ORIGINAL ARTICLE

A New Segmented Virus Associated with Human Febrile Illness in China

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ABSTRACT

BACKGROUND

In 2017, surveillance for tickborne diseases in China led to the identification of a patient who presented to a hospital in Inner Mongolia with a febrile illness that had an unknown cause. The clinical manifestation of the illness was similar to that of tickborne encephalitis virus (TBEV) infection, but neither TBEV RNA nor antibodies against the virus were detected.

METHODS

We obtained a blood specimen from the index patient and attempted to isolate and identify a causative pathogen, using genome sequence analysis and electron microscopy. We also initiated a heightened surveillance program in the same hospital to screen for other patients who presented with fever, headache, and a history of tick bites. We used reverse-transcriptase–polymerase-chain-reaction (RT-PCR) and cell-culture assays to detect the pathogen and immunofluorescence and neutralization assays to determine the levels of virus-specific antibodies in serum specimens from the patients.

RESULTS

We found that the index patient was infected with a previously unknown segmented RNA virus, which we designated Alongshan virus (ALSV) and which belongs to the jingmenvirus group of the family Flaviviridae. ALSV infection was confirmed by RT-PCR assay in 86 patients from Inner Mongolia and Heilongjiang who presented with fever, headache, and a history of tick bites. Serologic assays showed that seroconversion had occurred in all 19 patients for whom specimens were available from the acute phase and the convalescent phase of the illness.

CONCLUSIONS

A newly discovered segmented virus was found to be associated with a febrile illness in northeastern China. (Funded by the National Key Research and Development Program of China and the National Natural Science Foundation of China.)

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Routine surveillance for tickborne diseases in China led to the identification of a patient from the town of Alongshan who had a febrile illness with an unknown cause. An investigation was conducted to identify the pathogen that was causing this patient's illness. Analyses revealed a previously unknown segmented RNA virus, which we designated Alongshan virus (ALSV), that belongs to the unclassified jingmenvirus group in the family Flaviviridae, which includes the genera flavivirus, pestivirus, hepacivirus, and pegivirus.[1,2] ALSV was also detected in additional patients with fever and headache as well as in ticks and mosquitoes in the region (Fig. 1). In this article, we describe the discovery, isolation, and characterization of the virus.

Methods

Identification of the Index Patient and Heightened Surveillance

In April 2017, a 42-year-old female farmer from the town of Alongshan presented to a county hospital in Hulunbuir City in Inner Mongolia, China, with fever, headache, and a history of tick bites. The clinical features of the patient's illness were highly similar to those reported in cases of infectious tickborne encephalitis virus (TBEV)[3]; however, neither TBEV RNA nor antibodies against the virus were detected in blood specimens obtained from the patient. An investigation was conducted to identify the causative pathogen. Because it was suspected that the patient's illness was caused by a tickborne pathogen, a heightened

