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Genomic Characterization of Yogue, Kasokero, Issyk-Kul, Keterah, Gossas, and Thiafora Viruses: Nairoviruses Naturally Infecting Bats, Shrews, and Ticks

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Abstract. The genus *Nairovirus* of arthropod-borne bunyaviruses includes the important emerging human pathogen, Crimean–Congo hemorrhagic fever virus (CCHFV), as well as Nairobi sheep disease virus and many other poorly described viruses isolated from mammals, birds, and ticks. Here, we report genome sequence analysis of six nairoviruses: Thiafora virus (TFAV) that was isolated from a shrew in Senegal; Yogue (YOGV), Kasokero (KKOV), and Gossas (GOSV) viruses isolated from bats in Senegal and Uganda; Issyk-Kul virus (IKV) isolated from bats in Kyrgyzstan; and Keterah virus (KTRV) isolated from ticks infesting a bat in Malaysia. The S, M, and L genome segments of each virus were found to encode proteins corresponding to the nucleoprotein, polyglycoprotein, and polymerase protein of CCHFV. However, as observed in Leopards Hill virus (LPHV) and Erve virus (ERVV), polyglycoproteins encoded in the M segment lack sequences encoding the double-membrane-spanning CCHFV NSm protein. Amino acid sequence identities, complement-fixation tests, and phylogenetic analysis indicated that these viruses cluster into three groups comprising KKOV, YOGV, and LPHV from bats of the suborder Yingochiroptera; KTRV, IKV, and GOSV from bats of the suborder Yangochiroptera; and TFAV and ERVV from shrews (Soricomorpha: Soricidae). This reflects clade-specific host and vector associations that extend across the genus.

INTRODUCTION

Nairoviruses are arthropod-borne bunyaviruses that are transmitted primarily by ticks and some of which are important pathogens of humans and livestock. Crimean–Congo hemorrhagic fever virus (CCHFV) causes an emerging disease of humans with reported case fatality rates of 3–30%, typically associated with hemorrhage, shock, and multiorgan system failure within 2 weeks of the onset of symptoms.[1,2] CCHFV has a distribution that includes parts of Africa, the Middle East, eastern Europe, and Asia, and has been identified as an agent of significant public health concern.[2,3] Nairobi sheep disease virus (NSDV) causes hemorrhagic gastroenteritis in sheep and goats with mortality rates of up to 90%.[4,5] Together with Ganjam virus, which is considered to be a strain of the same virus, NSDV has a distribution that includes central and east Africa as well as South Asia and China.[6,7] At least 50 other nairoviruses have been isolated from ticks or vertebrate hosts and some have been associated with symptoms of disease in humans including fever, headache, and neurological disorders.[8] Many of these viruses remain poorly characterized.

The genus *Nairovirus* (family *Bunyaviridae*) presently comprises seven species (CCHFV, *Dugbe virus*, *Thiafora virus* [TFAV], *Qalyub virus*, *Hughes virus*, *Sakhalin virus*, and *Dera Ghazi Khan virus*) to which 35 viruses have been assigned on the basis of antigenic cross-reactions.[8,9] All nairoviruses within each of the corresponding serogroups are considered to be the same species.[10] Like other viruses in the family *Bunyaviridae*, nairoviruses have enveloped spherical virions and a negative-sense single-stranded RNA genome comprising three segments.[10] The L segment encodes a large protein (~450 kDa) with multiple highly conserved domains associated with replication and transcription, including the RNA-dependent RNA polymerase (RdRp), an ovarian-tumor (OTU)-like cysteine protease domain, and a zinc-finger domain.[11–13] The nairovirus M segment encodes a multiple-membrane-spanning polyglycoprotein that is processed by host peptidases to generate the mature envelope glycoproteins (Gn and Gc), a mucin-like protein, and other potential products.[14–17] The S segment encodes the viral nucleoprotein (N), subunits of which are organized in a head-to-tail manner to encapsidate the viral genome.[18] In CCHFV, the N protein has also been shown to have DNA endonuclease activity[19] and a conserved sequence motif that is characteristic of catalytic motif II of N6-adenine-specific DNA methylases.[8] The functions of these domains during infection are presently unclear.

