| Henan_CHN_20_2010       | 2010              | Henan      | Human   | JF682773                      | JF682774                      | JF682775                      |
| Henan_CHN_69_2010       | 2010              | Henan      | Human   | JF682776                      | JF682777                      | JF682778                      |
| JS2010_015_2010         | 2010              | Jiangsu    | Human   | JQ317172                      | JQ317173                      | JQ317174                      |
| JS2010_018_2010         | 2010              | Jiangsu    | Human   | JQ317175                      | JQ317176                      | JQ317177                      |
| JS2010_019_2010         | 2010              | Jiangsu    | Human   | JQ317178                      | JQ317179                      | JQ317180                      |
| JS2011_004              | 2011              | Jiangsu    | Human   | KC505123                      | KC505124                      | KC505125                      |
| JS2011_013_1            | 2011              | Jiangsu    | Human   | KC505126                      | KC505127                      | KC505128                      |
| JS2011_027              | 2011              | Jiangsu    | Human   | KC505129                      | KC505130                      | KC505131                      |
| JS2011_034              | 2011              | Jiangsu    | Human   | KC505132                      | KC505133                      | KC505134                      |
| JS2011_062              | 2011              | Jiangsu    | Human   | KC505135                      | KC505136                      | KC505137                      |
| JS2011_109              | 2011              | Jiangsu    | Human   | KC505138                      | KC505139                      | KC505140                      |
| JS2012_020              | 2012              | Jiangsu    | Human   | KC505141                      | KC505142                      | KC505143                      |
| JS2012_035              | 2012              | Jiangsu    | Human   | KC505144                      | KC505145                      | KC505146                      |
| JS2012_goat01           | 2012              | Jiangsu    | Goat    | KC473537                      | KC473538                      | KC473539                      |
| JS2012_tick01           | 2012              | Jiangsu    | Haemaphysalis longicornis     | KC473540                      | KC473541                      | KC473542                      |
| L_HGX_2010              | 2010              | Huaiyangshan | Human | HQ171187                      |                               |                               |
| L_HZM_2010              | 2010              | Huaiyangshan | Human | HQ171188                      |                               |                               |
| LWI_2009                | 2009              | Huaiyangshan | Human | HQ171186                      |                               |                               |
| LWQ_2010                | 2010              | Huaiyangshan | Human | HQ171190                      |                               |                               |
| L_WWG_2010              | 2010              | Huaiyangshan | Human | HQ171189                      |                               |                               |
| LN3_2010                | 2010              | Liaoning   | Human   | HQ141610                      |                               |                               |
| Phlebovirus_J53_2010    | 2010              | Jiangsu    | Human   | HQ141601                      | HQ141602                      | HQ141603                      |
| Phlebovirus_J54_2010    | 2010              | Jiangsu    | Human   | HQ141604                      | HQ141605                      | HQ141606                      |
| Phlebovirus_LN2_2010    | 2010              | Liaoning   | Human   | HQ141607                      | HQ141608                      | HQ141609                      |
| JS2010_014_2010         | 2010              | Jiangsu    | Human   | JQ317169                      | JQ317170                      | JQ317171                      |
**Table 1.** Cont.

