BRIEF REPORT

A New Phlebovirus Associated with Severe Febrile Illness in Missouri

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SUMMARY

Two men from northwestern Missouri independently presented to a medical facility with fever, fatigue, diarrhea, thrombocytopenia, and leukopenia, and both had been bitten by ticks 5 to 7 days before the onset of illness. *Ehrlichia chaffeensis* was suspected as the causal agent but was not found on serologic analysis, polymerase-chain-reaction (PCR) assay, or cell culture. Electron microscopy revealed viruses consistent with members of the Bunyaviridae family. Next-generation sequencing and phylogenetic analysis identified the viruses as novel members of the phlebovirus genus. Although Koch's postulates have not been completely fulfilled, we believe that this phlebovirus, which is novel in the Americas, is the cause of this clinical syndrome.

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THE PHLEBOVIRUS GENUS CONTAINS MORE THAN 70 ANTIGENICALLY DISTINCT viruses, which are divided into virus complexes according to whether they are borne by sand flies, mosquitoes, or ticks.[1] Sand-fly–borne viruses are found in the Americas, Asia, Africa, and the Mediterranean region, and infection with these viruses commonly results in a self-limiting 3-day fever, with the exception of Toscana virus, which can cause aseptic meningitis.[2] The prototype mosquito-borne phlebovirus is Rift Valley fever virus, which causes large-scale epizootics; human infection is often a self-limiting febrile illness that can progress to hepatitis, encephalitis, or hemorrhagic fever.[3] The only tickborne phlebovirus known to cause human disease is severe fever with thrombocytopenia syndrome virus (SFTSV), which was recently identified in central and northeastern China.[4]

CASE REPORTS

PATIENT 1

Patient 1 was a healthy 57-year-old man who lived on a 70-acre farm in northwestern Missouri. In early June 2009, he noticed a small nymphal tick embedded on his abdomen. The tick was subsequently removed with tweezers. There was no rash or localized itching. The following day, fever developed, which was followed by severe fatigue, headache, anorexia, nausea, and nonbloody diarrhea. Four days later, he was admitted to the hospital with a temperature of 37.9°C, which increased to 39.1°C the next day. Laboratory tests revealed a low white-cell count of 1900 cells per cubic millimeter, a low platelet count of 115,000 cells per cubic millimeter, and a low sodium level of 132 mmol per liter. Serum levels of liver aminotransferases were

slightly elevated, with an alanine aminotransferase level of 57 U per liter and an aspartate aminotransferase level of 44 U per liter. The serum level of C-reactive protein was elevated at 2.9 mg per deciliter. (Laboratory details are provided in Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org.)

The patient was hospitalized for 10 days. There was progression from moderate to severe thrombocytopenia, with a nadir of 37,000 cells per cubic millimeter on day 5 and 40,000 cells per cubic millimeter on days 6 and 7. Leukopenia continued throughout the hospitalization, with notable lymphopenia and mild neutropenia that progressed to moderate neutropenia on day 7 (Fig. 1A). Band forms were detected on days 2 and 8. An erythrocyte sedimentation rate was within the normal range at 9 mm per hour, and the erythrocyte count and hemoglobin were unremarkable and stable. The hematocrit was slightly low during hospitalization (Fig. 1C). The prothrombin time and partial-thromboplastin time were normal.

Serum hepatic aminotransferase levels increased and peaked, with an alanine aminotransferase level of 315 U per liter and an aspartate aminotransferase level of 431 U per liter on day 8 (Fig. 1B). Serum alkaline phosphatase levels rose within normal limits and peaked at 101 U per liter on day 9. Levels of creatinine and blood urea nitrogen remained normal. Urinalysis showed trace protein and 1+ ketones and was otherwise normal. Serum albumin levels were low, and serum sodium and calcium levels were mildly low.

On the second day of hospitalization, blood was sent to the Rickettsial Zoonoses Branch of the Centers for Disease Control and Prevention (CDC) and was subsequently shown to be negative for *E. chaffeensis*, *E. ewingii*, and rickettsiae of the spotted fever group on PCR assay. Serologic analysis later confirmed negative results of IgM and IgG assays for the spotted fever group and typhus. A rapid test for influenza A and B antigens was negative (Meridian Bioscience). Two blood cultures were sterile.

