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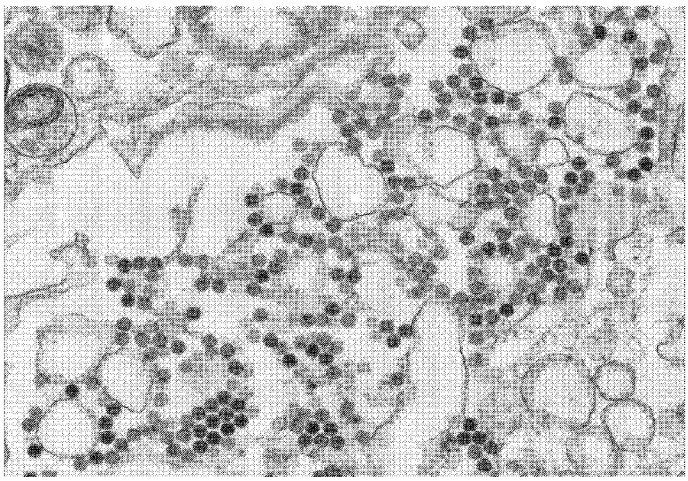
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[Continued on next page]

(54) Title: PATHOGENIC PHLEBOVIRUS ISOLATES AND COMPOSITIONS AND METHODS OF USE

FIG. 2



(57) Abstract: Described herein are the clinical and laboratory characteristics of two patients bitten by ticks and infected with a unique member of the genus *Phlebovirus* (family *Bunyaviridae*) with a proposed name of Heartland virus (HRTLV). Provided herein are nucleotide and amino acid sequences of the *Phlebovirus* isolates, primers and probes that specifically hybridize with the *Phlebovirus* isolates, and antibodies specific for the *Phlebovirus* proteins. Also provided are detection assays using the *Phlebovirus* nucleic acid molecules, proteins, probes, primers and antibodies. Further provided are recombinant *Phleboviruses* and their use for eliciting an immune response in a subject.

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PATHOGENIC PHLEBOVIRUS ISOLATES AND COMPOSITIONS AND METHODS OF USE

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 61/614,926, filed March 23, 2012, which is herein incorporated by reference in its entirety.

FIELD

This disclosure concerns the isolation, identification and sequencing of a unique *Phlebovirus*, and the use of the *Phlebovirus* nucleic acid molecules and proteins as detection and diagnostic reagents, as well as in reverse genetics and mini-genome reporter systems.

BACKGROUND

The genus *Phlebovirus* is one of five genera of the *Bunyaviridae* family. *Phleboviruses* are enveloped spherical viruses with icosahedral symmetry. The genome of *Phleboviruses* consists of three single-stranded RNA genome segments – small (S), medium (M) and large (L). The M and L segments use a negative sense coding strategy, while the S segment encodes two proteins using an ambisense strategy. The S segment encodes the non-structural protein NSs in the positive sense orientation and the nucleoprotein (NP) in the negative sense orientation; each protein is translated from a subgenomic virus mRNA. The M segment encodes the glycoprotein precursor that is cleaved by host proteases into two structural domains – Gn and Gc. The L segment encodes the L protein, which functions as the RNA dependent RNA polymerase in primary and secondary transcription to generate mRNA and replicative intermediates, respectively.

There are approximately 70 named viruses in the *Phlebovirus* genus. These viruses are classified based on their serological relationships into two antigenic groups, the phlebotomus or sandfly fever group and the Uukuniemi group (Nichol *et*

al., *Virus Taxonomy: Classification and Nomenclature of Viruses. Eighth Report of the International Committee of the Taxonomy of Viruses*. Elsevier Academic Press, Genus *Phlebovirus*, pp. 706-716, 2005). *Phleboviruses* have a worldwide distribution and are transmitted by a wide variety of arthropods, including sandflies, mosquitoes and ticks. Several *Phleboviruses* have been linked to human disease, in some cases causing febrile illness, fever, hepatitis, meningitis, encephalitis or hemorrhagic syndrome.

Ticks (Acari: *Ixodidae*) transmit bacterial and viral pathogens to humans in the United States and elsewhere. Lyme disease, rickettsioses, ehrlichioses, tularemia, babesiosis, and Powassan virus infections of humans have all increased over the last decade. Reasons for these increases are multifactorial and include improved diagnosis, climate effects, and changes in land use (Roche *et al.*, First culture isolations of *Ehrlichia chaffeensis* from a Missouri patient. In: 22nd Annual Meeting of the Society for Vector Ecology. Ft. Collins, CO, 2008). An important species of tick that can transmit numerous pathogens is the lone star tick, *Amblyomma americanum*. In many parts of the country, humans are regularly exposed to this species and the pathogens it may carry (Goddard and Varela-Stokes, *Vet Parasitol* 160:1-12, 2009; Paddock and Yabsley, *Curr Top Microbiol Immunol* 315:289-324, 2007).

SUMMARY

Disclosed herein is the isolation and identification of a previously unidentified *Phlebovirus*. In particular, disclosed are two *Phlebovirus* isolates from patients who had recently been bitten by ticks. The sequences of all three genome segments of each isolate, as well as the amino acid sequences of the proteins encoded by the virus isolates, are further disclosed.

Provided herein is an isolated *Phlebovirus* comprising an S segment, an M segment and an L segment, wherein the nucleotide sequences of the S, M and L segments are at least 80% identical to the nucleotide sequences of the *Phlebovirus* isolates disclosed herein.

Further provided are isolated nucleic acid molecules of the *Phlebovirus* isolates and polypeptides encoded by the *Phlebovirus* isolates. Oligonucleotides, such as primers and probes, specific for a nucleic acid molecule of the *Phlebovirus* isolates are also provided by the present disclosure.

Also provided are isolated antibodies, or antigen-binding fragments thereof, that specifically bind the *Phlebovirus* isolates disclosed herein, or a polypeptide encoded by the *Phlebovirus* isolates.

Methods for detecting a *Phlebovirus*, *Phlebovirus* polypeptide, *Phlebovirus* nucleic acid molecule or *Phlebovirus*-specific antibody in a biological sample are also provided. The present disclosure further provides a method for identifying a subject infected with *Phlebovirus*.

Also provided are recombinant *Phleboviruses*, such as *Phleboviruses* encoding a reporter molecule and/or *Phleboviruses* comprising an attenuating mutation.

Immunogenic compositions comprising a recombinant *Phlebovirus* or *Phlebovirus* polypeptide and methods of eliciting an immune response against *Phlebovirus* using the immunogenic compositions are also provided by the present disclosure.

Also provided are *Phlebovirus* reverse genetics and mini-genome reporter systems. Further provided is a method of producing *Phlebovirus* pseudo-virus using the mini-genome reporter system.

The foregoing and other objects, features, and advantages of the invention will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a graph showing absolute values of white blood cell count and differential for Patient 1 during hospitalization. The normal limit is indicated with a dotted line. An asterisk denotes when virus was isolated from patient blood.

FIG. 1B is a graph showing absolute values of white blood cell count and differential for Patient 2 during hospitalization. The normal limit is indicated with a dotted line. An asterisk denotes when virus was isolated from patient blood.

FIG. 1C is a graph showing aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels for Patient 1 and Patient 2 during hospitalization. AST values are the dotted line and ALT values are the solid line. Normal values are indicated with the solid line (ALT) and the dashed line (AST) at the bottom of the graph.

FIG. 1D is a graph showing hematocrit values and platelet counts during hospitalization. Hematocrit values are on the left y-axis and indicated by the two lower lines. Platelet counts are on the right y-axis and indicated by the two upper lines. Patient 1 is indicated with a circle and Patient 2 is indicated with a square. Normal values are indicated by dotted lines at the center of the graph.

FIG. 2 is a thin section electron microscopy image of infected VeroE6 cells, revealing spherical virion particles averaging 86 nm in diameter.

FIGS. 3A-3D show phylogenetic analyses of nucleoprotein (FIG. 3A), NSs (FIG. 3B), glycoprotein (FIG. 3C) and polymerase (FIG. 3D) amino acid sequences of representative members of the *Phlebovirus* genus. GenBank™ accession numbers are as follows. Nucleoprotein: Gouleako HQ541736, Uukuniemi M33551, Catch Me Cave EU274384, SFTSV HM745932, Sandfly Sicilian J04418, Massilia EU725773, Sandfly Naples Sabin EF201829, Toscana FJ153286, Punta Toro EF201835, Chandiru HM119409, RFV ZH501 DQ380149, RFV Entebbe DQ380156, Salobo 578142 EF201815, Salobo 416992 EF201816, Joa EF201819, Frijoles EF201820. Nonstructural NSs: Uukuniemi M33551, SFTSV HM745932, Massilia EU725773, Sandfly Naples Sabin EF201829, Toscana FJ153286, Punta Toro EF201835, Chandiru HM119409, Sandfly Sicilian J04418, RFV ZH501 DQ380149, RFV Entebbe DQ380156, Salobo 578142 EF201815, Salobo 416992 EF201816, Joa EF201819, Frijoles EF201820. Glycoprotein: Uukuniemi M17417, Massilia EU725772, Toscana EU003180, RFV ZH501 DQ380200, SFTSV HM745931, Punta Toro Adames DQ363407, Chandiru HM119408, Sandfly Sicilian

U30500, Punta Toro M11156. Polymerase: RFV ZH501 DQ375406, RFV ZH548 DQ375403, Toscana AR France EF656363, Sandfly Sicilian GQ847513, Uukuniemi D10759, Chandiru HM119407, Massilia EU725771, SFTSV HM745930, Gouleako HQ541738.

FIGS. 4A-4B are tables showing laboratory results of Patient 1 (FIG. 4A) and Patient 2 (FIG. 4B) during hospitalization.

FIGS. 5A-5B are an amino acid alignment of NP from *Phlebovirus* isolate #1 (Mo4), *Phlebovirus* isolate #2 (Mo7) and several different *Phlebovirus* species.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. The sequence listing is submitted as an ASCII text file, created on March 12, 2013, 226 KB, which is incorporated by reference herein. In the accompanying sequence listing:

SEQ ID NO: 1 is the nucleotide sequence of the S segment of *Phlebovirus* isolate #1.

SEQ ID NO: 2 is the nucleotide sequence of the M segment of *Phlebovirus* isolate #1.

SEQ ID NO: 3 is the nucleotide sequence of the L segment of *Phlebovirus* isolate #1.

SEQ ID NO: 4 is the amino acid sequence of the nucleoprotein (NP) of *Phlebovirus* isolate #1.

SEQ ID NO: 5 is the amino acid sequence of the NSs protein of *Phlebovirus* isolate #1.

SEQ ID NO: 6 is the amino acid sequence of the glycoprotein (GP) polyprotein of *Phlebovirus* isolate #1.

SEQ ID NO: 7 is the amino acid sequence of the polymerase protein of *Phlebovirus* isolate #1.

SEQ ID NO: 8 is the nucleotide sequence of the S segment of *Phlebovirus* isolate #2.

SEQ ID NO: 9 is the nucleotide sequence of the M segment of *Phlebovirus* isolate #2.

SEQ ID NO: 10 is the nucleotide sequence of the L segment of *Phlebovirus* isolate #2.

SEQ ID NO: 11 is the amino acid sequence of the nucleoprotein (NP) of *Phlebovirus* isolate #2.

SEQ ID NO: 12 is the amino acid sequence of the NSs protein of *Phlebovirus* isolate #2.

SEQ ID NO: 13 is the amino acid sequence of the glycoprotein (GP) polyprotein of *Phlebovirus* isolate #2.

SEQ ID NO: 14 is the amino acid sequence of the polymerase protein of *Phlebovirus* isolate #2.

SEQ ID NOs: 15-23 are nucleotide sequences of primers and probes used for qRT-PCR.

SEQ ID NO: 24 is the amino acid sequence of NP from Sandfly fever Sicilian virus (SFSV; GenBank™ Accession No. J04418).

SEQ ID NO: 25 is the amino acid sequence of NP from Rift Valley fever virus (RFV) strain ZH-501 (GenBank™ Accession No. DQ380149).

SEQ ID NO: 26 is the amino acid sequence of NP from *Phlebovirus* sp. Be An 578142 (GenBank™ Accession No. EF201815).

SEQ ID NO: 27 is the amino acid sequence of NP from *Phlebovirus* sp. Be An 416992 (GenBank™ Accession No. EF201816).

SEQ ID NO: 28 is the amino acid sequence of NP from *Phlebovirus* sp. Be Ar 371637 (GenBank™ Accession No. EF201820).

SEQ ID NO: 29 is the amino acid sequence of NP from *Phlebovirus* sp. VP161A (GenBank™ Accession No. EF201819).

SEQ ID NO: 30 is the amino acid sequence of NP from Sandfly fever Naples virus Sabin strain (GenBank™ Accession No. EF201829).

SEQ ID NO: 31 is the amino acid sequence of NP from Massilia virus strain W (GenBank™ Accession No. EU725773).

SEQ ID NO: 32 is the amino acid sequence of NP from Toscana virus isolate H/IMTSSA (GenBank™ Accession No. FJ153286).

SEQ ID NO: 33 is the amino acid sequence of NP from Uukuniemi (UUK) virus (GenBank™ Accession No. M33551).

SEQ ID NO: 34 is the amino acid sequence of NP from *Phlebovirus* HB29/China/2010 (GenBank™ Accession No. HM745932), which is also known as severe fever with thrombocytopenia syndrome virus (SFTSV).

SEQ ID NO: 35 is the amino acid sequence of NP (partial) from Gabek Forest virus (GenBank™ Accession No. FJ235927).

SEQ ID NO: 36 is the amino acid sequence of NP from Punta Toro virus (PTV) strain Adames (GenBank™ Accession No. EF201835).

SEQ ID NO: 37 is the amino acid sequence of NP from Rift Valley fever virus (RFV) strain Entebbe (GenBank™ Accession No. DQ380156).

SEQ ID NO: 38 is the amino acid sequence of NP from Chandiru virus (GenBank™ Accession No. HM119409).

SEQ ID NO: 39 is the amino acid sequence of NP (partial) from Rio Grande virus (GenBank™ Accession No. FJ235929).

SEQ ID NO: 40 is the nucleotide sequence of the pcMo4GnGc vector.

SEQ ID NO: 41 is the nucleotide sequence of the pcMo4L vector.

SEQ ID NO: 42 is the nucleotide sequence of the pcMo4NP vector.

SEQ ID NO: 43 is the nucleotide sequence of the pcMo4Morf vector.

SEQ ID NO: 44 is the nucleotide sequence of the pLCK-Mo4Lvc vector.

SEQ ID NO: 45 is the nucleotide sequence of the pLCK-Mo4Mvc vector.

SEQ ID NO: 46 is the nucleotide sequence of the pLCK-Mo4Svc vector.

SEQ ID NO: 47 is the nucleotide sequence of the pLCK-Mo4SvcDelNSs_GLuc vector.

SEQ ID NO: 48 is the nucleotide sequence of the pLCK-Mo4M_EGFPBlastNeg vector.

SEQ ID NO: 49 is the nucleotide sequence of the pLCK-Mo7Lvc vector.

SEQ ID NO: 50 is the nucleotide sequence of the pLCK-Mo7Mvc vector.

SEQ ID NO: 51 is the nucleotide sequence of the pLCK-Mo7Svc vector.

SEQ ID NOs: 52 and 53 are primer sequences.

DETAILED DESCRIPTION

I. Abbreviations

ALT	alanine aminotransferase
AST	aspartate aminotransferase
cDNA	complementary DNA
GP	glycoprotein
HRTLV	Heartland virus
NGS	next generation sequencing
NP	nucleoprotein
NS	non-structural
ORF	open reading frame
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RVF	Rift Valley fever
SFTSV	severe fever with thrombocytopenia syndrome virus
UUK	Uukuniemi virus

II. Terms and Methods

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers

(ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided:

Adjuvant: A substance or vehicle that non-specifically enhances the immune response to an antigen. Adjuvants can include a suspension of minerals (alum, aluminum hydroxide, or phosphate) on which antigen is adsorbed; or water-in-oil emulsion in which antigen solution is emulsified in mineral oil (for example, Freund's incomplete adjuvant), sometimes with the inclusion of killed mycobacteria (Freund's complete adjuvant) to further enhance antigenicity. Immunostimulatory oligonucleotides (such as those including a CpG motif) can also be used as adjuvants (for example, see U.S. Patent Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; 6,339,068; 6,406,705; and 6,429,199). Adjuvants also include biological molecules, such as costimulatory molecules. Exemplary biological adjuvants include IL-2, RANTES, GM-CSF, TNF-α, IFN-γ, G-CSF, LFA-3, CD72, B7-1, B7-2, OX-40L and 41 BBL.

Administer: To give, apply or bring the composition into contact with the subject. Administration can be accomplished by any of a number of routes, such as, for example, topical, oral, intranasal, subcutaneous, intramuscular, intraperitoneal, intravenous and intrathecal.

Ambisense: Refers to a genome or genomic segments having both positive sense and negative sense portions. For example, the S segment of a *Phlebovirus* is ambisense, encoding nucleoprotein (NP) in the negative sense and the non-structural protein (NSs) in the positive sense.

Antibody: A polypeptide ligand comprising at least a light chain or heavy chain immunoglobulin variable region which specifically recognizes and binds an epitope of an antigen. Antibodies are composed of a heavy and a light chain, each of which has a variable region, termed the variable heavy (V_H) region and the variable light (V_L) region. Together, the V_H region and the V_L region are responsible for binding the antigen recognized by the antibody.

Antibodies include intact immunoglobulins and the variants and portions of antibodies well known in the art, such as Fab fragments, Fab' fragments, $F(ab)'_2$ fragments, single chain Fv proteins ("scFv"), and disulfide stabilized Fv proteins ("dsFv"). A scFv protein is a fusion protein in which a light chain variable region of an immunoglobulin and a heavy chain variable region of an immunoglobulin are bound by a linker, while in dsFvs, the chains have been mutated to introduce a disulfide bond to stabilize the association of the chains. The term also includes genetically engineered forms such as chimeric antibodies (for example, humanized murine antibodies), heteroconjugate antibodies (such as, bispecific antibodies). See also, *Pierce Catalog and Handbook*, 1994-1995 (Pierce Chemical Co., Rockford, IL); Kuby, J., *Immunology*, 3rd Ed., W. H. Freeman & Co., New York, 1997.

Typically, a naturally occurring immunoglobulin has heavy (H) chains and light (L) chains interconnected by disulfide bonds. There are two types of light chain, lambda (λ) and kappa (k). There are five main heavy chain classes (or isotypes) which determine the functional activity of an antibody molecule: IgM, IgD, IgG, IgA and IgE.

Each heavy and light chain contains a constant region and a variable region (the regions are also known as "domains"). References to "V_H" or "VH" refer to the variable region of an immunoglobulin heavy chain, including that of an Fv, scFv, dsFv or Fab. References to "V_L" or "VL" refer to the variable region of an immunoglobulin light chain, including that of an Fv, scFv, dsFv or Fab.

