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Original article

Complete genome sequencing of Kaisodi virus isolated from ticks in India belonging to *Phlebovirus* genus, family *Phenuiviridae*

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ABSTRACT

An unknown virus was repeatedly isolated from hard tick (*Haemaphysalis spinigera*) during a proactive arbovirus survey in ticks conducted in 1957, in India. The virus remained uncharacterized for a long time. The passages of this virus in different vertebrate and invertebrate cells along with human and monkey-derived cell culture showed no cytopathic effect. It was identified later to be a member of Kaisodi group among *Phlebovirus* genus in the family *Phenuiviridae* (Order: Bunyavirales) by serological methods. Due to its genomic diversity, sequencing of this virus was a challenge for a while. In this study, we were able to sequence the complete genome of this virus isolate using next-generation sequencing (NGS) platform. The unknown virus was identified to be Kaisodi virus (KASDV) using NGS analysis. *De novo* genome assembly derived three genomic segments for the KASDV which encode for RNA-dependent RNA polymerase, glycoprotein precursor, and nucleoprotein. Functional as well as conserved domains for Kaisodi serogroup viruses were predicted and compared to a known representative of the genus *Phlebovirus*. The phylogenetic tree revealed its closeness to Silverwater virus, of Kaisodi serogroup with nucleotide (69%, 62%, and 61%) and amino acid (52%, 51%, and 62%) identity for L, M, and S segment, respectively. The study demonstrates the presence of a conserved motif (^{72}TRGNK^{76}) around the RNA binding motif region in tick-borne phleboviruses. The intergenic region encompassing the S segment of Kaisodi serogroup was GC-rich whereas the other *Phlebovirus* had AT-rich genome. KASDV has the largest intergenic region and larger loops, suggesting stem-loops formed due to larger loops as a possible factor for instability and cause of transcription termination. This paper also describes the real-time RT-PCR and RT-PCR assays developed and used for the detection of KASDV RNA in ticks from Karnataka, Kerala and Maharashtra State, India. The KASDV positivity observed in the recently collected tick pools indicates that the KASDV, isolated from Karnataka state in 1957, is also circulating in the adjoining Kerala state. On the basis of the current study, it should be possible to develop diagnostic assays which would facilitate an in-depth field survey exploring the veterinary and medical significance of KASDV.

1. Introduction

Serological, morphological and biochemical features classify *Phenuiviridae* (previously known as *Bunyaviridae)* family into five genera namely *Orthobunyavirus, Hantavirus Phlebovirus*, *Nairovirus,* and *Tospovirus* (Saimons et al., 1990). Sand flies, mosquitoes or ticks are responsible for the transmission of phleboviruses (Saimons et al., 1990). The genus *Phlebovirus* consists of nine species and at least 70 different serogroups (Moriconi et al., 2017). All phleboviruses have single-stranded tri-segmented RNA genomes; L, M and S segments, which encode for RNA-dependent RNA polymerase (RdRp), glycoprotein and nucleoprotein, respectively. The glycoprotein gene of phleboviruses also encodes for NSm protein, which is absent in tick-borne viruses (Elliott and Brennan, 2014). The S-segment of phleboviruses has a unique ambisense arrangement to code nucleocapsid protein (N) and a non-structural protein (NSs) (Saimons et al., 1990; Ihara et al., 1984; Marriott et al., 1989). For phleboviruses, the size of RdRp, glycoprotein precursor and ambisense nucleoprotein (non-structural and nucleocapsid) is approximately 2120 amino acid (aa), 1010 aa, 275 aa, and 247 aa residues respectively (Elliott and Schmaljohn, 2014).

In 1957, during the outbreak investigation of Kyasanur Forest Disease (KFD) at Sagar, Shimoga district, Karnataka State, India, Kaisodi virus [KASDV] was isolated from the hard ticks, *Haemaphysalis spinigera* (Bhatt et al., 1966; Pavri and Casals, 1966). The virus derives

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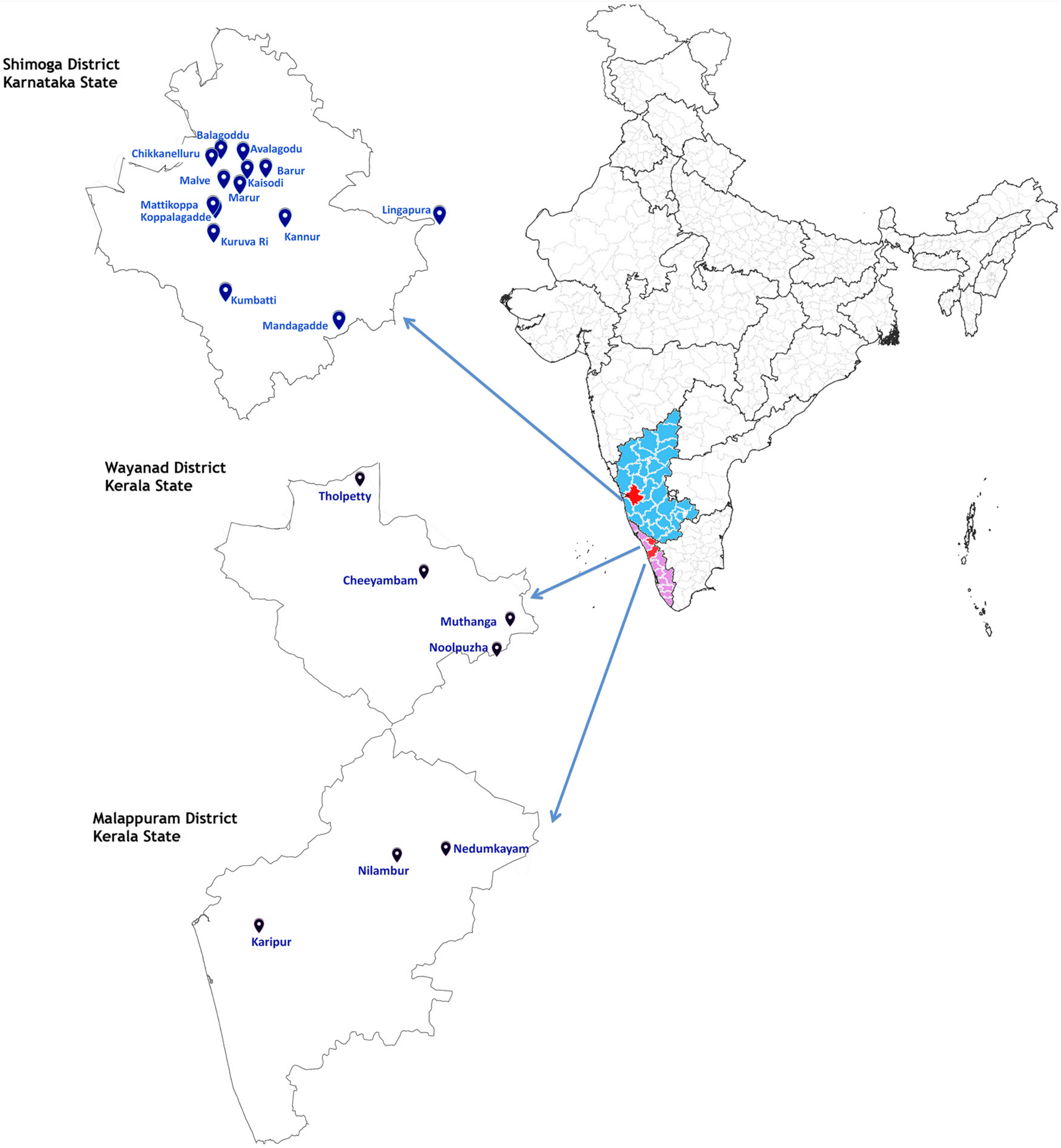

