Characterization of the Bhanja Serogroup Viruses (Bunyaviridae): a Novel Species of the Genus Phlebovirus and Its Relationship with Other Emerging Tick-Borne Phleboviruses


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Bhanja virus (BHAV) and its antigenically close relatives Forecariah virus (FORV), Kismayo virus (KISV), and Palma virus (PALV) are thought to be members of the family Bunyaviridae, but they have not been assigned to a genus or species. Despite their broad geographical distribution and reports that BHAV causes sporadic cases of febrile illness and encephalitis in humans, the public health importance of the Bhanja serogroup viruses remains unclear, due in part to the lack of sequence and biochemical information for the virus proteins. In order to better define the molecular characteristics of this group, we determined the full-length sequences of the L, M, and S genome segments of multiple isolates of BHAV as well as FORV and PALV. The genome structures of these Bhanja viruses are similar to those of viruses belonging to the genus Phlebovirus. Functional domains and amino acid motifs in the viral proteins that are conserved among other known phleboviruses were also identified in proteins of the BHAV group. Phylogenetic and serological analyses revealed that the BHAVs are most closely related to the novel emerging tick-borne phleboviruses severe fever with thrombocytopenia syndrome virus and Heartland virus, which have recently been implicated as causing severe acute febrile illnesses associated with thrombocytopenia in humans in China and the United States. Our results indicate that the Bhanja serogroup viruses constitute a single novel species in the genus Phlebovirus. The results of this study should facilitate epidemiological surveillance for other, similar tick-borne phleboviruses that may represent unrecognized causes of febrile illness in humans.

The family Bunyaviridae constitutes the largest group of RNA viruses, with more than 350 viruses identified (1). Bunyaviruses, particularly those belonging to the genera Orthobunyavirus, Phlebovirus, and Nairovirus, are found worldwide and are capable of replicating alternately in vertebrates and arthropods. Different viruses in these three genera are transmitted to vertebrates by the bites of infected mosquitoes, ticks, phlebotomine sand flies, or culicoid mites. Bunyaviruses cause a number of important diseases in humans and in livestock (2).

Bunyaviruses possess a genome consisting of three negative-stranded RNA segments: the L segment, encoding the RNA-dependent RNA polymerase (RdRp); the M segment, encoding the envelope glycoproteins (GPs) Gn and Gc; and the S segment, encoding a nucleocapsid protein (N). The M and/or S segments of some viruses also encode nonstructural proteins (NSm and/or NSs). Despite the discovery of numerous bunyaviruses that are pathogenic to humans and/or domestic animals, genetic information about these viruses is still rather limited. This lack of knowledge has hampered our understanding of many biological and ecological aspects of these viruses, and it has impeded the development of much-needed diagnostic tools. Several novel bunyaviruses causing disease in human and domestic animals have recently emerged in Western Europe, China, and the United States. These include severe fever with thrombocytopenia syndrome virus (SFTSV; also known as Huaiyangshan virus or Henan fever virus), Schmallenberg virus (SBV), and Heartland virus. SFTSV is a novel tick-borne phlebovirus (TBPV) that emerged recently in China and causes a severe and sometimes fatal febrile illness with thrombocytopenia and hemorrhagic manifestations in humans (3–5). SBV is midge borne and appeared recently in Western Europe, where it causes congenital malformations and reduced milk production in cattle, goats, and sheep (6). Heartland virus is a novel phlebovirus (presumably tick borne) that was recently reported in the central United States, where it is associated with a severe febrile illness in humans (7). The continual emergence of new pathogenic bunyaviruses and the propensity of these segmented viruses to undergo reassortment in nature (8–12) emphasize the need for a more comprehensive understanding of their genetics.