A "monoclonal antibody" is an antibody produced by a single clone of B-lymphocytes or by a cell into which the light and heavy chain genes of a single antibody have been transfected. Monoclonal antibodies are produced by methods known to those of skill in the art, for instance by making hybrid antibody-forming cells from a fusion of myeloma cells with immune spleen cells. Monoclonal antibodies include humanized monoclonal antibodies.

A "chimeric antibody" has framework residues from one species, such as human, and CDRs (which generally confer antigen binding) from another species, such as a murine antibody.

A "humanized" immunoglobulin is an immunoglobulin including a human framework region and one or more complementarity determining regions (CDRs) from a non-human (for example a mouse, rat, or synthetic) immunoglobulin. The non-human immunoglobulin providing the CDRs is termed a "donor," and the human immunoglobulin providing the framework is termed an "acceptor." Generally, all parts of a humanized immunoglobulin, except possibly the CDRs, are substantially identical to corresponding parts of natural human immunoglobulin sequences. A "humanized antibody" is an antibody comprising a humanized light chain and a humanized heavy chain immunoglobulin. A humanized antibody binds to the same antigen as the donor antibody that provides the CDRs. Humanized immunoglobulins can be constructed by means of genetic engineering (see for example, U.S. Patent No. 5,585,089).

A "human" antibody (also called a "fully human" antibody) is an antibody that includes human framework regions and all of the CDRs from a human immunoglobulin. In one example, the framework and the CDRs are from the same originating human heavy and/or light chain amino acid sequence. However, frameworks from one human antibody can be engineered to include CDRs from a different human antibody. All parts of a human immunoglobulin are substantially identical to corresponding parts of natural human immunoglobulin sequences.

Antigen: A compound, composition, or substance that can stimulate the production of antibodies or a T-cell response in an animal, including compositions that are injected or absorbed into an animal. An antigen reacts with the products of specific humoral or cellular immunity.

Anti-genomic: As used herein, "anti-genomic" refers to a genomic segment of a *Phlebovirus* in the orientation opposite to the viral genome. For example, *Phleboviruses* are negative-sense RNA viruses. Thus, "anti-genomic" refers to the positive-sense orientation (or virus complementary sense), while "genomic" refers to the negative-sense orientation of a gene segment.

Attenuated: In the context of a live virus, the virus is attenuated if its ability to infect a cell or subject and/or its ability to produce disease is reduced (for

example, eliminated) compared to a wild-type virus. Typically, an attenuated virus retains at least some capacity to elicit an immune response following administration to an immunocompetent subject. In some cases, an attenuated virus is capable of eliciting a protective immune response without causing any signs or symptoms of infection. In some embodiments, the ability of an attenuated virus to cause disease in a subject is reduced at least about 10%, at least about 25%, at least about 50%, at least about 75% or at least about 90% relative to wild-type virus. Accordingly, an "**attenuating mutation**" is a mutation in the viral genome and/or an encoded polypeptide that results in an attenuated virus.

Biological sample: A sample obtained from a subject (such as a human or veterinary subject). Biological samples, include, for example, fluid, cell and/or tissue samples. In some embodiments herein, the biological sample is a fluid sample. Fluid samples include, but are not limited to, serum, blood, plasma, urine, feces, saliva, cerebral spinal fluid (CSF) or other bodily fluid. Biological samples can also refer to cells or tissue samples, such as biopsy samples, tissue sections or isolated leukocytes.

Contacting: Placement in direct physical association; includes both in solid and liquid form. "Contacting" is often used interchangeably with "exposed." In some cases, "contacting" includes transfecting, such as transfecting a nucleic acid molecule into a cell. In other examples, "contacting" refers to incubating a molecule (such as an antibody) with a biological sample.

Fluorophore: A chemical compound, which when excited by exposure to a particular wavelength of light, emits light (*i.e.*, fluoresces), for example at a different wavelength. In some embodiments herein, a probe is labeled with a fluorophore, such as at the 5' end of the probe. Probes used for real-time PCR assays typically include a fluorophore and a quencher. Fluorophores suitable for use with real-time PCR assays, such as TaqMan™ PCR, include, but are not limited to, 6-carboxyfluorescein (FAM), tetrachlorofluorescein (TET), tetramethylrhodamine (TMR), hexachlorofluorescein (HEX), JOE, ROX, CAL Fluor™, Pulsar™, Quasar™, Texas Red™, Cy™3 and Cy™5.

Hybridization: Oligonucleotides and their analogs hybridize by hydrogen bonding, which includes Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary bases. Generally, nucleic acid consists of nitrogenous bases that are either pyrimidines (cytosine (C), uracil (U), and thymine (T)) or purines (adenine (A) and guanine (G)). These nitrogenous bases form hydrogen bonds between a pyrimidine and a purine, and the bonding of the pyrimidine to the purine is referred to as "base pairing." More specifically, A will hydrogen bond to T or U, and G will bond to C. "Complementary" refers to the base pairing that occurs between two distinct nucleic acid sequences or two distinct regions of the same nucleic acid sequence.

"**Specifically hybridizable**" and "specifically complementary" are terms that indicate a sufficient degree of complementarity such that stable and specific binding occurs between the oligonucleotide (or its analog) and the DNA or RNA target. The oligonucleotide or oligonucleotide analog need not be 100% complementary to its target sequence to be specifically hybridizable. An oligonucleotide or analog is specifically hybridizable when binding of the oligonucleotide or analog to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide or analog to non-target sequences under conditions where specific binding is desired, for example under physiological conditions in the case of *in vivo* assays or systems. Such binding is referred to as specific hybridization.

Hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the hybridization method of choice and the composition and length of the hybridizing nucleic acid sequences. Generally, the temperature of hybridization and the ionic strength (especially the Na^+ and/or Mg^{++} concentration) of the hybridization buffer will determine the stringency of hybridization, though wash times also influence stringency. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed by Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd

ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, chapters 9 and 11; and Ausubel *et al. Short Protocols in Molecular Biology*, 4th ed., John Wiley & Sons, Inc., 1999.

For purposes of the present disclosure, "stringent conditions" encompass conditions under which hybridization will only occur if there is less than 25% mismatch between the hybridization molecule and the target sequence. "Stringent conditions" may be broken down into particular levels of stringency for more precise definition. Thus, as used herein, "moderate stringency" conditions are those under which molecules with more than 25% sequence mismatch will not hybridize; conditions of "medium stringency" are those under which molecules with more than 15% mismatch will not hybridize, and conditions of "high stringency" are those under which sequences with more than 10% mismatch will not hybridize. Conditions of "very high stringency" are those under which sequences with more than 6% mismatch will not hybridize.

"Specific hybridization" refers to the binding, duplexing, or hybridizing of a molecule only or substantially only to a particular nucleotide sequence when that sequence is present in a complex mixture (for example, total cellular DNA or RNA). Specific hybridization may also occur under conditions of varying stringency.

Immune response: A response of a cell of the immune system, such as a B-cell, T-cell, macrophage or polymorphonucleocyte, to a stimulus such as an antigen. An immune response can include any cell of the body involved in a host defense response, including for example, an epithelial cell that secretes an interferon or a cytokine. An immune response includes, but is not limited to, an innate immune response or inflammation. As used herein, a protective immune response refers to an immune response that protects a subject from infection (prevents infection or prevents the development of disease associated with infection).

Immunogen: A compound, composition, or substance which is capable, under appropriate conditions, of stimulating an immune response, such as the production of antibodies or a T-cell response in an animal, including compositions

that are injected or absorbed into an animal. As used herein, an "**immunogenic composition**" is a composition comprising an immunogen.

Immunize: To render a subject protected from an infectious disease, such as by vaccination.

Isolated: An "isolated" biological component (such as a nucleic acid, protein or virus) has been substantially separated or purified away from other biological components (such as cell debris, or other proteins or nucleic acids). Biological components that have been "isolated" include those components purified by standard purification methods. The term also embraces recombinant nucleic acids, proteins or viruses, as well as chemically synthesized nucleic acids or peptides.

Oligonucleotide: A short nucleic acid polymer. Oligonucleotides are generally less than 100 nucleotides in length. In some embodiments herein, the oligonucleotide is 8-100, 10-50, 12-40, 16-30 or 18-24 nucleotides in length. In particular examples, the oligonucleotide is 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides in length.

ORF (open reading frame): A series of nucleotide triplets (codons) coding for amino acids without any termination codons. These sequences are usually translatable into a peptide.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame.

Pharmaceutically acceptable carrier: The pharmaceutically acceptable carriers (vehicles) useful in this disclosure are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of one or more therapeutic compounds or molecules, such as one or more recombinant *Phleboviruses*, and additional pharmaceutical agents.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (for example, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

***Phlebovirus*:** One of five genera of the *Bunyaviridae* family. *Phleboviruses* are enveloped spherical viruses with icosahedral symmetry. The genome of *Phleboviruses* consists of three single-stranded RNA genome segments – small (S), medium (M) and large (L). The M and L segments are negative sense RNA strands, while the S segment is ambisense RNA. The S segment encodes the non-structural protein NSs in the positive sense orientation and the nucleoprotein (NP) in the negative sense orientation. The M segment encodes the glycoprotein precursor that is cleaved by host proteases into two structural domains – Gn and Gc. The L segment encodes the L protein, which functions as the RNA dependent RNA polymerase in primary and secondary transcription to generate mRNA and replicative intermediates, respectively. *Phleboviruses* have a worldwide distribution and are transmitted by a wide variety of arthropods, including sandflies, mosquitoes and ticks. Several *Phleboviruses* have been linked to human disease, in some cases causing febrile illness, fever, hepatitis, meningitis, encephalitis or hemorrhagic syndrome.

Polypeptide: A polymer in which the monomers are amino acid residues which are joined together through amide bonds. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used. The terms "polypeptide" or "protein" as used herein are intended to encompass any

amino acid sequence and include modified sequences such as glycoproteins. The term "polypeptide" is specifically intended to cover naturally occurring proteins, as well as those which are recombinantly or synthetically produced. The term "residue" or "amino acid residue" includes reference to an amino acid that is incorporated into a protein, polypeptide, or peptide.

Conservative amino acid substitutions are those substitutions that, when made, least interfere with the properties of the original protein, that is, the structure and especially the function of the protein is conserved and not significantly changed by such substitutions. Examples of conservative substitutions are shown below.

Original Residue	Conservative Substitutions
Ala	Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
His	Asn; Gln
Ile	Leu, Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Ile
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

Conservative substitutions generally maintain (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain.

The substitutions which in general are expected to produce the greatest changes in protein properties will be non-conservative, for instance changes in which (a) a hydrophilic residue, for example, seryl or threonyl, is substituted for (or

by) a hydrophobic residue, for example, leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, for example, lysyl, arginyl, or histadyl, is substituted for (or by) an electronegative residue, for example, glutamyl or aspartyl; or (d) a residue having a bulky side chain, for example, phenylalanine, is substituted for (or by) one not having a side chain, for example, glycine.

Preventing, treating or ameliorating a disease: "Preventing" a disease refers to inhibiting the full development of a disease. "Treating" refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop. "Ameliorating" refers to the reduction in the number or severity of signs or symptoms of a disease.

Probes and primers: A probe comprises an isolated nucleic acid molecule attached to a detectable label or other reporter molecule. Typical labels include radioactive isotopes, enzyme substrates, co-factors, ligands, chemiluminescent or fluorescent agents, haptens, and enzymes. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed, for example, in Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989 and Ausubel *et al. Short Protocols in Molecular Biology*, 4th ed., John Wiley & Sons, Inc., 1999.

Primers are short nucleic acid molecules, for instance DNA oligonucleotides 10 nucleotides or more in length, for example that hybridize to contiguous complementary nucleotides or a sequence to be amplified. Longer DNA oligonucleotides may be about 12, 15, 18, 20, 25, 30, or 50 nucleotides or more in length. Primers can be annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, and then the primer extended along the target DNA strand by a DNA polymerase enzyme. Primer pairs can be used for amplification of a nucleic acid sequence, for example, by the polymerase chain reaction (PCR) or other nucleic-acid amplification methods known in the art. Other examples of amplification include strand displacement amplification, as disclosed in U.S. Patent No. 5,744,311;

transcription-free isothermal amplification, as disclosed in U.S. Patent No. 6,033,881; repair chain reaction amplification, as disclosed in WO 90/01069; ligase chain reaction amplification; gap filling ligase chain reaction amplification, as disclosed in 5,427,930; and NASBA™ RNA transcription-free amplification, as disclosed in U.S. Patent No. 6,025,134.

Methods for preparing and using nucleic acid probes and primers are described, for example, in Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989; Ausubel *et al. Short Protocols in Molecular Biology*, 4th ed., John Wiley & Sons, Inc., 1999; and Innis *et al. PCR Protocols, A Guide to Methods and Applications*, Academic Press, Inc., San Diego, CA, 1990. Amplification primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, MA).

Quencher: A substance that absorbs excitation energy from a fluorophore when in close proximity. Probes used for real-time PCR assays, such as TaqMan™ PCR, typically include a fluorophore and a quencher. Quenchers suitable for use with real-time PCR assays include, but are not limited to, ZEN™, Iowa Black™ FQ, tetramethylrhodamine (TAMRA), black hole quencher (BHQ)1, BHQ2, BHQ3 and 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL). In some examples, a probe contains two quenchers. In one non-limiting example, the probe contains both ZEN™ and Iowa Black™ FQ.

Recombinant: A recombinant nucleic acid, protein or virus is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, for example, by genetic engineering techniques. For example, a recombinant *Phlebovirus* can be generated using a reverse genetics system. In some examples, the recombinant *Phleboviruses* comprise

one or more deletions in a viral virulence factor, such as NSs. In other examples, the recombinant RVF viruses comprise a heterologous gene, such as a reporter gene.

Reporter gene: A reporter gene is a gene operably linked to another gene or nucleic acid sequence of interest (such as a promoter sequence). Reporter genes are used to determine whether the gene or nucleic acid of interest is expressed in a cell or has been activated in a cell. Reporter genes typically have easily identifiable characteristics, such as fluorescence, or easily assayed products, such as an enzyme. Reporter genes can also confer antibiotic resistance to a host cell or tissue. Reporter genes include, for example, GFP (or eGFP) or other fluorescence genes, luciferase, β-galactosidase and alkaline phosphatase.

Sequence identity: The similarity between amino acid or nucleic acid sequences is expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology); the higher the percentage, the more similar the two sequences are. Homologs or variants of a given gene or protein will possess a relatively high degree of sequence identity when aligned using standard methods.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith and Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman and Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85:2444, 1988; Higgins and Sharp, *Gene* 73:237-244, 1988; Higgins and Sharp, *CABIOS* 5:151-153, 1989; Corpet *et al.*, *Nucleic Acids Research* 16:10881-10890, 1988; and Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85:2444, 1988. Altschul *et al.*, *Nature Genet.* 6:119-129, 1994.

The NCBI Basic Local Alignment Search Tool (BLAST™) (Altschul *et al.*, *J. Mol. Biol.* 215:403-410, 1990) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MD) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx.

In some embodiments herein, provided are nucleotide or amino acid sequences at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to any one of SEQ ID NOs: 1-51.

Subject: Living multi-cellular vertebrate organisms, a category that includes both human and non-human mammals.

Therapeutically effective amount: A quantity of a specified agent sufficient to achieve a desired effect in a subject being treated with that agent. For example, this may be the amount of a recombinant *Phlebovirus* useful for eliciting an immune response in a subject and/or for preventing infection by *Phlebovirus*. Ideally, in the context of the present disclosure, a therapeutically effective amount of a recombinant *Phlebovirus* is an amount sufficient to increase resistance to, prevent, ameliorate, and/or treat infection caused by *Phlebovirus* in a subject without causing a substantial cytotoxic effect in the subject. The effective amount of a recombinant *Phlebovirus* useful for increasing resistance to, preventing, ameliorating, and/or treating infection in a subject will be dependent on, for example, the subject being treated, the manner of administration of the therapeutic composition and other factors.

Vaccine: A preparation of immunogenic material capable of stimulating an immune response, administered for the prevention, amelioration, or treatment of infectious or other types of disease. The immunogenic material may include attenuated or killed microorganisms (such as attenuated viruses), or antigenic proteins, peptides or DNA derived from them. Vaccines may elicit both prophylactic (preventative) and therapeutic responses. Methods of administration vary according to the vaccine, but may include inoculation, ingestion, inhalation or other forms of administration. Inoculations can be delivered by any of a number of routes, including parenteral, such as intravenous, subcutaneous or intramuscular. Vaccines may be administered with an adjuvant to boost the immune response.

Vector: A nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector may include nucleic acid sequences that permit it to replicate in a host cell, such as an origin of replication (DNA sequences

that participate in initiating DNA synthesis). A vector may also include one or more selectable marker genes and other genetic elements known in the art.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Hence "comprising A or B" means including A, or B, or A and B. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. All GenBank™ Accession numbers are incorporated by reference herein as they appear in the database on February 29, 2012. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

III. Overview of Several Embodiments

Disclosed herein is the discovery of a new member of the genus *Phlebovirus* (family *Bunyaviridae*) with a proposed name of Heartland virus (HRTLV). HRTLV is associated with severe febrile illness following tick bite. Described are the clinical and laboratory characteristics of two patients bitten by ticks and infected with HRTLV. In particular, provided herein are the complete nucleotide sequences of all three genome segments of two *Phlebovirus* isolates, as well as the amino acid sequences of the proteins encoded by each isolate, including the NP, GP, NSs and polymerase (L) proteins. Oligonucleotides, such as primers and probes, that

specifically hybridize with the *Phlebovirus* isolates, and antibodies specific for the *Phlebovirus* proteins are further provided. Also provided are diagnostic and detection assays using the *Phlebovirus* nucleic acid molecules, proteins, probes, primers and antibodies. Further provided are recombinant *Phleboviruses*, such as recombinant Phleboviruses encoding a reporter molecule and/or comprising an attenuating mutation, and their use for eliciting an immune response in a subject. Also provided are reverse genetics and mini-genome reporters systems using plasmids containing nucleic acid sequence derived from the *Phlebovirus* isolates.

In some embodiments, infection with a *Phlebovirus* disclosed herein is associated with recent tick bite and/or one or more of the following clinical features: thrombocytopenia, leukopenia, fever, and decreased calcium level. In some embodiments, the *Phlebovirus* disclosed herein has an average viral particle diameter of approximately 80-90 nm, such as about 86 nm.

A. *Phlebovirus* Isolates, Nucleic Acid Molecules and Polypeptides

Provided herein are isolated *Phleboviruses* comprising an S segment, an M segment and an L segment at least 80% identical to the *Phlebovirus* isolates disclosed herein. In some embodiments, the nucleotide sequence of the S segment is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 1 or SEQ ID NO: 8; the nucleotide sequence of the M segment is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 2 or SEQ ID NO: 9; the nucleotide sequence of the L segment is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 3 or SEQ ID NO: 10; or any combination thereof.