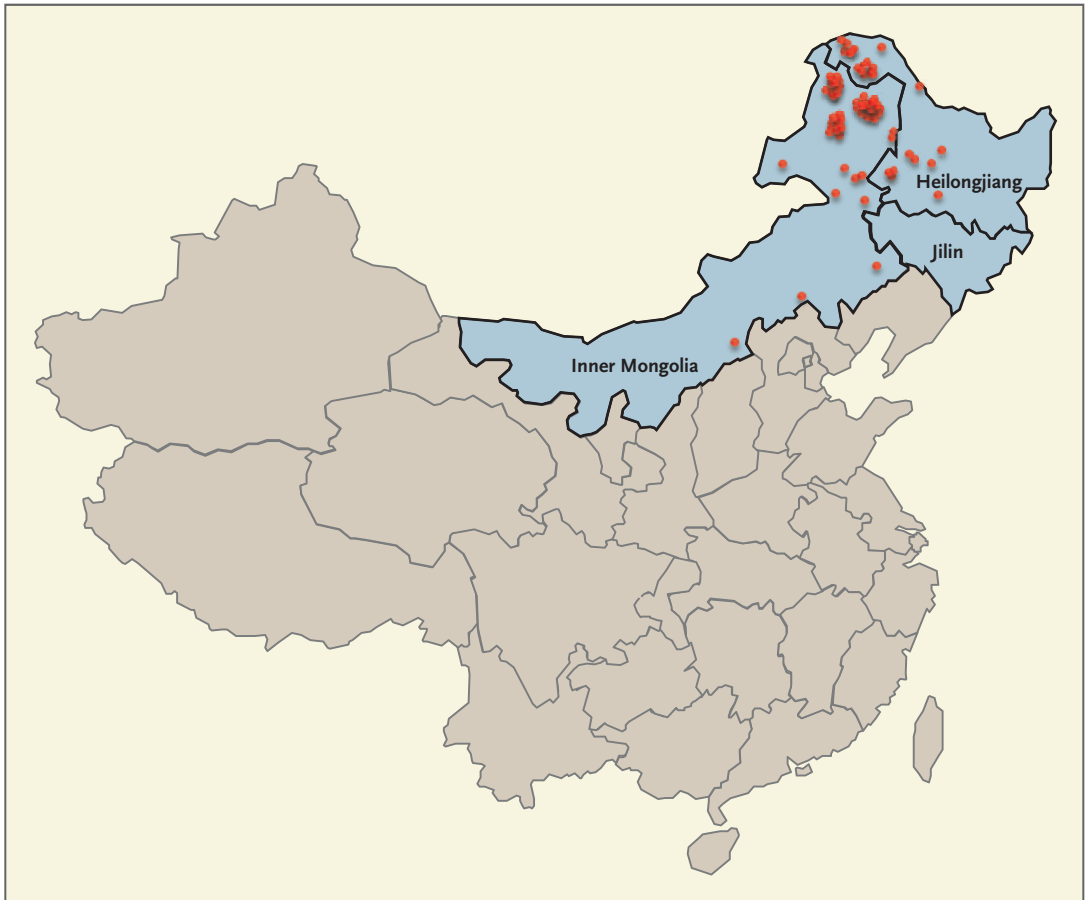

Figure 1. Geographic Distribution of Patients with Alongshan Virus (ALSV) in China.
Areas where surveillance of tickborne pathogens was carried out are shown in blue. Red circles indicate the locations of the patients with confirmed ALSV infection (geographic information is missing for 2 of the 86 patients).

surveillance program was initiated in the same hospital to identify patients with fever, headache, and a history of tick bites. We obtained serum specimens from these patients. We also obtained serum specimens from 100 healthy persons who were living in the area, and we collected ticks and mosquitoes from the region for testing. The study was approved by the ethics committee of the Inner Mongolia General Forestry Hospital, and all participants involved in the study provided written informed consent.

ISOLATION OF AN UNKNOWN PATHOGEN

A blood specimen was obtained from the index patient on day 4 after the onset of illness. The specimen was centrifuged at 12,000 rpm for 10 minutes; the supernatant was then diluted 10 times in minimal essential medium and inoculated onto monolayers of African green monkey kidney (Vero) cells, mouse hepatoma (Hepa 1-6) cells, hamster kidney (BHK-21) cells, human malignant glioma (U-87MG) cells, and human foreskin fibroblast (HFF) cells. The cells were cultured at 37°C in 5% carbon dioxide and observed daily to monitor for cytopathic effect.

ELECTRON MICROSCOPY

The infected Vero cells that showed cytopathic effect were prepared for electron-microscopic analysis.[4] The culture supernatants were collected for virus purification by means of discontinuous sucrose gradient centrifugation. The culture pellets were then fixed with a 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate buffer, postfixed with 1% osmium tetroxide, dehydrated in increasing concentrations of ethanol solutions, and embedded in epoxy resin. The ultrathin sections were stained with 2% uranyl acetate and lead citrate and were then examined with a transmission electron microscope.

GENETIC ANALYSIS

The viral RNA genome was extracted from the virus-infected cell culture with the use of a QIAamp Viral RNA Mini Kit (Qiagen). The genome was sequenced by means of the sequence-independent, single-primer amplification method.[5,6] The sequences of the 5′ and 3′ regions of the viral RNA were determined by rapid amplification of complementary DNA ends. Polymerase-chain-reaction product was cloned into the pMD18-T cloning vector (Takara) and sequenced according to standard methods. Phylogenetic analyses were performed with the Molecular Evolutionary Genetics Analysis software, version 5, and Bayesian Evolutionary Analysis Sampling Trees software, version 1.6.