The patient was empirically placed on doxycycline (100 mg) intravenously twice daily for 14 days for suspected ehrlichiosis. Nonbloody diarrhea persisted through the fourth day of hospitalization. Stool specimens were negative for leukocytes, *Clostridium difficile* toxins, and salmonella, shigella, and campylobacter species. The results of two-dimensional echocardiography and chest radiography were normal.

The patient has reported fatigue and recurrent headaches in the 2 years since his hospitalization, but these symptoms cannot be clearly attributed to the viral infection. In addition, he initially had short-term memory difficulty, which has slowly improved, and anorexia, which resolved 4 to 6 weeks after discharge.

PATIENT 2

Patient 2 was a 67-year-old man with a 5-year history of type 2 diabetes who was otherwise healthy. He lived on an approximately 100-acre farm in northwestern Missouri. While on his property in early 2009, he received an average of 20 tick bites daily for approximately 2 weeks. He removed the embedded ticks with his fingers and tweezers. The last tick bite was noticed 1 week before hospitalization. Approximately 4 days before hospitalization, subjective fever, fatigue, and anorexia developed. Additional symptoms included myalgia, dry cough, and nonbloody diarrhea. No rash was noted before or during hospitalization.

On hospital admission in June 2009, his temperature was 38.1°C and reached 39.1°C the following day. Laboratory studies that were conducted on admission showed a low white-cell count of 2100 cells per cubic millimeter, a low platelet count of 78,000 cells per cubic millimeter, and a slightly elevated aspartate aminotransferase level of 54 U per liter (Fig. 1A, 1B, and 1C). The serum sodium level was slightly low at 130 mmol per liter, as was the calcium level at 7.8 mg per deciliter (1.95 mmol per liter). The results of urinalysis were normal.

The patient was hospitalized for 12 days. After day 2, thrombocytopenia progressed from moderate to severe, with a nadir of 34,000 cells per cubic millimeter on days 5 and 6. Platelet numbers increased starting on day 8 and reached a normal level by day 11 (Fig. 1C). Testing for antiplatelet antibodies was negative. Leukopenia continued until day 10, with mild neutropenia progressing to moderate neutropenia on days 6 to 8 (Fig. 1A). Band forms were present on days 2 to 7, and lymphocytes gradually increased to a normal range by day 8 (Fig. 1A). Erythrocyte counts and hemoglobin were within normal limits, and the hematocrit was slightly low throughout hospitalization. The prothrombin time, partial thromboplastin time, and fibrinogen levels were nor-