In some examples, the nucleotide sequence of the S segment comprises or consists of SEQ ID NO: 1 or SEQ ID NO: 8; the nucleotide sequence of the M segment comprises or consists of SEQ ID NO: 2 or SEQ ID NO: 9; the nucleotide

sequence of the L segment comprises or consists of SEQ ID NO: 3 or SEQ ID NO: 10; or any combination thereof.

In particular non-limiting examples, the S segment comprises or consists of SEQ ID NO: 1, the M segment comprises or consists of SEQ ID NO: 2 and the L segment comprises or consists of SEQ ID NO: 3; or the S segment comprises or consists of SEQ ID NO: 8, the M segment comprises or consists of SEQ ID NO: 9 and the L segment comprises or consists of SEQ ID NO: 10.

Further provided are isolated polypeptides encoded by a *Phlebovirus* disclosed herein. In some embodiments, the amino acid sequence of the *Phlebovirus*-encoded polypeptide is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 4, 5, 6, 7, 11, 12, 13 or 14. In particular examples, the amino acid sequence of the *Phlebovirus* polypeptide comprises or consists of SEQ ID NO: 4, 5, 6, 7, 11, 12, 13 or 14.

Also provided are isolated polypeptides, wherein the amino acid sequence of the polypeptide is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 4, 5, 6, 7, 11, 12, 13 or 14. In some embodiments, the amino acid sequence of the polypeptide comprises or consists of SEQ ID NO: 4, 5, 6, 7, 11, 12, 13 or 14.

Further provided are isolated nucleic acid molecules. In some embodiments, the nucleotide sequence of the nucleic acid molecule is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 1, 2, 3, 8, 9, or 10. In some examples, the nucleotide sequence of the nucleic acid molecule comprises or consists of SEQ ID NO: 1, 2, 3, 8, 9, or 10.

Vectors comprising any of the nucleic acid molecules disclosed herein are further provided by the present disclosure. The vector can be any suitable vector, such as a plasmid vector or a viral vector. In some embodiments, the vector comprises a promoter, an origin of replication and/or a selectable marker. In some

examples, the nucleic acid molecule of the vector is operably linked to a promoter. Also provided are host cells comprising such vectors.

Also provided are oligonucleotides that specifically hybridize with a *Phlebovirus* nucleic acid molecule. Oligonucleotides are generally less than 100 nucleotides in length. In some embodiments, the oligonucleotide is less than 80, less than 60, less than 40 or less than 30 nucleotides in length. In other embodiments, the oligonucleotide is 8-100, 10-50, 12-40, 16-30 or 18-24 nucleotides in length. In particular examples, the oligonucleotide is 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides in length. In one non-limiting example, the oligonucleotide is 12 to 40 nucleotides in length. In another non-limiting example, the oligonucleotide is 18 to 24 nucleotides in length.

In some embodiments, the oligonucleotide is at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 15, 16, 17, 18, 19, 20, 21, 22 or 23. In some examples, the nucleotide sequence of the oligonucleotide comprises or consists of SEQ ID NO: 15, 16, 17, 18, 19, 20, 21, 22 or 23.

In some embodiments, the oligonucleotide comprises a fluorophore. Numerous fluorophores are known in the art and an appropriate fluorophore can be selected by a skilled artisan based on the intended use of the oligonucleotide. As one example, for real-time PCR assays, such as TaqMan™ PCR, exemplary fluorophores include, but are not limited to, FAM, TET, TMR, HEX, JOE, ROX, CAL Fluor™, Pulsar™, Quasar™, Texas Red™, Cy™3 and Cy™5.

In some embodiments, the oligonucleotide comprises a quencher. In some instances, the oligonucleotide comprises more than one quencher, such as two quenchers. A suitable quencher (or quenchers) can be selected by one of skill in the art depending on the intended purpose of the oligonucleotide. As one example, for real-time PCR assays, such as TaqMan™ PCR, exemplary quenchers include, but are not limited to, ZEN™, Iowa Black™ FQ, TAMRA, BHQ1, BHQ2, BHQ3 and DABCYL. In some examples, the oligonucleotide includes two quenchers, such as ZEN™ and Iowa Black™ FQ.

In one non-limiting example, the oligonucleotide, such as when the oligonucleotide will be used as a probe, comprises a fluorophore and a quencher. In another non-limiting example, the oligonucleotide comprises a fluorophore and two quenchers.

B. *Phlebovirus*-Specific Antibodies

Provided herein are isolated antibodies, or antigen-binding fragments thereof, that specifically bind to the *Phlebovirus* isolates and/or the *Phlebovirus* polypeptides disclosed herein. In some embodiments, the antibody is a polyclonal antibody. In other embodiments, the antibody is a monoclonal antibody.

In some embodiments, the antibody or antigen-binding fragment thereof specifically binds an epitope of a *Phlebovirus* virion, and can thus detect virus particles. In some embodiments, the antibody or antigen-binding fragment thereof specifically binds a linear epitope, or conformational epitope, or both, of a *Phlebovirus* polypeptide.

In some embodiments, the antibody or antigen-binding fragment thereof specifically binds to a NSs, NP, GP or L protein of *Phlebovirus*. In particular examples, the antibody or antigen-binding fragment thereof specifically binds a polypeptide that is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 4, 5, 6, 7, 11, 12, 13 or 14. In other particular examples, the antibody or antigen-binding fragment thereof specifically binds a polypeptide with an amino acid sequence comprising or consisting of SEQ ID NO: 4, 5, 6, 7, 11, 12, 13 or 14.

In some embodiments, the antigen-binding fragment is an Fab, Fab', $F(ab)'_2$ scFv or dsFv. In some embodiments, the antibodies are mouse, rat or rabbit antibodies. In other embodiments, the antibodies are humanized antibodies or fully human antibodies. In other embodiments, the antibodies are chimeric antibodies.

Methods of generating monoclonal and polyclonal antibodies are well known in the art and are described in section IV below.

C. Methods for the Diagnosis and Detection of *Phlebovirus*

The isolation and sequencing of the *Phlebovirus* isolates disclosed herein has enabled the development of a series of assays that can be used for the detection of a *Phlebovirus* in a biological sample and/or the diagnosis of a *Phlebovirus* infection in a subject.

Provided herein is a method for detecting a *Phlebovirus* or *Phlebovirus* polypeptide in a biological sample using a *Phlebovirus*-specific antibody disclosed herein. In some embodiments, the method includes contacting the biological sample with a *Phlebovirus*-specific antibody, or antigen-binding fragment thereof; and detecting binding of the antibody or antigen-binding fragment to the biological sample. Binding of the antibody or antigen-binding fragment to the biological sample indicates the presence of the *Phlebovirus* or the *Phlebovirus* polypeptide in the biological sample.

In some embodiments of the detection method, the antibody or antigen-binding fragment thereof specifically binds to a NSs, NP, GP or L protein of *Phlebovirus*. In particular examples, the antibody or antigen-binding fragment thereof specifically binds a polypeptide that is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 4, 5, 6, 7, 11, 12, 13 or 14. In other particular examples, the antibody or antigen-binding fragment thereof specifically binds a polypeptide with an amino acid sequence comprising or consisting of SEQ ID NO: 4, 5, 6, 7, 11, 12, 13 or 14.

Also provided is a method for detecting *Phlebovirus*-specific antibodies in a biological sample using the *Phlebovirus* polypeptides disclosed herein. In some embodiments, the method includes contacting the biological sample with a *Phlebovirus*-specific polypeptide; and detecting binding of the polypeptide to the biological sample. Binding of the polypeptide to the biological sample indicates the presence of the *Phlebovirus*-specific antibodies in a biological sample.

In some embodiments of the detection method, the *Phlebovirus* polypeptide is a NSs, NP, GP or L protein. In particular examples, the amino acid sequence of

the *Phlebovirus* polypeptide is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 4, 5, 6, 7, 11, 12, 13 or 14. In other particular examples, the amino acid sequence of the *Phlebovirus* polypeptide comprises or consists of SEQ ID NO: 4, 5, 6, 7, 11, 12, 13 or 14.

Detection assays based on binding of a polypeptide to an antibody are well known in the art and include, for example, ELISA, Western blot, fluorescence activated cell sorting (FACS), radioimmunoassay and immunohistochemistry. As is well known to one of skill in the art, in some cases the detection assay further includes the step of contacting an antigen-antibody complex with a detection reagent, such as a labeled secondary antibody (*e.g.*, an anti-isotype antibody, such as an anti-IgG antibody), or in the case of a sandwich ELISA, a second antibody that recognizes the same antigen as the first antibody and is labeled for detection. Secondary antibodies can also be conjugated to magnetic beads to allow for magnetic sorting. In other cases, the primary antibody is directly labeled. Directly labeled antibodies can be used for a variety of detection assays, such as FACS.

Further provided is a method for detecting a *Phlebovirus* nucleic acid molecule in a biological sample using an oligonucleotide probe specific for the *Phlebovirus* nucleic acid molecules disclosed herein. In some embodiments, the method includes contacting the biological sample with an oligonucleotide probe that specifically hybridizes with a *Phlebovirus* nucleic acid molecule; and detecting hybridization of the probe with the biological sample. Hybridization of the probe to the biological sample indicates the presence of the *Phlebovirus* nucleic acid molecule in the biological sample.

In some embodiments, the biological sample is a nucleic acid amplification product obtained by a method comprising isolating RNA from the biological sample; reverse transcribing the RNA to generate cDNA; and amplifying the cDNA using a pair of primers that specifically hybridize to a *Phlebovirus* nucleic acid molecule, thereby producing a nucleic acid amplification product.

Also provided is a method for identifying a subject infected with a *Phlebovirus* using a pair of primers that specifically hybridize with a *Phlebovirus* nucleic acid molecule disclosed herein. In some embodiments, the method includes isolating RNA from a biological sample obtained from the subject; reverse transcribing the RNA to generate cDNA; amplifying the cDNA using a pair of primers that specifically hybridize to a *Phlebovirus* nucleic acid molecule; and detecting an amplification product. Detection of the amplification product identifies the subject as infected with the *Phlebovirus*.

In some embodiments of the nucleic acid-based detection methods, the nucleotide sequence of the *Phlebovirus* nucleic acid molecule is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 1, 2, 3, 8, 9, or 10. In some examples, the nucleotide sequence of the *Phlebovirus* nucleic acid molecule comprises or consists of SEQ ID NO: 1, 2, 3, 8, 9, or 10.

In some embodiments of the nucleic acid-based detection methods, the pair of primers comprises: (i) a first primer having a nucleotide sequence at least 85%, at least 90% or at least 95% identical to SEQ ID NO: 16 and a second primer having a nucleotide sequence at least 85%, at least 90% or at least 95% identical to SEQ ID NO: 17; (ii) a first primer having a nucleotide sequence comprising or consisting of SEQ ID NO: 16 and a second primer having a nucleotide sequence comprising or consisting of SEQ ID NO: 17; (iii) a first primer having a nucleotide sequence at least 85%, at least 90% or at least 95% identical to SEQ ID NO: 19 and a second primer having a nucleotide sequence at least 85%, at least 90% or at least 95% identical to SEQ ID NO: 20; (iv) a first primer having a nucleotide sequence comprising or consisting of SEQ ID NO: 19 and a second primer having a nucleotide sequence comprising or consisting of SEQ ID NO: 20; (v) a first primer having a nucleotide sequence at least 85%, at least 90% or at least 95% identical to SEQ ID NO: 22 and a second primer having a nucleotide sequence at least 85%, at least 90% or at least 95% identical to SEQ ID NO: 23; or (iv) a first primer having a

nucleotide sequence comprising or consisting of SEQ ID NO: 22 and a second primer having a nucleotide sequence comprising or consisting of SEQ ID NO: 23.

In some embodiments, detecting the amplification product comprises hybridizing the amplification product to a probe. In some examples, the probe comprises a nucleotide sequence at least 85%, at least 90% or at least 95% identical to SEQ ID NO: 15, SEQ ID NO: 18 or SEQ ID NO: 21. In particular non-limiting examples, the probe comprises a nucleotide sequence comprising or consisting of SEQ ID NO: 15, SEQ ID NO: 18 or SEQ ID NO: 21.

In some embodiments, the probe comprises a fluorophore, a quencher or both. In one non-limiting example, the probe comprises a fluorophore and two quenchers.

Methods of detecting specific nucleic acid molecules in a sample using polymerase chain reaction (PCR) are well known in the art. In some embodiments, the PCR detection method is a real-time PCR method, such as TaqMan™ PCR. TaqMan™ PCR assays typically use self-quenching probes, and in some cases, include a fluorophore and two quenchers. In some instances when a probe contains two quenchers, a first quencher is placed at the 3' end of the probe and a second quencher is inserted into the oligonucleotide, such as by using a linker. The fluorophore is typically placed at the 5' end of the oligonucleotide probe. During the annealing phase of each PCR cycle, the primers and double-quenched probe both bind complementary sections of the DNA. During the elongation phase, polymerization of the new DNA strand is initiated from the primers. Once the polymerase reaches the bound probe, its 5' to 3' exonuclease activity degrades the probe, thereby physically separating the quencher from the fluorophore. As a result, fluorescence can be measured and will increase in real-time with the exponential increase in PCR product.

In some embodiments of the detection and diagnosis assays, the method further includes the step of obtaining a biological sample from a subject. In some examples, the sample is obtained directly from the subject and used in one of the above-described assays. In other examples, the sample is obtained indirectly

without directly removing the biological sample from the subject. In some cases where the sample is obtained indirectly, the sample is obtained from, for example, a clinician or laboratory personnel.

In some embodiments, the biological sample is a cell or tissue sample, such as a biopsy sample, bone marrow aspirate or isolated leukocytes. In other embodiments, the biological sample is a bodily fluid sample. In some examples, the bodily fluid sample comprises serum, blood, plasma, urine, feces, saliva or cerebral spinal fluid.

D. Recombinant *Phleboviruses*

Further provided herein are recombinant *Phleboviruses*. In some embodiments, the genome of the recombinant *Phlebovirus* comprises an S segment having a nucleotide sequence at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 1 or SEQ ID NO: 8; an M segment having a nucleotide sequence at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 2 or SEQ ID NO: 9; and an L segment having a nucleotide sequence at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 3 or SEQ ID NO: 10.

In some examples, the recombinant Phlebovirus comprises a deletion, such as a deletion of the NSs open reading frame (ORF). In particular examples, the deleted ORF is replaced with a reporter gene, such as a gene encoding a fluorescent protein or an antibiotic resistance gene.

In some examples, the recombinant *Phlebovirus* comprises at least one attenuating mutation. The attenuating mutation can be any insertion, deletion or substitution that results in a decrease in virus infectivity or virus-induced disease. The attenuating mutation can be in any of the gene segments and/or viral proteins. In some examples, the attenuating mutation results in an alteration or deletion of a virulence protein, such as NSs.

Recombinant *Phleboviruses* can be generated, for example, using a reverse genetics system. Reverse genetics systems for *Phleboviruses* are known in the art and are described in PCT Publication No. WO 2009/082647 and U.S. Patent No. 8,084,248. An exemplary reverse genetics system is also described below and in Example 3.

E. Reverse Genetics System

Provided herein is a reverse genetics system for producing recombinant *Phlebovirus*, comprising a first plasmid that contains an anti-genomic copy of an S segment, a second plasmid that contains an anti-genomic copy of an M segment and a third plasmid that contains an anti-genomic copy of an L segment, wherein each plasmid comprises a T7 promoter and a hepatitis delta virus ribozyme. In some embodiments, the nucleotide sequence of the first plasmid is at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 46 (pLCK-Mo4Svc), SEQ ID NO: 47 (pLCK-Mo4SvcDelNSs_GLuc) or SEQ ID NO: 51 (pLCK-Mo7Svc); or comprises or consists of the nucleotide sequence of SEQ ID NO: 46, SEQ ID NO: 47 or SEQ ID NO: 51. In some embodiments, the nucleotide sequence of the second plasmid is at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%identical to SEQ ID NO: 45 (pLCK-Mo4Mvc), SEQ ID NO: 48 (pLCK-Mo4M_EGFPBlastNeg) or SEQ ID NO: 50 (pLCK-Mo7Mvc), or comprises or consists of the nucleotide sequence of SEQ ID NO: 45, SEQ ID NO: 48 or SEQ ID NO: 50. In some embodiments, the nucleotide sequence of the third plasmid is at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%identical to SEQ ID NO: 44 (pLCK-Mo4Lv) or SEQ ID NO: 49 (pLCK-Mo7Lvc), or comprises or consists of the nucleotide sequence of SEQ ID NO: 44 or SEQ ID NO: 49.

Also provided are nucleic acid molecules comprising an anti-genomic copy of the L, M or S segment of *Phlebovirus* isolate #1 (Mo4) or *Phlebovirus* isolate #2 (Mo7), or a modified version thereof. In some embodiments, the nucleic acid molecule comprises a nucleotide sequence that is at least 80%, at least 85%, at least

90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to (i) nucleotides 107-6474 of SEQ ID NO: 44 (Mo4 anti-genomic L segment); (ii) nucleotides 107-3533 of SEQ ID NO: 45 (Mo4 anti-genomic M segment); (iii) nucleotides 107-1878 of SEQ ID NO: 46 (Mo4 anti-genomic S segment); (iv) nucleotides 107-1536 of SEQ ID NO: 47 (Mo4 anti-genomic S segment in which the NSs ORF is replaced by luciferase); (v) nucleotides 107-6474 of SEQ ID NO: 49 (Mo7 anti-genomic L segment); nucleotides 107-3533 of SEQ ID NO: 50 (Mo7 anti-genomic M segment); or nucleotides 107-1878 of SEQ ID NO: 51 (Mo7 anti-genomic S segment). In some examples, the nucleic acid molecule comprises a vector.

F. Mini-Genome Reporter System

Also provided herein is a *Phlebovirus* mini-genome reporter system, which can be used, for example, to detect viral replication, package pseudo-virus and to screen for antiviral compounds. In some embodiments, the mini-genome reporter system comprises three plasmids. The first plasmid comprises a reporter gene flanked by UTR sequences of a *Phlebovirus* S, M or L segment. The first plasmid need not contain the complete UTR sequences of the gene segment, but generally includes at least the first 15 nucleotides of 3' UTR and the last 15 nucleotides of 5' UTR sequence. The second plasmid encodes a *Phlebovirus* NP. For example, the second plasmid can contain an anti-genomic copy of the S segment, which encodes NP, or the second plasmid can be an expression vector encoding NP. The third plasmid encodes a *Phlebovirus* L protein. For example, the third plasmid can contain an anti-genomic copy of the L segment, which encodes the L protein, or the third plasmid can be an expression vector encoding the L protein.

In some examples, the first plasmid encodes an EGFP-blasticidin reporter flanked by M segment UTRs. In one non-limiting example, the first plasmid is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 48 (pLCK-Mo4M_EGFPBlastNeg); or comprises or consists of SEQ ID NO: 48.