SEROLOGIC ANALYSIS

After the virus was successfully isolated, an immunofluorescence assay was developed to detect virus-specific antibodies in serum specimens obtained from hospitalized patients and healthy participants.[7] In brief, monolayers of Vero cells infected with the strain isolated from the index patient were trypsinized and spotted onto 8-well slides. The slides were incubated with serial dilutions, by a factor of two, of patient serum specimens (starting at 1:20) and probed with fluorescein isothiocyanate–conjugated goat anti-human IgG. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Immunofluorescence assay end-point titers are reported as the reciprocal of the highest dilution of serum that produced virus-specific fluorescence. A titer of 1:20 was considered positive.

Patient serum specimens were also prepared for microneutralization assay. Specimens were serially diluted and were then mixed with an equal volume of viral solution containing 100 median tissue-culture infectious doses and incubated at 37°C for 1.5 hours. The mixture was then added in quadruplicate to a 96-well plate that had been seeded with 1×10^4 Vero cells per well 12 hours before infection. After the plates were incubated at 37°C in 5% carbon dioxide for 7 days, immunofluorescence assay was used to detect viral infection. Microneutralization end-point titers are reported as the reciprocal of the highest dilution of serum that could inhibit viral infection.[8]

RT-PCR TESTING

Viral RNA was extracted from whole-blood specimens that were obtained from hospitalized patients and from homogenized ticks and mosquitoes. The RNA was then amplified with the use of nested reverse-transcriptase polymerase chain reaction (RT-PCR) with virus-specific primers, and the PCR products were sequenced to verify the positive results.

RESULTS

ISOLATION OF THE VIRUS

We isolated ALSV strain H3 from the index patient. We later isolated four additional strains from blood specimens obtained from hospitalized patients during the acute phase of the illness.

Of the cell lines that were inoculated with the serum specimen obtained from the index patient, cytopathic effect associated with ALSV was detected only in the Vero cells (Fig. 2). After 2 weeks of incubation, light microscopy showed that inoculated Vero cells had become rounded and detached from the flasks, and there were some granular particles in the cytoplasm. Negative-stain electron microscopy showed that these virions were enveloped, spherical (or nearly spherical) particles, with a diameter of approximately 80 to 100 nm, which indicated that ALSV had morphologic similarity to viruses in the genus flavivirus. Viral particles could be seen in the cytoplasm of infected Vero cells on transmission electron microscopy (Fig. 2F). After several passages, cytopathic effect could typically be observed in Vero cells after 4 days of incubation.

MOLECULAR CHARACTERIZATION

We obtained the complete genome of ALSV strain H3 and the partial sequences of 27 additional isolates obtained from patients, ticks, and mosquitoes (Table S3 in the Supplementary Appendix, available with the full text of this article at NEJM.org). Sequence analysis showed that all isolates were closely related, with 96% nucleotide identity (Fig. S2 in the Supplementary Appendix).

The genomic segments of ALSV were numbered 1 to 4 on the basis of the numbering convention for the genome of the Jingmen tick virus (JMTV).[1] The complete genome of ALSV included 11,350 nucleotides; the terminals of the four segments were similar to those of JMTV counterparts.[1] In addition, segments 1 and 3 were monocistronic, whereas segments 2 and 4 were bicistronic. Segment 1 contained 2995 nucleotides and one open reading frame that encoded 914 amino acids of the nonstructural protein NS1. This was similar to the NS5 proteins of flaviviruses and was characterized by the conserved motifs of RNA-dependent RNA polymerase and methyltransferase that are typical of flaviviruses. Segment 3 contained 2811 nucleotides that encoded NS2 and resembled the NS2b–NS3 complex of flaviviruses.

The nonflavivirus proteins in ALSV included viral proteins VP1, VP2, and VP3. VP1 had two overlapping open reading frames (VP1a and VP1b), which were encoded in segment 2 (2811 nucleotides); one predicted signal peptide; two potential N-linked glycosylation sites; and three transmembrane regions. VP2 and VP3 were encoded by overlapping open reading frames in segment 4 (2738 nucleotides). VP2 was a small protein with one predicted signal peptide, and VP3 was a membrane protein with nine predicted transmembrane domains.

Phylogenetic analysis of genomic sequences showed that ALSV was related to the segmented viruses of the unclassified jingmenvirus group in the family Flaviviridae. To confirm this, we performed an additional phylogenetic analysis of deduced amino acid sequences, which showed that ALSV and JMTV formed a different monophyletic group within the diverse Flaviviridae family (Fig. 3). Specifically, ALSV, JMTV, and Guaico Culex virus (GCXV) were clustered together, and they showed a closer relationship to members of the genus flavivirus than to the other genera.