| Isolated strain       | Year of isolation | Location | Host               | NCBI Access number (L segment) | NCBI Access number (M segment) | NCBI Access number (S segment) |
|-----------------------|-------------------|----------|--------------------|--------------------------------|--------------------------------|--------------------------------|
| SDLZtick12_2010       | 2010              | Shandong | Haemaphysalis longicornis | JQ684871                      | JQ684872                      | JQ684873                      |
| SPL003A               | Japan Human       |          |                    |                                |                                |                                |
| SPL004A               | Japan Human       |          |                    |                                |                                |                                |
| SPL005A               | Japan Human       |          |                    |                                |                                |                                |
| SPL010A               | Japan Human       |          |                    |                                |                                |                                |
| SPL032A               | Japan Human       |          |                    |                                |                                |                                |
| SPL035A               | Japan Human       |          |                    |                                |                                |                                |
| XCO-A112L             | 2010              | Hubei    | Haemaphysalis longicornis | JF906056                      | JF906057                      |                                |
| YG1                   | Japan Human       |          |                    |                                |                                |                                |
| YG51_2011             | 2011              | Henan    | Human              |                                |                                |                                |
| YNY1_2011             | 2011              | Henan    | Human              |                                |                                |                                |
| YPQ133_2011           | 2011              | Henan    | Human              |                                |                                |                                |
| YPQ2_2011             | 2011              | Henan    | Human              |                                |                                |                                |
| YPQ5_2011             | 2011              | Henan    | Human              |                                |                                |                                |
| YSC19_2011            | 2011              | Henan    | Human              |                                |                                |                                |
| YSC3_2011             | 2011              | Henan    | Human              |                                |                                |                                |
| YSH9_2011             | 2011              | Henan    | Human              |                                |                                |                                |
| YXX1_2011             | 2011              | Henan    | Human              |                                |                                |                                |
| YXX2_2011             | 2011              | Henan    | Human              |                                |                                |                                |
| 2010_CBX              | 2010              | Huaiyangshan |                |                                |                                |                                |
| 2010_FQM              | 2010              | Huaiyangshan |                |                                |                                |                                |
| 2010_LZR              | 2010              | Huaiyangshan |                |                                |                                |                                |
| 2010_T112             | 2010              | Huaiyangshan |                |                                |                                |                                |
| 2010_WJ               | 2010              | Huaiyangshan |                |                                |                                |                                |
| 2010_WQ               | 2010              | Huaiyangshan |                |                                |                                |                                |
| 2010_WSQ              | 2010              | Huaiyangshan |                |                                |                                |                                |
| 2010_WWG              | 2010              | Huaiyangshan |                |                                |                                |                                |
| 2010_WWX              | 2010              | Huaiyangshan |                |                                |                                |                                |
| 2010_ZGQ              | 2010              | Huaiyangshan |                |                                |                                |                                |
| M_HZM_2010            | 2010              | Huaiyangshan |                |                                |                                |                                |
| M_WSQ_2010            | 2010              | Huaiyangshan |                |                                |                                |                                |
| Phlebovirus_LN3_2010  | 2010              | Liaoning | Human              |                                |                                |                                |
| S_HGX_2010            | 2010              | Huaiyangshan |                |                                |                                |                                |
| S_HZM_2010            | 2010              | Huaiyangshan |                |                                |                                |                                |
| S_WU_2009             | 2009              | Huaiyangshan |                |                                |                                |                                |
| S_WUQ_2010            | 2010              | Huaiyangshan |                |                                |                                |                                |
| S_WWG_2010            | 2010              | Huaiyangshan |                |                                |                                |                                |
| SDLZCattle01_2011     | 2011              | Shandong | Cattle             |                                |                                |                                |
| SDLZDog01_2011        | 2011              | Shandong | Dog                |                                |                                |                                |
| SDLZP01_2011          | 2011              | Shandong | Human              |                                |                                |                                |
| SDLZP02_2011          | 2011              | Shandong | Human              |                                |                                |                                |
| SDLZP03_2011          | 2011              | Shandong | Human              |                                |                                |                                |
| SDLZP04_2011          | 2011              | Shandong | Human              |                                |                                |                                |
| SDLZP05_2011          | 2011              | Shandong | Human              |                                |                                |                                |
| SDLZP06_2011          | 2011              | Shandong | Human              |                                |                                |                                |
| SDLZP07_2011          | 2011              | Shandong | Human              |                                |                                |                                |
| SDLZP08_2011          | 2011              | Shandong | Human              |                                |                                |                                |
Figure 1. Bayesian coalescent and Maximum likelihood (ML) analysis of FTLSV based on the L segment. Maximum clade credibility and Maximum likelihood tree is shown with posterior probability values and bootstrap value depicted at the nodes. Phylogenetic analysis was carried out using MrBayes 3.2.1 and Mega software 5, respectively. Designations of the different FTLSV phylogroups (A, B, C, D, E, F clade) are as indicated, based on previously defined assignments. These sequences are described in more detail in Table 1. The sequences from Japan were included in this analysis.

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| Isolated strain          | Year of isolation | Location | Host | NCBI Access number (L segment) | NCBI Access number (M segment) | NCBI Access number (S segment) |
|--------------------------|-------------------|----------|------|--------------------------------|--------------------------------|--------------------------------|
| SDLZP09_2011             | 2011              | Shandong | Human|                                |                                | JQ693012                       |
| SDLZSheep01_2011         | 2011              | Shandong | Sheep|                                |                                | JQ693002                       |
| SDPLP01_2011             | 2011              | Shandong | Human|                                |                                | JQ693013                       |
10^-4 (5.56 × 10^-5–6.67 × 10^-6) and L = 1.95 × 10^-4 (5.01 × 10^-5–3.37 × 10^-6) nucleotide substitutions/site/year. The MRCA of the current viruses identified today as FTLSV is estimated to have existed 117 (95% HPD = 36–241) years before present based the M segment analysis, 159 (95% HPD = 54–321) years before present based on the L segment analysis, and 195 (95% HPD = 28–455) years before present based on the S segment analysis.