In this article, we report the complete genome sequences of six nairoviruses that were isolated from bats and a shrew in Africa, a bat in Central Asia, and ticks feeding on a bat in Southeast Asia. We show that they share a distinctive genome organization that lacks sequences encoding an NSm protein and they cluster phylogenetically according to vertebrate host to form distinct sub-clades within the genus.

MATERIALS AND METHODS

Description of viruses. The identification; current taxonomic assignment; date, place, and source of isolation; disease association; and Genbank numbers of the viruses included in this study are shown in Table 1. TFAV from Senegal is serologically related to the previously characterized and sequenced Erve virus (ERVV) from France and each was isolated from a shrew (*Crocidura* spp.).[20–22] TFAV and ERVV were previously assigned to the species *Thiafora virus*.[23] Yogue virus (YOGV) and Kasokero virus (KKOV) are unclassified nairoviruses isolated from Egyptian fruit bats (*Rousettus*

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Table 1
Viruses used in this study

Virus	Strain	Species	Serogroup	Date of isolation	Place of isolation	Species of isolation	Other	Genbank
Crimean–Congo hemorrhagic fever (CCHFV)	IbAr10200	*Crimean–Congo hemorrhagic fever virus*	CCHF	11.05.1966	Sokoto, Nigeria	*Hyalomma excavatum* (ticks) collected from a camel	Severe hemorrhagic fever in humans	NC_005300 NC_005301 NC_005303
Thiafora (TFAV)	AnD 11411	*Thiafora virus*	Thiafora	17.2.1971	Bandia, Senegal	*Crocidura* sp. (shrew)		KR537450 KR537451 KR537452
Erve (ERVV)	Brest/An221	*Thiafora virus*	Thiafora	5.5.1982	Saulges, Mayenne, France	*Crocidura russula* (white-toed shrew)	Human infections—associated with headache	JF911697 JF911698 JF911699
Keterah (KTRV)	P61361	Not assigned	Keterah	11.2.1966	Keterah, Kelantan, Malaysia	*Argae pusillus* (tick) from *Scotophilus kuhlii* (*temmincki*) (lesser Asian yellow house bat)		KR537447 KR537448 KR537449
Issyk-Kul (IKV)	LEIV-315K	Not assigned	Keterah	15.5.1970	Dzety Oguzsk Region, Kyrgyzstan	*Nyctalus noctula* (common noctule bat)	Fever in humans	KR537441 KR537442 KR537443
	LEZ 86-787			1986	Germany	*Argus vespertlionis* (ticks)		
Gossas (GOSV)	DakAnD 401	Not assigned	Keterah	19.11.1964	Gossas, Senegal	*Tadarida* sp. (bat)	Formerly classified as a possible rhabdovirus	KR534876 KR534877 KR534878
Yogue (YOGV)	DakAnD 56	Not assigned	Yogue	19.6.1968	Bandia, Senegal	*Rousettus aegyptiacus* (Egyptian fruit bat)		KR537453 KR537454 KR537455
Kasokero (KKOV)	Z-52963 and Z-52969	Not assigned	Yogue	XX.8.1977	Masaka District, Uganda	*Rousettus aegyptiacus* (Egyptian fruit bat)	Human laboratory cases	KR537444 KR537445 KR537446
Leopards Hill (LPHV)	11SB17	Not assigned	New	29.11.2011	Lusaka, Zambia	*Hipposideros gigas* (leaf-nosed bats)	Hemorrhagic gastroenteritis and severe hepatic disease in mice	NC_025831 NC_025832 NC_025833

