· Rapid Communication ·

Hemorrhagic fever caused by a novel tick-borne *Bunyavirus* in Huaiyangshan, China

Yong-Zhen Zhang[1+*], Dun-Jin Zhou[2+*], Yanwen Xiong[1+], Xiao-Ping Chen[1+], Yong-Wen He[3+], Qiangzheng Sun[1+], Bin Yu[2+], Juan Li[1+], Yong-An Dai[4+], Jun-Hua Tian[2+], Xin-Cheng Qin[1+], Dong Jin[1], Zhigang Cui[1], Xue-Lian Luo[1], Wei Li[3], Shan Lu[1], Wen Wang[1], Jin-Song Peng[2], Wen-Ping Guo[1], Ming-Hui Li[1], Zhen-Jun Li[1], Shaomin Zhang[1], Chen Chen[1], Yan Wang[1], Menno D. de Jong[5], Jianguo Xu[1*]

[1] State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Changping Liuzi 5, 102206, Beijing, China

[2] Wuhan Center for Disease Control and Prevention, 430022, Wuhan, Hubei Province, China

[3] Department of Infectious Diseases, Affiliated Union Hospital of Tongji Medical College, Huazhong University of Science and Technology, 430022, Wuhan, Hubei province, China

[4] Department of Infectious Diseases, Zhongnan Hospital, Wuhan University, 430071, Wuhan, Hubei province, China

[5] Department of Medical Microbiology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

[+] Contributed equally to this work.

[*] Correspondence to Dr. Jianguo Xu (Email: xujianguo@icdc.cn) and Dr. Yong-Zhen Zhang (Email: yongzhenzhang@sohu.com), State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Changping Liuzi 5, Beijing, 102206, China; Dun-Jin Zhou (Email: zdj@whcdc.org), Wuhan Center for Disease Control and Prevention, 430022, Wuhan, Hubei Province, China.

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Abstract

Background:

From April to July in 2009 and 2010, unexplained severe hemorrhagic fever-like illnesses occurred in farmers from the Huaiyangshan mountains range.

Methods:

Clinical specimens (blood, urine, feces, and throat swabs) from suspected patients were obtained and stored. Mosquitoes and ticks in affected regions were collected. Virus was isolated from 2 patients and characterized by whole genome sequencing. Virus detection in additional patients and arthropods was done by virus-specific reverse transcription (RT) PCR. Clinical and epidemiological data of RT-PCR confirmed patients were analyzed.

Results:

An unknown virus was isolated from blood of two patients and from *Haemaphysalis* ticks collected from dogs. Whole genome sequence analysis identified the virus as a novel member of the family *Bunyaviridae*, most closely related to the viruses of the genus *Phlebovirus* within which it forms a separate lineage. Subsequently, infection was confirmed by RT-PCR in 33 of 58 suspected patients. The illness in these patients was characterized by fever, severe malaise, nausea, vomiting, and diarrhea. Prominent laboratory findings included low white cell- and platelet counts, coagulation disturbances, and elevation of liver enzymes. Hemorrhagic complications were observed in 3 cases, 5 (15%) patients died.

Conclusions:

A novel tick-borne *Bunyavirus* causing life-threatening hemorrhagic fever in humans has emerged in the Huaiyangshan mountain areas of China. Further studies are needed to determine the epidemiology, geographic distribution and vertebrate animal ecology of this virus.

Introduction

Viral hemorrhagic fever is a clinical syndrome characterized by acute fever and bleeding disorders, and caused by zoonotic and arthropod-borne RNA viruses, including *Filoviridae* (e.g., Ebola hemorrhagic fever, Marburg hemorrhagic fever), *Arenaviridae* (e.g., Lassa fever), *Flaviviridae* (e.g., Dengue hemorrhagic fever (DHF), Yellow fever and *Bunyaviridae* (e.g., Crimean-Congo hemorrhagic fever (CCHF), Hantavirus-associated hemorrhagic fever with renal syndrome (HFRS). Currently, HFRS, CCHF, DHF, and Chikungunya fever are endemic in China.[1, 2] The emergence of novel or previously unrecognized hemorrhagic fever-associated viruses is not unlikely in areas where people, animals and arthropod vectors live in close proximity.

From April to July in 2009 and 2010, several patients with hemorrhagic fever-like illnesses of unknown etiology were admitted to hospitals in Wuhan city, the capital of Hubei province in central China. All patients were living in rural areas of the Huaiyangshan mountains region. A novel virus was isolated from the two patients' blood as well as from *Haemaphysalis* ticks collected from dogs in the same region. Full genome characterization identified this virus as a novel member of the family *Bunyaviridae*. We herein present the genetic characterization of the virus and the clinical, virological and epidemiological data of 33 laboratory-confirmed infected patients.

Materials and Methods

Case definitions and clinical and laboratory data

Patients presenting with acute onset of fever (>37.5 ℃), leucopenia (<4.0 × 10E9/L), thrombocytopenia (<100 × 10E9/L), who were admitted to Union Hospital or Zhongnan Hospital in Wuhan city (catchment population 61 million), were considered as suspected cases if no other causes (including hantavirus and *Anaplasma phagocytophilum*) were apparent by routine investigations. Confirmed cases were defined by RT-PCR detection of the novel virus in the patient's blood. During admission, blood specimens, and when feasible, feces, urine and throat swabs were collected and stored at -80 ℃ until further processing. Clinical and laboratory data were retrieved from clinical records of confirmed patients. Patients from 2009 were identified retrospectively, active case-finding was initiated in April 2010.

The study was approved by the ethics committee of National Institute of Communicable Disease Control and Prevention, according to medical research regulations of the Ministry of Health, China. Oral informed consent was obtained from all patients. The first and last author designed the study. All authors contributed to collection or analysis of data and vouch for the quality and accuracy of these. The first and subsequent drafts were written by the first and last three authors who also decided to publish the paper with mutual agreement of all authors.

Collecting ticks and mosquitoes

Some patients had a history of tick bites and ticks were even found on some patients during admission. In May 2010, ticks were therefore collected from cattle, dogs, and goats in four villages where laboratory-confirmed patients were living. Ticks collected from each animal were kept alive in separate vials and labeled, with collection points noted, and were identified by morphological examination and further confirmed by sequence analysis of mitochondrial 12S rDNA.[3] They were homogenized for virus isolation and viral RNA detection by RT-PCR. In the same area, mosquitoes were also collected by electric traps in cattle and pig barns, and identified by morphological examination.

Isolation of virus with Vero E6 cells

Whole blood from patients and supernatants of homogenized ticks were used to inoculate Vero E6 cell monolayers as described previously.[4] After 2 weeks of incubation, trypsinized cells were fixed onto glass slides for detection of virus antigens by indirect immunofluorescence assay (IFA) using the patients' sera as the primary antibody. Isolates were further identified by sequence-independent amplification of nucleic acids and sequence analysis (see below). Ultrastructural characteristics of the isolates grown in Vero E6 cells or enriched by ultracentrifugation were observed by a transmission electron microscope.