Comparison of the amino acids further confirmed that ALSV is genetically distinct from other jingmenviruses. The NS3 and NS5 proteins of ALSV were both more closely related to those of JMTV (79.1 to 80.9% similarity) than to those of flaviviruses (14.5 to 22.8% similarity). However, the viral proteins of ALSV and JMTV shared only 23.7 to 74.9% similarity. More details on molecular characterization are provided in the Supplementary Appendix.

EPIDEMIOLOGIC INVESTIGATION

From May through September 2017, we identified 374 patients who presented to the hospital with fever, headache, and a history of tick bites. Patients were considered to have ALSV infection if ALSV or viral RNA was detected in blood specimens or if seroconversion was observed (i.e., a quadrupling in serum IgG antibodies against ALSV from the acute phase to the convalescent phase of the illness as determined by means of immunofluorescence assay or neutralization testing). Patients with other laboratory-

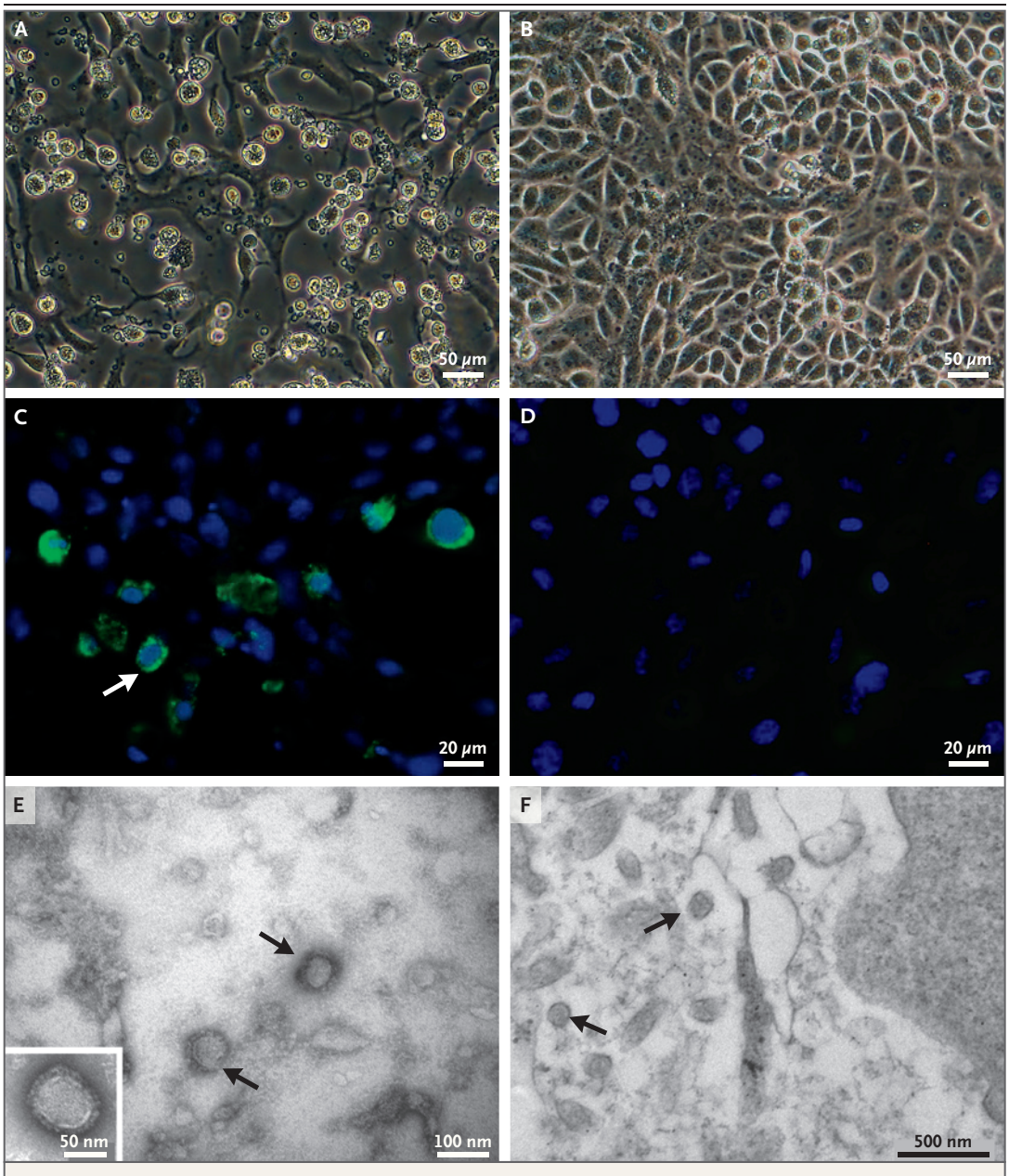

Figure 2. Morphologic Features of ALSV as Seen on Microscopy.

Serum specimens from a patient with ALSV infection were used to inoculate Vero cells. Light microscopy shows virus-induced cellular changes in infected Vero cells (Panel A), and Panel B shows the uninfected cells. Immunofluorescence assay reveals the virus in infected Vero cells (Panel C, arrow), and Panel D shows the uninfected cells. Panel E shows negatively stained virions (arrows) purified from ALSV-infected Vero cells; the inset shows an enlarged view of a virion. In Panel F, transmission electron microscopy shows virions (arrows) in the cytoplasm of infected Vero cells.

confirmed infections were not included in the study. A total of 86 patients with complete medical records had ALSV infection as confirmed by nested RT-PCR (Fig. S1 in the Supplementary Appendix). Of these patients, 60 were from Inner Mongolia and 24 were from Heilongjiang, and the location was unknown for 2 patients (Fig. 1). Ticks were commonly found in the patients'

working environments; however, no evidence of other tickborne infections or diseases — including severe fever with thrombocytopenia syndrome virus (SFTSV), TBEV, *Borrelia burgdorferi* sensu lato, *Borrelia miyamotoi*, anaplasmosis, babesiosis, or rickettsiosis — was identified in these patients (Table S5 in the Supplementary Appendix). Most ALSV infections occurred from May through July (70 patients [81%]). Of the 86 patients with confirmed ALSV, 63 (73%) were men, 84 (98%) were farmers or