aegyptiacus) in Africa.[20,23,24] KKOV was subsequently isolated from sick laboratory workers with an illness characterized by headache, diarrhea, myalgia, and arthralgia.[24] Gossas virus (GOSV) was isolated from a free-tailed bat (*Tadarida* sp.) in Senegal and was originally identified as a rhabdovirus.[20,25] However, subsequent sequence analysis has revealed that this was due to laboratory contamination of the sample with Nkolbisson virus (family *Rhabdoviridae*).[26] Keterah virus (KTRV) is an unclassified nairovirus isolated from tick larvae (*Argus pusillus*) taken from a lesser Asian house bat (*Scotophilus temmincki*) in Malaysia.[27] KTRV is closely related antigenically to Issyk-Kul virus (IKV),[20] another unclassified nairovirus first isolated from organs and blood of several bat species (*Nyctalus noctula, Myotis blythii, Vespertilio serotinus*, and *Vespertilio pipistrellus*) and bat ticks (*Argas vespertilionis*) collected in Kyrgyzstan.[28] The prototype IKV strain (LEIV-315K) was obtained from pooled organs of a common noctule bat (*N. noctula*). IKV was subsequently isolated from bats (*V. pipistrellus*) in Tadzhikistan where it was also associated with an outbreak of febrile illness in humans.[29] IKV strain LEZ 86-1784 was obtained from F. Rodhain of Institut Pasteur in Paris. It was isolated from bat ticks (*A. vespertilionis*) collected near Berlin in Germany in 1986 by H. Sinnecker. Leopards Hill virus (LPHV) was isolated from giant leaf-nosed bats (*Hipposideros gigas*) in Zambia and shown experimentally to cause hemorrhagic gastroenteritis and severe hepatic disease in mice.[30] Genbank accession numbers of partial L gene sequences of other nairoviruses used in phylogenetic analyses are provided in Figure 1.

Growth and passage history. The six nairoviruses sequenced in this study were obtained from the World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch, Galveston, TX and the Institut Pasteur, Paris, France. Each of the viruses was initially isolated by intracranial inoculation of newborn mice and most have been maintained by serial passage in suckling mice (SM).[27] Strains and passage histories of viruses sequenced were as follows: GOSV Dak An D401 (SM, BHK-BSR2), IKV strain LEIV-315K (SM7), KKOV strain UgZ52969 (SM8), KTRV strain P6-1361 (SM12), TFAV strain Dak An D11411 (SM6, Vero2), and YOGV strain DakAn D5634 (SM3).

Preparation of samples for sequencing. For TFAV, fluid medium from a culture of infected Vero cells was used for RNA extraction and sequencing. The source of viral RNA for the other five nairoviruses was filtered homogenates of infected mouse brain that were prepared as 10% suspensions in phosphate-buffered saline (PBS), pH 7.4.

Extraction of viral RNA. GOSV and TFAV were grown in BHK-BSR and Vero cell monolayers, respectively. Supernatants