Serologic analysis

Sera from patients were tested for specific IgG antibody by IFA, using Vero E6 cells infected

with the new virus as antigen, and non-infected cells as control. Sera were also tested for the presence of antibodies against hantaviruses (strains 76–118 and L99) and *A. phagocytophilum* as described previously.[5,6]

Whole genome sequencing and diagnostic reverse transcription (RT) PCR

Sequence-independent single primer amplification was used to identify viral nucleic acids of unknown genetic sequence.[7] Whole genome sequences were subsequently generated by primer walking strategies. Based on generated whole genome sequences, two nested RT-PCR methods were designed, one targeted at the L segment, generating a 914 bp fragment (nt 2208–3121), and one at the S segment, generating a 601 bp fragment (nt 63–663) (Table 1). The PCR products were gel-purified and cloned into pMD18-T vector. DNA sequencing was performed with the ABI-PRISM Dye Termination Sequencing kit on an ABI 3730 genetic analyzer (Applied Biosystems, Carlsbad, CA, USA). At least 2 cDNA clones were used to determine each viral sequence.

Table 1. Specific primers used to amplify the whole genome of Huaiyanshan virus.

Primer	Primer Sequence (5′-3′)	Base position	Amplicon size(bp)
L Segment			
5-Ter-F1	ACACAAAGACGCCCAGATGA	1-20	1132
L1112-R1	GACATCTAGAAGGCACTTTGC	1132-1112	
5-Ter-F2	ACGCCCAGATGAACTTGGAA	9-28	1062
PT1050-R2	AGCCTGAGTCGGTCTTGATGT	1070-1050	
L968-F1	AGGAGCAACAAGCAAACATCAT	968-989	1305
L2253-R1	CAATGTACCAGCTGTTCACCA	2273-2253	
P991-F2	GAATCGAGGGACAGTCAAAC	991-1010	1250
P2221-R2	TTGTGGACCGGCCTGAGATG	2240-2221	
L2136-F1*	TGGATTGCATGGTGCGAATTG	2136-2156	1020
L3135-R1*	GATCAGATGACCTAGACTCAG	3155-3135	
Seq2F2*	GAACATTCCATGCCATCTCAG	2208-2228	914
Seq1R2*	GTGAGCTAAAAACCTTAGGTC	3121-3101	
L3031-F1	CATGCCAGCYAAATTCCACAG	3031-3051	1125
L4135-R1	TTCTTGTTGGCAGCTCTCCTG	4155-4135	
L3058-F2	TTGGGCTGCCATTTCCATGTT	3058-3078	1073
P4111-R2	GGACTCCAGGATTCTCATCT	4130-4111	
PN4010-F1	GGAACTCTCAGCCACTCTGTT	4010-4030	974
L4963-R1	GTAGAGAAGGCCTCTATGATC	4983-4963	
PN4071-F2	TGAACAGGATGGGCCTTCCTG	4071-4091	884
L4934-R2	CTTGTACTCCTCAGTGTATGG	4954-4934	
L4843-F1	TAGCCTAGAAGCTGAGAAGAG	4843-4863	900
P5722-R1	CCTTGGGTCTTCCTATCATTT	5742-5722	
P4891-F2	TATCTCCATCCTCAAGCATGT	4891-4911	829
P5699-R2	AGGAGAACTGAGGCATGTGA	5719-5699	
L5577-F1	ACACTGATATATCAGAGTCAGC	5577-5598	792
3-Ter-R1	ACACAAAGACCGCCCAGATC	6368-6349	
P5617-F2	CAATCGAGACCTCTTCTCCT	5617-5636	744
3-Ter-R2	ACCGCCCAGATCTTAAGGAA	6360-6341	
M Segment			
M-F1	ACACAAAGACGGCCAACAATG	1-21	630
M611-R1	GTCTGGGAATTCACTTTGGC	630-611	
M-F2	ACGGCCAACAATGATGAAAGTC	9-30	596
M585-R2	TCTTCAGCTCCAGAAATGTC	604-585	
M526-F1	GATAGGGTTCTCTGGATAGG	526-545	455
M960-R1	ATCCCTCCATATGACACAACG	980-960	
M543-F2	AGGTGATGTTGCTTGTCAGC	543-562	402
M924-R2	TGCACAAGTGAGCATCTACAC	944-924	
M810-F1	GTRCAAGAGAGCTCATCCAAG	760-780	614
M1354-R1	TTCTTTGCAGGGTAGCACTG	1373-1354	
M838-F2	TGTGCTAYAAGGAAGGGACTG	788-808	565
M1331-R2	GGATTTTTTAGAAACTCACGAC	1352-1331	
M1267-F1	GAATTCACATTTGAGGGTAGTT	1267-1288	678
M1922-R1	ACCGGGCATCAGGAACAAAAT	1942-1922	
M1285-F2	AGTTGCATGTTCCCAGATGG	1285-1304	589
M1853-R2	TGAGACATTTTGAGTCYGGAC	1873-1853	
M1705-F1	TGTGATGAGATGGTCCATGCT	1705-1725	626
M2311-R1	ACCTCCATATCTGAGCCCAA	2330-2311	
M1751-F2	AAGGGAGCGGAAATATGAAGG	1751-1771	553
M2284-R2	ACACTCACACCCTTGAAGAC	2303-2284	
M2220-F1	AGAGCTAACAATGCCCTCAG	2220-2239	581
M2780-R1	AGACTTTGGCGCCTGTCATGC	2800-2780	
M2247-F2	GAGGACATTCCACCCCATGA	2247-2266	516
M2742-R2	ACATTTGTCACYTCCCCTGTG	2762-2742	
M2655-F1	CCCCCTGGACATCACAGCTAT	2655-2675	460
M3095-R1	CCCATTGCCAAACAAGGCAT	3114-3095	
M2690-F2	CTGTYAATTATAGAGGCCTTCG	2691-2711	387
M3058-R2	CTTGGGATATTTGCCCCTGT	3077-3058	
M2943-F1	ACTGAACTGTGGGGGACATG	2943-2962	435
M3358-R1	ACACAAAGACCGGCCAACACT	3378-3358	
M3014-F2	TTGTGGATGGCAGCTACATG	3014-3033	351
M3343-R2	AACACTTCAAYRGAACCTCCAT	3365-3344	
S Segment			
S1-F1	ACACAAAGAACCCCCAAAAAAGG	1-23	711
S692-R1	CCCTTGGCCTTCAGCCACTT	711-692	
S9-F2	AACCCCCAAAAAAGGAAAGACG	9-30	661
S650-R2	AAGACAGAGTTCACAGCAGC	669-650	
S562-F1	GCAAGATGCCTTCACCAAGA	562-581	462
S1003-R1	ACAGTGTCTTGGATGAGGATG	1023-1003	
S603-F2	GAGCCAGCAAGACAGAAGTT	603-622	374
S956-R2	CCAYATCTGATGGCACACTAT	976-956	
S829-F1	GACAAAATTAGACCTCCTTCG	829-849	643
S1452-R1	TGTACTACAAGGACATGAGG	1471-1452	
S857-F2	ACCAATGGCTGGCCAATCTCT	857-877	494
S1341-R2	TCAAGAACAGCTGGGCAATG	1350-1341	
S1259-F1	TATCATGTCCCCTTCAAAAAG	1259-1279	488
S1725-R1	ACACAAAGAACCCCCTTCATTT	1746-1725	
S1313-F2	GGCCCCGCCAGTTCTCTCT	1313-1331	426
S1717-R2	AACCCCCTTCATTTGGAAACCA	1738-1717	
Stest-F1*	ATGTCAGAGTGGTCCAGGATT	44-64	675
Stest-R1*	AAGGATTCCCTTGGCCTTCA	718-699	
Stest-F2*	TTGCAGTGGAGTTTGGTGAGC	63-83	601
Stest-R2*	GAGTTCACAGCAGCATGGAG	663-644	

* Primes used for clinical sample detection.