forestry workers who lived in hilly or wooded areas and worked in fields, and 82 (95%) had a clear history of tick bites before the onset of disease. The ages of the patients ranged from 24 to 77 years, and 58 patients (67%) were 40 to 60 years of age (Table S1 in the Supplementary Appendix). The start of the incubation period was determined on the basis of patients' reports, and the duration was generally 3 to 7 days, although in some cases it was as short as 1 day or as long as 10 days.

RT-PCR assay did not detect ALSV or antibodies against ALSV in any of the serum specimens obtained from 100 healthy persons in the region. ALSV RNA was detected by means of RT-PCR in *Ixodes persulcatus* ticks collected from wooded and hilly areas where the patients were bitten, with a prevalence of 6.5% in Inner Mongolia and 3.7% in Heilongjiang (Table S6 in the Supplementary Appendix). ALSV RNA was also detected by means of RT-PCR in mosquitoes in the province of Jilin. There was no epidemiologic evidence of person-to-person transmission of ALSV. The virus isolated from ticks was inoculated onto Vero cells, and the RNA sequences were found to be closely related to those in specimens obtained from patients (Fig. S2 in the Supplementary Appendix).

CLINICAL SYMPTOMS AND TREATMENT

The clinical symptoms of ALSV infection were nonspecific. The index patient presented with fever (temperatures of 38.5 to 39.2°C), persistent moderate headache, fatigue, and nausea, accompanied by cough and pharyngeal discomfort; the right cervical lymph nodes were also enlarged and painful. The most common symptoms in the patients assessed in this study were headache (69 patients) and fever (67 patients). Other clinical findings included fatigue (51 patients), depression (32 patients), coma (30 patients), poor appetite (27 patients), nausea (26 patients), myalgia or arthralgia (23 patients), and rash or petechiae (22 patients) (Table 1).

Laboratory testing showed that the most common abnormal findings in patients were elevated or decreased levels of lactate dehydrogenase (in 68% of patients) and elevated levels of high-sensitivity C-reactive protein (in 50%). Organ failure was relatively uncommon. Liver injury was observed in approximately 25% of patients, and muscle injury in approximately 10%, as indicated by elevated levels of serum aspartate aminotransferase (in 29% of patients), alanine aminotransferase (in 25% of patients), lactate dehydrogenase (in 20%), and creatine kinase (in 9%) (Table S2 in the Supplementary Appendix).