At present, 40 viruses listed as members of the family Bunyaviridae have not been assigned to genera or approved as species, due to the lack of genetic information and/or serologic cross-reactivity with other known bunyaviruses (1). Bhanja virus (BHAV), Forecariah virus (FORV), and Kismayo virus (KISV) are included in this group of unassigned bunyaviruses, although they are antigenically related to one another based on serologic tests and form a Bhanja serogroup together with Palma virus (PALV), which has not been assigned to the Bhanja group in the current list.
co’s modified Eagle medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco, Life Technologies). Two strains of BHAV (IG690 [20] and IbAr2709 [29]), KISV strain LEIV3641A (14), and PALV strain PoTi4.92 (30) were obtained from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at the University of Texas Medical Branch (UTMB), Galveston. BHAV strain R-1819 (25) and FORV strain DakArk4927 (15) were provided by the Division of Vector-Borne Infectious Diseases (DVBD), National Center for Infectious Diseases, Centers for Disease Control and Prevention (Table 1).

Viruses were grown in Vero E6 cells or DH82 cells in DMEM supplemented with 2% FCS, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin (Gibco, Life Technologies), and 10 μg/ml MycoKill AB (GE Healthcare). Virus growth was monitored based on the appearance and progression of cytopathic effect (CPE) in cells. When advanced CPE was observed, the culture supernatants were harvested for further experiments. Virus titers were determined by plaque assays in Vero E6 cells. Experiments with these viruses were performed in the biosafety level 2 (BSL-2) and BSL-3 laboratories at the Rocky Mountain Laboratories (RML), Division of Intramural Research (DIR), National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Hamilton, MT, and the University of Texas Medical Branch, Galveston.

Serological tests. Antigens for use in serological tests were prepared from frozen lysates of Vero cells or from newborn mouse brains infected with the indicated viruses (Table 1). Complement fixation (CF) tests were carried out using antigens prepared from infected mouse brains and extracted by the sucrose-acetone method (31). We were unable to obtain good hemagglutinating activity with mouse brain antigens prepared for many of the tick-associated bunyaviruses, so frozen harvests of infected Vero cell cultures (undiluted) were used as an alternative antigen in the hemagglutination inhibition (HI) tests. The CF and HI tests were performed by the microtiter technique, as described previously (31). CF titers were recorded as the reciprocals of the highest antibody/highest antigen dilutions giving 3+ or 4+ fixation of complement on a scale of 0 to 4+. HI antibody titers of 1:20 or greater were considered positive.

Immune reagents. Virus-specific antibodies (mouse hyperimmune ascitic fluids and rabbit hyperimmune sera) used in serological tests were obtained from the WRCEVA collection at the UTMB. The methods used to prepare mouse immune ascitic fluids (MIAFs) have been described previously (31,32). The immunizing antigens were 10% homogenates of infected newborn mouse brain in phosphate-buffered saline (PBS); MIAFs were made in adult ICR mice given four weekly intraperitoneal injections of the mouse brain antigen mixed with Freund’s adjuvant (31,32). Sarcoma 180 cells (ATCC) were used to induce ascites (31). A rabbit hyperimmune serum to SFTSV was also prepared, using the same immunization schedule, except that the immunogen was infected rabbit kidney cells grown in a medium with 5% rabbit serum instead of bovine serum. All animal work was done at the UTMB under an approved animal protocol.

In addition, convalescent-phase sera from three severe fever with thrombocytopenia syndrome (SFTS) patients were included in HI tests.
These sera were obtained by one of us (X.-J.Y.) during field studies in China (5).

Electron microscopy. DH82 cells infected with BHAV IbAr2709 were harvested 24 h postinfection. Cells were washed with PBS and were fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.4) overnight at room temperature, according to a previously published protocol (33). After embedding, cells were sectioned into 70-nm layers with a diamond knife, poststained with 1% uranyl acetate and 1% lead citrate, and examined with a model H7500 electron microscope (Hitachi High-Technologies) at 80 kV. Digital images were collected with an XR100 charge-coupled-device camera (Advanced Microscopy Techniques).