FTLSV probably originated from Huaiyangshan in the 18th century

To determine the geographic origins and movements of FTLSV in China, the results of the Bayesian phylogeographic analyses were summarized by annotating the MCC tree nodes with their most probable location states via color labeling (Figure 4). The colors of the tree branches represent the most probable locations of their associated viral lineages (i.e., those supported by the highest probabilities), and a color change between two connected nodes implies a probable migration event. Simultaneous spatiotemporal pattern of FTLSV movements in China since the time of the MRCA was also visualized in Google Earth. The phylogeographic analysis showed the Huaiyangshan area appears to have been the origin of FTLSV in China (Figure 5). This was confirmed by the MCC tree, which showed that the MRCA of genotypes A–E originated in Huaiyangshan. The root state posterior probabilities for all locations ranged between 9.21% for Liaoning and 37.40% for Huaiyangshan. These dispersal histories are summarized in Figure 5, which shows snapshots of the dispersal patterns between locations from 1793 to 2012. Two main spread routes were observed (Figure 5A, Figure 5B, Figure 5C). FTLSV initially spread from the Huaiyangshan region into the surrounding provinces: eastward to Anhui and Jiangsu Provinces, and northward to Liaoning and Shandong Provinces. Thereafter, FTLSV is predicted to have spread from the Jiangsu Province to Liaoning, Shandong, and Anhui Provinces. In a further investigation of these diffusion patterns, the BF test identified three well-supported (BF > 5) migration routes for FTLSV (Figure 5D): from...
Huaiyangshan to Jiangsu (BF = 9.74), from Jiangsu to Shandong (BF = 3.35), and from Jiangsu to Liaoning (BF = 3.17).

The extent of geographic structure in the FTLSV L phylogeny

Both the AI and PS statistics indicated that there was very strong geographic clustering of FTLSV strains by province or area of origin (P<0.05) (Table 2). When the extent of phylogenetic clustering of individual province was tested, geographic clustering was significant for Huaiyangshan and Jiangsu except for Anhui, Liaoning, and Shandong, for which there was evidence of significant gene flow to and from other regions of China (P>0.05). These results suggest that the genetic diversity of FTLSV in Huaiyangshan and Jiangsu is shaped primarily by in situ evolution.

Figure 3. Bayesian coalescent analysis of FTLSV based on the S segment. Maximum clade credibility and Maximum likelihood tree is shown with posterior probability values and bootstrap value depicted at the nodes. Phylogenetic analysis was carried out using Mrbayes 3.2.1 and Mega software 5, respectively. Designations of the different FTLSV phylogroups (A,B,C,E,F clade) are as indicated, based on previously defined assignments. These sequences are described in more detail in Table 1. The sequences from Japan were included in this analysis.

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Figure 4. Maximum clade credibility tree based on L genes summarized for FTLSV were generated using the geospatial Bayesian analysis. Posterior clade probabilities for key nodes were shown. The colors of the branches correspond to their probable geographic location as calculated using the geospatial analysis.

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Figure 5. (A–C) Inferred migration graph for FTLSV in China and its reflection of the events reconstructed from the MCC tree for 1806, 1928 and 1985. The maps are based on satellite pictures made available in Google Earth. (D) The FTLSV migration routes inferred under the discrete phylogeographic diffusion model using a BF test. Rates supported by $3 \leq BF < 10$.

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Table 2. Extent of geographic clustering, as indicated by Al and PS statistics.

| Statistic                | Observed value | Null value | P value |
|--------------------------|----------------|------------|---------|
|                          | Mean           | Lower 95% CI | Upper 95% CI | Mean           | Lower 95% CI | Upper 95% CI |
| Among province or Area   |                |            |         |                |            |             |
| AI                       | 1.18           | 1.02       | 1.36    | 4.22           | 3.13       | 5.05        | 0.00        |
| PS                       | 13.00          | 13.00      | 13.00   | 24.98          | 22.36      | 27.83       | 0.00        |
| Anhui vs others          |                |            |         |                |            |             |
| AI                       | 0.48           | 0.33       | 0.59    | 0.82           | 0.41       | 1.21        | 0.21        |
| PS                       | 4.00           | 4.00       | 4.00    | 3.90           | 3.00       | 4.00        | 1.00        |
| Huaiyangshan vs others   |                |            |         |                |            |             |
| AI                       | 0.61           | 0.58       | 0.69    | 3.38           | 2.42       | 4.38        | 0.00        |
| PS                       | 7.01           | 7.00       | 7.00    | 20.41          | 17.60      | 23.81       | 0.00        |
| Jiangsu vs others        |                |            |         |                |            |             |
| AI                       | 0.58           | 0.43       | 0.68    | 3.16           | 2.36       | 3.97        | 0.00        |
| PS                       | 6.02           | 6.00       | 6.00    | 17.74          | 15.44      | 20.00       | 0.00        |
| Liaoning vs others       |                |            |         |                |            |             |
| AI                       | 0.08           | 0.08       | 0.08    | 0.43           | 0.10       | 0.63        | 0.02        |
| PS                       | 2.00           | 2.00       | 2.00    | 2.00           | 2.00       | 2.00        | 1.00        |
| Shandong vs others       |                |            |         |                |            |             |
| AI                       | 0.62           | 0.60       | 0.70    | 0.63           | 0.25       | 0.91        | 0.48        |
| PS                       | 3.00           | 3.00       | 3.00    | 2.95           | 3.00       | 3.00        | 1.00        |