Imaging of the central nervous system showed mild ischemic demyelination of the white matter of the brain (7 of 54 patients), with a normal pattern in most patients (47 of 54 patients). Cerebrospinal fluid was obtained from 2 patients; the findings were normal in 1 patient, and there were abnormalities in the findings in the other patient, including slight turbidity and an elevated white-cell count (296×10^6 per liter [15% neutrophils, 83% lymphocytes, and 2% monocytes]), total protein (0.95 g per liter), and adenosine deaminase (2.5 U per liter).

All 86 patients were treated empirically with a combination of ribavirin and benzylpenicillin sodium for 3 to 5 days. Ribavirin was administered intravenously (0.5 g per day), and benzylpenicillin sodium intramuscularly (2 million units per day). For some patients with severe headache, vinpocetine (20 mg per day) and sulfotanshinone sodium (50 mg per day) were administered intravenously for 3 to 5 days. The symptoms usually resolved after 6 to 8 days of treatment, and all patients had complete clinical recovery. Hospital stay time was approximately 10 to 14 days (median, 11 days [interquartile range, 10 to 13]). Neither permanent clinical complications nor death occurred among patients with confirmed infection.

SEROLOGIC ANALYSES

A total of 19 patients who had ALSV infection as confirmed by RT-PCR assay and who had available serum specimens from the acute and the convalescent phases were assessed for seroconversion with the use of immunofluorescence