RNA isolation. After centrifugation of cell culture supernatants twice at 2,500 × g to remove debris, samples were concentrated to a small final volume (<1,000 μL) using an Amicon Ultra-15 centrifugal filter unit with an Ultracel-50 membrane (Millipore). Concentrated supernatants were lysed in 3 volumes of TRIzol LS reagent (Life Technologies), and RNA was extracted using a modified guanidine thiocyanate method (34). To 500 μL of lysate, 147 μL of 1-bromo-3-chloropropane (Sigma) was added, and the phases were separated by centrifugation for 20 min at 4°C. An equal amount of guanidine isothiocyanate–containing RLT buffer (Qiagen) was added to the aqueous phase, and RNA was subsequently DNase treated and purified by using RNeasy columns according to the manufacturer’s protocol (Qiagen).

cDNA synthesis. cDNA was synthesized using a modification of the protocol described by Palacios et al. (35). Briefly, the first-strand cDNA was synthesized using the SuperScript III reverse transcriptase system (Invitrogen). PCRs were performed in triplicate in two phases: the initial cycles were performed at a low annealing temperature of 25°C and were followed by cycles at a more stringent annealing temperature of 55°C. The cycling conditions were as follows: 95°C for 2 min; 5 cycles at 95°C for 15 s, 25°C for 1 min, and 72°C for 1 min; 25 cycles at 95°C for 1 s, 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 1 min. Replica PCR products were pooled and purified using the QiAquick kit according to the manufacturer’s protocol (Qiagen). The final PCR products were visualized by gel electrophoresis on a 2% SeaKem agarose gel (Lonza), quantified by UV spectrophotometry at 260 nm and 280 nm, and used as the template for sequencing on the 454 Genome Sequencer FLX system (454 Life Sciences).

High-throughput sequencing and data analysis. cDNA samples were quantitated using Picogreen reagent (Life Technologies) and were prepared according to the Rapid Library Preparation Method Manual: GS FLX Titanium Series, by 454 Life Sciences (36). A pool consisting of 12 samples = N-3 = a defined 17-mer primer (5′-GTT TCC CAG TAG GTC TCN NNN NNN -CGC CGT primer and a primer targeting a specific 17-mer sequence (5′-CGC GTG TTC CCA GTA GTC CTG-3′) by modification of a procedure described by Palacios et al. (35). Five microliters of each ss-cDNA template was used as the template in 50-μL reaction mixtures consisting of 0.2 μM primer mix, 1 X Platinum Taq polymerase buffer, 2.5 U Platinum Taq polymerase, 0.2 mM each dNTP, and 1.5 mM MgCl2 (Invitrogen). PCRs were performed in triplicate in two phases: the initial cycles were performed at a low annealing temperature of 25°C and were followed by cycles at a more stringent annealing temperature of 55°C. The cycling conditions were as follows: 95°C for 2 min; 5 cycles at 95°C for 15 s, 25°C for 1 min, and 72°C for 1 min; 25 cycles at 95°C for 1 s, 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 1 min. Replica PCR products were pooled and purified using the QiAquick kit according to the manufacturer’s protocol (Qiagen). The final PCR products were visualized by gel electrophoresis on a 2% SeaKem agarose gel (Lonza), quantified by UV spectrophotometry at 260 nm and 280 nm, and used as the template for sequencing on the 454 Genome Sequencer FLX system (454 Life Sciences).

Table 2: Results of complement fixation tests with Bhanja group viruses

<table>
<thead>
<tr>
<th>Antigen</th>
<th>CF titer a with antibody against</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHAV</td>
<td>1,024/512 256/512 256/512 16/128</td>
</tr>
<tr>
<td>PALV</td>
<td>1,024/128 512/128 256/128 8/32</td>
</tr>
<tr>
<td>FORV</td>
<td>1,024/32 512/32 512/32 0</td>
</tr>
<tr>
<td>KISV</td>
<td>16/128 16/128 0 1,024/128</td>
</tr>
</tbody>
</table>

a Expressed as the reciprocal of the highest antibody dilution/reciprocal of the highest antigen dilution. 0, <8/8/8.