Fig. 1. Map of Karnataka state, India depicting the regions of KASDV isolation from ticks.

its name from the village where it was first isolated. During 1960–1964, twenty-eight isolates of KASDV were isolated from the tick pools collected from KFD affected area, depicted in Fig. 1 (Kaisodi, Koppalagadde, Marur, Malve, Balagodu, Mitlikoppa villages, Shimoga district, Karnataka State, India) (Bhatt et al., 1966). Later, during 1965–1970, KASDV was isolated from two more tick species, *Haemaphysalis turturis* (mammalian tick) and *Haemaphysalis wellingtoni* (bird ectoparasites) (Lingapura, Manddagadde, Kumbatti, Avalagodu, Kuruvari, Barur and chikkanellur villages near a small forest at Kuruvari, about 8 miles south of Sagar panchayat, Shimoga district) (Sreenivasan et al., 1973). Since *H. spinigera* is found to infect both mammals and birds, attempts were made to find its natural host. Some species of birds, rodents, monkeys, and cattle demonstrated the presence of KASDV neutralizing antibodies indicating infection with KASDV (Sreenivasan et al., 1973). Despite many KASDV isolations from tick samples, reports of KASDV isolation in the human serum sample is still not reported. It is important to mention that no specific survey has been carried out to understand the role of this virus in human infections.

A recent phylogenetic study of L segment of tick-borne phleboviruses (TBPVs) has demonstrated three clusters among this group of viruses including; the Severe Fever Thrombocytopenia Syndrome (SFTS) group, the Bhanja group (BHAV), and the Uukuniemi group (UUEV). Recently, a novel group named Kaisodi was proposed based on partial L segment analysis of various TBPVs and its recognition as a *Phlebovirus* (Matsuno et al., 2015). The Kaisodi serogroup consists of KASDV [India], Silverwater virus (SILV) [Canada], Lanjan virus (LJNV) [Malaysia] and Khasan virus (KHAV) [Russia] (Matsuno et al., 2015). Serologically also, SILV, LJNV, and KASDV are placed in the same group (Mahy, 2009). The glycoprotein segment was found to be more diverse than the S segment in *Phlebovirus* group. Among the S segment genes, N gene was found to be more conserved than the NSs whereas non-structural proteins show group-specificity suggesting their role in pathogenicity (Xu et al., 2007).

In the last few decades, some phleboviruses have been isolated in India from birds, ticks, bats, animals, and humans (Yadav et al., 2017). Some of these viruses like Sand-fly Naples virus, Sicilian-fly virus, Bhanja virus, and Palma virus are found to be associated with human and cattle diseases (Yadav et al., 2017). Recently in a proactive study, the novel Malsoor virus (MALV) was isolated from *Rousettus* species of bats from Maharashtra state, India (Mourya et al., 2014). MALV had the highest identity with SFTS virus. Human relevance of the KASDV virus is yet to be established (Shete et al., 2017). Since there is a continuous emergence of various phleboviruses from different parts of the world, it is likely that many of the phleboviruses are yet to be discovered and characterized in India. In this study, a prototype strain of KASDV (IG-14132) was sequenced using next-generation sequencing (NGS) method and characterized to understand its relationship with other phleboviruses. Indigenously developed real-time RT-PCR and RT-PCR assay was used for the screening of the ticks collected from KFD endemic areas of Kerala, Karnataka and Maharashtra state and found negative for KFDV. The positivity of the KASDV in ticks emphasized the need for screening of human and animal samples for a better understanding of virus infectivity in these hosts.

2. Materials and methods

2.1. Virus isolation: in-vivo and in-vitro method

Lyophilized vial of mice brain suspension of KASDV (G-14132) isolated from the hard tick, *Haemaphysalis spinigera*, in 1957 from Kannur, Karnataka state was used in this study. KASDV-infected mice brain was re-suspended in bovine serum albumin phosphate saline (pH: 7.4) (BAPS) and centrifuged at 4500 RPM (Thermofisher scientific, Model ST-40R) for 5 min; the supernatant was collected and used for inoculation. The group of infant mice [n = 8] was intracerebrally inoculated with 20 μl of the virus suspension per mouse. Post inoculation, mice were observed twice daily for any clinical signs. After experimental endpoints were reached which included no feeding and significantly reduced movement, they were euthanized using isoflurane (> 5%) followed by cervical dislocation. Their brains were harvested and 10% mice brain suspension was prepared in 1.25% BAPS. The virus stock was stored at −80 °C until further use. All the experiments carried out during this study were approved by Institutional Animal Ethics Committee (Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Registration No. 43/GO/C/1999/CPCSEA) and Institutional Biosafety Committee (IBSC).

The mouse brain virus stock was used for understanding the susceptibility of different cell types towards this virus. The monolayer of 70% confluent cells were infected with the virus stock and cytopathic effect (CPE) were monitored on mammalian cells of Baby Hamster Kidney-21 (BHK-21), Rhabdomyosarcoma (RD), Porcine kidney (PS), Vero CCL81 clone, indigenously developed *Pipistrellus* bat embryonic cell line (Mourya et al., 2013) and C6/36 cell line (arthropod *Aedes albopictus* cell line) till the 7th day after inoculation using phase contrast microscopy.

2.2. Complete genome sequencing of the KASDV using next-generation sequencing platform

Extraction of the RNA was performed from the mouse brain suspension of the KASDV using QIAmp Viral RNA extraction kit (Qiagen) as per the manufacturer's protocol with slight modifications. The concentration of extracted RNA was quantified by Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies) using Qubit RNA High Sensitivity (HS) kit and then stored at −80 °C until use. The host ribosomal RNA was depleted using NEBNext rRNA depletion kit (New England Biolabs). This RNA was further purified using Agencourt AMPure XP beads (Beckman Coulter). The depleted RNA was quantified with Qubit RNA High Sensitivity (HS) kit. The RNA library was prepared using the TruSeq Stranded mRNA LT Library preparation kit (Illumina) according to the manufacturer's instructions for the depleted RNA. The RNA library was quantified by using KAPA DNA library quantification kit (Kapa Biosystems) and normalized to 1.8 pM (picomolar). The normalized library was loaded on the Illumina Miniseq platform. After the completion of the run, FASTQ files were imported and analyzed using CLC Genomics Workbench software Version 10 (CLC, Qiagen).

RNA contigs were generated from paired-end reads, using the *de-novo* assembly program implemented in CLC Genomics Workbench. Virus identification from the RNA contigs was made using the BLAST program. BLASTx was used to identify putative protein function based on sequence homology. Once the virus was identified, reference mapping was done using the reference sequence from GenBank.

2.3. Molecular characterization and phylogenetic analysis of the KASDV

Different groups of *Phlebovirus* sequences for SILV, Huangpi Tick Virus 2 (HPTV), KHAV, UUEV, BHAV, and SFTSV were retrieved from the GenBank database for comparison. These sequences were further translated and used for comparing protein sequences. The reference nucleotide sequence downloaded from GenBank for characterizing KASDV are as follows: L segment: KF892046 (KHAV), NC_031138 (HPTV), KM114257 (SILV), NC_005214 (UUEV), NC_027140 (BHAV), NC_018136 (SFTSV); M segment: KF892047 (KHAV), NC_031139 (HPTV), KM114255 (SILV), NC_005220 (UUEV), NC_027141 (BHAV), NC_018138 (SFTSV); and S segment: KF892048 (KHAV), NC_031140 (HPTV), KM114256 (SILV), NC_005221 (UUEV), NC_027142 (BHAV), NC_018137 (SFTSV).