In some examples, the second plasmid comprises a nucleotide sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to nucleotides 107-1878 of SEQ ID NO: 46 (Mo4 anti-genomic S segment); or comprises nucleotides 107-1878 of SEQ ID NO: 46. In other examples, the second plasmid comprises a nucleotide sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to nucleotides 107-1878 of SEQ ID NO: 51 (Mo7 anti-genomic S segment); or comprises nucleotides 107-1878 of SEQ ID NO: 51. In further examples, the second plasmid is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 46 or SEQ ID NO: 51; or comprises or consists of SEQ ID NO: 46 or SEQ ID NO: 51. In yet other examples, the second plasmid is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 42 (pcMo4NP); or comprises or consists of SEQ ID NO: 42.

In some examples, the third plasmid comprises a nucleotide sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to nucleotides 107-6474 of SEQ ID NO: 44 (Mo4 anti-genomic L segment); or comprises nucleotides 107-6474 of SEQ ID NO: 44. In other examples, the third plasmid comprises a nucleotide sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to nucleotides 107-6474 of SEQ ID NO: 49 (Mo7 anti-genomic L segment); or comprises nucleotides 107-6474 of SEQ ID NO: 49. In further examples, the second plasmid is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 44 or SEQ ID NO: 49; or comprises or consists of SEQ ID NO: 44 or SEQ ID NO: 49. In yet other examples, the third plasmid is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 41 (pcMo4L); or comprises or consists of SEQ ID NO: 41.

In some embodiments, the mini-genome reporter system further comprises a fourth plasmid encoding the *Phlebovirus* glycoproteins. In some examples, the fourth plasmid is an expression vector encoding GnGc. Thus, in particular examples, the fourth plasmid comprises a nucleotide sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 40; or comprises of consists of SEQ ID NO: 40. In other examples, the fourth plasmid is an expression vector encoding the complete M ORF. In particular examples, the fourth plasmid comprises a nucleotide sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 43; or comprises of consists of SEQ ID NO: 43.

Further provided is a method of producing *Phlebovirus* pseudo-virus, comprising transfecting the first, second, third and fourth plasmids into cells expressing T7 polymerase.

G. Immunogenic Compositions and Use Thereof

The *Phlebovirus* polypeptides and recombinant viruses described herein can be used in immunogenic compositions to elicit an immune response, such as to provide protection against infection by a *Phlebovirus*.

Provided herein are immunogenic compositions comprising a *Phlebovirus* polypeptide as described herein, or an immunogenic fragment thereof, and a pharmaceutically acceptable carrier. The immunogenic composition can further include an adjuvant. In some examples, the immunogenic composition comprises a *Phlebovirus* NSs, NP, GP or L protein. In particular examples, the amino acid sequence of the *Phlebovirus* polypeptide is at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 4, 5, 6, 7, 11, 12, 13 or 14. In other particular examples, the amino acid sequence of the *Phlebovirus* polypeptide comprises or consists of SEQ ID NO: 4, 5, 6, 7, 11, 12, 13 or 14. In yet other examples, the polypeptide comprises an immunogenic fragment of SEQ ID NO: 4, 5, 6, 7, 11, 12, 13 or 14.

Also provided are immunogenic compositions comprising a recombinant *Phlebovirus* as described herein and a pharmaceutically acceptable carrier. The immunogenic composition optionally includes an adjuvant. In some embodiments, the recombinant *Phlebovirus* comprises an S segment having a nucleotide sequence at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 1 or SEQ ID NO: 8; an M segment having a nucleotide sequence at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 2 or SEQ ID NO: 9; and an L segment having a nucleotide sequence at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 3 or SEQ ID NO: 10.

Further provided is a method of eliciting an immune response against *Phlebovirus* in a subject by administering to the subject a therapeutically effective amount of a recombinant *Phlebovirus*, a *Phlebovirus* polypeptide, or an immunogenic composition as disclosed herein. In some embodiments, the subject is administered the recombinant *Phlebovirus*, *Phlebovirus* polypeptide, or immunogenic composition prophylactically to prevent infection by a Phlebovirus. In other cases, the recombinant *Phlebovirus*, *Phlebovirus* polypeptide, or immunogenic composition is administered as a treatment for *Phlebovirus* infection.

Also provided is a method of immunizing a subject against *Phlebovirus* infection by administering to the subject a therapeutically effective amount of a recombinant *Phlebovirus*, a *Phlebovirus* polypeptide, or an immunogenic composition as disclosed herein.

IV. Methods of Producing *Phlebovirus*-Specific Antibodies

Provided by the present disclosure are antibodies or antibody fragments that specifically bind the *Phlebovirus* isolates and/or *Phlebovirus* polypeptides disclosed herein. The *Phlebovirus*-specific antibodies can be used, for example, in assays for the detection of a *Phlebovirus* or *Phlebovirus* polypeptide in a biological sample. Exemplary immunodetection assays include the enzyme-linked immunosorbent

assay (ELISA), Western blot, radioimmunoassay (RIA) and immunohistochemistry (IHC) assay. Methods of performing such assays are well known in the art and are briefly described above.

In some embodiments, provided are polyclonal antibodies specific for a *Phlebovirus* or *Phlebovirus* polypeptide. In other embodiments, provided are monoclonal antibodies specific for a *Phlebovirus* or *Phlebovirus* polypeptide. In some examples, the polyclonal or monoclonal antibody (or antigen-binding fragment thereof) recognizes a *Phlebovirus* particle, such as a particle of one of the *Phlebovirus* isolates disclosed herein. In other examples, the polyclonal or monoclonal antibody (or antigen-binding fragment thereof) specifically binds a *Phlebovirus* polypeptide disclosed herein.

Methods of making polyclonal and monoclonal antibodies are well known, and are described below. Polyclonal antibodies, antibodies which consist essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody preparations, are contemplated. The preparation of polyclonal antibodies is well known to those skilled in the art (see, for example, Green *et al.*, "Production of Polyclonal Antisera," in: *Immunochemical Protocols*, pages 1-5, Manson, ed., Humana Press, 1992; Coligan *et al.*, "Production of Polyclonal Antisera in Rabbits, Rats, Mice and Hamsters," in: *Current Protocols in Immunology*, section 2.4.1, 1992). For example, an antigen (such as a *Phlebovirus* polypeptide or fragment thereof) is administered to a host animal such as, but not limited to, a rabbit, mouse or rat, to induce the production of antisera containing polyclonal antibodies specific for the antigen. Various adjuvants can be used to increase the immunological response, depending on the host species. Exemplary adjuvants include Freund's (complete and incomplete) adjuvant, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins and dinitrophenol. Such adjuvants are well known in the art.

The preparation of monoclonal antibodies likewise is conventional (see, for example, Kohler & Milstein, *Nature* 256:495, 1975; Coligan *et al.*, sections 2.5.1-

2.6.7; and Harlow *et al.* in: *Antibodies: a Laboratory Manual*, page 726, Cold Spring Harbor Pub., 1988). Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an antigen, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B lymphocytes, fusing the B lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures. Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography (see, *e.g.*, Coligan *et al.*, sections 2.7.1-2.7.12 and sections 2.9.1-2.9.3; Barnes *et al.*, Purification of Immunoglobulin G (IgG), in: *Methods in Molecular Biology*, Vol. 10, pages 79-104, Humana Press, 1992).

Methods of *in vitro* and *in vivo* multiplication of monoclonal antibodies are well known to those skilled in the art. Multiplication *in vitro* may be carried out in suitable culture media such as Dulbecco's Modified Eagle Medium or RPMI 1640 medium, optionally supplemented by a mammalian serum such as fetal calf serum or trace elements and growth-sustaining supplements such as normal mouse peritoneal exudate cells, spleen cells, thymocytes or bone marrow macrophages. Production *in vitro* provides relatively pure antibody preparations and allows scale-up to yield large amounts of the desired antibodies. Large-scale hybridoma cultivation can be carried out by homogenous suspension culture in an airlift reactor, in a continuous stirrer reactor, or in immobilized or entrapped cell culture. Multiplication *in vivo* may be carried out by injecting cell clones into mammals histocompatible with the parent cells, such as syngeneic mice, to cause growth of antibody-producing tumors. Optionally, the animals are primed with a hydrocarbon, especially oils such as pristane (tetramethylpentadecane) prior to injection. After one to three weeks, the desired monoclonal antibody is recovered from the body fluid of the animal.

Antibodies can also be derived from a subhuman primate antibody. General techniques for raising therapeutically useful antibodies in baboons can be found, for

example, in PCT Publication No. WO 91/11465; and Losman *et al.*, *Int. J. Cancer* 46:310, 1990.

Alternatively, an antibody that specifically binds a *Phlebovirus* polypeptide can be derived from a humanized monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementarity determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain, and then substituting human residues in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, by Orlandi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 86:3833, 1989. Techniques for producing humanized monoclonal antibodies are described, for example, by Jones *et al.*, *Nature* 321:522, 1986; Riechmann *et al.*, *Nature* 332:323, 1988; Verhoeyen *et al.*, *Science* 239:1534, 1988; Carter *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 89:4285, 1992; Sandhu, *Crit. Rev. Biotech.* 12:437, 1992; and Singer *et al.*, *J. Immunol.* 150:2844, 1993.

Antibodies can also be derived from human antibody fragments isolated from a combinatorial immunoglobulin library. See, for example, Barbas *et al.*, in: *Methods: a Companion to Methods in Enzymology*, Vol. 2, page 119, 1991; Winter *et al.*, *Ann. Rev. Immunol.* 12:433, 1994. Cloning and expression vectors that are useful for producing a human immunoglobulin phage library can be obtained, for example, from Stratagene Cloning Systems (La Jolla, CA).

In addition, antibodies can be derived from a human monoclonal antibody. Such antibodies are obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain loci are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human

antibodies from transgenic mice are described by Green *et al.*, *Nature Genet.* 7:13, 1994; Lonberg *et al.*, *Nature* 368:856, 1994; and Taylor *et al.*, *Int. Immunol.* 6:579, 1994.

Antibodies include intact molecules as well as fragments thereof, such as Fab, $F(ab')_2$, and Fv which are capable of binding the epitopic determinant. These antibody fragments retain some ability to selectively bind with their antigen and are defined as follows:

(1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain;

(2) Fab', the fragment of an antibody molecule can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule;

(3) $(Fab')_2$, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; $F(ab')_2$ is a dimer of two Fab' fragments held together by two disulfide bonds;

(4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and

(5) Single chain antibody, defined as a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

Methods of making these fragments are known in the art (see for example, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988). An epitope is any antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or

sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

Antibody fragments can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted $F(ab')_2$. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly (see U.S. Patents No. 4,036,945 and U.S. Patent No. 4,331,647, and references contained therein; Nisonhoff *et al.*, *Arch. Biochem. Biophys.* 89:230, 1960; Porter, *Biochem. J.* 73:119, 1959; Edelman *et al.*, *Methods in Enzymology*, Vol. 1, page 422, Academic Press, 1967; and Coligan *et al.* at sections 2.8.1-2.8.10 and 2.10.1-2.10.4).

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

For example, Fv fragments comprise an association of V_H and V_L chains. This association may be noncovalent (Inbar *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 69:2659, 1972). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde (see, for example, Sandhu, *Crit. Rev. Biotech.* 12:437, 1992). Preferably, the Fv fragments comprise V_H and V_L chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells

synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are known in the art (see Whitlow *et al.*, *Methods: a Companion to Methods in Enzymology*, Vol. 2, page 97, 1991; Bird *et al.*, *Science* 242:423, 1988; U.S. Patent No. 4,946,778; Pack *et al.*, *Bio/Technology* 11:1271, 1993; and Sandhu, *Crit. Rev. Biotech.* 12:437, 1992).

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells (Larrick *et al.*, *Methods: a Companion to Methods in Enzymology*, Vol. 2, page 106, 1991).

Antibodies can be prepared using an intact polypeptide or fragments containing small peptides of interest as the immunizing antigen. The polypeptide or a peptide used to immunize an animal can be derived from substantially purified polypeptide produced in host cells, *in vitro* translated cDNA, or chemical synthesis which can be conjugated to a carrier protein, if desired. Such commonly used carriers which are chemically coupled to the peptide include keyhole limpet hemocyanin, thyroglobulin, bovine serum albumin, and tetanus toxoid. The coupled peptide is then used to immunize the animal (*e.g.*, a mouse, a rat, or a rabbit).

Monoclonal antibodies can also be prepared by well-known recombinant methods or using phage display. In some embodiments, monoclonal antibodies are generated using any phage display method known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. In some cases, the phage displays antigen binding fragments, such as Fab and Fv or disulfide-bond stabilized Fv, expressed from a repertoire or combinatorial antibody library (*e.g.*, human or murine). Phage expressing an antigen binding fragment is generally selected using labeled antigen or antigen bound or captured to a solid surface or bead. Phages used in these methods are typically filamentous phage, such as M13

phage. Generally, the antigen binding fragments are expressed as a recombinantly fused protein to either the phage gene III or gene VIII protein. Exemplary phage display methods are described in Brinkman *et al.* (*J Immunol Methods* 182:41-50, 1995), Ames *et al.* (*J Immunol Methods* 184:177-186, 1995), Kettleborough *et al.* (*Eur J Immunol* 24:952-958, 1994), Persic *et al.* (*Gene* 187:9-18, 1997), Burton *et al.* (*Advances in Immunology*, 57:191-280, 1994), and in PCT Publication Nos. WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and in U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108.

After phage selection, the antibody coding regions from the phage are isolated and can be used to generate whole antibodies, including human antibodies, or any other desired fragments, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria.

Polyclonal or monoclonal antibodies can be further purified, for example, by binding to and elution from a matrix to which the polypeptide or a peptide to which the antibodies were raised is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies (see, for example, Coligan *et al.*, Unit 9, *Current Protocols in Immunology*, Wiley Interscience, 1991).

The following examples are provided to illustrate certain particular features and/or embodiments. These examples should not be construed to limit the disclosure to the particular features or embodiments described.

EXAMPLES

Example 1: Patient Reports and Methods

This example describes the patients from which a novel *Phlebovirus* was isolated and the methods used for isolation and identification of the virus.

Patient 1

Patient 1 is a healthy 57-year-old male who lives on a 70-acre farm in northwestern Missouri with horses and cats. In early June 2009, he noticed a small nymphal tick embedded on his abdomen, which his wife removed with tweezers. He had not applied tick repellant, nor did he know how long the tick had been embedded. There was no rash or localized itching. The following day, he noticed a fever and his symptoms subsequently progressed to severe fatigue, headache, anorexia, nausea, and non-bloody diarrhea. Four days later, he was admitted to hospital. He denied visual changes or symptoms of neuropathy, cough, shortness of breath, lymph node enlargement, vomiting or abdominal pain. His temperature was 37.9°C and peaked at 38.2°C. The next day, his temperature reached 39.1°C. Initial laboratory results showed a low white blood cell count of 1900 cells/μL (normal 4500-11000 cells/μL), low platelet count of 115 x 10^3 cells/μL (normal 150-450 x 10^3cells/μL), and low sodium 132 mEq/L (normal 136-146 mEq/L) (FIG. 4). Serum levels of liver transaminases were slightly elevated with an ALT of 57 U/L (normal 10-40 U/L) and AST of 44 (normal 10-30 U/L). A serum C reactive protein of 2.9 mg/dL (normal < 0.9 mg/dL) was found.

The patient was hospitalized for 10 days. Moderate thrombocytopenia progressed to severe with a nadir of 37 x 10^3 cells/μL at day 5 and 40 x 10^3 cells/μL at days 6 and 7. Leukopenia continued throughout hospitalization with notable lymphopenia and mild neutropenia that progressed to moderate neutropenia at day 7 (FIG. 1A). Band forms were detected at day 2 and day 8. An erythrocyte sedimentation rate of 9 mm/hr (normal, 0-15 mm/hr), erythrocyte count, and hemoglobin were unremarkable and stable. Hematocrit was slightly low during hospitalization (FIG. 1D and FIG. 4). Prothrombin time and partial thromboplastin time were normal (FIG. 4).

Serum hepatic transaminases increased during hospitalization and peaked with an ALT of 315 U/L and AST of 431 U/L at day 8 (FIG. 1C). Alkaline phosphatase levels rose within normal limits and peaked at 101 U/L (normal 25-100

U/L) at day 9. Creatine and blood urea nitrogen levels remained normal. A urinalysis demonstrated trace protein, 1+ ketones and was otherwise normal. Serum albumin levels were low as well as mildly low serum sodium and calcium levels (FIG. 4).

Tests specific for influenza A and B and *Borrelia* were negative. EDTA treated blood was sent to CDC for testing on the second day of hospitalization and subsequently shown to be negative for *Ehrlichia chaffeensis*, *Ehrlihia ewingii*, and spotted fever group rickettsiae by PCR. Serology confirmed negative results for spotted fever group and typhus in an IgM and IgG assay.

On the second day of hospitalization, the patient was empirically placed on doxycycline (100 mg) by vein twice daily for a total of 14 days for suspected ehrlichiosis. Non-bloody diarrhea persisted through the fourth day of hospitalization. Stool specimens were negative for leukocytes, *Salmonella*, *Shigella*, and *Campylobacter* and *Clostridium difficile* toxins. A 2-D echocardiogram and chest x-ray were unremarkable.

Since hospital discharge, the patient has experienced fatigue and generalized headaches on an almost daily basis, which have persisted for over 2 years. Additionally, he had short-term memory difficulty, which slowly improved, and anorexia that resolved after the initial 4-6 weeks following discharge.

The *Phlebovirus* isolated from Patient 1 is referred to herein as *Phlebovirus* isolate #2 or "Mo7."

Patient 2

Patient 2 is a 67-year-old male with a 5 year history of type 2 diabetes, but otherwise healthy. He lives on an approximately 100-acre farm in northwestern Missouri with numerous horses, dogs, and cats. In the spring of 2009, he received an average of 15-20 tick bites daily for approximately 2 weeks while building a fence on his property. He had not used tick repellant. He removed the embedded ticks with his fingers and tweezers. The last tick bite occurred 1 week before hospitalization. Approximately 4-5 days before hospitalization he developed

subjective fever, fatigue, and anorexia. Additional symptoms included myalgias, dry cough, and non-bloody diarrhea. No rash was noted before or during hospitalization.

On hospital entry in June 2009, his temperature was 37.1°C and reached a maximum of 38.1°C that day. The following day his temperature reached 39.1°C. Laboratory studies conducted on admission were notable for a low white blood count of 2100 cells/μL, a low platelet count of 78 x 10^3 cells/μL, and an elevated AST of 54 U/L (FIG. 1B-1D). The serum sodium was slightly low at 130 mEq/L and calcium low at 7.8 mEq/L. A urinalysis was unremarkable.

The patient was hospitalized for 12 days. After day 2, moderate thrombocytopenia progressed to severe with a nadir of 34 x 10^3cells/μL at days 5 and 6. Platelet numbers began to increase at day 8 and reached normal by day 11 (FIG. 1D). He tested negative for anti-platelet antibodies. Leukopenia continued until day 10 with mild neutropenia progressing to moderate neutropenia at day 6 to day 8 (FIG. 1B). Band forms were present at days 2 to 7 and lymphocytes gradually increased into a normal range by day 8 (FIG. 1B). Erythrocyte counts and hemoglobin were within normal limits, and the hematocrit was slightly low throughout hospitalization (FIG. 4). Prothrombin time, partial thromboplastin time and fibrinogen concentration were normal, but serum D-dimer elevated at 4.08 mg/L (FIG. 4).