Sequence alignment and phylogenetic analysis. The nucleotide sequences obtained for each genome segment, or the deduced amino acid sequences of each of the open reading frames (ORFs), were aligned with the representative sequences of other known phleboviruses from GenBank (see Table S1 in the supplemental material) using MUSCLE as implemented in MEGA, version 5 (40). Phylogenetic trees were constructed using the neighbor-joining (NJ) and maximum likelihood (ML) methods. For NJ analysis, the Tamura 3-parameter model and the Poisson model built into MEGA 5 were used for nucleotide sequences and amino acid sequences, respectively. The robustness of the nodes was tested by 2,000 bootstrap replications. For ML analysis, RAxML was used with 1,000 bootstrap replications. The general time-reversible (GTR) + Γ + I model was selected for tree searches of nucleotide and amino acid sequences.

Nucleotide sequence accession numbers. The genome sequences of all Bhanja group viruses were deposited in GenBank under the following accession numbers (S segment, M segment, and L segment): JX961619, JX961620, and JX961621 (BHAV IG690); JX961622, JX961623, and JX961624 (BHAV R-1819); JX961616, JX961617, and JX961618 (BHAV IbAr2709); JX961625, JX961626, and JX961627 (FORV DaArk4927); and JX961628, JX961629, and JX961630 (PALV PoTi4.92).

RESULTS AND DISCUSSION

Serological studies. Table 2 shows the results of CF tests comparing four Bhanja group viruses: BHAV (strain IG690), PALV, FORV, and KISV. The CF test indicates the degree of serological divergence of the N protein. In this test, BHAV,
PALV, and FORV were essentially indistinguishable. KISV was distinct, although there was some cross-reactivity between BHAV, PALV, and KISV.

Table 3 gives the results of the HI test comparing Uukuniemi virus (UUKV), BHAV, and Heartland virus antigens against UUKV, BHAV, and SFTSV MIAF, as well as hyperimmune SFTSV rabbit serum and convalescent-phase sera from three human cases of SFTS. To date, we have not produced an SFTSV preparation with potent hemagglutination activity or a Heartland virus MIAF. Despite these limitations, a close antigenic relationship between Heartland virus and SFTSV was demonstrated by the HI test. The BHAV antigen also reacted with the mouse and rabbit antibodies against SFTSV, as well as with the convalescent-phase sera from human SFTS patients. The anti-UUKV antibody also re-

**Table 3** Results of hemagglutination inhibition tests, comparing Uukuniemi virus, Bhanja virus, SFTSV, and Heartland virus

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Uukuniemi virus</th>
<th>Bhanja virus</th>
<th>Heartland virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Uukuniemi virus</td>
<td>1:320</td>
<td>0</td>
<td>1:20</td>
</tr>
<tr>
<td>Anti-Bhanja virus</td>
<td>0</td>
<td>1:320</td>
<td>1:20</td>
</tr>
<tr>
<td>Anti-SFTSV (mouse)</td>
<td>0</td>
<td>1:40</td>
<td>1:1,280</td>
</tr>
<tr>
<td>Anti-SFTSV (rabbit)</td>
<td>NT</td>
<td>1:40</td>
<td>1:320</td>
</tr>
<tr>
<td>SFTS serum 1 (human)</td>
<td>0</td>
<td>1:80</td>
<td>1:160</td>
</tr>
<tr>
<td>SFTS serum 2 (human)</td>
<td>0</td>
<td>1:20</td>
<td>1:40</td>
</tr>
<tr>
<td>SFTS serum 3 (human)</td>
<td>0</td>
<td>1:40</td>
<td>1:80</td>
</tr>
</tbody>
</table>

*NT, not tested; 0, <1:20.

*SFTS convalescent-phase human serum.

**FIG 2** Growth of Bhanja viruses in cell lines. (A and B) The growth of Bhanja virus (BHAV) bAr2709 in DH82 cells was confirmed by electron microscopy; (A) Virions in an intracellular vacuole; (B) inclusion bodies. Bars, 100 nm. (C) Three isolates of BHAV and an isolate of Palma virus (PALV) were tested for growth in Vero E6 and DH82 cells. The cells were infected at an MOI of 0.05, and the supernatants of the cells at each time point were titrated by a plaque assay using Vero E6 cells. The experiments were performed in triplicate. Error bars indicate the standard errors of the means.