The characteristic RdRp domain was identified in the L segment by aligning the amino acid sequence of the reference sequences in CLC Genomics Workbench Version 10 (Qiagen). Presence of different N-glycosylation sites and the trans-membrane structure was predicted to characterize the M segment. Amino acid (aa) sequences for the glycoprotein precursor were analyzed using NetNGlyc11, version 1.0 (www.cbs.dtu.dk/services/NetNGlyc), to determine the presence of N-linked glycosylation sites (Gupta et al., 2004). Transmembrane domains were predicted using MEMSAT3 server (UCL) (Jones, 2007). Intergenic regions of S segment RNA for the different groups of the virus were aligned, and their secondary structure was predicted using the default setting of CLC Genomics Workbench Version 10 (Qiagen). Information on the 3D structure of the phleboviruses is only available for Rift Valley fever virus (RVFV), SFTS and Sand-fly fever Naples virus mostly for Gc and nucleocapsid protein (Berman et al., 2000), and are transmitted by mosquitoes, ticks, and sandflies respectively. 3D structures for some *Phlebovirus* are available for Gc and nucleocapsid in Protein Data Bank. Hence an attempt was made for predicting the 3D structure of Gc and nucleocapsid protein for KASDV and a closely related virus of Kaisodi serogroup using the online SWISS-MODEL workstation in an automated mode. For these two proteins, models were subjected to PROCHECK analyses at SWISS-MODEL structural assessment server (http://swissmodel.expasy.org/workspace/?func=%20tools_structureassessment1). Visualization of the protein structures and imagery was generated using the DS Visualizer. Surface electrostatics analyses were carried out using NOCH software (Shil et al., 2011). Recombination analysis was performed using the RDP4 software. The sequence was considered to be recombinant if recombination was predicted by four different algorithms implemented in RDP (Martin et al., 2015).

We have phylogenetically analyzed the large set of sequences i.e.182 for L segment, 171 for M segment and 170 for nucleocapsid gene, belonging to *Phlebovirus* genera. For performing phylogenetic analysis, complete sequences of various phleboviruses for all the three segments were retrieved from GenBank. These sequences were aligned using the ClustalW algorithm as implemented by MEGA version 7.0. A phylogenetic tree for the aligned nucleotide sequence was generated for L, M, and S (nucleocapsid gene) segments in MEGA using Maximum Likelihood method along with 1000 bootstrap replications (Kumar et al., 2016). General Time Reversible (GTR) along with gamma distribution and invariant site model has been used as the substitution model for the nucleotide sequence to obtain the tree.

2.4. Screening of tick samples for detection of KASDV using real-time RT-PCR and RT-PCR based assay

Real-time RT-PCR and RT-PCR assays specific to nucleocapsid (N) gene were standardized to detect Kaisodi viral RNA. The primers were designed from the KASDV sequence obtained through NGS. The N gene (genomic location 1–789 region) of the S segment sequence was used to design primers for real-time RT-PCR of 154 bp and RT-PCR assay of 309 bp. Real-time RT-PCR was performed using SuperScript III Platinum One-Step qRT-PCR kit (Invitrogen) and primers (Forward: (nucleotide (nt) 298) 5′-CGCTCCAGGAGCTAAGAAAGG-3′(nt 319), Reverse: (nt 429)5′-GCACATCAGCTACCAAGTGTAGC-3′(nt452) and, probe: (nt 388) 5′-CATGGACGTTGGCCACTCTGG-3′(nt 409)). The probe was labeled with 6-carboxyfluorescein (FAM) at 5′ end and Black Hole Quencher 1, 3′ (BHQ1) quencher at 3′ end. The real-time RT-PCR conditions were: 50 °C for 30 min; 95 °C for 2 min and 40 cycles of 95 °C for 15 s; 50 °C for 1 min. RT-PCR based analysis was performed using primers (Forward: (nt 121) 5′-TGGTGGAGCTCTATGCCTACCAG-3′(nt 144) and Reverse: (nt 429) 5′-GCACATCAGCTACCAAGTGTAGC-3′(nt 452)) and specified cycling conditions (50 °C for 30 min; 94 °C for 2 min and 35 cycles of 94 °C for 30 s; 52 °C for 30 s; 68 °C for 30 s). KASDV derived from KASDV infected *PS* cells was 10-fold serially diluted and used to determine the sensitivity of both assays.

Testing assessed the specificity of both the assays with viral RNAs of endemic/reported tick-borne viruses of India (Crimean Congo Hemorrhagic Fever (CCHF), Malsoor, Thottapalayam, Catque and Kyasanur Forest Disease (KFD) viruses). Cross-reactivity was also checked for control mouse brain suspension, tissue culture fluids of Vero CCL-81, Vero E6, BHK-21 and PS cells, three negative control human serum samples and KFD and CCHF virus negative tick samples.

A total of 577 tick pools samples belonging to *Haemaphysalis* sp. (*H. spinigera and H. turtusis*) were tested to see the presence of Kyasanur Forest Disease virus (KFDV). The ticks were collected from different districts of Kerala (73 from Malappuram district, 232 from Wayanad district), Karnataka (51 from Shimoga District), and Maharashtra (221 from Sindhudurg district) states of India during the year 2013–2017 (Fig. 1). RNA samples from these ticks were tested for the presence of KFDV as described previously and negative samples were frozen and stored at −80 °C (Mourya et al., 2012). The samples that were negative for KFDV were tested for the presence of Kaisodi viral RNA by real-time RT-PCR. The samples were first screened using a real-time RT-PCR assay. Subsequently, the real-time RT-PCR positive samples were confirmed using RT-PCR assay and amplicons sequencing. The amplified RT-PCR products were sequenced, and sequences were deposited in GenBank.

3. Results

3.1. Virus isolation in mice and susceptibility of vertebrate and invertebrate cell lines to the KASDV

No sickness in the infant mice inoculated with KASDV suspension was observed till 4th day post-infection (PID). On 5th PID, a slight abdominal swelling was observed in 2 mice, while food consumption was partially reduced in all infected mice. On 6th PID, mice were found to be lethargic with reduced mobility. The food consumption was significantly reduced. On 7th PID, severe sickness was noticed; abdominal swelling and eventually the food intake was completely stopped by the mice. Mice were euthanized, and their brains were harvested and used for viral RNA extraction.

In the cell culture susceptibility experiments only PS cells demonstrated slight changes in cells morphology, from 4th PID onwards while the rest of the cells showed no cytopathic effect (CPE). Typically, these included rounding of cells, shrinking and progressive cell detachment.

3.2. Complete genome (S, M and L segments) sequencing of the KASDV using next-generation sequencing platform

The preliminary RNA data obtained for KASDV was analyzed using CLC genomic workbench (Qiagen). Contiguous sequences (Contigs) were assembled using a *de novo* assembly program with the paired-end reads for RNA. RNA reads gave 8184 contigs with an average length of 495nucleotides. The N75 and N50 for RNA were 382 and 471 respectively. These RNA contigs sequences were analyzed using the BLAST program and showed higher homology to *Phleboviruses* genus in an initial search. Three-segment of the *Phlebovirus* genome; L segment encoding RNA polymerase (6475 nt), M segment encoding Glycoprotein precursor (3232 nt) and S segment encoding nucleoprotein (1767 nt) were retrieved. RNA polymerase (L segment) and glycoprotein precursor (M segment) were of 2138 and 1011 aa in length respectively. The retrieved nucleoprotein segment retrieved encoded two proteins, nucleocapsid protein and non-structural protein of lengths 247 and 277 aa respectively. These sequences were deposited in the GenBank database, and their accession numbers are MG581739 (L segment), MG581740 (M segment), and MG581741 (S segment).