Phylogenetic analysis

Viral gene segment sequences and tick mtDNA sequences were aligned by using the ClustalW2 program. The nucleotide (nt) and amino acid (aa) identities were calculated by using the DNAStar program. Neighbor-Joining (NJ) optimality criteria were used for phylogenetic reconstruction using PAUP* (version 4b10). For NJ, we used a transition/transversion ratio (Ts/Tv) of 2 : 1. Heuristic searches were performed for both optimality criteria, with 1000 (NJ) random stepwise additions and tree bisection reconnection (TBR) branch swapping. Node support value was evaluated with 1000 bootstraps for NJ trees. Viral and tick mtDNA sequences available in GenBank were used for phylogenetic relationships.

Results

Isolation and genetic characterization of virus from patients

Blood from two patients in 2010 (patients 2010-2 and 2010-13, Tables 2 and 3), of whom one died, were inoculated onto Vero E6 cells. At 14 day post-infection, virus-positive cells were found by PCR, IFA and electron microscopy (Figure 1). The two isolates were designated Huaiyangshan-Human-1 and Huaiyangshan-Human-2 respectively. We determined the whole genome sequences from the two isolates. The negative-sense RNA genome consisted of 11,492 nt and revealed a genomic structure characteristic of the family *Bunyaviridae*, consisting of 3 separate segments: a 1746 nt small (S) segment, a 3378 nt medium (M) segment, and a 6368 nt large (L) segment (Figure 2A). Phylogenetic analysis of the complete L segment sequences revealed that the two isolates were closely related to each other (95.95% nt identity, 99.14% aa identity), but were genetically distinct from any of the known Bunyaviruses. Highest similarity was observed with Uukuniemi virus (D10759) (38.83% at nt level and 28.57% at aa level) and other members of the genus *Phlebovirus* (up to 32.47% at nt level and 39.57% at aa level) within which the new 'Huaiyangshan virus' formed a separate lineage in the S- and L trees (Figure 2B-D). The trees based on the S and M segment sequences had similar topologies as those based on the complete L segment sequences.

Retrospective and prospective hospital-based case finding in 2009 and 2010

Blood samples from 37 patients in 2009 who fulfilled our criteria of suspected cases were submitted to National Institute for Communicable Disease Control and Prevention, China CDC. Of these, 'Huaiyangshan virus' RNA could be detected by RT-PCR and sequencing in 23 (62.2%) specimens obtained at day 2 to 22 from onset of the illness. Of the 23 PCR-confirmed cases, serum was available from 9 patients all of which tested positive for virus-specific IgG antibody by IFA. No virus-specific antibody was detected in the available sera from 3 PCR-negative suspected patients.

Between April 15 and July 20, 2010, a total of 21 suspected patients were prospectively investigated. Both L and S segment sequences were detected in blood of 20 (95.2%) patients collected at day 1-40 from illness onset (Table 4). Virus-specific IgG antibodies could be detected in all 20 PCR-positive patients but not in the remaining PCR-negative suspected patient. Paired sera were available from 12 patients, which all showed at least four fold increases in IgG antibody titers (Table 5). Antibodies against hantaviruses or *A. phagocytophilum* were not detectable in any of the patients in 2009 and 2010.

Clinical presentation of the disease

The clinical records of a total of 33 confirmed cases, including 13 in 2009 and 20 in 2010, were analyzed in detail (Tables 2 and 7). Patients were all previously healthy, aged 37 to 78 years (mean 55 years), and equally distributed between the male and female sex. The patients were admitted after 2 to 15 days (median 7 days) of illness with fever (100%) and varying rates of non-specific symptoms including severe malaise (48%), nausea (39%), vomiting (36%), diarrhea (42%), and myalgia or arthralgia (24%). Hemorrhagic complications were noted in 3 patients, including severe vaginal bleeding in 2 cases and gum bleeding in the third.

Laboratory abnormalities included low blood white cell counts (29/32), low platelet counts (30/32), increased thrombin times (19/21), activated partial thromboplastin times (22/23), and high levels of lactate dehydrogenase (LDH, 27/29), creatine kinase (CK, 22/28), and blood urea nitrogen (BUN, 9/28) (Tables 3 and 8). Increased hepatic transaminase levels were observed in all patients, including elevated levels of

Table 2. Individual clinical symptoms and signs in Huaiyangshan virus infected patients in Henan and Hubei provinces, China, 2009-2010.[a]

Patient No.	Age	Sex	Fever	Malaise	Chills	Abdominal pain	Diarrhea	Nausea	Vomiting	Arthralgia/ myalgia	Headache	Hemorrhagic complications	Days of Hospitalization[b]
2009-1	59	M	+	-	-	-	-	-	-	-	-	-	9
2009-2	71	F	+	-	+	-	+	-	-	-	-	-	5
2009-3	59	F	+	+	+	-	+	+	-	-	-	-	7
2009-4	48	M	+	+	+	-	+	+	+	+	+	-	20
2009-5	57	M	+	+	+	+	-	-	-	-	-	-	12
2009-6	78	M	+	-	-	-	+	+	-	-	-	-	1
2009-7	52	F	+	-	-	-	-	-	-	-	-	-	12
2009-8	42	M	+	-	+	-	+	+	-	-	-	-	11
2009-9	56	F	+	+	-	-	+	+	+	-	+	+	21
2009-11	42	M	+	+	+	-	-	-	-	-	-	-	7
2009-12	45	F	+	+	-	-	+	+	+	-	-	-	4
2009-13	50	F	+	+	-	-	-	-	-	-	-	-	4
2009-14	63	M	+	+	-	+	-	+	-	+	+	-	4
2010-1	37	F	+	-	-	-	-	-	-	+	+	-	4
2010-2[c]	46	F	+	+	+	-	+	+	+	-	-	+	9
2010-3	53	F	+	+	+	-	-	+	+	-	-	-	9
2010-4	37	F	+	+	-	-	-	-	-	-	-	-	14
2010-5	52	M	+	-	-	-	-	-	-	-	-	-	1
2010-6	68	M	+	-	-	-	-	-	-	-	-	-	1
2010-7	61	M	+	-	-	-	+	-	+	-	-	-	11
2010-8	54	F	+	-	-	-	-	+	+	-	-	-	11
2010-9	44	F	+	-	-	-	-	-	-	-	-	-	10
2010-10	57	M	+	+	-	-	+	-	-	+	-	-	15
2010-11	69	F	+	+	-	-	-	-	-	-	-	-	11
2010-13[c]	63	M	+	+	+	-	-	-	+	-	-	-	4
2010-14	64	F	+	-	-	-	+	-	-	+	-	+	19
2010-15	59	M	+	+	+	-	+	-	+	+	+	-	19
2010-16	78	F	+	-	-	-	-	-	-	-	-	-	0.125
2010-17	47	M	+	-	-	-	-	-	-	-	-	-	12
2010-18	49	F	+	-	+	-	+	+	+	-	-	-	4
2010-19	55	F	+	+	+	-	-	+	+	+	-	-	10
2010-20	46	M	+	-	-	+	-	-	-	+	-	-	14
2010-21	51	F	+	-	+	-	+	+	+	-	-	-	9

[a]: Fatal cases are in bold. Remaining cases survived.