**FIG 3** Genome structure of Bhanja virus. (A) Schematic diagram of the coding strategy of Bhanja virus. BHAV has three genome segment RNAs with different lengths (nucleotide sequence lengths are shown on the right). The largest segment (L) encodes an RNA-dependent RNA polymerase (RdRp), and the second largest segment (M) encodes a glycoprotein (GP). The smallest RNA segment (S) encodes a nonstructural protein (NSs) and a nucleocapsid protein (N) in opposing directions. The amino acid (aa) sequence length of each protein is indicated above the corresponding open reading frame (shown as an open arrow). (B) Terminal conserved complementary sequences that constitute a “panhandle structure” in each genome segment. The upper strand in each alignment starts from the 3’ end of the genome, while the lower strand starts from the 5’ end. Vertical lines between the two strands indicate complementary nucleotides in the 3’ and 5’ ends. The conserved 3’ and 5’ terminal nucleotides of each genome segment are shaded. SFTSV, severe fever with thrombocytopenia syndrome virus; UUKV, Uukuniemi virus; RVFV, Rift Valley fever virus; GOUV, Gouleako virus (55). (C) The predicted secondary structure of the intergenic region between the two ORFs of the S segment RNA was calculated using CLC Main Workbench (CLC bio). Each number indicates the nucleotide position from the 3’ end of the virus genome.
acted slightly with the Heartland virus antigen. These data support other recent work (41) indicating that on the basis of serologic relationships, BHAV, SFTSV, and Heartland virus are all members of an expanded Uukuniemi group within the genus Phlebovirus.

Growth of Bhanja virus in vertebrate cells. The growth characteristics of several Bhanja group viruses were determined in both DH82 and Vero cells. BHAV, PALV, and FORV all produced cytopathic effect (CPE) and plaques in Vero and DH82 cells. In contrast, no CPE or plaques were observed in either of these two vertebrate cell lines with KISV. RT-PCR primers, based on the nucleotide and deduced amino acid sequences of these S segments showed homology with S segment nucleotide sequences and N cleavage site. The nucleotide sequences of the L and M genome segments contained two open reading frames in opposing orientations (antisense strategy). As with the L and M segments, the nucleotide and deduced amino acid sequences of their ORFs showed homology with the L segments (RdRp) and M segments (GPs Gn and Gc) of other phleboviruses (see Tables S2 and S3 in the supplemental material). The small segment (S segment; 1,866 nt) segments (the L and M segments) each contained one long deduced open reading frame (ORF) in the negative-sense orientation. The nucleotide sequences of the L and M genome segments and the deduced amino acid sequences of their ORFs showed homology with the L segments (RdRp) and M segments (GPs Gn and Gc) of other phleboviruses (see Tables S2 and S3 in the supplemental material). The small segment (S segment; 1,866 nt) for BHAV R-1819 and PALV PoTi4.92 and 1,871 nt for the others) contained two open reading frames in opposing orientations (ambisense coding strategy). As with the L and M segments, the nucleotide and deduced amino acid sequences of these S segments showed homology with S segment nucleotide sequences and N and NSs amino acid sequences of other known phleboviruses (see Tables S4 and S5 in the supplemental material).

In order to form “panhandle structures,” the 3’ and 5’ ends of the bunyavirus genome have complementary sequences (42), which are conserved within the Phlebovirus genus. Bhanja group viruses also have conserved sequences at their genome termini, and the sequences of the five terminal nucleotides (3’-UGUGU and ACACA-5’) are identical to those of other members of the