RNA dependent RNA polymerases have conserved motifs that are required to carry out the process of replication and transcription. These motifs may be identical or to some extent dissimilar in their amino acid sequences around the motif. We have analyzed these characteristic motifs to identify the presence of unique RdRp motifs in the *Phlebovirus* group. Characteristic signature feature for RdRp (L segment) of the *Bunyaviridae* family was identified with few differences in the analyzed phleboviruses group. In Kaisodi group viruses, the sequence DxxKWS in motif A is replaced by DAxTWN, where lysine is replaced by threonine while in case of Uukuniemi and the SFTS reference sequences lysine is present at that position. Similarly, in Kaisodi group of viruses, motif B has the amino acid sequence (^{1095}QGILHFTSS^{1103}) whereas in Uukuniemi virus the motif B has the sequence (QGxxxYxSS). Rest of the motif C (SDD), motif D (K) and motif E (EFxSE) remained the same as depicted in Fig. 2.

Polyprotein encoded by the M segment of the KASDV is cleaved into two proteins, the Gc and Gn, similar to UUEV or SFTS virus (Palacios et al., 2013). Fig. 3 depicts the different glycosylation sites and the predicted trans-membrane region for the reference group of viruses. MEMSAT3 predicted three trans-membrane helical domains, which is in agreement with the previously published literature (Palacios et al., 2013). However, for the KASDV, a four-helical trans-membrane domain was predicted indicating the difference in the tertiary structure as compared to other reference sequences. It is observed that KASDV along with its serogroup has little variation in the predicted glycosylation pattern and a similar pattern is observed in other reference protein (Palacios et al., 2013).

Amino acids required for the RNA binding motif, i.e., R66, K69, and K76 were also present in the nucleocapsid protein of KASDV, as described in previous work (Matsuno et al., 2015; Ferron et al., 2011). Aligning of nucleocapsid sequences with reference sequences in the study demonstrated that tick-borne *Phlebovirus* had a conserved motif (^{72}TRGNK^{76}) around the RNA binding motif region. We further analyzed the non-coding regions between the S segments for the Kaisodi group along with the reference sequences of the BHAV virus, UUEV, and SFTS virus. We observed that the intergenic region encompassing S segment of Kaisodi serogroup are GC rich whereas the other *Phlebovirus* used in the analysis have AT-rich genome and have the largest intergenic region (Fig. 4). It is reported that intergenic regions of UUEV viruses are capable of forming a secondary hairpin-like structure (Giorgi et al., 1991). Secondary structure prediction for the intergenic region within the S segment also demonstrated the potential of forming a hairpin-like structure, suggesting the similar mechanism of transcription termination amongst Kaisodi serogroup. An interesting feature observed in Kaisodi serogroup viruses is that they have larger loops as compared to the other phleboviruses analyzed in the study, depicted in

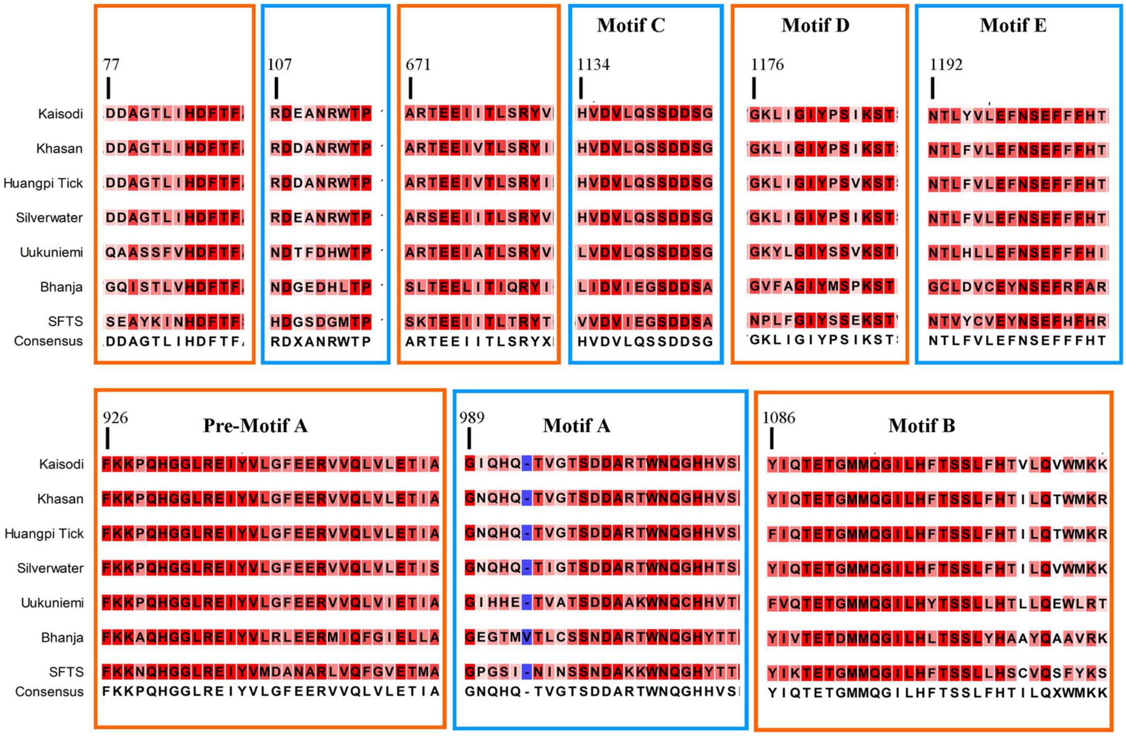

Fig. 2. Comparison of KASDV L segment amino acids with different phleboviruses.