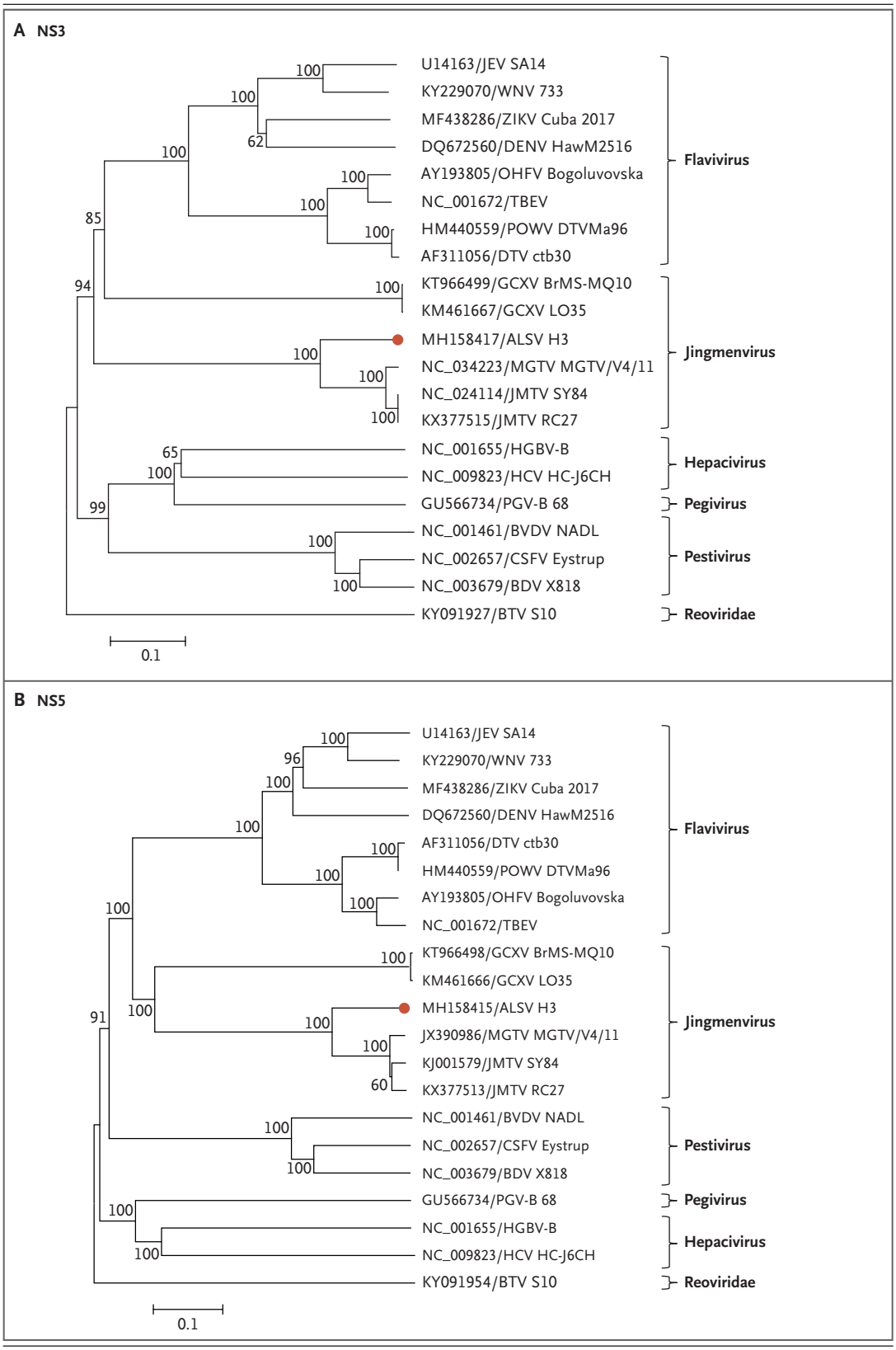

Figure 3 (facing page). Phylogenetic Analysis of ALSV and Other Viruses.

Shown are phylogenetic trees of the NS3 (Panel A) and NS5 (Panel B) proteins. The newly discovered ALSV is included (red dot in each panel). The phylogenetic trees were generated with Molecular Evolutionary Genetics Analysis software, version 5. The coding regions for NS3 in segment 3 and NS5 in segment 1 were analyzed by means of the neighbor-joining method with Poisson correction and complete deletion of gaps. Bootstrap testing (1000 replicates) was performed, and the bootstrap values are indicated. Sequences are named according to GenBank accession number, virus, and strain (where applicable). The scale bar in each panel indicates 0.1 substitutions per site. BDV denotes border disease virus, BTV bluetongue virus, BVDV bovine viral diarrhea virus, CSFV classical swine fever virus, DENV dengue virus, DTV deer tick virus, GCXV Guaico Culex virus, HCV hepatitis C virus, HGBV-B hepatitis GB virus B, JEV Japanese encephalitis virus, JMTV Jingmen tick virus, MGTV Mogiana tick virus, OHFV Omsk hemorrhagic fever virus, TBEV tickborne encephalitis virus, PGV-B pegivirus B, POWV Powassan virus, WNV West Nile virus, and ZIKV Zika virus.

Table 1. Clinical Characteristics of Hospitalized Patients Infected with Alongshan Virus (ALSV).*

Characteristic	No. of Patients (%) (N=86)
Headache	69 (80)
Fever	67 (78)
Fatigue	51 (59)
Depression	32 (37)
Coma	30 (35)
Poor appetite	27 (31)
Nausea	26 (30)
Myalgia or arthralgia	23 (27)
Rash or petechiae	22 (26)
Cough	10 (12)
Vomiting	10 (12)
Lymphadenopathy	8 (9)
Abdominal pain or tenderness	7 (8)
Chills	5 (6)
Diarrhea	2 (2)

* Shown are prospectively collected clinical characteristics of patients with laboratory-confirmed ALSV infection and complete medical records.

assay and microneutralization. Seroconversion (defined by an antibody titer in the convalescent-phase serum specimen that was at least 4 times as high as the titer in the acute-phase specimen) was detected in all 19 patients (Table 2). These findings showed that a high titer of neutralizing antibodies developed in the convalescent phase, and a humoral immune response to ALSV was induced.

DISCUSSION

Our findings suggest that ALSV, a newly identified segmented virus in the family Flaviviridae, may be associated with a febrile illness in patients in Inner Mongolia and Heilongjiang, China. Furthermore, we found that ALSV infected multiple human cell lines, induced pathologic changes in mice, and caused inflammatory responses in patients (Figs. S11 through S14 in the Supplementary Appendix).

Although ALSV is genetically different from JMTV, it is more closely related to JMTV than it is to other jingmenviruses. JMTV was first isolated from *Rhipicephalus microplus* in China, and it has a wide range of hosts, including cattle, dogs, and goats, and a wide geographic distribution in China.[1] JMTV variants have also been identified in other countries. For example, a variant of the Mogiana tick virus (MGTV) that was related to JMTV variants was isolated from *R. microplus* in Brazil.[9,10] In addition, the JMTV variant RC27 was isolated from a red colobus monkey in Uganda, the genome of which shows 88 to 92% identity with JMTV strain SY84 and MGTV.[11] These data show that JMTV or variants of the virus may be more widely distributed than previously estimated.