[b]: The median time of hospitalization was 9 (0.125-21) days.

[c]: Patients from whom the two viruses (Huaiyangshan-Human-1 and Huaiyangshan-Human-2) were isolated.

aspartate aminotransferase (AST) levels in 27 of 30 patients and increased alanine aminotransferase (ALT) levels in 28 of 31 patients. The presence of occult blood and protein in urine were noted in 7 of 13 and 14 of 20 patients respectively.

Beside in blood, viral RNA could be detected in throat swabs from 12 of 19 (63%) patients, urine from 11 of 16 (69%) and feces from 6 of 13 (46%) patients (Table 4). In 3 of 13 patients, virus could be detected in all 3 specimens.

Five of 33 (15.2%) patients died, with ages ranging from 50 to 78 (mean 62) years. These deaths occurred soon after admission (3 hours to 4 days). The cause of death was cerebral hemorrhage in one patient, and multi-organ failure in four cases. In these fatal cases, levels of liver enzymes, LDH, and CK, as well as APTT and TT values were all significantly elevated. Surviving patients were discharged without apparent sequelae after 1 to 21 (median 10.5) days of hospitalization.

Geographic distribution and epidemiological features

All 33 confirmed patients analyzed in detail were farmers, involved in domestic animal herding, tea collection and other agricultural activities, living in the villages along the low Huaiyangshan mountain areas (Figure 3). The Huaiyangshan mountains are composed of three mountains, Tongbaishan Mountain, Dabieshan Mountain, and

Table 3. Individual laboratory findings in Huaiyangshan virus-infected patients in Henan and Hubei provinces, China, 2009–2010.*

Patient No.	WBC (10^9/L)	LYMPH %	NEUT %	PLT (10^9/L)	HGB (g/L)	ALT (U/L)	AST (U/L)	ALB (g/L)	APTT (s)	TT (s)	LDH (U/L)	CK (U/L)	BUN (mmol/L)	CR (μmol/L)
2009–1	2.89	49.1	32.5	13	143	119	146	31.3	/	/	439	256	3.6	68.8
2009–2	3.80	23.8	23.8	117	136	29	71	36.9	/	/	216	144	6.4	101.0
2009–3	2.30	37.6	36.3	40	137	69	351	41.6	49.1	72.0	2033	1625	10.1	120.0
2009–4	0.59	54.2	35.6	30	129	195	620	24.7	40.5	19.0	354	92	6.1	108.8
2009–5	1.20	43.3	45.73	66	145	147	423	29.9	55.4	22.1	516	218	3.9	80.4
2009–6	1.20	13.0	82.3	40	121	74	275	26.3	/	/	702	355	6.1	91.0
2009–7	0.90	40.3	50.7	45	135	/	96	35.4	31.4	51.6	298	92	10.4	110.3
2009–8	2.39	25.9	65.3	11	161	136	431	27.1	39.9	21.5	/	/	13.9	219.4
2009–9	0.99	29.3	61.6	50	55	43	32	28.1	51.6	52.6	1226	868	3.7	62.5
2009–11	1.15	28.7	67.0	41	154	48	133	34.1	40.1	27.1	1512	/	6.7	118.4
2009–12	1.35	47.4	33.4	13	65	42	88	39.5	38.3	19.0	/	/	5.0	110.9
2009–13	3.80	15.3	47.9	22	95	125	753	/	99.3	/	2331	318	12.6	213.6
2009–14	0.95	28.4	53.6	45	146	117	580	29.7	71.6	18.9	1953	1279	9.7	119.4
2010–1	2.65	58.5	36.4	102	136	59	67	/	35.5	19.5	334	107	/	/
2010–2†	3.16	0.89	57.2	32	102	178	314	40.7	46.0	23.1	440	228	14.9	173.8
2010–3	4.93	41.2	22.7	26	101	127	145	/	51.7	51.6	1737	464	5.33	74.1
2010–4	3.53	53.84	22.74	98	113	54	35	29.4	/	/	210	86	/	53.0
2010–5	1.29	/	/	11	/	619	1889	/	/	/	2779	6660	16.36	/
2010–6	1.65	32.9	64.5	69	138	/	/	/	/	/	/	/	/	/
2010–7	1.77	26.0	61.0	28	156	225	438	28.1	/	/	1109	1023	6.93	72.4
2010–8	1.25	54.7	35.8	6	108	127	305	34.5	45.9	29.7	446	596	3.00	47.5
2010–9	3.11	22.9	74.6	4	92	110	386	22.7	51.7	11.7	802	1225	4.86	96.6
2010–10	2.12	40.6	49.82	13	163	389	1310	35.3	76.4	/	2510	1911	6.39	71.5
2010–11	1.40	17.7	74.7	49	125	23	53	35.9	48.3	21.3	262	348	6.80	70.0
2010–13†	0.64	34.4	56.2	7	120	243	528	37.7	97.8	101.7	1598	1795	5.90	95.4
2010–14	0.61	4.67	94.3	12	48	44	184	27.3	55.4	17.0	765	1223	6.47	106.0
2010–15	1.45	13.40	83.0	39	110	223	590	28.0	62.3	15.8	467	14.30	8.44	133.0
2010–16	/	/	/	/	/	/	/	/	/	/	/	/	/	/
2010–17	1.56	34.0	60.2	51	145	33	87	35.3	/	19.5	436	929	/	91.0
2010–18	4.34	11.3	86.3	88	162	168	358	/	50.8	/	871	940	24.82	583.2
2010–19	7.73	28.6	59.7	47	128	77	40	35.8	/	/	279	52	1.90	45.0
2010–20	1.88	33.8	59.2	36	174	333	1502	28.6	66.7	112.9	3403	9805	6.32	77.2
2010–21	1.80	18.9	74.4	54	135	45	106	22.0	56.3	23.1	533	311	4.70	71.8
Normal values	4–10	20–40	50–70	100–300	110–150	0–40	0–45	35–50	25–35	12–16	109–245	38–174	2.9–8.2	44–133

*: Fatal cases are in bold. Remaining cases survived.

†: Patients from whom the two viruses (Huaiyangshan-Human-1 and Huaiyangshan-Human-2) were isolated.

Abbreviations are: WBC, White blood cell; LYMPH, Lymphocyte; NEUT, neutrophil; PLT, platelet; HGB, hemoglobin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; APTT, Activated partial thromboplastin time; TT, thrombin times; LDH, lactate dehydrogenase; CK, creatine kinase; BUN, blood urea nitrogen; BUN, blood urea nitrogen; CR, creatinine.

Zhangbaling Mountain, which border Henan, Hubei, and Anhui provinces of China. The region where confirmed cases originated from encompasses an area of 31, 300 km^2 with a population of approximately 12 million persons.

In 2009, all confirmed cases presented between April 23 and July 6 with a peak incidence in late May, and no patients were reported in the remaining months. In 2010, the first cases were again observed in April but have continued to occur up to the present time (25 October 2010).