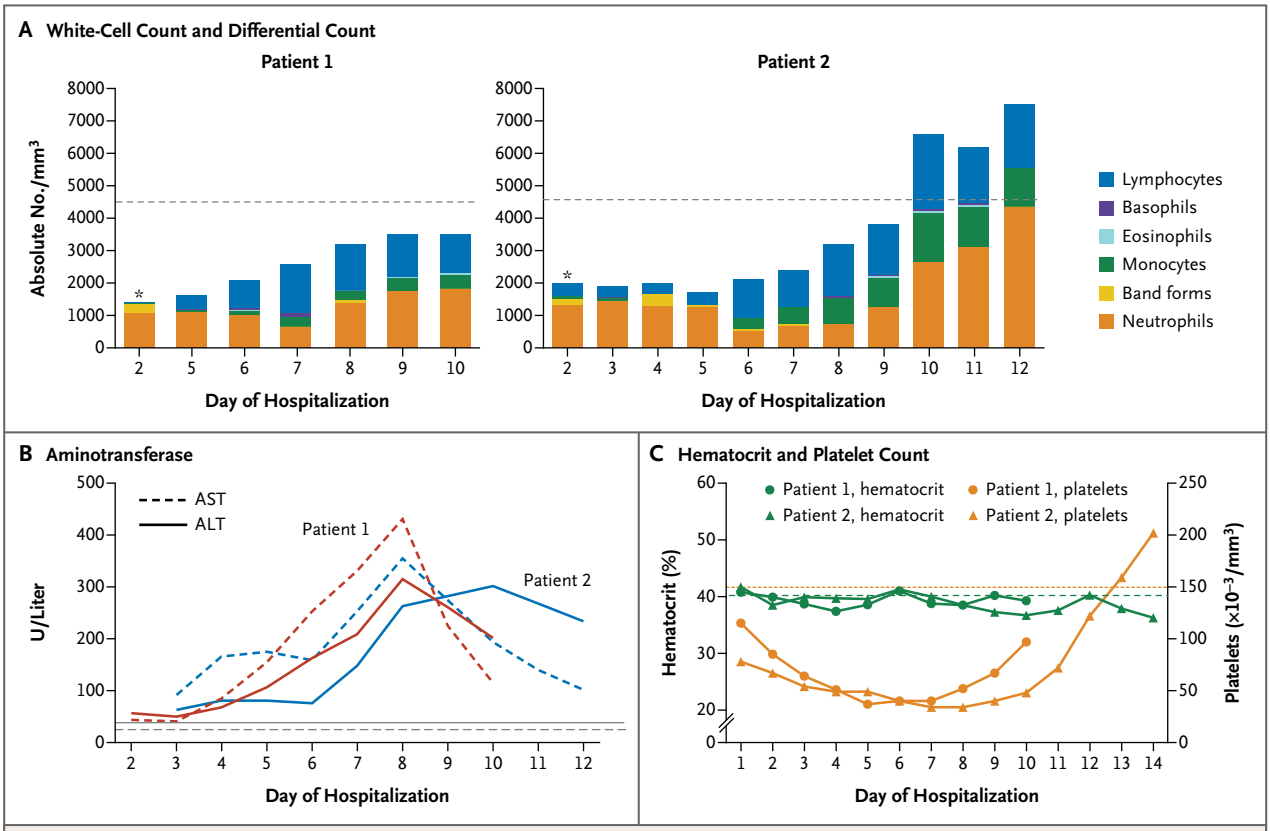

Figure 1. Laboratory Values for Patients 1 and 2.

Panel A shows the absolute values for the white-cell count and differential count for Patients 1 and 2 during hospitalization. The dashed lines indicate normal values. The asterisks indicate the day on which the novel virus was isolated from leukocytes obtained from the patients. Panel B shows levels of alanine aminotransferase (ALT, solid lines) and aspartate aminotransferase (AST, dashed lines) for the patients during hospitalization. The gray lines indicate normal values. Panel C shows hematocrit values (green) and platelet counts (gold) during the patients' hospitalization. The dashed lines indicate normal values.

mal, but the serum D-dimer level was elevated, at 4.08 mg per liter.

Blood was collected on day 2 of hospitalization and sent to the CDC. PCR results were found to be negative for *E. chaffeensis* and a range of ehrlichia and anaplasma species. Testing that was specific for borrelia antibody (Quest Diagnostics) was negative.

Alanine and aspartate aminotransferase levels were elevated and increased to 355 U per liter on day 8 and 302 U per liter on day 10, respectively (Fig. 1B). The alkaline phosphatase level was temporally high on day 10 but then resumed normal levels. Levels of creatinine and blood urea nitrogen remained normal. Levels of serum albumin and sodium remained low throughout hospitalization. Low serum calcium levels increased to normal by day 10. Results on chest radiography and abdominal ultrasonography were normal.

A bone marrow aspiration and biopsy were performed on day 2 of hospitalization. Trilineage hematopoiesis was detected, with less than 1% blasts and no ringed sideroblasts. There was notable defective development of erythrocytes (dyserythropoiesis) and megakaryocytes (dysmegakaryocytopoiesis). Flow cytometry confirmed 3 to 4% plasma cells with monoclonal lambda restriction, indicating response to infection. Cultures for fungi and mycobacteria were sterile.