Blood was collected on day 2 of hospitalization and sent to CDC. PCR results were found to be negative for *Ehrlichia chaffeensis* and a wide range of *Ehrichia* and *Anaplasma* species.

Aspartate and alanine aminotransferase levels were elevated and increased to 355 U/L at day 8 and 302 U/L at day 10, respectfully (FIG. 1C). Alkaline phosphatase was temporally high at day 10, but then resumed normal levels (FIG. 4). Creatinine and blood urea nitrogen levels remained normal. Serum albumin and sodium remained low throughout hospitalization. Low serum calcium concentrations increased to normal by day 10.

A bone marrow aspiration and biopsy was performed on day 2 of hospitalization. The peripheral smear found red cells to be normochromic and normocytic with the presence of ovalocytes along with acanthocytes and dacrocytes. Platelets showed normal morphology with no clumping or satellitosis. The bone marrow aspirate contained few cells. Red cells were normoblastic with frequent dysplastic forms of abnormal nuclear lobation and dyssynchrony between nuclear and cytoplasmic maturation and intracytoplasmic nuclear fragments. Granulocytogenesis was progressive and showed complete maturation. Blastocytes were less than 1% of the bone marrow population. Megakarocytes were present with micromegakarocytes and hypolobated forms noted. Plasma cells were 3-4% of the bone marrow cellularity. The myeloid to erythroid ratio of 3:1 was normal. Ringed sideroblasts were not seen. Flow cytometry demonstrated a 3% monoclonal plasma cell population with lambda light chain restriction. An evolving myelodysplastic syndrome could not be excluded. No infiltrates of infectious or neoplastic nature were noted.

Tests specific for Lyme disease were negative as well as cultures for fungus and acid-fast bacilli were negative. A chest radiograph and abdominal ultrasound were unremarkable. He was initially treated empirically with intravenous piperacillin/tazobactam, switched to ceftriaxone on hospital day 2, and to oral doxycycline (100 mg) twice daily on day 3 for suspected ehrlichiosis. He completed a course of doxycycline 100 mg twice daily for a total of 14 days.

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

The *Phlebovirus* isolated from Patient 2 is referred to herein as *Phlebovirus* isolate #1 or "Mo4."

Virus isolation

Leukocytes were separated from EDTA-treated blood in a Ficoll-histopaque gradient. The separated leukocytes were inoculated onto the canine monocyte cell line, DH82, cultivated in Eagle's MEM with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 10 mM HEPES and 10% fetal bovine serum (Childs *et al.*, *J Clin Microbiol* 37:2997-3000, 1999). Adherent and non-adherent cells were placed on glass slides by cytocentrifugation and examined using a modified rapid Wright-Giemsa stain (Diff-Quik). When cytologic changes were noted in the cells, media from the DH82 cell culture was collected, filtered to remove host cells, and inoculated onto VeroE6 cells grown in the same medium. When cytologic changes were noted in the recipient cells, infected VeroE6 cells were fixed in glutaraldehyde and processed into ultrathin sections and negative stained for transmission electron microscopy to visualize virus particles.

Metagenomic analysis to identify virus

Total RNA was isolated from infected cell culture media by TriPure™ extraction and subsequent RNA purification using RNAeasy™ columns followed by DNaseI treatment on the column (Qiagen). RNA was denatured at 75°C for 5 min and stored on ice. A random cDNA library was generated in a reverse transcription reaction using SuperScript III (Invitrogen) and random hexamer primers linked to an arbitrary 17-mer anchor sequence (GTTTCCCAGTAGGTCTCNNNNNNNN; SEQ ID NO: 52) at 42°C for 50 min and 70°C for 15 min (McMullan *et al.*, *Virology* 422:1-5, 2012). Residual RNA was removed by RNase H treatment at 37°C for 20 min and subsequent inactivation at 95°C for 5 min. The cDNA was randomly amplified by PCR using a primer specific for the 17-mer anchor sequence and the random hexamer primer with the anchor sequence and an extension of CGCC at the 5' end (CGCCGTTTCCCAGTAGGTCTC; SEQ ID NO: 53) in a 9:1 ratio with 8 cycles annealing at 25°C and 49 cycles annealing at 55°C. Products smaller than 70 nucleotides were removed using a MinElute™ column (Qiagen). Adapters for next

generation sequencing were ligated without fragmentation and sequencing was performed on a 454 FLX Genome Sequencer (Roche). Sequence reads were trimmed of adapter and primer sequences and searched using the BLASTx algorithm (CLC Genomics, NCBI). Sequences showing significant similarity were parsed using MEGAN (Huson *et al.*, *Genome Res* 17:377-386, 2007). Gaps in the virus genome were filled in using PCR and traditional Sanger sequencing. The 3' and 5' end of the virus were amplified in a 3' RACE reaction and sequenced. *Phlebovirus* sequences of each virus protein were aligned using MAFFT and phylogenic relationships inferred using the UPGMA method with 2,000 bootstrap replicates for statistical support (CLC Genomics, Geneious). Heartland virus (HRTLV) was proposed as the name for the newly discovered virus.

Analysis of biopsy specimens

Sections cut from formalin-fixed paraffin embedded bone marrow biopsy and clot specimens obtained from Patient 2 on day 2 of hospitalization were analyzed for the presence of Heartland virus by quantitative reverse transcription PCR (qRT-PCR) and immunohistochemistry. RNA was extracted from tissue sections and used as template in qRT-PCR assays, using primers and probes specific for the S, M, and L virus segments and human GAPDH, in a one-step reaction at 50°C for 30 min, 94°C 2 min, and 40 cycles of 94°C and 60°C 1 min (UltraSense, Invitrogen). The following probes and primers were used:

Sprobe: 6-fam/TCCCTCTTC/zen/TTCCCAAATGCCACC/IABKFQ (SEQ ID NO: 15)
S1: GATGCTTCCTTGGTTGCTG (SEQ ID NO: 16)
S2: TGCCAAATTCAGAGACCCTC (SEQ ID NO: 17)
Mprobe: 6-fam/ CAGGATGGC/zen/GCTGCATTAAAACACC/IABkFQ (SEQ ID NO: 18)
M1: ACACGATTGGATGGGTTCTC (SEQ ID NO: 19)
M2: TGGGCAAACAGGATGGAC (SEQ ID NO: 20)

Lprobe: 6-fam/ACCCCTGGA/zen/ATTGATAACACCGCC/IABkFQ (SEQ ID NO: 21)

L1: AACCCCTGCTTTCTGAGTG (SEQ ID NO: 22)

L2: ATTGGACTCATGGGCTTGG (SEQ ID NO: 23)

(6-fam = 6-carboxyfluorescein; zen = quencher; IABkFQ = Iowa Black™ FQ)

Ct values were related to known quantity of RNA standard generated from a T7 transcript of the S segment. The Heartland qRT-PCR assay for the S segment has a sensitivity of detection of approximately 2 molecules of *in vitro* transcribed RNA and an efficiency of 95.6% (slope -3.44).

An immunoalkaline phosphatase technique was performed using a 1:1000 dilution of hyperimmune rabbit serum reactive with Heartland virus nucleoprotein.

Example 2: Discovery of a new *Phlebovirus* associated with severe febrile illness following tick bite in two patients in Missouri

This example describes the isolation and identification of a novel *Phlebovirus* from patients reporting a recent tick bite.

Isolation of the virus

Initially, cell cultures showed cytopathic effects similar to those seen in early cultures of *Ehrlichia chaffeensis*. However, cellular vacuoles did not contain the typical bacterial microcolonies, known as morulae. Passage of the culture supernatant onto fresh DH82 cells yielded similar cytopathic effects in 9-11 days. Cytopathic changes were less evident, but also noted in the inoculated VeroE6 cell culture 9 days post infection. Thin section electron microscopy revealed enveloped particles averaging 86 nm in size, typical of a virus in the family *Bunyaviridae* (FIG. 2).

Genetic analysis of Heartland virus

To identify the virus, RNA from infected cell culture media was nonspecifically amplified and the cDNA products were sequenced using 454 next generation sequencing (NGS). Next, the non-redundant protein database was searched against all NGS reads translated in all 6 reading frames using the BLASTx algorithm and sequences significantly similar to those of viruses in the family *Bunyaviridae*, genus *Phlebovirus* were found. Contiguous sequences were assembled from the NGS reads and additional Sanger sequencing performed to elucidate the complete genomic sequence for the virus isolates from both patients. The two virus genomes were closely related with 98%, 95%, and 99% identity for the S, M and L segment respectfully at the nucleic acid level. The high genetic identity indicates that both patients were infected with the Heartland virus, but the numerous differences imply that the two patients were infected independently.

There are over 70 members of the *Phlebovirus* family that share a similar genome organization with a negative-sense tripartite coding strategy (Nichol *et al.*, Family Bunyaviradae. In: Fauquet *et al.* ed. Virus Taxomony: classification and nomenclature of viruses. San Diego, CA: Elsevier Academic Press; 2005:695-716). The L segment is 6,368 nucleotides in length and encodes a large RNA-dependent RNA polymerase with 3 regions and 6 motifs responsible for the process of transcription and replication; these regions are conserved among all phleboviruses and are present in Heartland virus. The M segment is 3427 nucleotides in length and encodes a polyprotein processed by host cell proteases into the virus glycoproteins Gn and Gc which are used for virion entry and assembly. There are 4 predicted N-linked glycosylation sites. The S segment is 1772 nucleotides and encodes the nucleoprotein that encapsidates the genomic RNA and a nonstructural NSs protein in an ambisense coding strategy. Each of the virus genome segments is potentially circularized by complementary base pairing of the genomic termini to form a panhandle structure.

Phylogenetic analysis

Phylogenetic analysis indicates Heartland virus is a distinct member of the genus *Phlebovirus*. Comparison of the aligned amino acid sequence of the polymerase, glycoproteins, nucleoprotein, and NSs protein all show Heartland virus to be distantly related to representative phlebovirus members of the Sicilian and Naples Sandfly, Joa, Salobo, Chandiru, Punta Toro, Rift Valley Fever, and Uukuniemi complexes (FIG. 3). The Heartland virus is most closely related to the Severe Fever with Thrombocytopenia Syndrome Virus (SFTSV), recently identified in central and northeastern China (Yu *et al.*, *N Engl J Med* 364:1523-1532, 2011; Xu *et al.*, *PLoS Pathog* 7:e1002369, 2011). This relationship is distant however, as pair-wise comparisons of the viral polymerase and nucleoprotein, the two most conserved virus proteins, show amino acid differences of 27% and 38.4%, respectively. The phlebovirus complexes are separated by at least 35% protein differences emphasizing the remote relationship between Heartland virus and SFTSV. An alignment of NP from several different Phleboviruses is shown in FIG. 5.

The genus *Phlebovirus* is divided into the sand fly- and mosquito-borne virus groups and the nonpathogenic tick-borne Uukuniemi (UUK) virus. Both Heartland virus and SFTSV exhibit a relatively close relationship with the tick- borne UUK for all virus proteins. Heartland virus was also distinct from an uncharacterized *Bunyavirus* isolated from a nymphal *Amblyomma americanum* tick embedded on a woodchuck in 1967 in western Kentucky, named the Lone Star virus (Kokernot *et al.*, *Am J Trop Med Hyg* 18:789-795, 1969). Comparison of the polymerase amino acid sequence shows Lone Star virus to share only 34% identity with Heartland virus, while SFTSV and Heartland virus share 73% identity. Heartland virus is the only pathogenic phlebovirus isolated from humans in the Americas.

Detection of virus RNA and antigen in patient bone marrow specimen

Heartland virus RNA was detected in sections of bone marrow biopsy and clot. Quantifying the copies of the housekeeping beta-2 microglobulin mRNA

indicate equivalent amounts of total RNA were compared. Immunohistochemical staining revealed the presence of virus nucleocapsid protein in hematopoietic cells of the bone marrow. In particular, megakarocytes were positive for virus antigen.

Discussion

Described herein is a new, potentially tick-borne, pathogenic virus in the United States. Heartland virus is a distinct member of the genus *Phlebovirus* and is most closely related to tick-borne phleboviruses, notably the recently isolated SFTSV. While clinical data based on two patients may not reflect the entire spectrum of illness associated with Heartland virus, both illnesses had a very similar clinical course. For instance, symptoms of infection for these two patients included fever, fatigue, anorexia, diarrhea, leukopenia, thrombocytopenia, and elevated hepatic transaminase levels. Both patients were viremic at day 2 of hospitalization, approximately 7 days after onset of symptoms. The temporal trends in white blood and platelet cell counts, and AST and ALT levels were strikingly similar in both patients. Both patients presented with neutropenia that continued to decline to levels below 700 at days 6 and 7 of hospitalization. Thrombocytopenia also continued until day 7 for both patients. Slightly elevated AST and ALT levels spiked at day 7 and day 8. After this time, there was an increase in circulating neutrophils, lymphocytes, monocytes, and platelets. Hepatic transaminase levels begin to decline. 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 SFTS. However, renal abnormalities are common for SFTS with 84% of patients having proteinuria and 59% with hematuria, in contrast to the patients in this report who showed normal creatine and blood urea nitrogen levels (Yu *et al.*, *N Engl J Med* 364:1523-1532, 2011). Coagulation abnormalities were also not observed despite a marked low platelet count whereas a minority of SFTS cases had elongated partial thromboplastin time, thrombin time, elevated fibrinogen levels and symptoms of gingival bleeding and hemorrhage with fatalities of disseminated

intravascular coagulation and cerebral hemorrhage (Gai *et al.*, *Clin Infect Dis* 54:249-252, 2012; Zhang *et al.*, *Clin Infect Dis* 54(4):527-533, 2011). There have been numerous reports of person-to-person transmission upon exposure to SFTSV infected blood and SFTSV has been detected in patient blood, throat swabs, urine and feces (Gai *et al.*, *Clin Infect Dis* 54:249-252, 2012; Zhang *et al.*, *Clin Infect Dis* 54(4):527-533, 2011; Bao *et al.*, *Clin Infect Dis* 53:1208-1214, 2011; Liu Y *et al.*, *Vector Borne Zoonotic Dis* 12(2):156-160, 2011). It remains to be determined if Heartland virus can be transmitted person-to–person; no family members of either patient reported symptoms resembling Heartland virus infection. It will be important to determine how patients acquire Heartland virus infection in order to promote risk reduction practices.

It should be noted that the clinical signs and symptoms that were observed, as well as epidemiologic exposures (tick bites), are similar to those of ehrlichiosis (Childs *et al.*, *J Clin Microbiol* 37:2997-3000, 1999). Heartland virus should be considered as a possible etiologic agent in these instances, particularly when a suspected ehrlichiosis does not improve within a few days with antibiotic treatment.

Although the virus has not been isolated from the tick as the tick specimens from the patients were not available, the presumed vector for Heartland virus is the lone star tick, *Amblyomma americanum*. Recent ecological studies of ticks captured in central and southern Missouri found 99.9% of the captured ticks to be *A. americanum* (Brown *et al.*, *Clin Infect Dis* 33:1586-1594, 2001). In northwestern Missouri, *A. americanum* is abundant and multiple isolates of *Ehrlichia chaffeensis* have originated from the regional hospital (Roche *et al.*, First culture isolations of Ehrlichia chaffeensis from a Missouri patient. In: 22nd Annual Meeting of the Society for Vector Ecology. Ft. Collins, CO, 2008; Paddock *et al.*, *Clin Infect Dis* 33:1586-1594, 2001). *A. americanum* is found throughout the southeastern and south central United States, extending up the Atlantic coast reaching all the way to Maine (Childs and Paddock, *Annu Rev Entomol* 48:307-337, 2003). Both the patients resided in fragmented deciduous forest and old fields, suitable habitats for *A. americanum*.

Heartland virus is the first pathogenic phlebovirus identified in the Americas. UUK virus, the prototypical tick-borne phlebovirus is considered nonpathogenic to humans. Members of the sand fly phlebovirus complex found in Asia, Africa, and around the Mediterranean Basin, commonly result in a self-limiting "3 day fever" with the exception of Toscana virus, which can causes aseptic meningitis (Depaquit *et al.*, *Euro Surveill* 15:19507, 2010). The mosquito-borne Rift Valley fever virus can cause large-scale epizootics; human infection is a self-limiting febrile illness that may progress to hepatitis, encephalitis, or hemorrhagic fever (Bird *et al.*, *J Am Vet Med Assoc* 234:883-893, 2009). The recent identification of the SFTSV from northeastern China, Catch-me-Cave virus from the subantarctic island of Macquarie, and the Finnish Uukuniemi virus demonstrates the extensive geographic distribution of viruses in this genus.

Example 3: Plasmids for Reverse Genetics and Mini-Genome Reporters

This example describes plasmids that were generated for a reverse genetics system, such as for the production of recombinant *Phlebovirus*, and for the development of mini-genome reporters, which can be used to screen for antiviral compounds and/or drug resistant virus mutants.

Description of plasmids

Plasmids for launching the reverse genetics system use the pSMART-LCKan backbone (Lucigen, Middleton, WI) altered to include (in the 5' to 3' direction) a T7 promoter, a single G nucleotide, a *Phlebovirus* S, M or L segment in the positive orientation (virus complementary sense or anti-genomic), hepatitis delta virus ribozyme and a T7 terminator. Plasmids for expression of viral proteins (NP, L, GnGc and the complete M ORF) use the pCAGGS vector. Table 1 below summarizes the plasmids that can be used for reverse genetics and mini-genome reporters.

Table 1: Description of Plasmids

Plasmid Name	Description	SEQ ID NO:
pcMo4GnGc	pCAGGS vector expressing GnGc	40
pcMo4L	pCAGGS vector expressing L protein	41
pcMo4NP	pCAGGS vector expressing NP	42
pcMo4Morf	pCAGGS vector expressing complete M ORF[1]	43
pLCK-Mo4Lvc	Mo4 L segment in the positive orientation	44
pLCK-Mo4Mvc	Mo4 M segment in the positive orientation	45
pLCK-Mo4Svc	Mo4 S segment in the positive orientation	46
pLCK-Mo4SvcDelNSs_GLuc	Mo4 S segment in the positive orientation with the NSs ORF replaced by Gaussia luciferase	47
pLCK-Mo4M_EGFPBlastNeg	Mo4 M segment UTRs flanking an EGFP-Blasticidin reporter	48
pLCK-Mo7Lvc	Mo7 L segment in the positive orientation	49
pLCK-Mo7Mvc	Mo7 M segment in the positive orientation	50
pLCK-Mo7Svc	Mo7 S segment in the positive orientation	51

[1]Includes a putative NSm and glycoproteins expressed as a polyprotein

Reverse genetics

Recombinant *Phleboviruses* can be generated using a reverse genetics system that parallels the reverse genetics system previously described for Rift Valley fever virus (see PCT Publication No. WO 2009/082647, which is herein incorporated by reference).