Vector studies

A total of 613 adult ticks, which belonging to two species (540 *Haemaphysalis longicornis*, 73 *Boophilus Microplus*) were collected from cattle, goats and dogs in 4 counties (Dawu, Xinzhou, Zengdu, Xinxian) of Hubei and Henan provinces where confirmed patients were living (Figure 3). Of that total, 57 *H. longicornis* ticks were collected from 2 cattle in Dawu; 111 ticks were from in Xinzhou [38 *H. longicornis* from 1 cow (6) and 2 dogs (32), 73 *B. Microplus* from 1 cow (69) and 2 dogs (4)]; 100

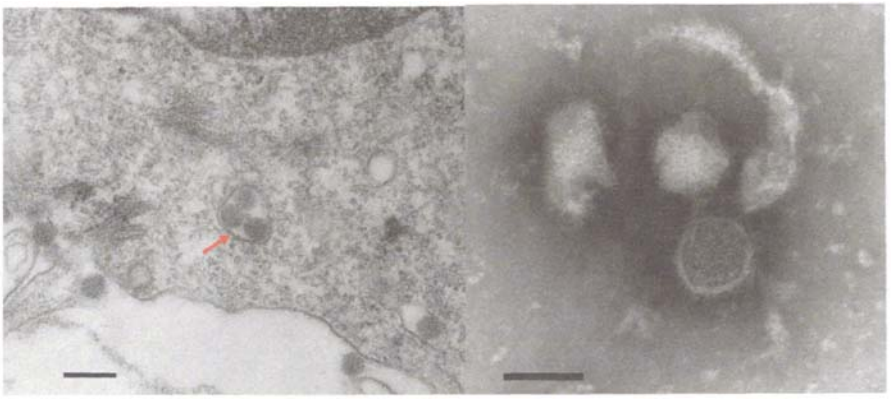

Figure 1. Ultrastructural Characteristics of Huaiyangshan virus Grown in Vero E6 Cells.
Left panel shows a thin-section electron-microscopical view of Huaiyangshan virions (arrow) in the lumen of an endoplasmic reticulum. Negative-stain electron microscopy (right panel) shows the stain-penetrated Huaiyangshan virions (sodium phosphotungstate stain). The bars represent 200 nm (left) and 100 nm (right) respectively.

Table 4. Detection of Huaiyangshan virus RNA by RT-PCR in the first and last available samples of the confirmed patients in 2010. [a]

Patient No.	RNA							
	Blood		Throat swab		Urine		Feces	
	Collected at day from onset of illness (last/first)	Virus	Collected at day from onset of illness (last/first)	Virus	Collected at day from onset of illness (last/first)	Virus	Collected at day from onset of illness (last/first)	virus
2010-1	7/7	+/+	7/7	-/-	9/9	-/-	9/9	+/+
2010-2 [b]	40/8	+/+	29/8	+/-	40/9	-/-	9/9	-/-
2010-3	9/9	+/+	10/9	+/+	NA		NA	
2010-4	16/10	+/+	10/10	+/+	16/11	-/-	18/18	-/-
2010-5	6/6	+/+	6/6	+/+	6/6	+/+	NA	
2010-6	10/10	+/+	10/10	+/+	NA		NA	
2010-7	25/7	+/+	17/10	+/-	25/11	+/+	15/11	+/-
2010-8	16/16	+/+	16/16	+/+	18/16	+/+	17/17	+/+
2010-9	12/12	+/+	12/12	-/-	14/12	+/+	NA	
2010-10	34/7	+/+	12/7	+/+	12/11	+/-	7/7	-/-
2010-11	13/5	+/+	13/5	-/-	14/5	+/+	5/5	-/-
2010-13 [b]	3/3	+/+	3/3	+/+	NA		NA	
2010-14	21/11	+/+	15/11	+/+	18/15	+/+	20/20	-/-
2010-15	18/7	+/+	15/8	+/+	8/8	+/+	18/8	+/+
2010-16	6/6	+/+	NA		NA		NA	
2010-17	16/16	+/+	16/16	+/+	19/17	-/-	19/17	-/-
2010-18	1/1	+/+	1/1	+/+	3/3	+/+	NA	
2010-19	11/8	+/+	11/8	+/+	12/10	+/+	12/10	+/+
2010-20	21/12	+/+	15/12	+/-	18/14	+/+	18/14	+/+
2010-21	25/8	+/+	15/9	-/-	9/9	+/+	10/10	+/+

Not: +, virus detected; -, virus detected negative; NA, no samples available.
[a]: Fatal cases are in bold. Remaining cases survived.
[b]: Patients from whom the two viruses (Huaiyangshan-Human-1 and Huaiyangshan-Human-2) were isolated.

H. longicornis ticks were from Zengdu (72 from 5 cattle and 28 from 7 goats); 345 *H. longicornis* ticks were from 3 cattle in Xinxian county. None of the animals from which ticks were collected showed signs of illness and there were no reports of a similar disease occurring in animals in the same region.

Huaiyangshan viral RNA was detected by nested RT-PCR in 4 pools of *H. longicornis* ticks, collected in each of the 4 counties. The strains were designated Huaiyangshan-Dawu-Cattle-Tick-1, Huaiyangshan-Zengdu-Cattle-Tick-1, Huaiyangshan-Xinxian-Cattle-Tick-1, and Huaiyangshan-Xinzhou-Dog-Tick-1.