The patient was initially treated empirically with intravenous piperacillin–tazobactam and was switched to ceftriaxone on hospital day 2 and to oral doxycycline (100 mg) twice daily on day 3 for suspected ehrlichiosis. He completed a 14-day course of doxycycline.

After hospital discharge, the patient noted fatigue, short-term memory difficulty, and anorexia. All the symptoms abated after 4 to 6 weeks and have not recurred in 2 years. Six months after discharge, the CDC confirmed the patient was negative for *E. chaffeensis* and *Anaplasma phagocytophilum* on IgG assay.

METHODS

CLINICAL SPECIMENS AND VIRUS CULTURE

EDTA-treated blood was collected and leukocytes separated with the use of Ficoll histopaque gradients and inoculated onto the canine monocyte cell line DH82.[5] Adherent and nonadherent cells were examined with the use of a modified rapid Wright–Giemsa stain (Diff-Quik). Culture supernatant was collected and transferred to Vero E6 cells and LLC-MK2 cells for virus propagation.

VIRUS GENOME SEQUENCING

Total RNA was extracted from infected cell culture media with the use of TriPure (Roche) and RNeasy (Qiagen) columns and nonspecifically amplified by means of random primers in a one-step reverse-transcriptase PCR reaction (SSIII RT–Platinum Taq HiFi Enzyme Mix, Invitrogen). Complementary DNA products were sequenced by means of next-generation sequencing (Roche 454) and analyzed with the use of bioinformatics tools.[6] (Details are provided in the Supplementary Appendix.)

RESULTS

ISOLATION OF A VIRUS FROM PATIENT LEUKOCYTES

Leukocytes were collected from both patients on day 2 of hospitalization and inoculated onto cultures of DH82 cells. These cultures showed cytopathic effects similar to early cultures of *E. chaffeensis*. However, cellular vacuoles did not contain bacterial morulae. Transfer of culture supernatants onto fresh DH82 cells resulted in similar cytopathic effects within 9 to 11 days. Cytopathic effects were less evident but also noted in Vero E6 cells 9 days after inoculation.

Studies were initiated to identify the suspected virus. Thin-section electron microscopy revealed enveloped particles averaging 86 nm in diameter, typical of a virus in the Bunyaviridae family (Fig. 2).

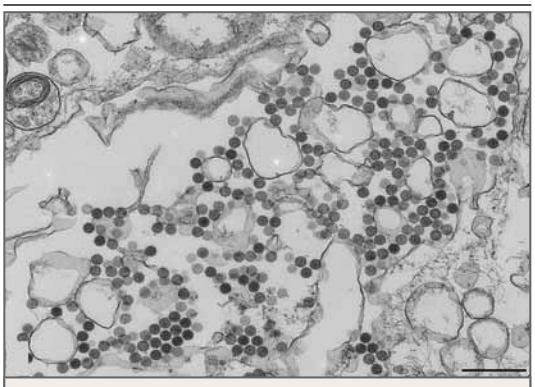

Figure 2. Thin-Section Electron Microscopy of Vero E6 Cells Revealing Virus Particles.

Extracellular enveloped, spherical virus particles with fairly homogeneous cores are visible in Vero E6 cells that were fixed in glutaraldehyde and processed for thin-section electron microscopy. Scale bar indicates 500 nm.

GENETIC ANALYSIS OF A NOVEL PHLEBOVIRUS

Total RNA was isolated from infected culture media and subjected to next-generation sequencing. The resulting full-length genome sequences were found to be similar to those of phleboviruses in the Bunyaviridae family, which are single-stranded, negative-sense RNA viruses comprised of three genome segments. We called this newly discovered virus the Heartland virus.

The phleboviruses share a similar genome organization.[7] The L segment is 6.4 kb in length and encodes a large RNA-dependent RNA polymerase. The M segment is 3.4 kb in length and encodes a polyprotein processed into the virus glycoproteins Gn and Gc, which are used for virion entry and assembly. The S segment is 1.7 kb in length and encodes the nucleoprotein that encapsidates the genomic RNA and a nonstructural (NSs) protein in an ambisense coding strategy. The genomes of virus isolates from both patients were sequenced in their entirety and found to be closely related, with 98%, 95%, and 99% identity for the S, M, and L virus segments, respectively. The high genetic identity indicates that both patients were infected with the same phlebovirus strain, but the differences between the isolates suggest that the two patients were infected independently.