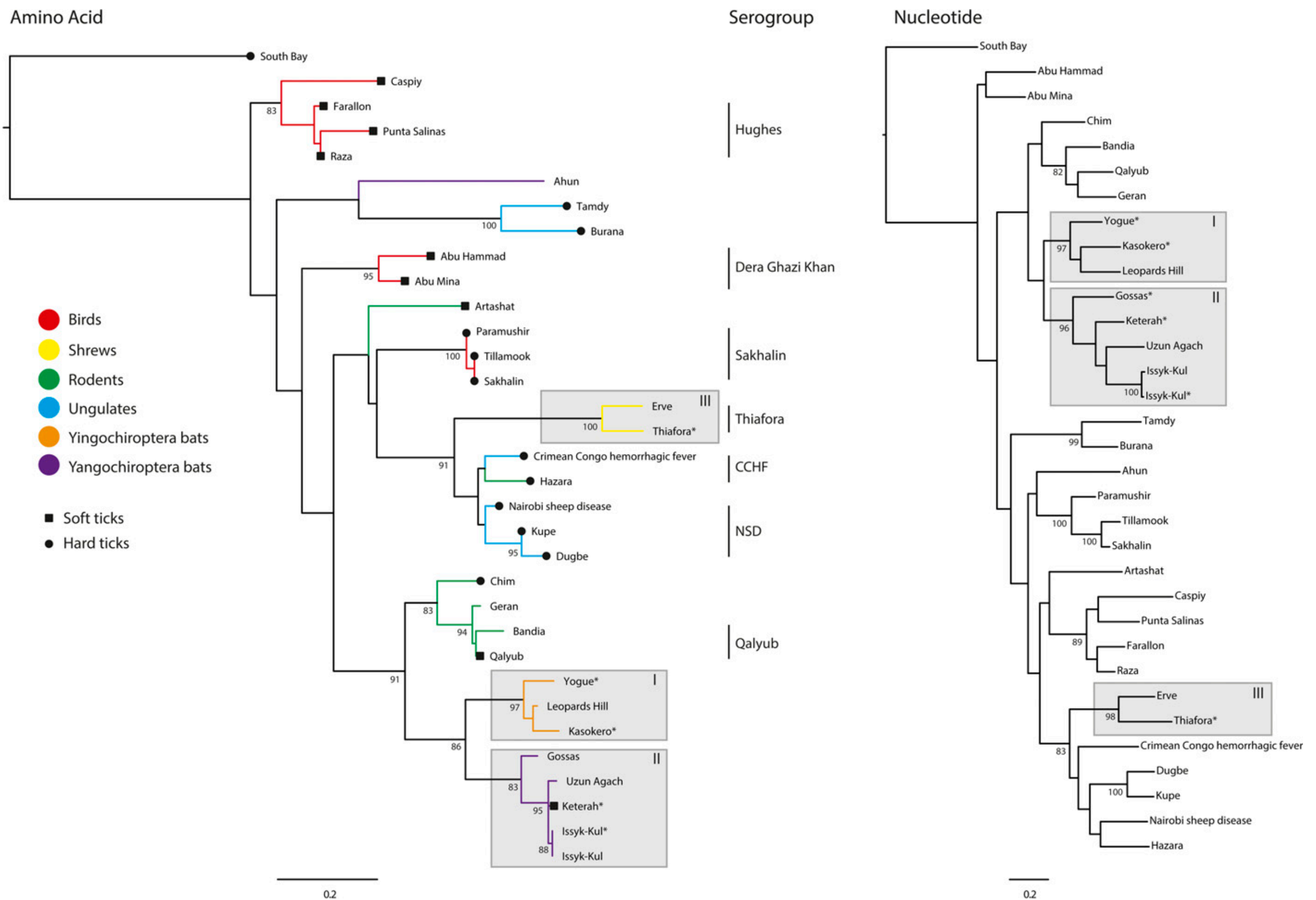

FIGURE 1. Phylogenetic trees of a partial sequence (466 nucleotides or 154 amino acids) of nairovirus L genes. The tree indicates the sources of isolation of the viruses from vertebrate hosts and hard or soft tick vectors. Current assignments to serogroups (species) are also shown. Genbank accession numbers: South Bay virus (KM048320), Caspiy virus (KF801658), Farallon virus (AY359523), Punte Salinas virus (AY359527), Raza virus (AY359529), Ahun virus (KF170224), Tamdy virus (KF801653), Burana virus (KF801651), Abu Hammad virus (AY357715), Abu Mina virus (AY357716), Artashat virus (KF801650), Paramushir virus (KF801657), Tillamook virus (AY359530), Sakhalin virus (KF801659), Erve virus (AY357719), Thiafora virus (KR537452), Crimean–Congo hemorrhagic fever virus (NC_005301), Hazara virus (AY359524), Nairobi sheep disease virus (KM464726), Kupe virus (EU257628), Dugbe virus (FJ422263), Chim virus (KF801656), Geran virus (KF801649), Bandia virus (AY357717), Qalyub virus (AY359528), Yogue virus (KR537455), Leopards Hill virus (AB842094), Kasokero virus (KR537446), Gossas virus (KR534878), Uzun Agach virus (KJ744032), Keterah virus (KR537449), and Issyk-Kul virus strain LEIV-315K (KF801652 and KR537443).

were harvested and clarified by low-speed centrifugation (2,000 *g*, 10 minutes at 4°C) once CPE was advanced. For other viruses, RNA was extracted directly from filtered homogenates of infected newborn mouse brains prepared as 10% suspensions in PBS, pH 7.4. To remove remaining cellular debris, all samples (cultured and filtered brain homogenates) were filtered through a 0.45 μM filter (EMD Millipore, Billerica, MA). Clarified supernatants were treated with a cocktail of DNases (14 U Turbo DNase [Ambion, Austin, TX], 20 U Benzonase [EMD Millipore, Billerica, MA], and 20 U RNase One [Promega, Madison, WI]) for 1 hour at 37°C. To reduce volume, 24 mL of supernatant was loaded on top of 8 mL of 30% sucrose (in TEN, pH 7.4) and centrifuged at 150,000 *g* for 4 hours at 4°C. The pellet was resuspended in 250 μL RNase/DNase and protease-free water (Ambion, Austin, TX). Viral RNA was then extracted using Trizol and resuspended in 50 μL RNase/DNase and protease-free water (Ambion, Austin, TX).