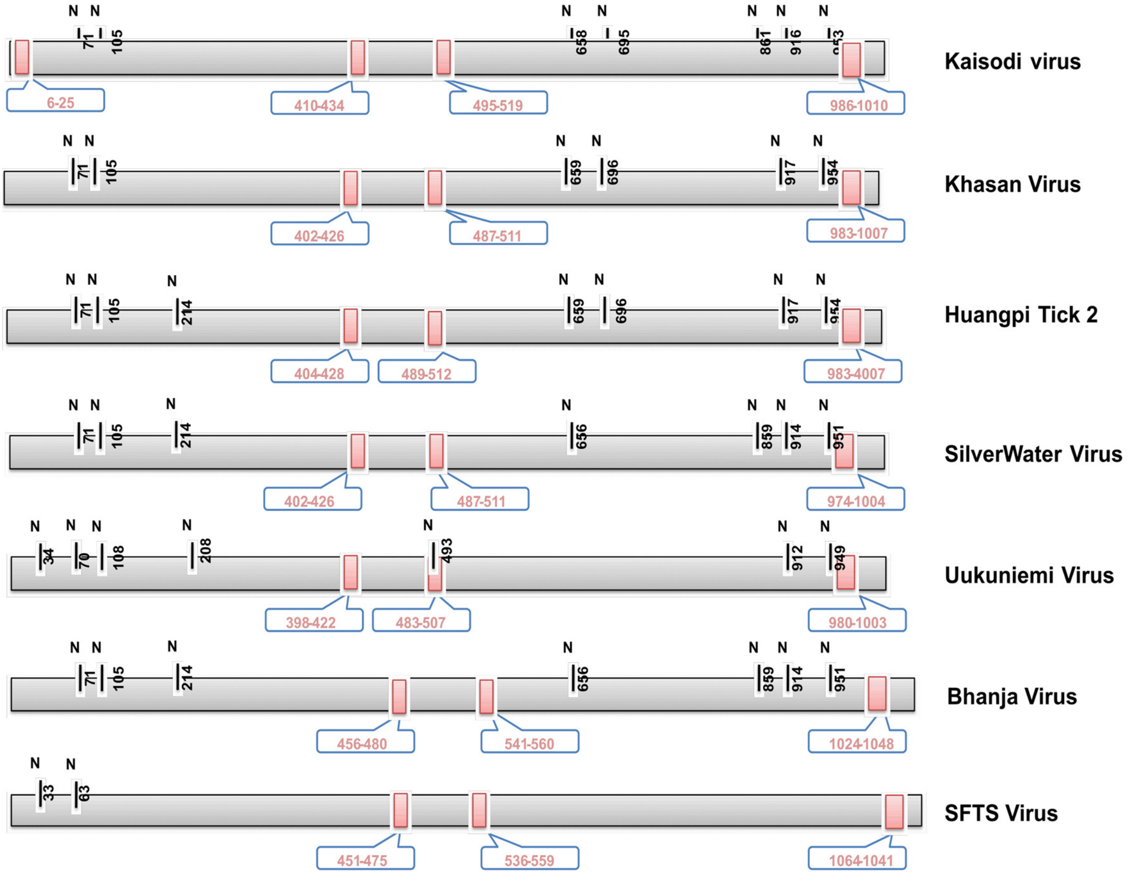

Fig. 3. Analysis of KASDV glycoprotein using different methods: Different positions of N-linked Glycosylation predicted using NETGLy 1.0 server are marked as N. MEMSAT3 server gave trans-membrane models depicted as a filled rectangle in the figure.

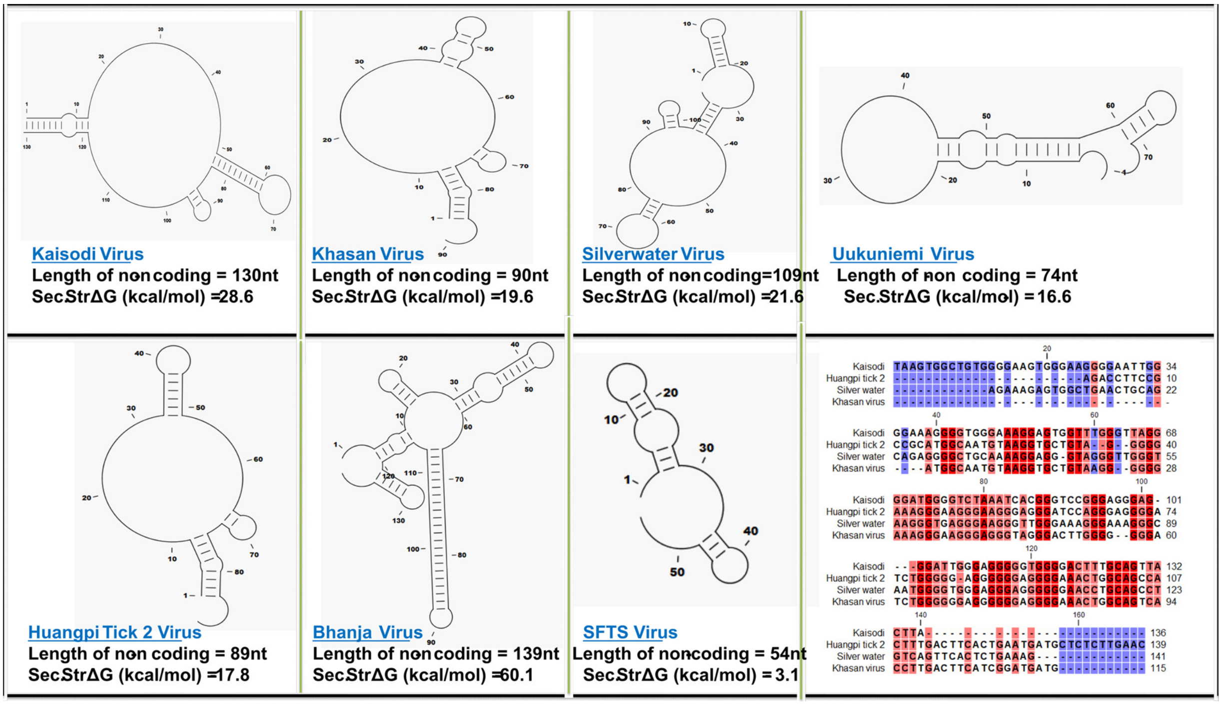

Fig. 4. Analysis of S segment for prediction of secondary structure for phlebovirus's non-coding region and its conserved sequence.

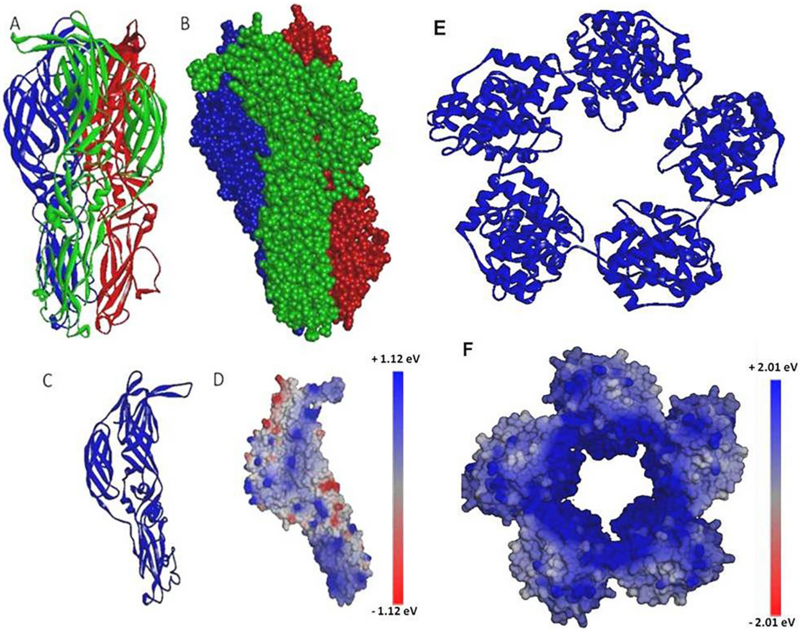

Fig. 5. 3D structure for Gc protein and nucleoprotein of KASDV: A) Predicted 3D structure of KASDV Gc protein trimer in Ribbon mode display: A-chain in blue, B-chain in Red and C-chain in Green. B) Gc-protein trimmer displayed with space fill mode. C) Fold of the A-chain secondary structures. D) Surface electrostatics of A-chain from NOCH software (Electrostatic potential range:-1.15 eV to + 1.15 eV). E) Multimeric form of KASDV nucleoprotein. F) Surface electrostatics as of KASDV obtained from NOCH software. Nucleoprotein in multimeric form (Electrostatic potential range:-2.01 eV to + 2.01 eV) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Fig. 4, suggested that stem-loop un-stability due to larger loops was a possible factor for transcription termination. However, detailed, experimental studies need to be carried out in future to concretize the above hypothesis.

The 3D structure of Gc protein and nucleoprotein of KASDV was obtained from the SWISSMODEL workstation (automated mode) using the known structure of RVFV Gc-protein (6EGU.pdb) and for nucleocapsid protein (4H5O.pdb) as template respectively. The template-target identity of AA composition was 27.5% and 36.9% for Gc and nucleocapsid protein, respectively. Ramachandran plot analyses of the predicted 3D structure revealed an occupancy of 98.5% of AA in the allowed regions and 1.5% in the generously allowed regions indicating that both the prediction are valid. The minimized energy of the structure (trimer) as obtained from SPDBV was −67663.89 kJ/mol, and for the nucleocapsid monomeric structure, it was −12444.8 kJ/mol. The structure for Gc was visualized as trimer as well as monomer depicted in Fig. 5(A–D) and nucleocapsid as multimer illustrated in Fig. 5(E–F). Similarly, the 3D structure for the Gc and nucleoproteins of the following viruses were also predicted: HPTV, KHAV, and SILV using the template mentioned above depicted in Fig. 6(A–B). The minimized energies for the predicted structures belonging to HPTV, KHAV, and SILV were found to be −67601.2 kJ/mol, −66134.9 kJ/mol and −66031.3 kJ/mol respectively, for Gc protein and 12,078.95 kJ/mol, −12406.68 kJ/mol and −10658.1 kJ/mol respectively for nucleocapsid protein.