Rescue of recombinant *Phleboviruses* is accomplished by simultaneous transfection of S, M and L anti-genomic plasmids into cells stably expressing T7 polymerase (for example, BSR-T7/5 cells). The genome segments of each plasmid are flanked by a T7 promoter, which enables generation of the primary RNA transcript, and the hepatitis delta virus ribozyme, which removes extraneous nucleotides from the 3' end of the primary transcriptional products. The T7 RNA polymerase generated transcripts are identical copies of the *Phlebovirus*-encoding plasmids, with the exception of an extra G nucleotide on the 5' end derived from the T7 promoter.

In one example, a recombinant *Phlebovirus* can be generated by transfection of the pLCK-Mo4Lvc, pLCK-Mo4Mvc and pLCK-Mo4Svc plasmids into BSR-

T7/5 cells (or any other cell type expressing T7 polymerase). In another example, a recombinant *Phlebovirus* can be generated by transfection of the pLCK-Mo7Lvc, pLCK-Mo7Mvc and pLCK-Mo7Svc plasmids. Alternatively, the Mo4 and Mo7 gene segment plasmids can be used in any combination so long as at least one S segment, at least one M segment and at least one L segment plasmid is transfected into the T7 polymerase expressing cells.

Recombinant *Phleboviruses* can also be generated using an S segment anti-genomic plasmid in which the NSs ORF is replaced by a reporter gene. Thus, in one example, the recombinant *Phlebovirus* is generated by transfection of the pLCK-Mo4Lvc, pLCK-Mo4Mvc and pLCK-Mo4SvcDelNSs_GLuc plasmids. In another example, the recombinant *Phlebovirus* is generated by transfection of the pLCK-Mo7Lvc, pLCK-Mo7Mvc and pLCK-Mo4SvcDelNSs_GLuc plasmid. Alternatively, a plasmid encoding the Mo7 S segment with the NSs ORF replaced by a reporter (such as Gaussia luciferase) can be generated and used to produce recombinant virus.

Furthermore, the S, M and L segment anti-genomic plasmids can be further altered to introduce any desired mutations (including deletions, additions and substitutions), such as to reporter viruses, produce attenuated virus and/or identify virulence factors.

Exemplary methods for producing recombinant virus using reverse genetics are described in PCT Publication No. WO 2009/082647. In one example, plasmids encoding *Phlebovirus* L, M and S segments are transfected in approximately 1 μg quantities with LT-1 (Mirus) at a ratio of 6:1 and transferred onto sub-confluent (approximately 60-70% confluent) monolayers of BSR-T7/5 cells stably expressing T7 polymerase (Buchholz *et al.*, *J. Virol.* 73(1):251-259, 1999). Four to five days post transfection, the cell supernatant is clarified by low speed centrifugation and passaged twice on confluent monolayers of Vero E6 cells. After passage and prior to use in subsequent experiments, the complete genome sequence of each rescued recombinant virus can be confirmed by previously described techniques (Bird *et al.*, *J. Virol.* 81:2805-2816, 2007).

Mini-genome reporters

Mini-genome reporter systems have been developed for several negative sense RNA viruses (see, for example, Jasenosky *et al.*, *Antimicrob Agents Chemother* 54(7):3007-3010, 2010; Freiberg *et al.*, *Virology* 370(1):33-44, 2008; Dumas *et al.*, *J Virol Methods* 142(1-2):59-66, 2007; Hoenen *et al.*, *Antiviral Res* 91(2):195-208, 2011). These systems allow for the development of cell lines that produce replicating virus. Such cell lines can be used, for example, to screen for compounds that inhibit viral replication, and to select for virus mutants that escape drug pressure.

To produce a *Phlebovirus* mini-genome system, a first plasmid contains a reporter gene that is flanked by viral UTRs from any of the three *Phlebovirus* gene segments. This plasmid is transfected into cells along with a plasmid or plasmids expressing the necessary replication factors, which for *Phlebovirus* are the NP and L proteins. As one example, the reporter gene can be an antibiotic resistance gene. Thus, after transfection of the reporter plasmid and plasmids expressing NP and L, cells that are undergoing viral replication can be selected based on antibiotic resistance. Cells can further be transfected with a plasmid encoding the *Phlebovirus* glycoproteins (GnGc) in order to package the replicating viral segments.

In some cases, the mini-genome reporter system includes a plasmid encoding an S segment (which encodes NP), a plasmid encoding an L segment (which encodes the L protein) and a reporter plasmid comprising an antibiotic resistance gene flanked by the M segment UTRs. As one example, the mini-genome reporter system uses the pLCK-Mo4Svc, pLCK-Mo4Lvc and pLCK-M_EGFPBlastNeg plasmids. Viral packaging can then be achieved by transfection of a plasmid encoding GnGc (such as the pcMo4GnGc plasmid) or a plasmid encoding the complete M ORF (such as the pcMo4Morf plasmid). In an alternative example, instead of using plasmids encoding the complete S and L segments, the mini-genome reporter system uses a plasmid expressing the L protein (such as pcMo4L) and a plasmid expressing NP (such as pcMo4NP). The selected combination of

plasmids is transfected into cells expressing T7 polymerase. Packaged virus can then be isolated and used to infect cells such as VeroE6, A549 or HuH7 cells.

In another example, one can evaluate viral replication only (without packaging) using a plasmid encoding the viral L segment (such as pLCK-Mo4Lvc or pLCK-Mo7vc) and a plasmid encoding an S segment in which the NSs ORF is replaced by a reporter (such as the pLCK-Mo4SvcDelNSs_GLuc plasmid).

In view of the many possible embodiments to which the principles of the disclosed invention may be applied, it should be recognized that the illustrated embodiments are only preferred examples of the invention and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

CLAIMS

1. An isolated *Phlebovirus* comprising an S segment, an M segment and an L segment, wherein:

(i) the nucleotide sequence of the S segment is at least 80%, at least 85%, at least 90%, or at least 95% identical to SEQ ID NO: 1 or SEQ ID NO: 8;

(ii) the nucleotide sequence of the M segment is at least 80%, at least 85%, at least 90%, or at least 95% identical to SEQ ID NO: 2 or SEQ ID NO: 9;

(iii) the nucleotide sequence of the L segment is at least 80%, at least 85%, at least 90%, or at least 95% identical to SEQ ID NO: 3 or SEQ ID NO: 10; or

(iv) any combination of (i), (ii) and (iii).

2. The isolated *Phlebovirus* of claim 1, wherein:

(i) the S segment comprises SEQ ID NO: 1, the M segment comprises SEQ ID NO: 2 and the L segment comprises SEQ ID NO: 3; or

(ii) the S segment comprises SEQ ID NO: 8, the M segment comprises SEQ ID NO: 9 and the L segment comprises SEQ ID NO: 10.

3. The isolated *Phlebovirus* of claim 1 or claim 2, wherein the virion particles of the *Phlebovirus* have an average diameter of about 80-90 nm.

4. The isolated *Phlebovirus* of claim 3, wherein the average diameter is about 86 nm.

5. An isolated polypeptide encoded by the *Phlebovirus* of any one of claims 1-4.

6. An isolated polypeptide wherein the amino acid sequence of the polypeptide is at least 80%, at least 85%, at least 90%, or at least 95% identical to SEQ ID NO: 4, 5, 6, 7, 11, 12, 13 or 14.

7. The isolated polypeptide of claim 6, wherein the amino acid sequence of the polypeptide comprises or consists of SEQ ID NO: 4, 5, 6, 7, 11, 12, 13 or 14.

8. An isolated nucleic acid molecule, wherein the nucleotide sequence of the nucleic acid molecule is at least 80%, at least 85%, at least 90%, or at least 95% identical to SEQ ID NO: 1, 2, 3, 8, 9, or 10.

9. The nucleic acid molecule of claim 8, wherein the nucleotide sequence of the nucleic acid molecule comprises or consists of SEQ ID NO: 1, 2, 3, 8, 9, or 10.

10. A vector comprising the nucleic acid molecule of claim 8 or claim 9.

11. A host cell comprising the nucleic acid molecule of claim 8 or claim 9, or the vector of claim 10.

12. An oligonucleotide 12 to 40 nucleotides in length, wherein the oligonucleotide specifically hybridizes with the nucleic acid molecule of claim 8 or claim 9.

13. The oligonucleotide of claim 12, wherein the nucleotide sequence of the oligonucleotide is at least 85%, at least 90% or at least 95% identical to SEQ ID NO: 15, 16, 17, 18, 19, 20, 21, 22 or 23.

14. The oligonucleotide of claim 13, wherein the nucleotide sequence of the oligonucleotide comprises or consists of SEQ ID NO: 15, 16, 17, 18, 19, 20, 21, 22 or 23.

15. The oligonucleotide of any one of claims 12-14, wherein the oligonucleotide is 18 to 24 nucleotides in length.

16. The oligonucleotide of any one of claims 12-15, wherein the oligonucleotide comprises a fluorophore, a quencher or both.

17. An isolated antibody or antigen-binding fragment thereof that specifically binds to the *Phlebovirus* of any one of claims 1-4 or the polypeptide of any one of claims 5-7.

18. The antibody or antigen-binding fragment of claim 17, wherein the antibody or antigen-binding fragment specifically binds to a nucleoprotein (NP), glycoprotein (GP), NSs or L protein of the *Phlebovirus*.

19. A method for detecting a *Phlebovirus* or *Phlebovirus* polypeptide in a biological sample, comprising:

(i) contacting the biological sample with the antibody of claim 17 or claim 18, or antigen-binding fragment thereof; and

(ii) detecting binding of the antibody or antigen-binding fragment to the biological sample, wherein binding of the antibody or antigen-binding fragment to the biological sample indicates the presence of the *Phlebovirus* or the *Phlebovirus* polypeptide in the biological sample.

20. A method for detecting *Phlebovirus*-specific antibodies in a biological sample, comprising:

(i) contacting the biological sample with the polypeptide of any one of claims 5-7; and

(ii) detecting binding of the polypeptide to the biological sample, wherein binding of the polypeptide to the biological sample indicates the presence of the *Phlebovirus*-specific antibodies in a biological sample.

21. A method for detecting a *Phlebovirus* nucleic acid molecule in a biological sample, comprising:

(i) contacting the biological sample with an oligonucleotide probe that specifically hybridizes with the nucleic acid molecule of claim 8 or claim 9; and

(ii) detecting hybridization of the probe with the biological sample, wherein hybridization of the probe to the biological sample indicates the presence of the *Phlebovirus* nucleic acid molecule in the biological sample.

22. The method of claim 21, wherein the biological sample is a nucleic acid amplification product obtained by a method comprising:

(i) isolating RNA from the biological sample;

(ii) reverse transcribing the RNA to generate cDNA; and

(iii) amplifying the cDNA using a pair of primers that specifically hybridize to the isolated nucleic acid molecule of claim 8 or claim 9, thereby producing a nucleic acid amplification product.

23. A method for identifying a subject infected with the *Phlebovirus* of any one of claims 1-4, comprising:

(i) isolating RNA from a biological sample obtained from the subject;

(ii) reverse transcribing the RNA to generate cDNA;

(iii) amplifying the cDNA using a pair of primers that specifically hybridize to the isolated nucleic acid molecule of claim 8 or claim 9; and

(iv) detecting an amplification product from step (iii), wherein detection of the amplification product identifies the subject as infected with the *Phlebovirus*.

24. The method of claim 23, wherein detecting the amplification product comprises hybridizing the amplification product to a probe.

25. The method of any one of claims 22-24, wherein the pair of primers comprises:

(i) a first primer having a nucleotide sequence at least 85%, at least 90% or at least 95% identical to SEQ ID NO: 16 and a second primer having a nucleotide sequence at least 85%, at least 90% or at least 95% identical to SEQ ID NO: 17;

(ii) a first primer having a nucleotide sequence comprising or consisting of SEQ ID NO: 16 and a second primer having a nucleotide sequence comprising or consisting of SEQ ID NO: 17;

(iii) a first primer having a nucleotide sequence at least 85%, at least 90% or at least 95% identical to SEQ ID NO: 19 and a second primer having a nucleotide sequence at least 85%, at least 90% or at least 95% identical to SEQ ID NO: 20;

(iv) a first primer having a nucleotide sequence comprising or consisting of SEQ ID NO: 19 and a second primer having a nucleotide sequence comprising or consisting of SEQ ID NO: 20;

(v) a first primer having a nucleotide sequence at least 85%, at least 90% or at least 95% identical to SEQ ID NO: 22 and a second primer having a nucleotide sequence at least 85%, at least 90% or at least 95% identical to SEQ ID NO: 23; or

(iv) a first primer having a nucleotide sequence comprising or consisting of SEQ ID NO: 22 and a second primer having a nucleotide sequence comprising or consisting of SEQ ID NO: 23.

26. The method of any one of claims 21, 22, 24 and 25, wherein the probe comprises a nucleotide sequence at least 85%, at least 90% or at least 95% identical to SEQ ID NO: 15, SEQ ID NO: 18 or SEQ ID NO: 21.

27. The method of claim 26, wherein the probe comprises a nucleotide sequence comprising or consisting of SEQ ID NO: 15, SEQ ID NO: 18 or SEQ ID NO: 21.

28. The method of any one of claims 21, 22 and 24-27, wherein the probe comprises a fluorophore, a quencher or both.

29. The method of any one of claims 19-28, further comprising obtaining a biological sample from the subject prior to step (i).

30. A recombinant *Phlebovirus*, wherein the genome of the recombinant *Phlebovirus* comprises:

(i) an S segment having a nucleotide sequence at least 95% identical to SEQ ID NO: 1 or SEQ ID NO: 8;

(ii) an M segment having a nucleotide sequence at least 95% identical to SEQ ID NO: 2 or SEQ ID NO: 9; and

(iii) an L segment having a nucleotide sequence at least 95% identical to SEQ ID NO: 3 or SEQ ID NO: 10.

31. The recombinant *Phlebovirus* of claim 30, wherein the S segment comprises a deletion of the NSs open reading frame (ORF), or the NSs ORF is replaced by the ORF of a reporter gene.

32. The recombinant *Phlebovirus* of claim 30 or claim 31, comprising at least one attenuating mutation.

33. An immunogenic composition comprising the recombinant *Phlebovirus* of claim 32 and a pharmaceutically acceptable carrier.

34. A method of eliciting an immune response against *Phlebovirus* in a subject, comprising administering to the subject a therapeutically effective amount of the recombinant *Phlebovirus* of claim 32 or the immunogenic composition of claim 33.

35. A *Phlebovirus* reverse genetics system, comprising a first plasmid comprising an anti-genomic *Phlebovirus* S segment, a second plasmid comprising an anti-genomic *Phlebovirus* M segment and a third plasmid comprising an anti-genomic *Phlebovirus* L segment, wherein each plasmid comprises a T7 promoter and a hepatitis delta virus ribozyme, and wherein:

(i) the first plasmid comprises a nucleotide sequence at least 80% identical to nucleotides 107-1878 of SEQ ID NO: 46, nucleotides 107-1536 of SEQ ID NO: 47, or nucleotides 107-1878 of SEQ ID NO: 51;

(ii) the second plasmid comprises a nucleotide sequence at least 80% identical to nucleotides 107-3533 of SEQ ID NO: 45 or nucleotides 107-3533 of SEQ ID NO: 50; and

(iii) the third plasmid comprises a nucleotide sequence at least 80% identical to nucleotides 107-6474 of SEQ ID NO: 44 or nucleotides 107-6474 of SEQ ID NO: 49.

36. A *Phlebovirus* mini-genome reporter system, comprising a first plasmid comprising a reporter gene flanked by UTR sequences of a *Phlebovirus* S, M or L segment, a second plasmid encoding a *Phlebovirus* NP, and a third plasmid encoding a *Phlebovirus* L protein, wherein:

(i) the second plasmid comprises a nucleotide sequence at least 80% identical to nucleotides 107-1878 of SEQ ID NO: 46 or nucleotides 107-1878 of SEQ ID NO: 51; and

(ii) the third plasmid comprises a nucleotide sequence at least 80% identical to nucleotides 107-6474 of SEQ ID NO: 44 or nucleotides 107-6474 of SEQ ID NO: 49.

37. The *Phlebovirus* mini-genome reporter system of claim 36, further comprising a fourth plasmid, wherein the fourth plasmid encodes *Phlebovirus* GnGc.

38. A method of producing *Phlebovirus* pseudo-virus, comprising transfecting cells expressing T7 polymerase with the first, second, third and fourth plasmids of the *Phlebovirus* mini-genome reporter system of claim 37.