![Comparison of the glycoproteins among tick-borne phleboviruses. (Top) Schematic diagrams of the predicted structure of GP for Bhanja virus IG690 (BHAV), severe fever with thrombocytopenia syndrome virus (SFTSV), and Uukuniemi virus (UUKV). The length of the total amino acid (aa) sequence for each protein is given on the right. Signal sequences (shaded rectangles) were predicted with SignalP 4.0, and transmembrane domains (filled rectangles) were predicted with MEMSAT3. Each region or domain starts from the amino acid position indicated above the rectangle and ends at the position indicated below the rectangle. N-linked glycosylation sites were predicted with NetNGlyc, version 1.0, and are indicated by the letter “Y” above each diagram. (Bottom) Alignment of the amino acid sequences of the areas around the transmembrane region of Gn (left) and the cytoplasmic tail of Gc (right) in tick-borne phleboviruses. Numbers above the alignment indicate the amino acid position in the BHAV IG690 GP. FORV, Forecariah virus; PALV, Palma virus. (Left) The deduced transmembrane domains are boxed, and the Golgi retention motif of UUKV GP (47) is indicated by black dots under the alignment. (Right) The conserved lysine at the third amino acid position from the C terminus of GP is shaded.
The intergenic regions of Punta Toro virus and UUKV, located between the N and NSs ORFs, have been reported to potentially constitute a hairpin-like secondary structure (43, 44). Bhanja group viruses have conserved complementary nucleotide sequences in the region, which, in theory, are also able to constitute a hairpin-like structure (Fig. 3C). While the TBPVs have similar secondary structures in this region, the nucleotide sequences are not conserved. It is noteworthy that the S segment genome RNAs of other phleboviruses, such as Rift Valley fever virus (RVFV), do not form such a secondary structure, suggesting that the mechanism of transcription termination differs among phleboviruses (44). Overall, the characteristics of the genome RNA segments clearly indicated that Bhanja group viruses are members of the genus *Phlebovirus* (Fig. 3B). The intergenic regions of Punta Toro virus and UUKV, located between the N and NSs ORFs, have been reported to potentially constitute a hairpin-like secondary structure (43, 44). Bhanja group viruses have conserved complementary nucleotide sequences in the region, which, in theory, are also able to constitute a hairpin-like structure (Fig. 3C). While the TBPVs have similar secondary structures in this region, the nucleotide sequences are not conserved. It is noteworthy that the S segment genome RNAs of other phleboviruses, such as Rift Valley fever virus (RVFV), do not form such a secondary structure, suggesting that the mechanism of transcription termination differs among phleboviruses (44). Overall, the characteristics of the genome RNA segments clearly indicated that Bhanja group viruses are members of the genus *Phlebovirus*.

Next, we compared the amino acid sequences of the Bhanja group viruses with those of other phleboviruses, mainly the TBPVs. These amino acid sequence-based predictions revealed that the BHAV GP contains two potential transmembrane domains and two signal sequences. Like the viruses belonging to the Uukuniemi group, the BHAV GP ORF does not contain a non-structural protein NSm in a pre-glycoprotein coding region (45) (Fig. 4). The transmembrane topology of the BHAV GP was predicted to be similar to those of the SFTSV and UUKV GPs, suggesting that it uses similar maturation machinery and that the GP will be cleaved into two membrane glycoproteins, Gn and Gc, which then constitute the heterodimer, as shown for UUKV GP maturation (45). Interestingly, the amino acids downstream of the first transmembrane domain, which form the cytoplasmic tail of Gn and are essential for the retention of the GP by the Golgi complex, are not conserved among phleboviruses (46, 47). Similarly, although the cytoplasmic tail of Gn was conserved among the Bhanja group viruses, these sequences differed from those in other TBPVs (Fig. 4). The cytoplasmic tail of Gc is involved in the budding of virions and the intracellular trafficking of the GPs (47). The lysine in the third position from the C terminus in the cytoplasmic tail of Gc, which is critical for the trafficking of the UUKV Gc, is also conserved among Bhanja group viruses and the other TBPVs (Fig. 4). These results suggest that the GP of BHAV retains many of the functions associated with other phlebovirus GPs.