For all these predictions, the Ramachandran plot analyses revealed

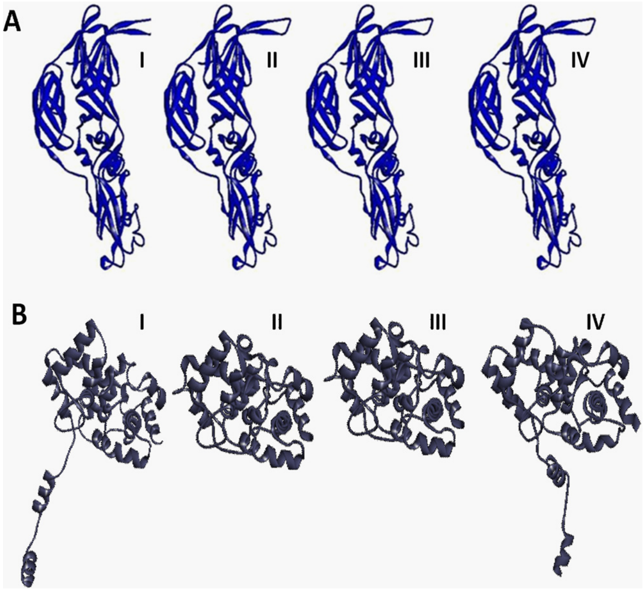

Fig. 6. Predicted 3D structures of Gc Protein and Nucleoprotein monomer for different phleboviruses: Predicted 3D structures of (A) Gc Protein and (B) Nucleoprotein monomers for I) KASDV, II) HPTV, III) KHAV and IV) SILV.

occupancy of > 98.5% (allowed + generously allowed regions). Structural comparison of KASDV Gc protein (A-chains) and KASDV nucleoprotein monomer with that of HPTV, KHAV and SILV was conducted by superimposition method (backbone atoms) as implemented in SPDBV and evaluated based on the root mean squared distance (RMSD) values. The RMSD values for KASDV vs. HPTV, KASDV vs. KHAV and KASDV vs. SILV were found to be 0.4 Å, 0.59 Å and 0.54 Å respectively and for nucleocapsid protein was 0.81 Å, 0.78 Å and 1.43 Å respectively. Also, the RMSD values for HPTV vs. KHAV and HPTV vs. SILV were 0.24 Å and 1.8 Å while that of KHAV vs. SILV was 1.8 Å for Gc protein. Also, the RMSD values predicted nucleocapsid protein for HPTV vs. KHAV and HPTV vs. SILV were 0.54 Å and 0.57 Å while that of KHAV vs. SILV was 0.41 Å.

The nucleotide-based phylogenetic tree was generated for the three segments L, M and S using MEGA 7.0 software (Mourya et al., 2012). Our observation was similar to the suggestion made by Matsuno et al., group (Matsuno et al., 2015), in which KASDV formed a small cluster with other phleboviruses similar to its lineage. We observed that L, M and S segments for KHAV, SILV and HPTV formed a separate cluster along with KASDV, which was different from UUEV group depicted in Fig. 7(A–C). We also observed that tick-borne viruses (UUEV and KASDV group) formed a cluster with sandfly-borne viruses and not with other tick-borne viruses (BHAV and SFTS group), which indicates a recent common ancestor between them. Table 1 shows the overall nucleotide identity between different groups concerning the KASDV, which also supports the above result. Table 2 depicts the nucleotide, and amino acid identity observed within the Kaisodi serogroup used in this study. We did not observe any recombination for the three genomic segments of KASDV.

3.3. *Screening of tick samples for detection of KASDV using real-time RT-PCR and RT-PCR based assay*

Both the assays were found to be equally sensitive and could detect up to 10^{-8} dilutions of the virus stock derived from KASDV infected *PS* cells. In case of real-time RT-PCR, a Ct value of 36 was considered to be cut-off based on assay standardization. In the case of RT-PCR, a product of 308 bp of nucleotide was obtained, which was further confirmed by sequencing.

Both the real-time RT-PCR and RT-PCR assays were found to be specific for KASDV upon testing with viral RNAs of CCHF, Malsoor, Thottapalayam, Catque and KFD viruses, extracted RNA from virus negative mouse brain suspension, tissue culture fluids from VeroCCL81, VeroE6, BHK21 and PS cells, three negative human serum samples, and three KFDV/CCHFV negative tick samples.

Real-time RT-PCR was performed for all the 577 tick samples of which 221 samples from Sindhudurg district, Maharashtra were found to be negative for KASDV. Eight of 73 samples from Malappuram district (Karipur, Nilambur, and Nedumkayam villages) and 15 out of 232 from Wayanad district (Tholpetty, Cheeyambam, Muthanga, and Noolpulzha villages) of Kerala state were found to be positive by real-time RT-PCR. There was no positivity found in 51 tick samples screened from Karnataka. All the 23 real-time RT-PCR positive tick samples were further analyzed using RT-PCR followed by agarose gel electrophoresis and sequencing. Out of 23 samples tested, though amplicons of a specific size (308 bp) were seen in all samples, the sequence could be obtained from only four samples tested positive, and their sequences were obtained using the Sanger sequencing method. Larger positivity was observed in RT-PCR compared to real-time PCR since real-time PCR is more sensitive than developed RT-PCR for this virus. The pairwise analysis of the sequences identified them to be KASDV. The accession numbers for the obtained Kaisodi sequences from ticks are from MG982665 - MG982668.

4. Discussion

Arthropod-borne infections are of major concern worldwide and India being a tropical country, it has the same impact of arthropod-borne infections like other countries. The climatic changes along with the increased urbanization, emergence, and re-emergence of have led to increased prevalence of arboviral diseases in India spread by arboviruses such as Dengue (Flavivirus), Chandipura (Rhabdovirus), Chikungunya (Alphavirus), Crimean-Congo Hemorrhagic Fever (CCHF) (Orthonairovirus), and KFD (Flavivirus). The increase in tick-borne infections is directly associated with deforestation. The tick-borne diseases are spreading to newer areas at an alarming rate, and clear evidence is seen from detection of KFD virus in five states and deadly disease CCHF in three states of India (Chaubal et al., 2018; Roy et al., 2016). Arthropod-borne infections are more prevalent during tropical months than other months, which has highlighted the need for increased surveillance of vector-borne infections in India. In this study, we have characterized one such tick-borne virus KASDV on the basis of its full genome sequence. KASDV has been isolated from *Haemophysalis* ticks earlier from the KFD endemic areas of Karnataka state. Later on, detailed studies were focused on KFD virus, but not much work was done on the KASDV to know if this virus is present in newer areas of KFD endemic states.

Detection of KASDV, from *Haemaphysalis* spp., were incidentally from the same vector as Kyasanur Forest Disease virus which suggested a probable co-existence and co-transmission of both the viruses in Kerala state, India. The absence of KASDV in tick samples from Karnataka and Maharashtra state doesn't mean that this virus has disappeared in these states and may otherwise indicate inadequate sample size. The prevalence of *Haemaphysalis* spp. in Maharashtra and Karnataka states suggest that the KASDV may be prevalent in these states. Therefore, further surveillance of tick samples would be required for a better understanding of the transmission pattern of KASDV.