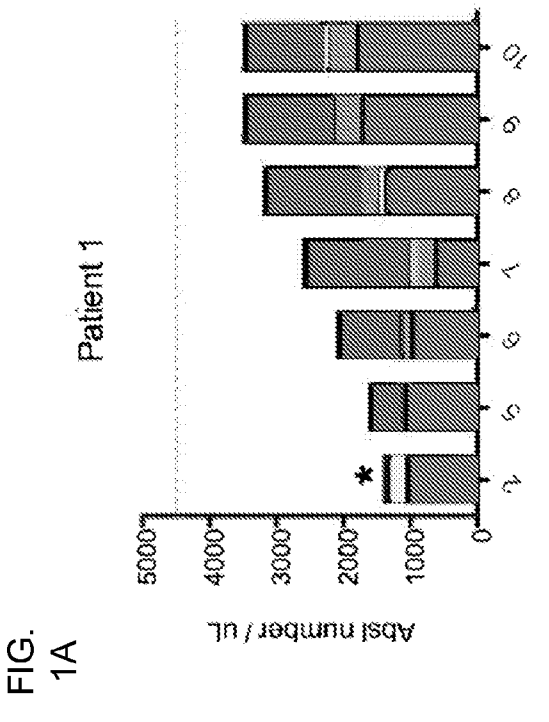


FIG. 1A

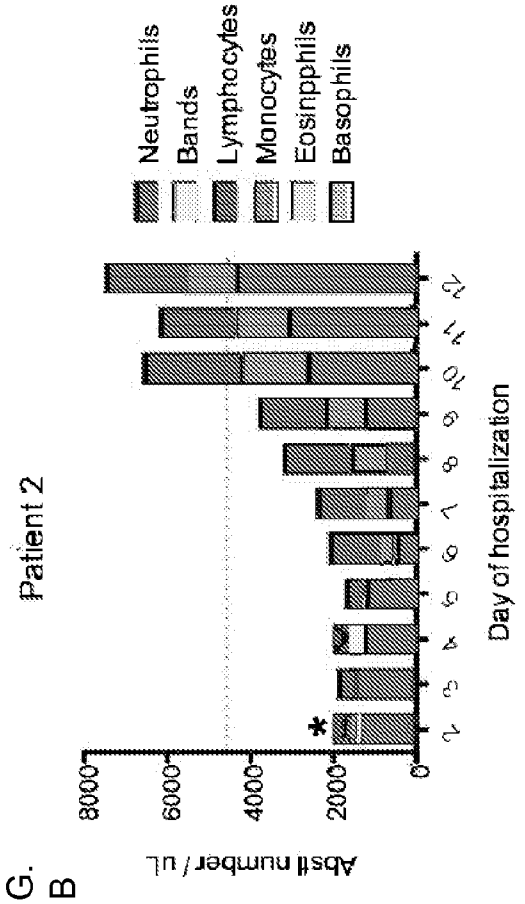


FIG. 1B

FIG. 1C

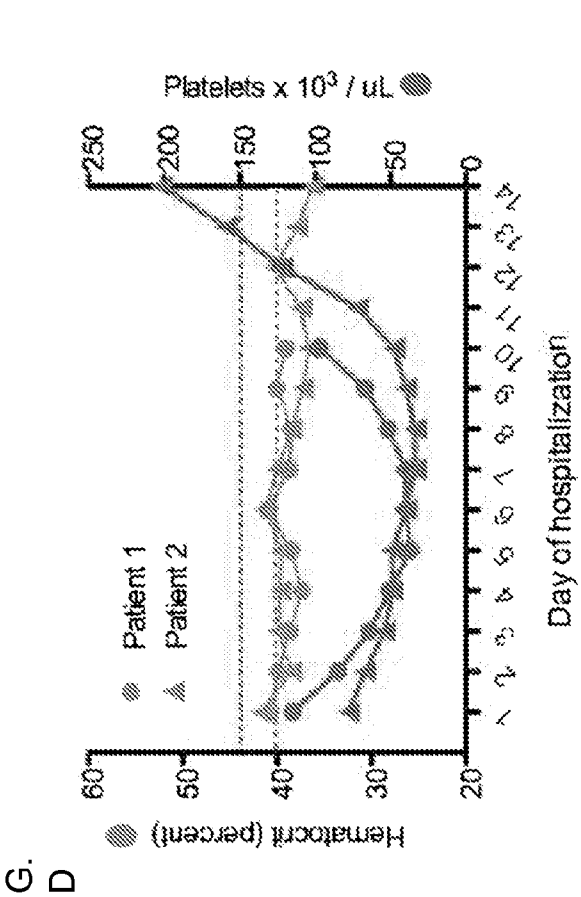


FIG. 1D

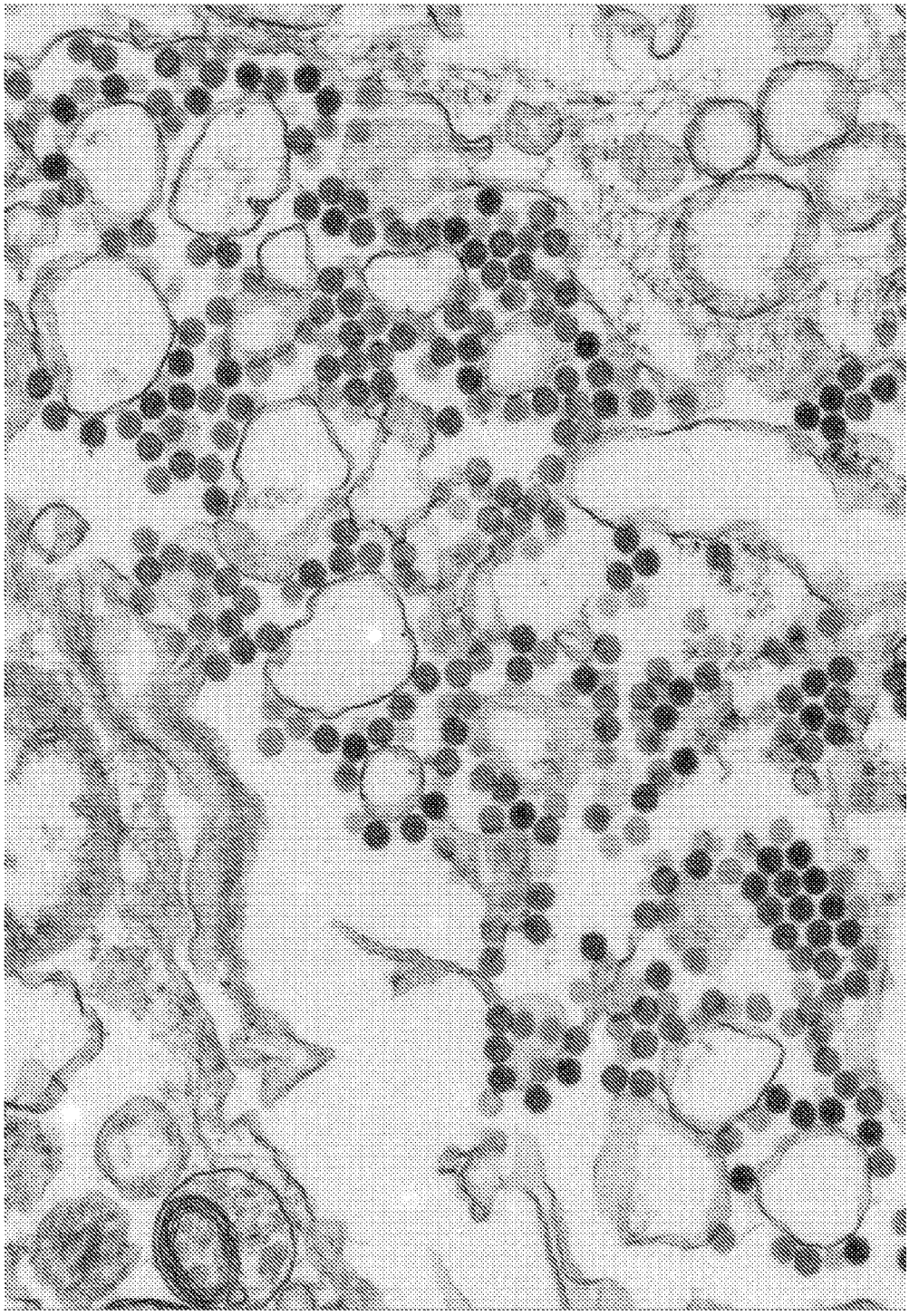


FIG. 2

FIG. 3A

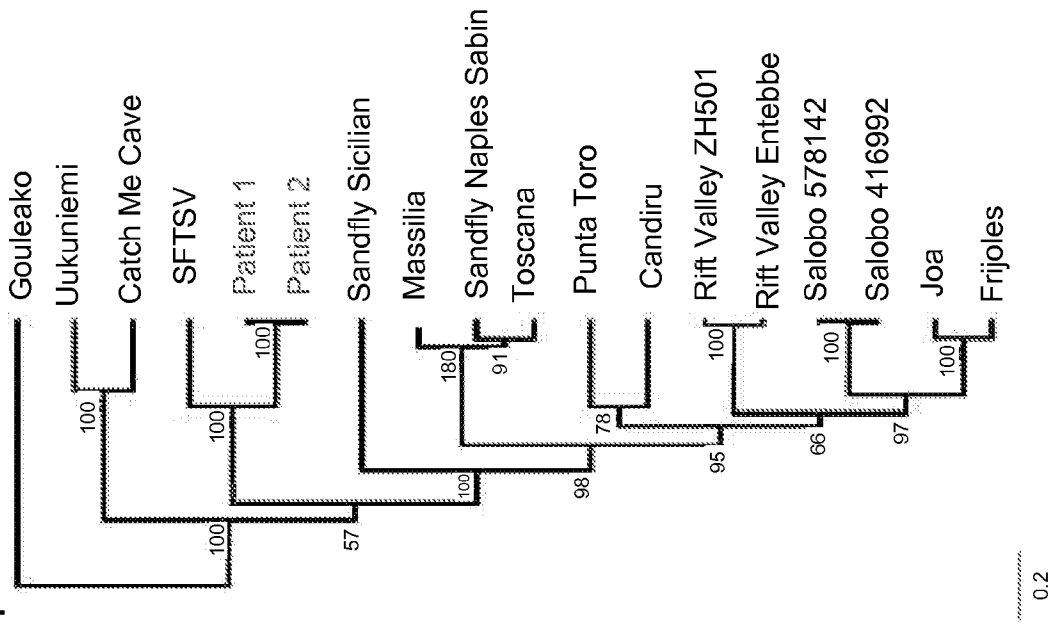

Nucleoprotein
Gouleako
Uukuniemi
Catch Me Cave
SFTSV
Patient 1
Patient 2
Sandfly Sicilian
Massilia
Sandfly Naples Sabin
Toscana
Punta Toro
Candiru
Rift Valley ZH501
Rift Valley Entebbe
Salobo 578142
Salobo 416992
Joa
Frijoles
0.2

FIG. 3B

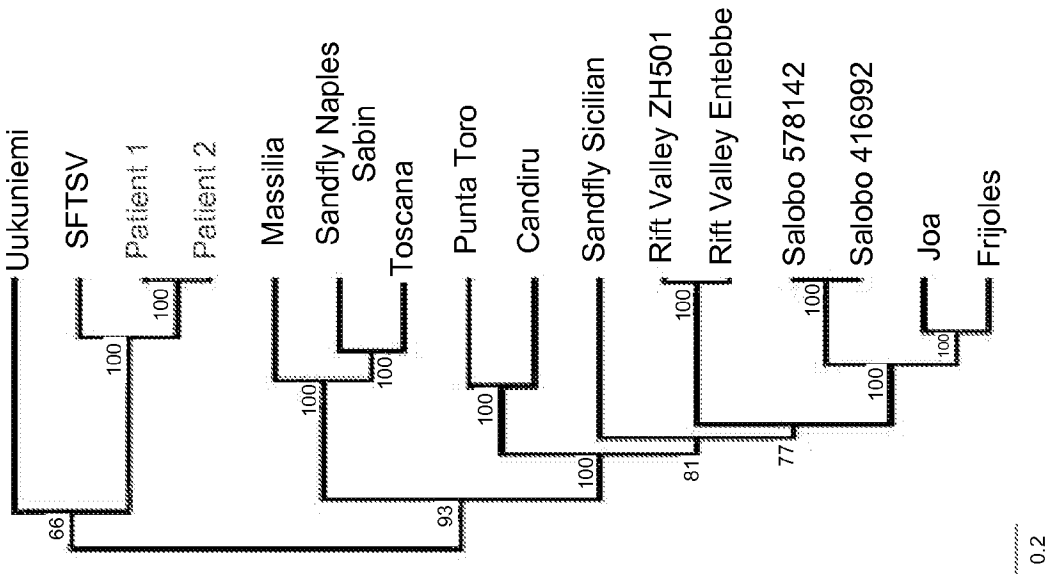

Nonstructural S
Uukuniemi
SFTSV
Patient 1
Patient 2
Massilia
Sandfly Naples Sabin
Toscana
Punta Toro
Candiru
Sandfly Sicilian
Rift Valley ZH501
Rift Valley Entebbe
Salobo 578142
Salobo 416992
Joa
Frijoles
0.2

FIG. 3D
Polymerase

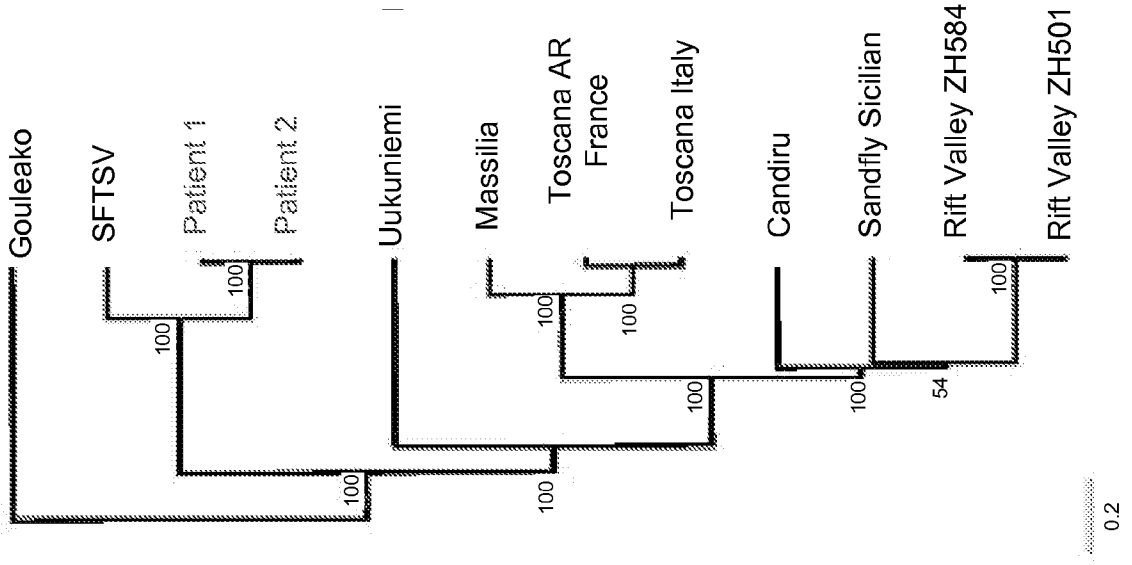


FIG. 3C
Glycoprotein

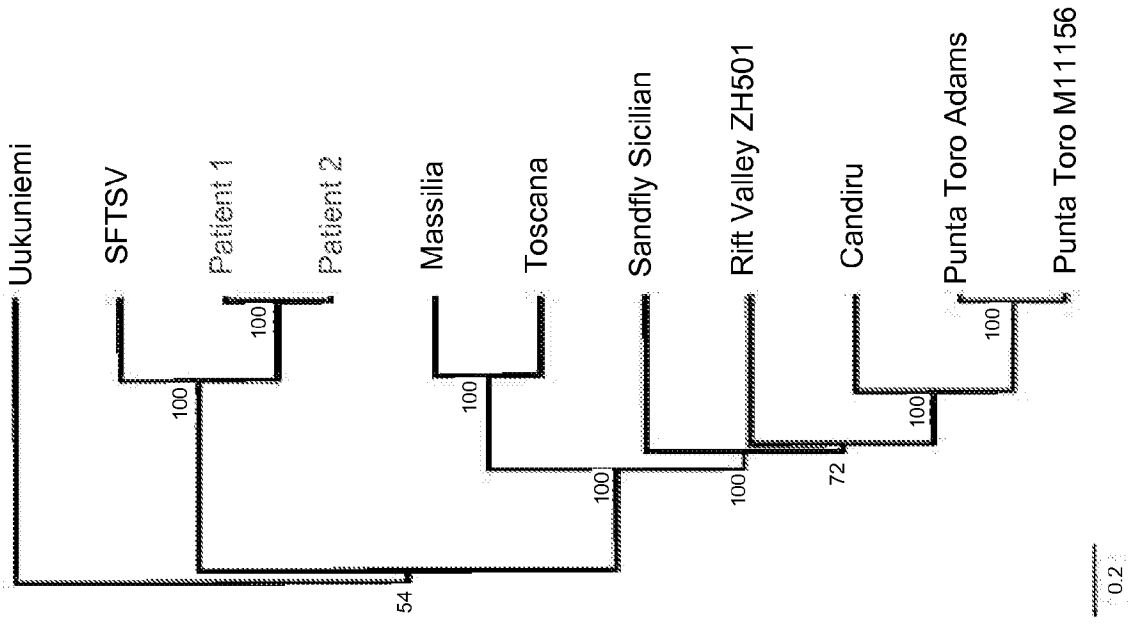


FIG. 4A

Patient 1	Normal Range	6/18/2009	6/19/2009	6/20/2009	6/21/2009	6/22/2009	6/23/2009	6/24/2009	6/25/2009	6/26/2009	6/27/2009
Day of admission		1	2	3	4	5	6	7	8	9	10
White-cell count ($x10^3$/mL)	4.5-11	1.9	1.4	1.2	1.5	1.6	2.1	2.6	3.2	3.5	3.5
Differential Absolute Number											
Neutrophils	3020-5820		1078			1104	1008	650	1376	1750	1820
Band forms	150-400		252						96		
Lymphocytes	1500-3000		28			432	882	1534	1440	1330	1190
Monocytes	285-500		42			64	126	286	288	385	420
Eosinophils	50-150					0	21	0	0	35	70
Basophils	15-50					16	63	130	0	0	0
Hematocrit (%)	40-52 (males)	40.8	39.9	38.7	37.2	38.6	41	38.8	38.5	40.2	39.3
Hemoglobin (g/dL)	13.5-17.5 (males)	14.7	14.2	13.7	13.3	13.9	14.4	13.4	13.3	13.9	13.5
Platelet count ($x10^3$ cells/mL)	150-450	115	85	64	52	37	40	40	52	57	97
Erythrocyte count ($x10^6$ cells/ mL)	4.3-5.7 (males)	4.62	4.54	4.4	4.27	4.42	4.66	4.35	4.28	4.46	4.38
Mean corpuscular volume (fL)	80-100	88	88	88	88	87	88	89	90	90	90
Mean corpuscular hemoglobin (pg/cell)	26-34	31.8	31.3	32.1	31.1	31.4	30.9	30.8	31.1	30.3	30.8
Mean corpuscular hemoglobin centration (%Hb/cell)	31-37	36	35.6	35.4	35.6	36	35.1	34.5	34.5	33.6	34.4
Red blood cell distribution width (%)	11-14	12.9	12.9	12.9	12.9	13.1	13.3	13.4	13.6	13.4	13.3
Prothrombin time						10					
seconds	9-13					0.92					
International normalized ratio	0.9-1.2					29.9					
Activated partial thromboplastin time (sec)	25-35										
Fibrinogen (mg/dl)	200-400										
D-Dimer (mg/L)	0-3.0										
Total protein (g/dL)	6.4-8.3		6.7	6.1	5.8	5.9	6.3	6.1	6.5	6.5	6.6
Albumin (g/dL)	3.4-4.8		3.4	3	2.9	2.9	3.1	2.9	2.8	2.9	2.9
Calcium (mg/dL)	8.6-10	8	7.7	7.4	7.2	7.4					
Bilirubin Total (mg/dL)	0.3-1.2		0.3	0.3	0.4	0.4	0.9	1	0.8	0.7	0.7
Alkaline Phosphatase (U/L)	25-100		71	71	65	70	72	88	98	101	99
Sodium (mEq/L)	136-146	132	131	132	128	131					
Potassium (mEq/L)	3.5-5.1	3.5	3.6	3.5	3.2	4.2					
Chloride (mEq/L)	98-106	100	99	100	100	101					
Total CO_2 (mEq/L)	23-29	23	22	24	22	23					
Creatinine	0.7-1.3 (males)	1.4	1.4	1.3	1.2	1.1					
ALT (U/L)	10-40 (males)		57	50	68	107	163	209	315	261	202
AST (U/L)	10-30		44	41	85	155	253	331	431	225	135
Glucose Lvl (mg/dL)	80-115	88	101	99	135	111					
BUN (mg/dL)	6-20	17	17	18	14	12					
Bilirubin Conjugated (Direct) (mg/dL)	0-0.2					0.1	0.4	0.7	0.4	0.3	0.2
Magnesium (mEq/L)	1.3-2.1			1.7	1.5						