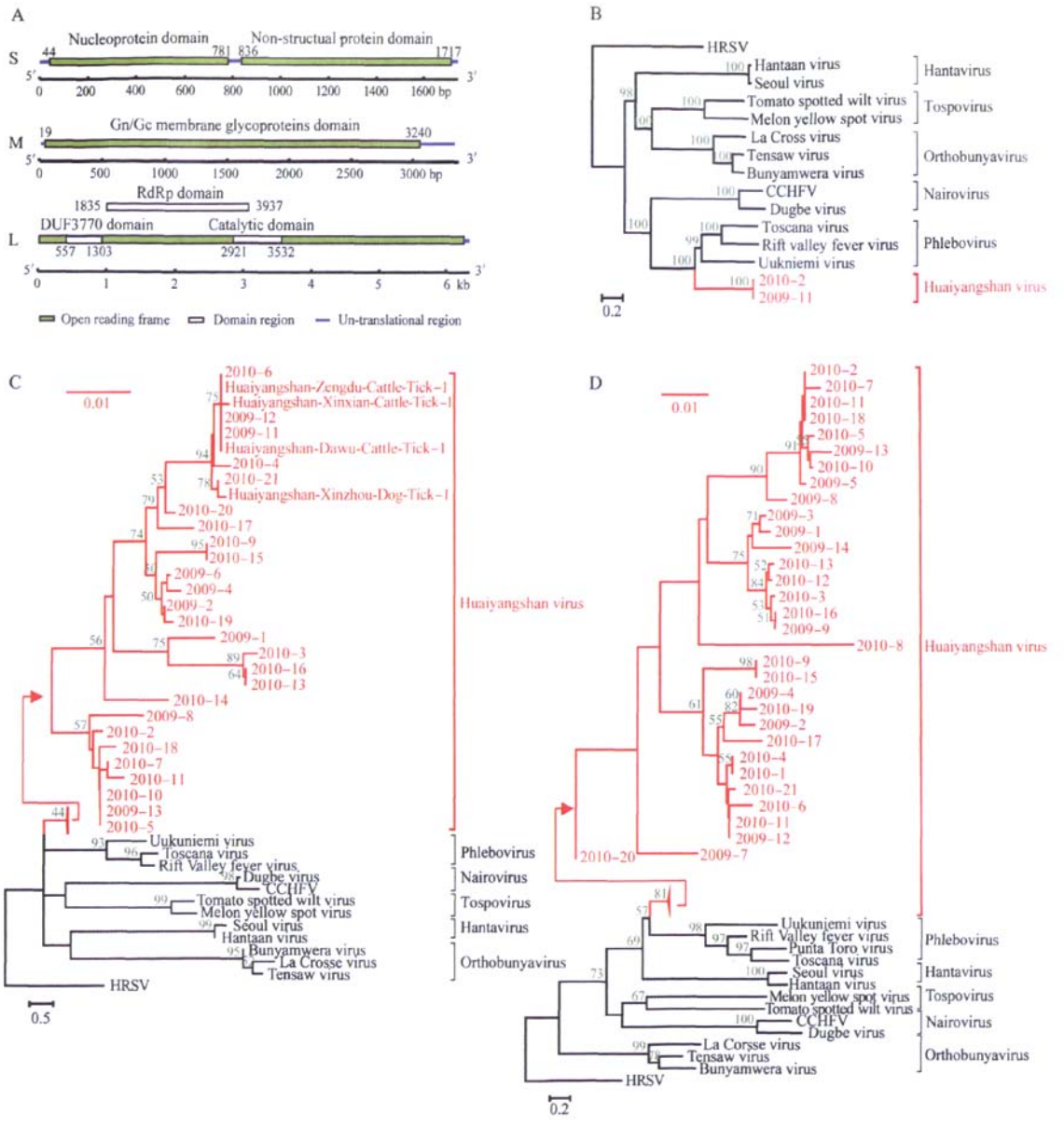

Figure 2. Genetic Characteristics of Huaiyangshan virus and Its Relationship with Other Known Members of Family *Bunyaviridae*. Panel A shows the genetic structure of S, M, and L segment sequences of Huaiyangshan virus. Phylogenetic relationship of Huaiyangshan virus with other member of family *Bunyaviridae* is shown in panel B. The tree was based on the complete L segment sequences by using neighbor-joining (NJ) method. Human respiratory syncytial virus (HRSV) was used as the outgroup. The genetic diversity of Huiayangshan viruses detected from human blood and tick samples is shown the phylogenetic trees based on the partial L (in panel C) and partial S (in panel D) segment sequences, and rooted with HRSV. Numbers (>50%) at the branch nodes indicate bootstrap values obtained by NJ analysis analyses. Colors (green and red) highlight Huaiyangshan viruses obtained in this study. The GenBank accession numbers are shown in Table 6.

The latter strain was also isolated in cell culture from homogenized viral RNA-positive ticks collected from Xinzhou. Phylogenetic analysis of 12S rDNA sequences revealed that all 4 virus positive ticks belong to *H. longicornis* (Figure 4). No viral RNA was found in the *B. Microplus* ticks. Furthermore, no virus was detected in more than 2000 mosquitoes (*Anopheles sinensis*, *Armigeres subalbatus*, *Culex tritaeniorhynchus*) from the same region.

Genetic diversity of the new viruses from patients and ticks

Partial S segment sequences were recovered from 32 of 33 patients, and partial L segment sequences from 26 of 33 patients and 4 tick samples. Sequence analysis showed that the S and L segment

Table 5. IgG antibody titers against Huaiyangshan virus in paired sera from 12 patients in 2010 as measured by indirect IFA.

Patient No.	First specimen (days after illness onset)	Last specimen (days after illness onset)	Fold increase
2010-2	1:4 (8)	1:1024 (40)	256
2010-4	1:16 (10)	1:256 (16)	16
2010-7	1:16 (7)	1:64 (25)	4
2010-8	1:64 (16)	1:1024 (52)	16
2010-9	1:64 (12)	1:256 (48)	4
2010-10	1:4 (7)	1:256 (34)	64
2010-11	1:16 (5)	1:64 (13)	4
2010-14	1:4 (11)	1:1024 (21)	256
2010-15	1:16 (7)	1:256 (18)	16
2010-19	1:64 (8)	1: 256 (11)	4
2010-20	1:64 (12)	1:256 (21)	4
2010-21	1:16 (8)	1:256 (25)	16

sequences recovered from human specimens were closely related, with 93.68% to 100% nt identity and 97.99% to 100% aa identity. In addition, the sequences of partial L segments recovered from ticks were closely related to those from patients, with more than 95.41% nt identity and 98.47% aa identity. Phylogenetic analyses of these partial segment sequences revealed that, while strains from patients clustered together, three different sub-lineages could be distinguished (Figure 2C-D).

Discussion

During investigations of unexplained severe febrile illnesses occurring in the Huaiyanshan mountains range in central China, a previously unknown virus was isolated from the blood of patients. Whole genome sequence analyses identified this virus from patients as a new member of the family *Bunyaviridae*, for which we propose the name Huaiyangshan virus (HYSV). Subsequently, HYSV was isolated from ticks collected from dogs in the same region, indicating that HYSV may be transmitted by ticks.

The clinical illness caused by HYSV presents with non-specific symptoms and signs, including high fever, severe malaise, nausea, vomiting and diarrhea. Laboratory abnormalities share several features with other viral hemorrhagic fevers,[8] such as leucopenia, severe thrombocytopenia, and coagulation abnormalities suggestive of disseminated intravascular coagulation (DIC). Manifest bleeding occurred in a

Table 6. The GenBank accession numbers of viral sequences obtained in this study and those retrieved from GenBank.

Virus strains	S Segment	M Segment	L Segment
Patient No. 2009-1	HQ419211	-	HM459827
Patient No. 2009-2	HQ419212	-	HM459824
Patient No. 2009-3	HQ419213	-	-
Patient No. 2009-4	HQ419214	-	HM459828
Patient No. 2009-5	HQ419215	-	-
Patient No. 2009-6	-	-	HM459832
Patient No. 2009-7	HQ419216	-	-
Patient No. 2009-8	HQ419217	-	HM459822
Patient No. 2009-9	HQ419218	-	-
Patient No. 2009-11	HQ171191*	HQ419229*	HQ171186*
Patient No. 2009-12	HQ419219	-	HM459835
Patient No. 2009-13	HQ419220	-	HM459836
Patient No. 2009-14	HQ419221	-	-
Patient No. 2010-1	HQ179732	-	-
Patient No. 2010-2	HQ171195*	HQ419236*	HQ171190*
Patient No. 2010-3	HQ179729	-	HQ179699
Patient No. 2010-4	HQ171192*	-	HQ171187*
Patient No. 2010-5	HQ179735	-	HQ179704
Patient No. 2010-6	HQ179734	-	HQ179703
Patient No. 2010-7	HQ179731	-	HQ179701
Patient No. 2010-8	HQ179739	-	-
Patient No. 2010-9	HQ179738	-	HQ179707
Patient No. 2010-10	HQ179730	-	HQ179700
Patient No. 2010-11	HQ179733	-	HQ179702
Patient No. 2010-13	HQ419239*	-	HQ419226*
Patient No. 2010-14	HQ179742	-	HQ179710
Patient No. 2010-15	HQ171194*	-	HQ171189*
Patient No. 2010-16	HQ171193*	-	HQ171188*
Patient No. 2010-17	HQ179737	-	HQ179706
Patient No. 2010-18	HQ179740	-	HQ179708
Patient No. 2010-19	HQ179744	-	HQ179712
Patient No. 2010-20	HQ179743	-	HQ179711
Patient No. 2010-21	HQ179736	-	HQ179705
Huaiyangshan-Zengdu-Cattle-Tick-1	-	-	HQ419223
Huaiyangshan-Xinxian-Cattle-Tick-1	-	-	HQ419225
Huaiyangshan-Dawu-Cattle-Tick-1	-	-	HQ419224
Huaiyangshan-Xinzhou-Cattle-Tick-1	-	-	HQ419222
Hantaan virus#	AF329390	M14627	X55901
Seoul virus#	AY766368	AF035833	AF189155
Tomato spotted wilt virus#	AY744480	AB190818	AY070218
Melon yellow spot virus#	AB038343	NC_008307	AB453906
La Crosse virus#	GU596389	EF485031	GU596378
Tensaw virus#	FJ943507	FJ943508	FJ943510
Bunyamwera virus#	AJ697960	M11852	X14383
CCHFV#	GQ862372	AF467768	GU477492
Dugbe virus#	AF434165	M94133	DVU15018
Toscana virus#	X53794	X89628	X68414
Rift Valley fever virus#	EU709748	DQ380200	X56464
Uukuniemi virus#	M33551	M17417	D10759
HRSV#	DQ780568	AY728170	U27298