In the RdRp sequences of BHAVs, conserved functional motifs of negative-stranded RNA virus RNA polymerases (Fig. 5A) were identified: all three dipeptides (HD at positions 81 and 82, PD at positions 114 and 115, and RY at positions 678 and 679 of BHAV IG690 RdRp) found in arenavirus and bunyavirus polymerases are also conserved in the RdRp’s of BHAV and the other TBPVs (i.e., SFTSV and UUKV). Six motifs that have been identified in a broad range of negative-stranded RNA virus RdRp’s have also been identified in TBPV RdRp’s. In the N protein sequences, critical amino acid residues for the RNA-binding motif, as identified in the crystal structure of the N protein of RVFV (48, 49), were also found in TBPV N proteins (Fig. 5B). The amino acid residues R64, K67, and K74 of the RVFV N protein are conserved among phleboviruses, including UUKV. Sequence alignment of the RNA-binding region
shows that the three amino acid residues and the region around R64 are all highly conserved among the TBPVs. The conservation of these functional or potentially functional amino acids in the Bhanja virus N and RdRp proteins indicates a similarity in the RNA replication and RNA-packaging machineries between BHAVs and other phleboviruses. The amino acid sequences of phlebovirus NSs proteins, which inhibit host innate immune responses (50, 51), are more divergent than the other protein sequences (see Tables S2 to S5 in the supplemental material). Further analysis may reveal both common and different strategies of NSs to suppress host responses among phleboviruses.

Phylogenetic and serological evidence for the classification of Bhanja group viruses into a novel species in the genus Phlebovirus that is closely related to SFTSV. Phylogenetic trees based on the nucleotide sequences of the L, M, and S genome segments were constructed to define the molecular relationships between Bhanja group viruses and other phleboviruses (Fig. 6). Two additional BHAV and PALV sequences (M3811 and M3443, respectively), recently determined by Dilcher et al. (52), were also included in the construction of these phylogenetic trees. In all three phylogenetic trees (L, M, and S segments), the Bhanja group viruses constitute a single clade closely related to the clades formed by SFTSV and Heartland virus. Despite the similar conclusions regarding the genetic relationship between BHAV and SFTSV drawn by Dilcher et al. (52) and in our study, the phylogenetic dendrograms produced by these two studies were slightly different, which might reflect the selection of the evolutionary model, outgroup, and/or number of sequences used to construct the tree. Recently, Palacios et al. determined and reported additional full-length genome sequences of viruses belong to the UUKV group (41) (the sequence data are available in GenBank). Exploiting the additional data reported by Palacios et al., Dilcher et al., and the present study may provide a more confident classification of TBPVs based on molecular biological approaches. The other phleboviruses (such as RVFV, sandfly fever Naples virus [SFNV], and sandfly fever Sicilian virus [SFSV]), which are transmitted by mosquitoites or sand flies, appeared in a different branch distinct from the clades formed by BHAVs and SFTSVs. While the clades formed by other TBPVs, including Uukuniemi group viruses, were clearly divergent from the clades of sand fly- or mosquito-borne phleboviruses in trees based on the L and M segment sequences, clades of the TBPVs and sandfly- or mosquito-borne phleboviruses were not clearly separated in the trees based on the S segment sequences. This partial difference in tree topology between the L and M segments, on the one hand, and the S segments, on the other (i.e., instability of the UUKV clade), may be due to different evolutionary processes affecting the N protein versus the other proteins, as shown in the phylogenetic trees based on amino acid sequences (Fig. 7), suggesting that the N gene of Uukuniemi group viruses may evolve by selection under different environmental conditions (e.g., vector and mammalian host). The phylogenetic trees for BHAVs, SFTSV, and Heartland virus based on the amino acid sequences of the RdRp, GP, and NSs showed topologies similar to those of the trees based on nucleotide sequences. The Bhanja group viruses, SFTS and Heartland viruses, shared a most recent common ancestor among viruses in this genus and constitute a cluster distinct from the other viruses (Fig. 7). The results of HI tests (Table 3), which reflect mainly antigenic similarities and differences among the GPs of phleboviruses, also support a close relationship between BHAV, SFTSV, and Heartland virus. Antiserum against BHAV reacted with homologous antigens (BHAV IG690) but not with the UUKV antigen, and vice
versa. In contrast, antisera to SFTSV and convalescent-phase sera from SFTS patients reacted with the BHAV and Heartland virus antigens. These serological and phylogenetic results support the classification of BHAVs and PALV into a novel species of the genus *Phlebovirus*. However, although the serological relationship of KISV to BHAV is closer than that to the other TBPVs (Table 2), taxonomic assignment of KISV to the genus *Phlebovirus* still remains elusive due to the lack of sequence data. Thus, at present, KISV can be identified only as a "probable phlebovirus."