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rather than extensive migration. However, the number of sequences from Anhui, Liaoning and Shandong was small, which may bias the result.

Discussion

FTLSV represents a threat to public health, and understanding the how the causative virus sustains and spreads in the environment is important to implementing effective prevention and control measures. Currently, however, the mechanisms underlying the emergence and spread of FTLSV are unknown. The virus was first reported in 2007 [9]; at least 11 provinces in China including Beijing [32], and three countries, Korea [33], Japan (AB817979–AB818002) FTLSV-infected cases. These reports demonstrated the regional threat of this pathogen and indicate that enhanced surveillance is needed to gain a better understanding of its evolutionary history, geographic spread and evolving virulence. Using a powerful and robust Bayesian phylogenetic and phylogeographic approach, we reconstructed the spatial and temporal spread of FTLSV in China.

The phylogenetic relationships between strains circulating in China and Japan were confirmed as a part of this study, as were identification of the five genotypes already identified as circulating in China, consistent with the results reported by Zhang and Lam et al [4,31]. For our comparison, FTLSV sequences were mainly derived from viruses isolated from Henan, Hubei, Jiangsu, Anhui, Liaoning and Shandong Provinces, and the close phylogenetic relationship among them indicated that the movement of FTLSV is common in China. At least two possible reasons for unnumbered movement of FTLSV: 1) there are no obvious physical barriers to prevent viral transmission, as the locations all correspond to plains or hilly regions of China; and 2) these regions share similar enzootic vectors and potential primate and non-primate reservoirs.

Several papers have reported the isolation of FTLSV from two tick species (Haemaphysalis longicornis and Rhipicephalus microplus) and domestic animals such as sheep, goat and cattle [4,7,8]. Furthermore, the analysis performed in this study suggest that FTLSV strains from Japan share a common ancestor with viral genotypes circulating in China, indicating that the virus has crossed the sea between China and Japan, forming the Japanese lineage.

Evolution of FTLSV has impacted the sequence of the three viral RNA segments, creating unique viral genetic types and S, M and L RNA segment phylogenetic topologies indicate that FTLSV reassortment (particularly in the S segment) is not uncommon. Genetic reassortment can profoundly affect the pathogenicity and diversity of RNA viruses [34,35,36,37,38,39]. For example, reassortment among influenza virus strains (another RNA virus with a segmented genome) results in antigenic shift and the emergence of new pandemic strains [40]. The S segment of FTLSV encodes the N protein, which binds viral RNAs and encapsidates them in to ribonucleoprotein complexes (RNP) as well as the NS protein, which prevents the host interferon response by interfering with the cellular mRNAs and protein synthesis machineries of host cells. Reassortment of the S segment, therefore, could influence the replication and immunogenicity of the virus. The existence of many circulating genotypes in China and the wide distribution of potential vectors may increase the opportunity for reassortment to occur in FTLSV. Surveillance for detection of viruses with enhanced pathogenicity is critical to implementing control and prevention measures reducing the impact of FTLSV infection.

Purifying selection also appears to be another potential force in the evolution of FTLSV, although some sites were also found to be under positive selection. These results indicated that nucleotide fixation events are due primarily to genetic drift and stochastic processes, which may be important in the evolution of FTLSV. Strong purifying selection can maintain evidence of sequence homology long after saturation has occurred at synonymous sites [41,42]. But purifying selection can obscure the age of viral lineages (e.g., as observed with measles, Ebola, and avian influenza viruses) [41,42]. This issue is a potential limitation to the work reported here. Unfortunately, there is not a answer to the problem of underestimating the tMRCA due to purifying selection in molecular clock analysis of virus evolution. However, with advances currently being made in the development of increasingly more realistic evolutionary models, the bias imposed by the purifying selection may be solved in the foreseeable future. Still, given the apparent recent history of FTLSV, it is possible that the presence of purifying selection may not have a significant impact of the current evolution of the virus.