* Complete genome obtained in this study.
\# GenBank accession numbers retrieved from GenBank.

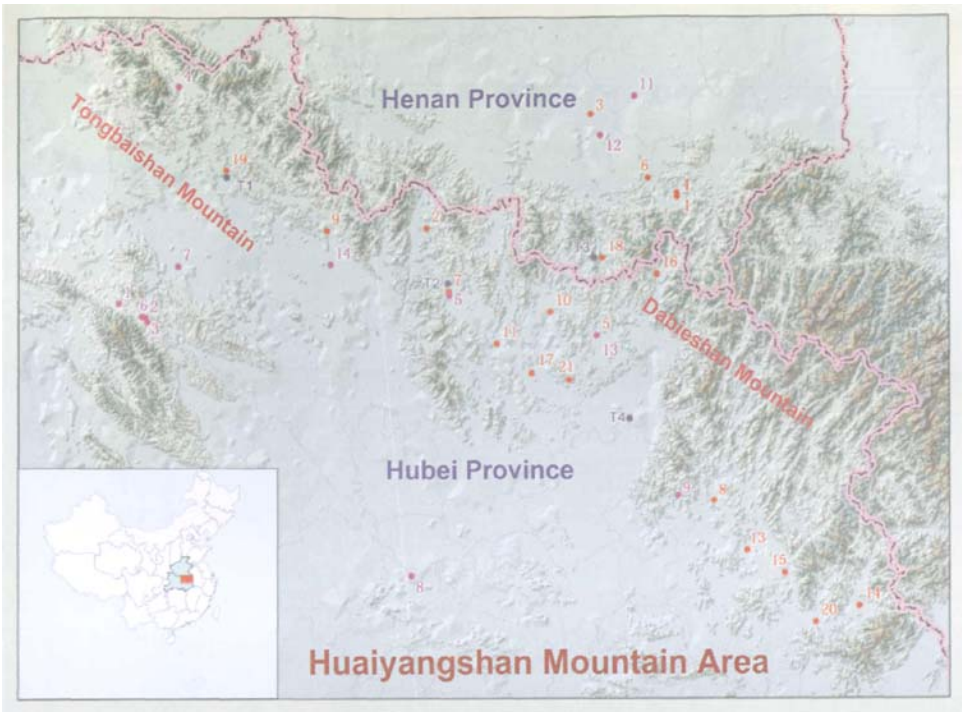

Figure 3. Geographic Distribution of Hemorrhagic Fever caused by Huaiyangshan virus.

The map shows the geographic distribution of hemorrhagic fever with liver damage occurred in Huaiyangshan mountain areas in China in 2009 and 2010. The purple and red dots represent the cases in 2009 and 2010 respectively. The number in purple and in red indicated patient identification number occurred in 2009 and 2010 respectively. The genetic information of the virus from the patients can be found in Figure 1. The first four numbers of the virus name were the year when cases occurred, and the two numbers after dash were patient identification number, indicating the order of onset of the illness in the specific year. Therefore, the information regarding to the patients can be found in Table 1 and 2 and Figure 1, based on patient identification number. In addition, T1, T2, T3, and T4 represent the sites in which Huaiyangshan Zengdu Cattle Tick 1, Huaiyangshan Dawu Cattle Tick 1 Huaiyangshan Xinxian Cattle Tick 1, and Huaiyangshan Xinzhou Dog Tick 1 were found from ticks, respectively.

Table 7. Clinical symptoms and signs in 33 Huaiyangshan virus-infected patients in Henan and Hubei provinces, China, 2009–2010.

Symptom or sign	No. of patients (%)	Symptom or sign	No. of patients (%)
Fever(>37.5 ℃)	33(100)	Nausea	13(39)
Malaise	16(48)	Vomiting	12(36)
Chills	13(39)	Arthralgia/myalgia	8(24)
Abdominal pain	3(9)	Headache	5(15)
Diarrhea	14(42)	Hemorrhagic complications	3(9)

minority of patients while occult hematuria was noted in most. Elevated blood levels of hepatic transaminases were seen in all patients, suggesting an important role of the liver as a primary target organ. Elevated LDH and CK levels were also observed in most patients, and renal function disturbances in some. These findings indicate systemic involvement which was also suggested by the detection of viral RNA in the throat, urine and feces in a substantial proportion of patients. Whether the detection of virus at these sites reflects blood contamination by occult

Table 8. Laboratory findings at presentation in Huaiyangshan virus-infected patients in Henan and Hubei provinces, China, 2009–2010.

Laboratory findings	Median	Range	Normal value
White cell count(×10E9/L)	1.71	0.59–7.73	4–10
Platelet count(×10E9/L)	39.5	4–117	100–300
Lymphocytes(%)	29.3	0.89–58.5	20–40
Neutrophils(%)	57.2	22.7–94.3	50–70
Hemoglobin(g/L)	135	48.8–174	110–150
ALT(U/L)	118	23–619	0–40
AST(U/L)	305	32–1889	0–45
Albumine(g/L)	30.6	22–41.6	35–50
APTT (s)	51.6	31.4–99.3	25–35
TT(s)	22.1	11.7–112.9	12–16
LDH(U/L)	702	210–3403	109–245
CK(U/L)	409.5	14.3–9805	38–174
BUN(mmol/L)	6.395	1.9–24.82	2.9–8.2
Creatinine(μmol/L)	95.4	45–583.2	44–133

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; APTT, Activated partial thromboplastin time; TT, thrombin times; LDH, lactate dehydrogenase; BUN, blood urea nitrogen.