PHYLOGENETIC ANALYSIS

Phylogenetic analysis of the aligned amino acid sequence of the polymerase, glycoprotein, nucleo-

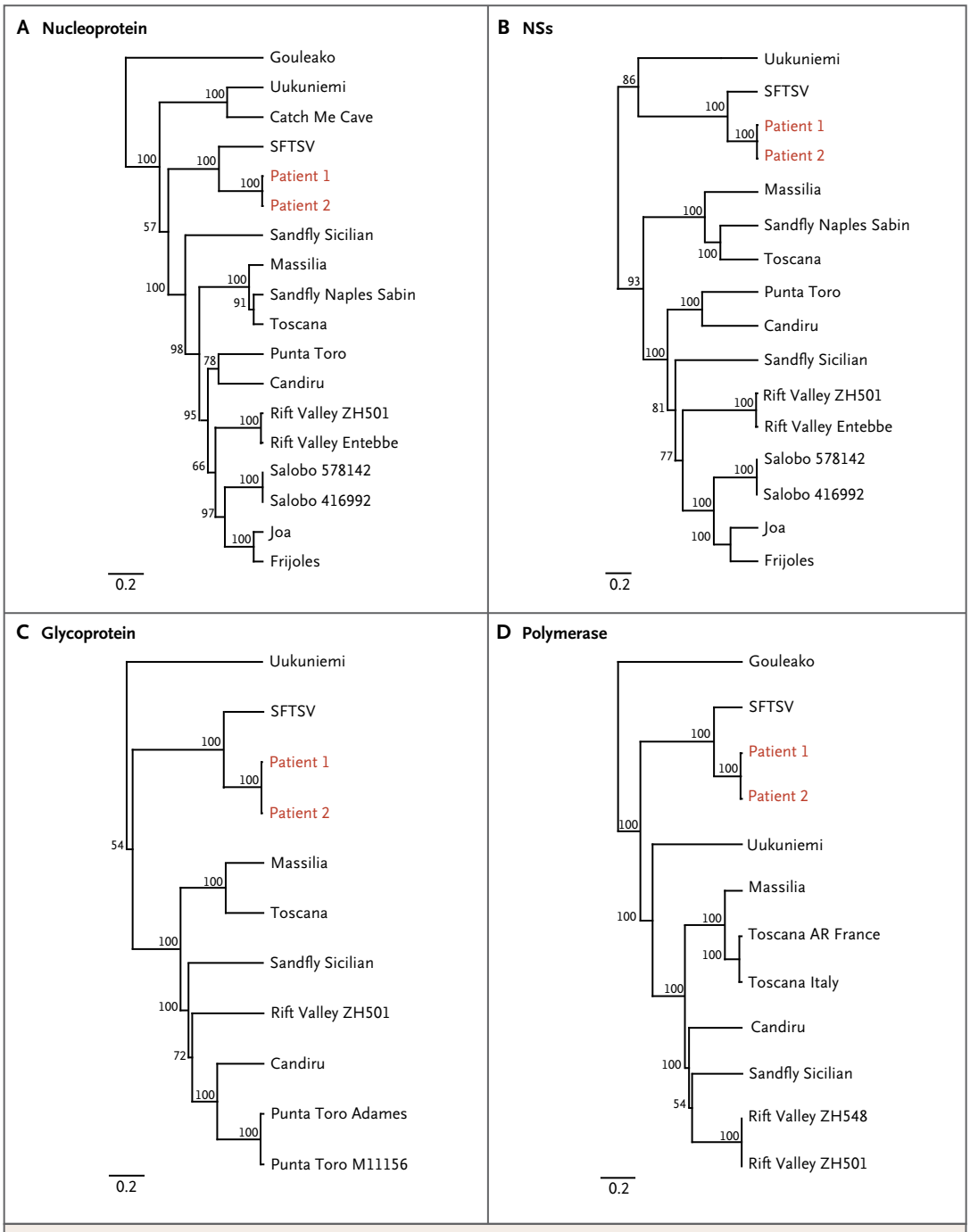

Figure 3. Phylogenetic Analyses of Amino Acid Sequencing of Representative Members of the Phlebovirus Genus.