ALSV infection should be differentiated from SFTSV, TBEV, human anaplasmosis, rickettsiosis, and leptospirosis. Patients with TBEV, SFTSV, and anaplasmosis, like patients with ALSV, present with fevers and headache, but infections of the central nervous system (meningitis or encephalitis) are common in patients with TBEV infection,[12] and leukopenia and thrombocytopenia may occur in patients with SFTSV or anaplasmosis.[13,14] Leptospirosis may be confused with ALSV infection because of the initial fever and headache that are associated with it, but common symptoms of leptospirosis, such as rash and jaundice, were not commonly found in patients with ALSV infection in our study.[15] The

Table 2. Results of Serologic Testing in 19 Patients with ALSV Infection.*

Patient No.	Area of Origin	Sex	Age	Time from Illness Onset to Specimen Collection		IFA†		MNT‡	
				AP	CP	AP	CP	AP	CP
			yr	*days*		*titer*		*titer*	
H102	Inner Mongolia	F	53	7	20	<20	160	<10	640
H131	Inner Mongolia	F	60	2	17	<20	>320	<10	640
H169	Inner Mongolia	F	51	4	49	<20	320	<10	160
H215	Heilongjiang	M	46	1	24	<20	160	<10	160
H218	Inner Mongolia	M	24	5	23	<20	160	<10	160
H266	Inner Mongolia	M	50	1	16	<20	80	<10	160
H274	Inner Mongolia	F	46	3	16	<20	160	<10	160
H275	Inner Mongolia	M	41	3	20	<20	160	<10	160
H358	Heilongjiang	F	67	10	34	<20	160	10	160
H384	Inner Mongolia	M	36	3	21	<20	320	<10	160
H416	Inner Mongolia	M	50	2	26	<20	320	<10	160
H467	Inner Mongolia	M	52	3	25	<20	320	<10	160
H468	Heilongjiang	M	47	4	19	<20	80	<10	160
H566	Inner Mongolia	M	75	5	25	<20	160	<10	160
H574	Heilongjiang	F	59	1	22	<20	320	<10	160
H599	Heilongjiang	F	60	2	24	<20	320	<10	320
H711	Heilongjiang	F	69	4	19	<20	80	<10	80
H1296	Inner Mongolia	M	43	3	23	<20	160	<10	160
H1367	Inner Mongolia	M	37	2	19	<20	160	<10	80

* All patients had ALSV infection as confirmed by reverse-transcriptase–polymerase-chain-reaction assay. AP denotes acute phase, and CP convalescent phase.
† Indirect immunofluorescence assay (IFA) end-point titers are reported as the reciprocal of the highest dilution of serum that produced ALSV-specific fluorescence.
‡ Microneutralization (MNT) end-point titers are reported as the reciprocal of the highest dilution of serum that could inhibit viral infection.

symptoms of rickettsiosis may include fever and headache and so may resemble those of ALSV infection, but the rash seen in some cases of rickettsiosis is typically observed on the feet and hands,[16] whereas in patients with ALSV infection, rash is usually seen on other areas of the body.

Ticks can transmit a diverse range of viruses, including TBEV and SFTSV, and in such cases there is some evidence of transovarial transmission.[17] We suspect that ALSV is transmitted by the tick *I. persulcatus*. Common hosts of *I. persulcatus* include most mammals (e.g., sheep, cattle, horses, dogs, rabbits, humans) and occasionally some birds. *I. persulcatus* is widely distributed in Asia and eastern Europe, including China, Korea, Japan, Mongolia, and Russia.[18] However, mosquitoes cannot be excluded as a possible vector. ALSV RNA was detected in mosquitoes in the province of Jilin in northeastern China, and the RNA from these mosquitoes was found to be genetically related to that from the patients and ticks we assessed (Table S7 and Fig. S2 in the Supplementary Appendix). Our findings suggest that ALSV may be the cause of a previously unknown febrile disease, and more studies should be conducted to determine the geographic distribution of this disease outside its current areas of identification.

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