Among the Bhanja group viruses, three geographic lineages (African, Asian, and European) might be implied by the phylogenetic analysis (Fig. 6) and the amino acid sequence identities (see

**FIG 7** Phylogenetic relationships among phlebovirus proteins. Maximum likelihood trees based on the amino acid sequences of each viral protein were constructed using the PROTGAMMA model. (A) RNA-dependent RNA polymerase (RdRp), encoded by the L segment; (B) glycoprotein, encoded by the M segment; (C) nucleocapsid protein (N), encoded by the S segment; (D) nonstructural protein (NSs), also encoded by the S segment. Numbers on the trees represent bootstrap values of 1,000 replicates. Abbreviations of taxa: BHAV, Bhanja virus; FORV, Forecariah virus; PALV, Palma virus; SFTSV, severe fever with thrombocytopenia syndrome virus; RVFV, Rift Valley fever virus.
Characterization of Bhanja Serogroup Viruses

Tables S2 to S5 in the supplemental material). As discussed above, the three phylogetic trees (L, M, and S segments) displayed different topologies within the Bhanja group virus branches, suggesting that different evolutionary processes have occurred among the three RNA genome segments of Bhanja group viruses (e.g., genetic reassortment). Nevertheless, Bhanja group viruses seem to be divided into African and Eurasian lineages. This may reflect or be defined by the geographic distribution of their tick vectors and/or mammalian reservoirs. In general, Bhanja group viruses have been isolated from multiple species of hard ticks. To date, the Eurasian Bhanja group viruses have been isolated only from the *Haemaphysalis* species of ticks, whereas BHAV group viruses of the African lineage have been isolated from several different genera of ticks, including *Amblyomma*, *Dermacentor*, *Rhipicephalus*, and *Hyalomma*. Further studies will be required to define the vector competence of different tick species for these viruses and the relationship between the geographic distributions of ticks and viruses.

Here we have shown a closer genetic and serological relationship of Bhanja group viruses to the more pathogenic SFTSV and Heartland virus than to UUKVs and the other phleboviruses. Although reported human cases of BHAV infection are rare, the close relationship between Bhanja group viruses, SFTSV, and Heartland virus may imply that Bhanja group viruses have pathogenic potential for humans. In addition, the broad geographical distribution of these TBPVs suggests the existence of other, still unrecognized but related TBPVs that could be pathogenic for humans. Further clarification of the evolutionary processes leading to the emergence of these known human pathogens, to prepare for the potential emergence of other novel, highly pathogenic TBPVs, would seem prudent. The genetic and serological data presented here should facilitate the development of diagnostic tools for the surveillance and recognition of tick-associated illnesses caused by unrecognized pathogens. Also, further investigations into the molecular biology of Bhanja group virus pathogenesis and transmission should contribute to a better understanding of the pathogenesis, ecology, and epidemiology of these viruses.

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The opinions, interpretations, conclusions, and recommendations presented here are those of the authors and are not necessarily endorsed by the NIH.

REFERENCES


22. Zakaryan VA, Gromeshevsky VL, Chubkova AI, Akopyan GS, Skvorotsve TM. 1974. Isolation of Bhanja virus from Dermacentor

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47. Overby AK, Popov VL, Pettersson RF, Neve EPA. 2007. The cytoplasmic tails of Uukuniemi virus (Bunyaviridae) Gs and Gc glycoproteins are important for intracellular targeting and the budding of virus-like particles. J. Virol. 81:12811–12815.