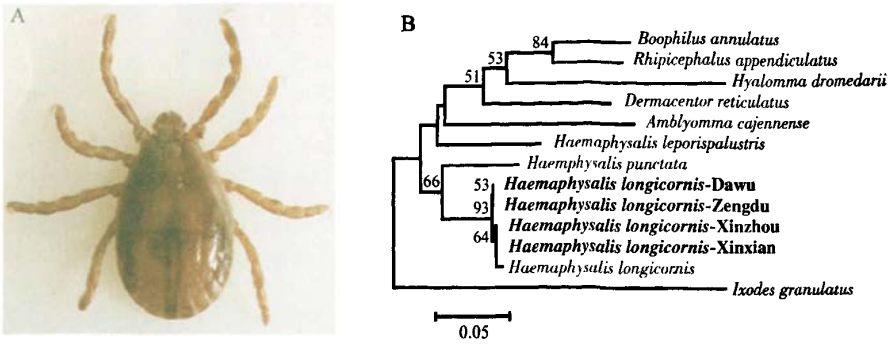

Figure 4. Adult *Haemaphysalis longicornis variabilis* and Its Phylogenetic Relationship with Other Ticks.

Panel A shows a female adult tick (*Haemaphysalis longicornis*) collected from dogs in Xinzhou county of Wuhan of Hubei Province, where confirmed patient was identified. Phylogenetic relationship of this species with other tick species is shown in panel B. Numbers (>50%) at the branch nodes indicate bootstrap values obtained by neighbor joining analysis. The sequences obtained in this study are closely related each other with overall nucleotide identities of 94.7 99.4%, and share a high homology (83.7 85.2%) with the known *H. longicornis* sequences (AF031853). The GenBank accession numbers of the other tick sequences are: *Haemphysalis longicornis* (AF031853), *Haemaphysalis puntata* (AF150032), *Haemaphysalis leporispalustris* (AM410574), *Amblyomma cajennense* (EU791583), *Boophilus annulatus* (U95866), *Rhipicephalus appendiculatus* (DQ901277), *Dermacentor reticulatus* (AF031849), *Hyalomma dromedarii* (HDU95874), *Ixodes granulatus* (DQ003014). The GenBank accession numbers of the tick sequences obtained in this study are: HQ219717–HQ219719 and HQ434625.

hemorrhages or active viral replication remains unclear. Further studies are clearly needed to provide insight into the pathogenesis of disease. Similarly, more studies are needed to determine the clinical spectrum of disease. In this hospital-based study, the case-fatality ration was 15%, with patients dying of hemorrhage or multi-organ failure. Community-based surveillance studies will determine to what extent asymptomatic or mild infections may occur.

HYSV which caused this disease is most closely related, albeit genetically quite distinct, to viruses of the genus *Phlebovirus*. The genus *Phlebovirus* currently consists of 53 known serotypes, which are divided into two major antigenic groups: the phlebotomus fever (or sandfly fever) group - which are transmitted by sandflies includes Rift Valley Fever virus and Toscana virus, and the Uukuniemi group which are transmitted by ticks.[9, 10] Although antibodies to Uukuniemi virus have been detected in humans, human disease caused by Uukuniemi viruses has not been demonstrated yet.[11] Phylogenetic analyses showed that the HYSV variants isolated from humans and ticks are closely related to each other, and form a separate lineage distinct from Uukuniemi virus and other phleboviruses (Figure 2C-D).

The viruses were detected in *H. longicornis* ticks collected from cattle and dogs in villages where laboratory-confirmed patients were living. As all patients were farmers, involved in cattle and goat herding, tea collection and other rural agricultural activities, high tick exposure of patients is likely. Indeed, some patients reported tick bites and ticks were found on some patients during admission. Moreover, tick densities are expected to be high during the April to July period when the illnesses occurred.[12] While sequence similarities to viruses recovered from patients may indeed implicate an important role of these ticks in transmission to humans, the precise arthropod ecology needs to be determined as does the possible existence of an animal reservoir for HYSV.

In summary, we report hemorrhagic fever-like illnesses caused by a novel tick-borne virus belonging to the family *Bunyaviridae* in villagers living in the Chinese Huaiyangshan mountain areas. Further studies are urgently needed to determine the precise clinical spectrum, pathogenesis, epidemiology, geographic distribution and vector ecology of this virus.

Acknowledgement

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Authors' words: On September 3, 2010, we submitted our manuscript to *the New England Journal of Medicine*. We first revised our manuscript according to the requirements from the editors and the suggestions of reviewers on October 9, 2010, and then resubmitted the revised manuscript to the journal on October 22, 2010. On November 16, 2010, the editor emailed us that our manuscript had been recommended for publication in *the New England Journal of Medicine* after improvement according to the editors' suggestions. The second revised manuscript was resubmitted to the journal on November 22, 2010. Unfortunately, the deputy editor suddenly informed us not to publish our manuscript in the journal more than 3 months later (March 7th, 2011).

作者的话 2010年9月3日我们将稿件投到*the New England Journal of Medicine*(新英格兰医学杂志)。2010年10月9日根据编辑部编辑与审稿专家的意见对稿件作第一次修改,我们于2010年10月22日修回。2010年11月16日收到编辑部的第二封要求修改稿件的信,并告诉我们已推荐在*the New England Journal of Medicine*上发表;我们于2010年11月22日修回。三个多月后的2011年3月7日*the New England Journal of Medicine*编辑部的副编辑突然发邮件告知不予发表。

中国淮阳山地区由新蜱传布尼亚病毒引起的出血热

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覃新程[1],金东[1],崔志刚[1],罗雪莲[1],李伟[3],卢珊[1],王文[1],彭劲松[2],郭文平[1],李明慧[1],李振军[1],
张少敏[1],陈晨[1],王艳[1],Menno D. de Jong[5],徐建国[1]

【摘要】 研究背景 2009—2010年4月至7月,我国淮阳山地区农民中出现不明原因的严重出血热样病例。**方法** 采集疑似病例的临床标本(血液、尿液、粪便和咽拭子),在患者所在地采集蚊和蜱。从2例患者的血液样本中分离到2株病毒,并进行全基因组测序分析。采用病毒特异的RT-PCR对其他病例及节肢动物进行病毒检测,对RT-PCR阳性病例的临床和流行病学资料进行分析。**结果** 从2例患者的血液及从犬身上采集到的长角血蜱中分离到1种未知病毒。全基因组测序分析发现该病毒为布尼亚病毒科的一个新成员,与白蛉病毒属最接近,是其中的一个新分支。58例疑似病例中33例RT-PCR结果阳性。病例临床特征主要表现为发热、乏力、恶心、呕吐和腹泻。主要实验室结果包括白细胞及血小板减少,凝血时间异常和肝酶升高。3例患者出现出血症状,5例死亡(死亡率15%)。**结论** 我国淮阳山地区存在着一种严重威胁生命健康的新蜱传布尼亚病毒。对于该病毒的流行病学、地理分布,以及脊椎动物宿主的生态学等需要作进一步的研究。

*桐柏山、大别山及张八岭总称淮阳山。

作者单位:1 中国疾病预防控制中心传染病预防控制所,北京,102206; 2 武汉市疾病预防控制中心,武汉,430022; 3. 华中科技大学同济医学院附属协和医院,武汉,430022; 4. 武汉大学附属中南医院,武汉,430071; 5.荷兰阿姆斯特丹大学医学中心,荷兰
张永振,周敦金,熊衍文,陈小萍,贺永文,孙强正,余滨,李娟,代永安,田俊华,覃新程同为第一作者
通信作者:徐建国,张永振,周敦金