Arthropod-borne bunyaviruses show distinct cellular tropism and cellular receptor-based entry into host cells (Albornoz et al., 2016). Antibodies against Silverwater virus have been detected from hare sera and human sera in 1961 (McLean and Larke, 1963), whereas Khasan virus was isolated from *Haemophysialis* ticks from a nursery of spotted deer and is not known to cause any disease in humans or animals (Beran and Steele, 1994). Though no CPE was observed in cell cultures (Vero CCL81, Vero E6, Bat embryo, and BHK cell lines), but the possibility of virus multiplication without causing CPE can't be denied, suggesting its

Fig. 7. Phylogenetic tree of S, M and L gene for KASDV in comparison to other phlebovirus: A: Phylogenetic tree of S segment nucleocapsid gene: A discrete gamma distribution model along with (+G, parameter = 1.6367 and [+I] was used to model the rate of evolutionary difference among 170 sequences for nucleocapsid gene belonging to S segment. Robustness of the tree was evaluated using a bootstrap method involving 1000 re-sampling. B: Phylogenetic tree of M segment glycoprotein precursor gene: A discrete gamma distribution model along with (+G, parameter = 2.4323 and + I, parameter = 2.09%] was used to model the rate of evolutionary difference among 171 sequences for glycoprotein precursor gene belonging to M segment. Robustness of the tree was evaluated using a bootstrap method involving 1000 re-sampling. C: Phylogenetic tree of L segment precursor gene: A discrete gamma distribution model along with (+G, parameter = 1.4034 and + I, parameter = 5.77%] was used to model the rate of evolutionary difference among 182 sequences for RdRp gene belonging to L segment. Robustness of the tree was evaluated using a bootstrap method involving 1000 re-sampling.

potential to infect animals. The possibility of virus multiplication without causing CPE raises a need for the KASDV specific surveys from clinical and field samples. Hence we have developed real-time RT-PCR, and RT-PCR based molecular diagnostic assays for the detection of KASDV. Development of real-time RT-PCR and RT-PCR would help in conducting KASDV specific surveys in animals and humans, thereby understanding the possible viremic and antibody profile of this virus in animals and humans.

The KASDV L segment showed some typical amino acid conservation in comparison to other representative members of *Phlebovirus* genus, e.g., a conserved motif of DARTWN as opposed to the DxxKWS in the motif-A present on RdRp protein, reported in the previous publication (Matsuno et al., 2013). Similarly, the motif-B present on RdRp protein had a conserved sequence ^{1095}QGILHFTSS^{1103} instead of aa motif QGxxxYxSS (Matsuno et al., 2013). The glycosylation pattern (N^{71}, N^{105}, N^{658}, N^{695}, N^{861}, N^{916} and N^{953}) and the predicted transmembrane domains were similar to the other phleboviruses showing a conserved nature of this genus (Matsuno et al., 2013).

The amino acids R66, K69, and K76 required for RNA binding motif were also identified in the nucleocapsid gene. Comparable similarities

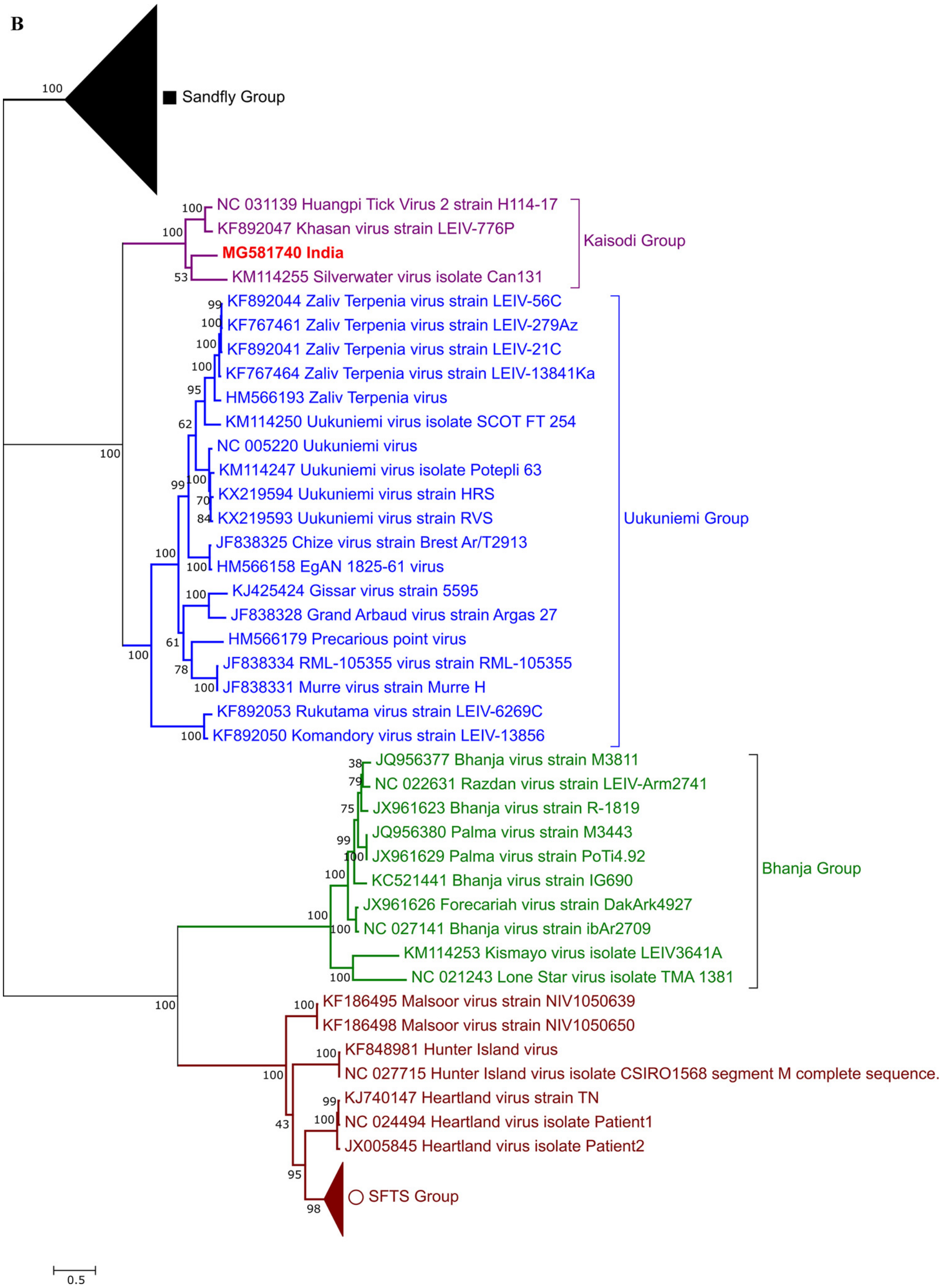

Fig. 7. (*continued*)

to reference KHAV, SILV, UUEV, BHAV, and SFTS virus was observed for RdRp, the trans-membrane domain of the glycoprotein, its glycosylation pattern and conserved residues on the nucleocapsid protein. This data suggest that KASDV has replication and RNA packaging strategies similar to other phleboviruses. Further, it is also observed that the amino acid identity for N gene is higher (66–70%) than for L and M segments (50–54%). N proteins help in the encapsidation process and change at amino acid levels has been reported to alter the replication and transcription of the assembled new virions (Walter et al., 2011). The presence of higher conservation of N gene may be associated to the role of N proteins which is also observed in for all the members of phleboviruses.