FIG. 4B

Patient 2														
6/4/2009	6/4/2009	6/5/2009	6/5/2009	6/6/2009	6/7/2009	6/8/2009	6/9/2009	6/10/2009	6/11/2009	6/12/2009	6/13/2009	6/14/2009	6/15/2009
1	1.5	2	2.5	3	4	5	6	7	8	9	10	11	12
2.1	1.7	1.6	2	1.9	2	1.7	2.1	2.4	3.2	3.8	6.6	6.2	7.5
			1280	1320	1444	1280	1241	504	672	736	1254	2640	3100	4350
			64	160	0	380	85	84	48	0	0	0	0	0
			240	420	361	340	374	1176	1152	1600	1558	3310	2736	1950
			16	100	95	0	0	336	528	800	912	1518	1240	1200
			0	0	0	0	0	0	0	0	38	66	62	0
			0	0	19	0	0	0	0	64	38	66	62	0
41.7	38.5	39.9	39.7	39.6	41.3	40	38.5	37.3	36.7	37.6	40.3	37.9	36.3
14.4	13.4	14.3	14	14.1	14.8	14.4	13.8	13.7	13.7	13.8	15	14.1	13.3
78	67	54	49	49	40	34	34	40	48	72	122	259	202
	4.35	4.6	4.5	4.59	4.75	4.64	4.48	4.43	4.39	4.55	4.87	4.52	4.27
	85	87	88	86	87	86	86	84	84	83	83	84	85
	30.8	31.1	31.1	30.7	31.2	31	30.8	30.9	31.2	30.3	30.8	31.2	31.1
	34.8	35.8	35.3	35.6	35.8	36	35.8	36.7	37.3	36.7	37.2	37.2	36.6
	13.4	13.3	13.2	13.2	13.2	13.3	13.2	13	12.8	12.8	12.7	12.9	13
		18.7											
		0.99											
		27.9											
		229.6											
		4.08											
				6.1	6.1	5.5	5.4	5.7	6		6.8	6.4	6.3
				3.3	3.1	2.8	2.6	2.8	2.9		3.2	3	2.9
	7.8			7.3	7.3	7.6	7.7	7.6	8.1	8.3	8.8	8.7	8.6
				0.5	0.5	0.5	0.8	1.1	1.1		0.9	0.7	0.6
				68	60	52	55	58	70		107	96	98
	136			125	127	129	127	131	131	133	132	133	134
	4.8			3.6	3.8	4.1	3.9	3.7	3.5	3.3	3.6	3.8	3.9
	97			92	92	96	96	97	98	97	97	98	100
	30			25	29	27	26	25	23	25	24	26	27
		2.3		1.1	1.2	1.1	1	0.9	0.8	0.8	0.8	0.8	0.8
	4.3			63	81	81	76	148	263		302	268	234
	54			92	166	175	159	253	355		194	140	102
				158	174	151	155	181	175	144	156	133	109
	130			16	16	18	16	12	10	15	22	23	21
	19												
						0.3	0.5	0.4	0.4			0.2	

FIG. 5A

```
SFSV_J04418                          MD...EYQKI AVEFGEQAID ETVIQDWLQA FAYQGFDART IIHNLVQ...
RFV_ZH501_DQ380149                   MD...NYQEL AIQFAAQAVD RNEIEQWVRE FAYQGFDARR VIELLKQ...
SALV_BeAn_578142_EF201815            MA...DYARI AVEFSGEAIN LAEIQGWVTD FAYQGFDARR IVELVQQ...
SALV_BeAn416992_EF201816             MA...DYARI AVEFSGEAIN LAEIQGWVTD FAYQGFDARR IVELVQQ...
BeAr371637_EF201820                  MT...DYAEI AVAFAGEPVN NAEVMGWVNE FAYEGFSAQR IIQLVQE...
VP161A_EF201819                      MT...DYADI AIAFAGEPIN NAEVMGWVNE FAYEGFNAQR IIQLVQE...
Sabin_EF201829                       MS.EENYREI ALAFLDEAAD SGTITAWVNE FAYQGFDPKR IVQLVKE...
Massila_W_EU725773                   MS.EDNYRTI ALAFLDESAD STTINAWVNE FAYQGFDPKR IVQLVKE...
Toscana_H_IMTSSA_FJ153286            MS.DENYRDI ALAFLDESAD SGTINAWVNE FAYQGFDPKR IVQLVKE...
UUK_S23_M33551                       MAMPENWVRF AIEISDAQWE EEEIREFINL FQYQGFDAAV VLSRIFELAK
SSFTSV_HB29_HM745932                 MS...EWSRI AVEFGEQQLN LTELEDFARE LAYEGLDPAL IIKKLKE...
Mo7                                  MT...DWSAI AVEIGNEPLD VPALVEFAKE IAYEGLDPAV IFGLLRE...
Mo4                                  MT...DWSAI AVEIGNEPLD VPALVEFAKE IAYEGLDPAV ILGLLRE...
Gabek_Forest_Virus_NP_partial        .......... .......... .......... .....FDPRI VVKLVSE...
PTV_Adames_EF201835                  MS....YEEI AVQFASESID EQAVAGWVTD FAYQGFDAKR VIALVKE...
RFV_Entebbe_DQ380156                 MD...NYQEL AIQFAAQAVD RNEIEQWVRE FAYQGFDARR VIELLKQ...
Candiru_HM119409                     MS....YEKL AVDIAGHEID ADTIKAWVQA FAYQGFDAKR VMELLVE...
RioGrandeVirusNPpartial              .......... .......... .......... .AYQGFDANR VVELVQE...

SFSV_J04418                          LG...GKSWE EDAKKMIILS LTRGNKPKKM VERMSPEGAR EVKSLVAKYK
RFV_ZH501_DQ380149                   YG...GADWE KDAKKMIVLA LTRGNKPRRM MMKMSKEGKA TVEALINKYK
SALV_BeAn_578142_EF201815            KG...GAGWK DDVKMMIVLC LTRGNKPTKM VEKMSPEGKV KVNRLISTYG
SALV_BeAn416992_EF201816             KG...GAGWK DDVKMMIVLC LTRGNKPTKM VEKMSPEGKV KVNRLISTYG
BeAr371637_EF201820                  RG...PQTWQ TDVKMMIVLA LTRGNKPAKM IEKMSAEGKK KATRLITMYN
VP161A_EF201819                      KG...PQTWQ TDVKMMIVLA LTRGNKPSKM IEKMSAEGKK KASRLITIYG
Sabin_EF201829                       RGTAKGRDWK KDVKMMIVLN LVRGNKPEAM MKKMSEKGAG IVAQLISVYQ
Massila_W_EU725773                   RGTAKGRDWK KDVKMMIVLN LVRGNKPESM MKKMSEKGAA IVTQLISTYQ
Toscana_H_IMTSSA_FJ153286            RGTAKGRDWK KDVKMMIVLN LVRGNKPEAM MKKMSEKGAS IVANLISVYQ
UUK_S23_M33551                       KADLSRDQML RDIRALITLH LTRGNKLSSI EKRLSEEGKK EFALLKARYQ
SSFTSV_HB29_HM745932                 TG...GDDWV KDTKFIIVFA LTRGNKIVKA SGKMSNSGSK RLMALQEKYG
Mo7                                  RG...GENWR NDVKYIIVFA LTRGNKIVKA CGKMSKKGAE RMTNLARVYE
Mo4                                  RG...GENWR NDVKYIIVFA LTRGNKIVKA CGKMSKKGAE RMTNLARVYE
Gabek_Forest_Virus_NP_partial        VE...G..WQ TDVKKMIILA LTRGNKPEKM VTKMSAKGRE EVAKLVKKYK
PTV_Adames_EF201835                  RG...GEDWK QDVKKMIVLS LTRGNKPNKM VLKMSDKGKA MVNELVLKYK
RFV_Entebbe_DQ380156                 YG...GADWE KDAKKMIVLA LTRGNKPRRM MMKMSKEGKA TVEALINKYK
Candiru_HM119409                     RG...GDDWV EDAKQMIILC LTRGNKPSKM MVKMSEKGKK IVQALVKRYS
RioGrandeVirusNPpartial              RA..KGRKWQ EDVKRMIILA LTRGNKPDKM RKKMSPEGIA VLDDLVKTYQ

SFSV_J04418                          IVEGRPGRNG ITLSRVLQPW LGGQSKLWKW LKTSYQSQGA QWTALCGQTY
RFV_ZH501_DQ380149                   LKEGNPSRDE LTLSRVAAAL AGWTCQALVV LSEWLPVTGT TMDGLSP.AY
SALV_BeAn_578142_EF201815            LKSGNPGRDD ITLSRVAAAF AGWTCQALNV LHPYLPVSGT TMDAISP.NY
SALV_BeAn416992_EF201816             LKSGNPGRDD ITLSRVAAAF AGWTCQALNV LHPYLPVSGT TMDAISP.NY
BeAr371637_EF201820                  LKSGNPGRDD LTLSRVASAF AGWTCQALAV LHPYLPVTGA SMDSISP.GY
VP161A_EF201819                      LKSGNPGRDD LTLSRIAAAF AGWTCQALAT LHPYLPVTGA AMDAISP.GY
Sabin_EF201829                       LKEGNPGRDT ITLSRVSAAF VPWTIQALRV LSDSLPVTGT TMDAIAGVTY
Massila_W_EU725773                   LKEGNPGRDT ITLSRVSAAF VPWTVQALKT LSESLPVTGT TMDSIAGTTY
Toscana_H_IMTSSA_FJ153286            LKEGNPGRDT ITLSRVSAAF VPWTVQALRV LSESLPVSGT TMDAIAGVTY
UUK_S23_M33551                       LVDKAKEAAD LTLSRIAIAN AGLTCRILPQ VVAHTAVTRS RMESLSA.DY
SSFTSV_HB29_HM745932                 LVERAETRLS ITPVRVAQSL PTWTCAAAAA LKEYLPVGPA VMNLKVE.NY
Mo7                                  LKENAVDRMA VTPVRVAQCL PTWTCAAAAA IKEYLPVGPA IMHNKIQ.GY
Mo4                                  LKENAVDRMA VTPVRVAQCL PTWTCAAAAA IKEYLPVGPA IMHNKIQ.GY
Gabek_Forest_Virus_NP_partial        LKSGNPGRND LTLSRVAAAF ASWTCNAIYH VQYYLPVTGN HMDAISK...
PTV_Adames_EF201835                  LKSGNPSRDD LTLSRITAAF AGWTCQAADY VQEYLPVTGR AMDTISS.GY
RFV_Entebbe_DQ380156                 LKEGNPSRDE LTLSRVAAAL AGWTCQALVV LSEWLPVTGT TMDGLSP.AY
Candiru_HM119409                     LKEGNPSRDD LTLSRVTAAL AGYTCQATEY VEEFLPVTGK NMDDLSK.NY
RioGrandeVirusNPpartial              LKSSSPGRDD LTLARIAAAF APWTCQATEA VENYMPVNGA ..........
```

FIG. 5B

```
SFSV_J04418                          PRQMMHPSFA GLI.DPSLDQ EDFNAVLDAH KLFLFMFSKT INVSLRGAQK
RFV_ZH501_DQ380149                   PRHMMHPSFA GMV.DPSLPG DYLRAILDAH SLYLLQFSRV INPNLRGRTK
SALV_BeAn_578142_EF201815            PRAMMHPCFA GLV.DQTIPT EYCQTIVDAM SVFLIQFSRT INKNLRGQPK
SALV_BeAn416992_EF201816             PRAMMHPCFA GLV.DQTIPT EYCQTIVDAM SVFLIQFSRT INKNLRGQPK
BeAr371637_EF201820                  PRAMMHPSFA GLI.DNSIPE AFLQTVVDAH ALYLLQFSRV INKNMRGQPK
VP161A_EF201819                      PRAMMHPSFA GLI.DNSIPE AYLQVVVDAH ALYLLQFSRV INRNMRGQPK
Sabin_EF201829                       PRAMMHPSFA GII.DLDLPN RAGEAIADAH GLFMLEFSKT INPSLRTKQP
Massila_W_EU725773                   PRCMMHPSFA GII.DLELPN NTGAMLADAH GLFMLEFSKT INPSLRTKQP
Toscana_H_IMTSSA_FJ153286            PRAMMHPSFA GII.DLDLPN GAGATIADAH GLFMIEFSKT INPSLRTKQA
UUK_S23_M33551                       PVCMMHNAFA GLI.DETLPE DSIKALVDAH RLYLLEFSRT INVKHRGMEA
SSFTSV_HB29_HM745932                 PPEMMCMAFG SLIPTAGVSE ATTKTLMEAY SLWQDAFTKT INVKMRGASK
Mo7                                  PLEMMCMAFG SLIPQADVSI EVIKDFMDAY SLWQDTFART INVDQRKMTK
Mo4                                  PLEMMCMAFG SLIPQADVSI EVIKDFMDAY SLWQDTFART INVDQRKMTK
Gabek_Forest_Virus_NP_partial        .......... .......... .......... .......... ..........
PTV_Adames_EF201835                  PRAMMHPSFA GLI.DQELPA DVLSEITQAH CLFMIQFSKT INPSLRGLSK
RFV_Entebbe_DQ380156                 PRHMMHPSFA GMV.DPSLPE DYLRAILDAH SLYLLQFSRV INPNLRGKTK
Candiru_HM119409                     PRAMMHPSFA GLI.DPKLPP DVLSTICDAF SLFMVQFSRT INPRNRGLSV
RioGrandeVirusNPpartial              .......... .......... .......... .......... ..........

SFSV_J04418                          RDIEESFSQP MLAAINSSFI DNTQRRAFLT KFGILTSGAR ATAVVKKIAE
RFV_ZH501_DQ380149                   EEVAATFTQP MNAAVNSNFI SHEKRREFLK AFGLVDSNGK PSAAVMAAAQ
SALV_BeAn_578142_EF201815            EVVIESFIQP MQAAMSSSFI APAERRKLMI ALGIVDANGK PSANVAAAAA
SALV_BeAn416992_EF201816             EVVIESFIQP MQAAMSSSFI APAERRKLMI ALGIVDANGK PSANVAAAAA
BeAr371637_EF201820                  SVVVSSFLQP MNAAIVSGFI SHDKRRKMLM AFGIVDQNGK PTQAVETAAK
VP161A_EF201819                      SVVVSSFLQP MNAAIVSGFI SNDRRRKMLM AFGIVDQNGK PTAAVESAAK
Sabin_EF201829                       NEIAATFEKP NMAAMSGRFF TREDKKKLLM AVGILNEDLV LTPAIVKCAE
Massila_W_EU725773                   NEIAATFEKP NMAAMTGRFF TRDDKKKLLI AIGVLDEDLV PNPAIEKCAE
Toscana_H_IMTSSA_FJ153286            NEVAATFEKP NMAAMSGRFF TREDKKKLLI AVGIIDEDLV LASAVVRSAE
UUK_S23_M33551                       KEILDANDSA LQAGLASSFL TPSQKRAYLL SFKLVDGNGK VNKAVQQAAT
SSFTSV_HB29_HM745932                 TEVYNSFRDP LHAAVNSVFF PNDVRVKWLK AKGILGPDGV PSRAAEVAAA
Mo7                                  AEVYAKFRDP LHAAVNSLFF PNATRISWLQ AKGLLTATKE ASGSVKAAAA
Mo4                                  AEVYAKFRDP LHAAVNSLFF PNATRISWLQ AKGLLTATKE ASGSVKAAAA
Gabek_Forest_Virus_NP_partial        .......... .......... .......... .......... ..........
PTV_Adames_EF201835                  DEIVSSFERP MQAAISSTFL TSANRRAMLK TLGIINDNLK PSSSTVSAAK
RFV_Entebbe_DQ380156                 EEVAATFTQP MNAAVNSSFI SHEKRREFLR AFGLVDSNGK PSAAVMAAAQ
Candiru_HM119409                     SEVASTFDRP INAAMNSSFI SGEQRKSFLR NLGILDENMQ PSNPVKAAAK
RioGrandeVirusNPpartial              .......... .......... .......... .......... ..........

SFSV_J04418                          VYRKLE...   (SEQ ID NO: 24)
RFV_ZH501_DQ380149                   AYKTAA...   (SEQ ID NO: 25)
SALV_BeAn_578142_EF201815            VFPRLL...   (SEQ ID NO: 26)
SALV_BeAn416992_EF201816             VFPRLL...   (SEQ ID NO: 27)
BeAr371637_EF201820                  AFMTIN...   (SEQ ID NO: 28)
VP161A_EF201819                      AFMTAV...   (SEQ ID NO: 29)
Sabin_EF201829                       KYCSKVGK.   (SEQ ID NO: 30)
Massila_W_EU725773                   KYKAKVGKV   (SEQ ID NO: 31)
Toscana_H_IMTSSA_FJ153286            KYRAKVGK.   (SEQ ID NO: 32)
UUK_S23_M33551                       VLRSLI...   (SEQ ID NO: 33)
SSFTSV_HB29_HM745932                 AYRNL....   (SEQ ID NO: 34)
Mo7                                  AYRNM....   (SEQ ID NO: 11)
Mo4                                  AYRNM....   (SEQ ID NO: 4)
Gabek_Forest_Virus_NP_partial        .........   (SEQ ID NO: 35)
PTV_Adames_EF201835                  VFRSL....   (SEQ ID NO: 36)
RFV_Entebbe_DQ380156                 AYKTAA...   (SEQ ID NO: 37)
Candiru_HM119409                     VFRGLK...   (SEQ ID NO: 38)
RioGrandeVirusNPpartial              .........   (SEQ ID NO: 39)
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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/033541

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N7/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/061752 A1 (3M INNOVATIVE PROPERTIES CO [US]; MILLER JESSE D [US]; PARTHASARATHY R) 14 May 2009 (2009-05-14) sequence 6	12,13
X	WO 2007/110869 A2 (TEL HASHOMER MEDICAL RES INFRA [IL]; GAK EVA [IL]; HALKIN HILLEL [IL]) 4 October 2007 (2007-10-04) pages 12, 13; example 1; sequence 15	12,13,15
X	WO 02/20548 A1 (UNIV WASHINGTON [US]; APPLIED PHYTOLOGICS INC [US]; LEWIS NORMAN G [US) 14 March 2002 (2002-03-14) sequence AAL38447	12,13,15
X	US 2005/048531 A1 (MITTMAN MICHAEL [US] ET AL) 3 March 2005 (2005-03-03) sequences 36377, 36378,	12,13

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 June 2013	27/06/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schulz, Regine

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/033541

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIANLI XU ET AL: "Metagenomic Analysis of Fever, Thrombocytopenia and Leukopenia Syndrome (FTLS) in Henan Province, China: Discovery of a New Bunyavirus", PLOS PATHOGENS, vol. 7, no. 11, 17 November 2011 (2011-11-17), page e1002369, XP055065594, DOI: 10.1371/journal.ppat.1002369 abstract	1-38

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2013/033541

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