Although the emergence of FTLSV and its association with FTLS was first confirmed during the summer of 2009 [1], similar symptoms had been reported by hospitals near Huaiyangshan since 2007 [1]. Consistent with this, these results indicate that FTLSV has been circulating in China for many years. The reconstructed epidemic history of FTLSV suggests that the virus is a recently emerged pathogen that originated around the 18th century. Bayesian analysis indicated that the most recent common ancestor of the FTLSV strains existed an estimated 117 to 195 year prior to 2012. Similar estimates were obtained for the L, M and S segments, indicating that these segments have co-evolved. Lam et al. [31] reported that FTLSV originated from a common ancestor and most likely existed between 1817 and 1895, which is somewhat more recent estimate than the one proposed in this analysis. This difference might be explained in two ways. First, the evolutionary rate of Rift Valley Fever Virus (RVFV) was used as a model for the FTLSV analysis of Lam et al. Although RVFV and FTLSV both belong to the family Bunyaviridae, RVFV is mosquito-borne while FTLSV is tick-borne, and mosquito-borne RNA viruses are believed to evolve more rapidly than tick-borne viruses within the same family [43,44]. Second, our analysis used full-length sequences of the three segments, and included many more sequences than were available in GenBank at the time of the previous study.

Bayesian phylogeographic analysis indicated that the Huaiyangshan area might be the origin of FTLSV. Huaiyangshan Mountain borders 
Henan and Hubei Provinces in China; these provinces are the source of the HuaHe River, which forms the dividing line between southern and northern China. This biologically diverse region has been and remains a home to cultural and economic exchanges between the two halves of China. As FTLSV has been detected in the circulating tick vectors Haemaphysalis longicornis and Rhipicephalus microplus, which are widely distributed across China and globally, conditions exist for FTLSV to have been transmitted from Huaiyangshan to neighboring provinces in China [4]. BaTS analysis detected strong structuring of the FTLSV phylogeny by provinces or area, suggesting that in situ evolution has played an important role in the maintenance of FTLSV in China. Additionally, the results of the BaTS analysis that considered the extent of population subdivision between individual provinces or areas and all others as a group indicate that for two of the five provinces or areas sampled (i.e., Huaiyangshan, and Jiangsu), the observed FTLSV genetic diversity was shaped primarily by in situ evolution as opposed to extensive migration.

This paper offers the first description of the movement of FTLSV in China. Our results indicate that this movement was slow during the early years of the spread, but has increasingly become more rapid in recent decades. Bayesian phylogeographic
analysis showed that FTLSV arose in Huaiyangshan between 1756 and 1817, and then moved into the Jiangsu and Anhui Provinces between 1773 and 1834. Over the next hundred years, FTLSV continued to circulate through Jiangsu, Anhui and the Huaiyangshan region. Once FTLSV reached Jiangsu Province, this province became a secondary source of FTLSV spread, as the virus moved from Jiangsu Province into Liaoning Province between 1905 and 1943. In the past 50 or 60 years FTLSV has moved more rapidly, as the MCC tree shows that many strains isolated from Shandong, Liaoning and Anhui were recently introduced from Huaiyangshan and Jiangsu. Finally, the Bayesian test provided statistical support for three transmission routes: from Huaiyangshan to Jiangsu, from Jiangsu to Shandong and from Jiangsu to Liaoning.

Collectively, these findings indicate that Huaiyangshan and Jiangsu may be the two major origins of FTLSV in China. Movement over large distances was observed, such as that between Liaoning and Jiangsu Province and Huaiyangshan (~1500 km). It is likely that an animal infected with FTLSV or carrying virus-infected ticks introduce FTLSV into Liaoning Province. The recent dramatic increases in unregulated wildlife trade, livestock import/export, and global human movement are likely to have created additional opportunities for spread of this severe disease. In addition, expanding urban populations have triggered ecosystem changes that may have promoted the emergence of FTLSV by increasing human exposure to the natural reservoir or vector.

In conclusion, our results provide new insights into the origin and spread of FTLSV in China. Further studies are needed to obtain complete FTLSV genomes from other countries in which the virus is endemic, and to elucidate the dynamics of the worldwide dispersion of FTLSV.

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Author Contributions

Conceived and designed the experiments: BX WC YJ XH LL YY. Performed the experiments: YD WW HW JS XT QL. Analyzed the data: XH LL YD. Contributed reagents/materials/methods analysis tools: XH LL YD. Wrote the paper: BX WC YJ XH LL YY.
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