The novel virus that was identified in Patients 1 and 2, a member of the phlebovirus genus, clusters with the tick-borne viruses and is most closely related to the severe fever with thrombocytopenia syndrome virus (SFTSV), which was recently identified in China. Analyses included sequencing of nucleoprotein (Panel A), nonstructural protein NSs (Panel B), glycoprotein (Panel C), and polymerase (Panel D). Phlebovirus sequences of each virus protein were aligned with the use of multiple alignment with fast Fourier transform (MAFFT), and phylogenic relationships were inferred with the use of the unweighted pair group method with arithmetic mean (UPGMA) method with 2000 bootstrap replicates for statistical support (CLC Genomics, Geneious). The GenBank accession numbers for the three segments for virus isolates from the two patients are provided in the Supplementary Appendix.

protein, and NSs protein suggested that the novel virus is a distinct member of the phlebovirus genus, clustering with the tickborne viruses and most closely related to SFTSV[4,8] (Fig. 3). This relationship is distant; however, pairwise comparisons of the viral polymerase and nucleoprotein (the two most conserved virus proteins) showed differences of 27% and 38%, respectively. Greater differences are found among the phlebovirus complexes that are borne by ticks, sand flies, and mosquitoes, which differ by at least 35%.

The novel virus was also distinct from an uncharacterized bunyavirus called lone star virus, which was isolated in 1967 from a nymphal *Amblyomma americanum* tick found on a woodchuck in western Kentucky.[9] Comparison of the polymerase amino acid sequence showed that lone star virus shared only 34% identity with the novel virus.

VIRAL RNA AND ANTIGEN IN BONE MARROW SPECIMEN

RNA of the novel virus was detected in bone marrow aspirate obtained from Patient 2. Immunohistochemical staining revealed the virus nucleocapsid protein in large mononuclear cells that did not resemble mature granulocytes, erythroid cells, or megakaryocytes. Staining was primarily cytoplasmic and seen in association with fragmented nuclear debris, which was a prominent finding in the biopsy specimen. No immunostaining was seen in control bone marrow–biopsy specimens or in normal rabbit serum used as a negative control (Fig. 4, and the Methods section in the Supplementary Appendix).

LONG-TERM PRESENCE OF REACTIVE IgG ANTIBODY

Patient serum samples were tested for the presence of antibodies reactive to the novel virus. In October 2011, more than 2 years after the onset of infection, blood was collected from both patients and serum samples were tested on enzyme-linked immunosorbent assay (ELISA) to detect IgG reactive with virus antigen (inactivated virus-infected cell lysate). Both serum samples were strongly positive, with titers of more than 6400.

DISCUSSION

Although Koch's postulates have not been completely fulfilled, our findings are consistent with the identification of a new pathogenic virus in the United States. This novel virus (which we called the Heartland virus) is a distinct member of the phlebovirus genus and is most closely related to tickborne phleboviruses, notably the recently isolated SFTSV. Clinical evaluations of the illness in the two patients who are described here probably do not reflect the entire spectrum of symptoms associated with this virus, yet both patients had a similar clinical course. Symptoms in the two patients included fever, fatigue, anorexia, and diarrhea.

Common laboratory findings were leukopenia with moderate neutropenia, thrombocytopenia, and elevated hepatic aminotransferase levels. Both patients had viremia on day 2 of hospitalization, approximately 7 days after the onset of symptoms. The temporal trends in white-cell and platelet counts and in aminotransferase levels were also strikingly similar between the two patients. Both patients presented with neutropenia that continued to decline to levels below 700 cells per cubic millimeter on days 6 and 7 of hospitalization. Thrombocytopenia continued until day 7 for both patients. Initially, the aminotransferase levels were only slightly elevated but spiked on days 7 and 8. After this time, there were increased levels of circulating neutrophils, lymphocytes, monocytes, and platelets, and aminotransferase levels began to normalize. Clinical evidence did not suggest respiratory or kidney involvement in either patient.