Dunn et al. observed that two conserved sequences exist in the S segment proximal to the termination site of orthobunyaviruses (Blakqori et al., 2012; Dunn et al., 1994). Further conserved motifs were observed at the 5′ UTR of Bunyamwera virus required for transcription termination (Barr et al., 2006; Blakqori et al., 2012;). Blakqori et al. studied the transcription termination in the Bunyamwera virus and proposed that the transcription terminal can be the same for two different genera of the virus (Blakqori et al., 2012). In this study, we observed the presence of the guanine-rich central region flanked on either side by two conserved motifs regions in KASD serogroup. The left side has region 1 (3′-ANGGGC-5′) and towards the right region 2 (3′-CACTTA/TGA/T-5′). Role of RNA hairpin structure in transcription termination has been experimentally demonstrated by Barr in the 3′UTR region of S segment of bunyaviruses (Barr, 2007). Secondary structure analysis for the intergenic region between the S segments demonstrated the presence of a loop, which is larger in the case of KASDV as compared to its other serogroup member. Presence of larger loop in the intergenic region of S segment led us to the hypothesis that the instability caused by this loop may influence transcription termination in case of KASDV. However, future experimental studies need to

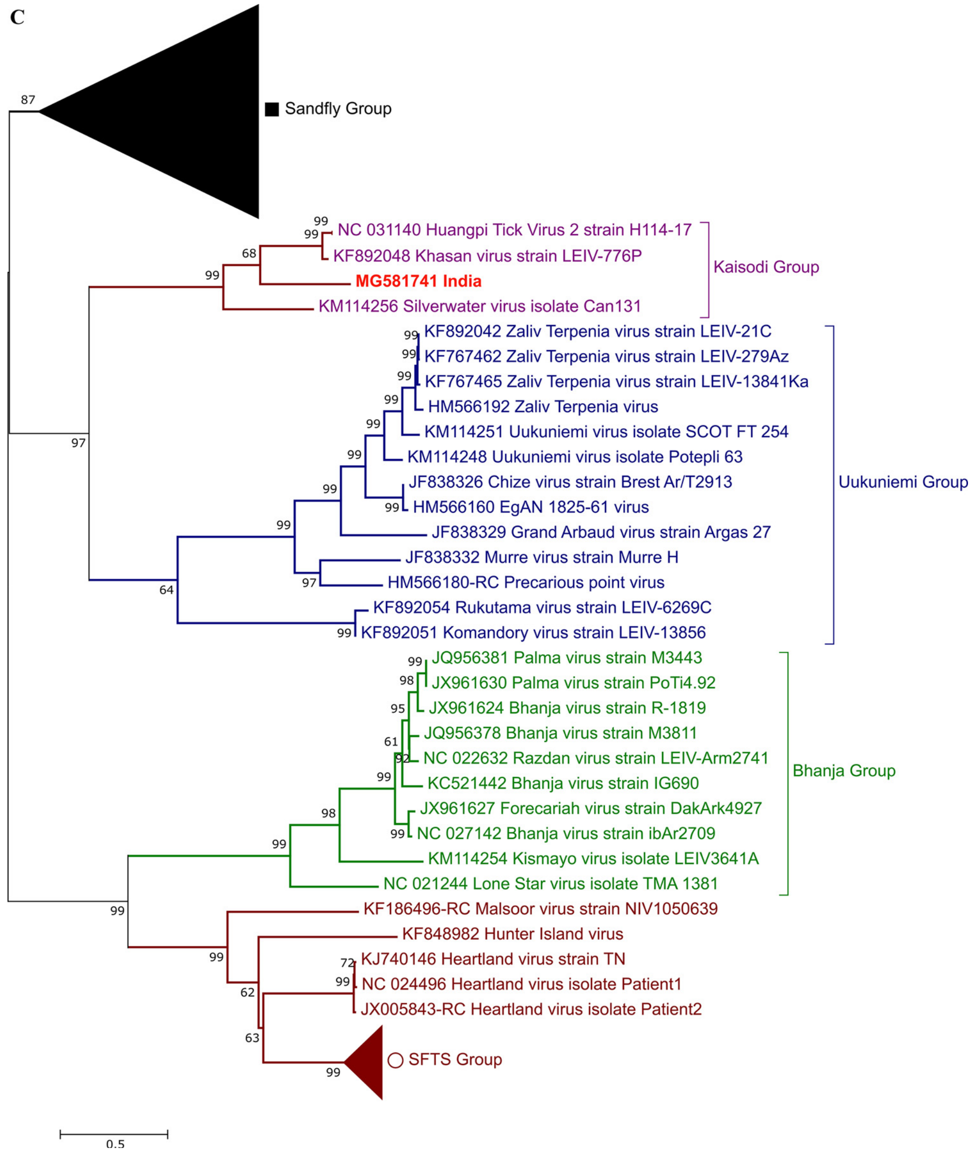

Fig. 7. (*continued*)

be performed to prove this possible mechanism.

Analysis of KASDV sequences with other phleboviruses shows that Kaisodi serogroup and UUEV group clustered along with the sand-fly-borne virus, thus suggests that there might be some common ancestor interconnecting the two different groups together.

To understand the structural fold of the proteins, 3D structure predictions were performed using homology-modeling techniques. The structures for Gc and nucleoprotein were predicted using the only available templates of RVFV among phleboviruses. For the KASDV Gc protein and KASDV nucleoprotein, amino acid identity with the template (RVFV Gc protein, 6EGU.pdb, and RVFV, 4HO.pdb) was ∼27% and ∼37% respectively. As evident from the structural comparison, the 3D conformation of Gc protein and nucleoprotein of KASDV is very similar to that of SILV (RMSD ∼ 0.54 Å) and SILV (RMSD ∼ 1.43 Å) respectively. The analyses are based on predictions, and therefore, have limitations and approximations, but the identity predicted cannot be denied. Only the experimental determination of protein structures can untangle the real properties and functional aspects of the proteins.

Tick-borne viruses are known to cause outbreaks of zoonotic infections worldwide. Complete genome sequencing and phylogenetic

Table 1
Percent nucleotide and amino acid identity of KASDV with different groups of the phlebovirus genus.

	Nucleotide Identity (%) for L, M and S segment			Amino acid Identity (%)for L, M and S segment		
Groups name of phlebovirus genus	L segment	M segment	N gene (nucleocapsid)	L segment	M segment	N gene (nucleocapsid)
Kaisodi	69–70	62–64	61–67	50–52	51–54	66–70
Uukuniemi	52–54	45–47	48–52	32–36	30–35	37–41
Bhanja	41–42	33–34	44–47	21–23	16–19	26–29
SFTS	43–45	33–37	43–48	22–26	14–21	28–40
Sand-fly	43–45	34–45	49–53	23–28	18–34	26–45

Table 2
Percent nucleotide and amino acid identity of KASDV with another member of Kaisodi serogroup.

Percent Identity of KASDV within Kaisodi serogroup for L, M and S segment						
	L segment (MG581739)		M segment (MG581740)		N* gene (MG581741)	
Virus details	Nucleotide (%)	AA (%)	Nucleotide (%)	AA (%)	Nucleotide (%)	AA (%)
Khasan virus	Not Available	Not Available	64.29	54.34	66.94	69.96
Silverwater virus	69.56	52.38	62.46	51.35	61.38	66.26
Huangpi tick virus 2	70.65	51.99	64.65	53.56	66.62	70.25

* N gene [Only nucleocapsid gene was compared not the entire S segment].

analysis of KASDV would facilitate in developing diagnostic tests so that potential infections in human and animal can be identified. The present communication puts forward a complete sequence for a novel phleboviruses, i.e., KASDV, its comparison with other available phleboviruses, and molecular diagnostic assays for the detection of KASDV, thereby paving the way for performing in-depth field study to know its relevance in animal and human populations.

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