Many of the clinical and laboratory facets of this illness are similar to those reported for the tickborne phlebovirus SFTSV.[2] However, we did not observe coagulation abnormalities despite a markedly low platelet count, whereas a minority of patients with SFTSV infection had an elongated partial thromboplastin time and thrombin time, an elevated fibrinogen level, and symptoms of gingival bleeding and hemorrhage, with fatalities from disseminated intravascular coagulation and cerebral hemorrhage.[10,11] There have been numerous reports of person-to-person transmission on exposure to SFTSV-infected blood, and SFTSV has been detected in blood, throat swabs, urine, and feces obtained from patients with the infection.[10-13] It remains to be determined whether the novel virus can be transmitted from person to person, since no family members or caregivers of either patient reported symptoms resembling those of the patients. It will be important to determine how patients acquire this viral infection in order to promote risk-reduction practices.

The clinical laboratory results, symptoms, and

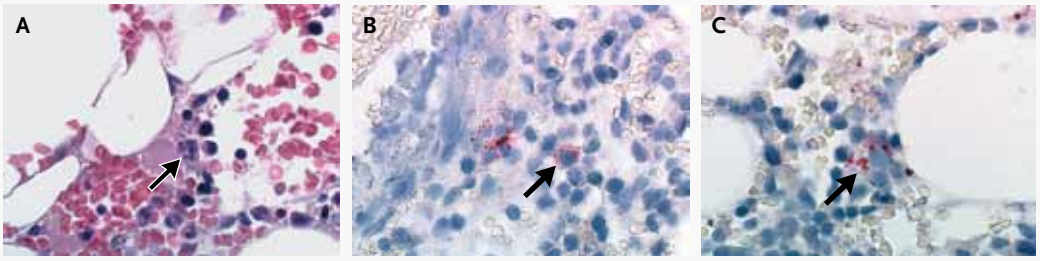

Figure 4. Histopathological and Immunohistochemical Staining of a Bone Marrow–Biopsy Sample from Patient 2.
Panel A shows fragmented nuclear debris (arrow) (hematoxylin and eosin). Panel B shows immunostaining of nucleocapsid protein from the novel phlebovirus within cytoplasm of large mononuclear cells (arrow). Panel C shows a higher-power image of the same sample indicating a granular immunostaining pattern (arrow). An indirect immunoalkaline phosphatase assay with naphthol fast-red substrate and light hematoxylin counterstaining was performed with the use of a 1:1000 dilution of hyperimmune rabbit serum reactive with the nucleoprotein of the novel phlebovirus (indicated in red).

occurrence of tick bite are similar to those of ehrlichiosis infections.[5] The novel virus should be considered as a possible etiologic agent in these instances, particularly when suspected ehrlichiosis does not improve within a few days of doxycycline treatment.

Although we did not isolate the novel virus from ticks, and tick specimens from the patients were not available, one potential vector is the lone star tick, *A. americanum*. Recent ecologic studies in central and southern Missouri found that 99.9% of captured ticks were *A. americanum*.[14] *A. americanum* is abundant in northwestern Missouri and found throughout the southeastern and south–central United States, extending up the Atlantic coast to Maine.[15] Both patients resided in areas with fragmented deciduous forest and old fields, suitable habitats for *A. americanum*. Studies will be required to determine the vector and potential hosts of this virus.

Although these two patients had severe disease, the incidence of infection with the novel virus and range of disease severity are currently unknown. Given the largely nonspecific symptoms observed, this virus could be a more common cause of human illness than is currently recognized. Epidemiologic and ecologic studies are needed to identify disease burden, risk factors for infection, and natural hosts of this new virus.

The findings and conclusions of this report are those of the authors and do not necessarily represent the views of the CDC.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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