Next-generation sequencing. Viral RNA (0.1–0.2 μg), quantified by Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA), was fragmented by incubation at 94°C for 8 minutes in 19.5 μL of fragmentation buffer (Illumina 15016648). First and second strand synthesis, adapter ligation, and amplification of the library were performed using the TruSeq RNA Sample Preparation kit (Illumina, San Diego, CA) under conditions prescribed by the manufacturer. Reads were assembled using ABySS (Michael Smith Genome Sciences Centre, Vancouver, Canada),[31] mapped back to the contigs using bowtie2 (John Hopkins University, Baltimore, MD),[32] and visually verified using the Integrated Genomics Viewer (Broad Institute, Boston, MA).[33] A total of 1.9, 7.9, 4.1, 6.7, 6.0, and 8.1 million reads were generated for the samples containing GOSV, IKV, KTRV, KKOV, YOGV, and TFAV, respectively. Reads mapping to the virus in each sample comprised ~80,000 (4%), ~740,000 (9.4%), 213,000 (5.2%), 1,600,000 (24%), 273,000 (4.5%), and 200,000 (2.5%), respectively.

Serological tests. Antigens used in complement-fixation (CF) tests and for immunizing animals were prepared from infected newborn mouse brains. CF antigens were prepared by the sucrose/acetone extraction method.[34] Hyperimmune

mouse ascitic fluids were prepared by four intraperitoneal injections, given at weekly intervals, with 10% suspensions of homogenized infected mouse brain in PBS mixed with Freund's complete adjuvant (first injection) or Freund's incomplete adjuvant (subsequent injections). Sarcoma 180 cells were given intraperitoneally 1 day after the final immunization to induce ascites formation. Complement fixation tests were conducted using a microassay described previously[35] using two units of guinea pig complement. Titers were recorded as the highest antibody dilutions giving 3+ or 4+ fixation of complement on a scale of 0–4+.

Phylogenetic analysis. Other than the viruses described earlier, full genome sequences are available for only five of the ~50 described nairoviruses (CCHFV, Hazara virus, NSDV, Dugbe virus, and Kupe virus). For most of the remaining viruses, only a short sequence fragment (< 450 nt) of a highly conserved region of the L segment is available; therefore, this region was selected for phylogenetic analysis. Amino acid and nucleotide sequence alignments were created from all available sequence data using ClustalW in Geneious v8.1.4 (www.geneious.com[36]) and refined manually. The resulting sequence alignments included 33 taxa and were 466 nucleotides or 154 amino acids in length. Maximum likelihood phylogenetic trees were constructed using PhyML 3.0,[37] employing the HKY85 nucleotide substitution model with invariant sites or the Whelan and Goldman (WAG) model of amino acid substitution, and a combination of nearest neighbor interchange (NNI) and subtree pruning and regrafting branch swapping. The phylogenetic robustness of each node was determined using 1,000 bootstrap replicates and NNI branch swapping.

RESULTS

Genome sequences of TFAV, YOGV, KKOV, GOSV, KTRV, and IKV (resequenced), including the complete coding regions of each of the three segments (L, M, and S), have been deposited in Genbank under accession numbers provided in Table 1. The complete genome sequence was determined for KKOV. For other viruses, only the extreme termini, which are highly conserved and anticomplementary among bunyavirus genome segments, were not determined. The genome sequences of LPHV, ERVV, and CCHFV were obtained from Genbank and used for comparative purposes (Table 1).

The S RNA segment in each of the viruses contains a single open reading frame encoding a protein with characteristics similar to those of the CCHFV N protein, for which the crystal structure has been resolved.[18,19,38,39] The nucleoproteins range in size from 485 to 673 amino acids (estimated 54.6–74.7 kDa). There was good alignment of each protein with the CCHFV N protein across each of the N-terminal and C-terminal components of the head domain and the central stalk domain (Figure 2), with amino acid sequence identity ranging from 36.2% to 44.8% (Table 2). The highest amino acid sequence identities across this conserved domain were between KKOV, YOGV, and LPHV (79.3–91.5%), IKV, KTRV, and GOSV (63.3–85.9%), and TFAV and ERVV (70.6%). However, as described previously for ERVV, the TFAV N protein features a long (198 aa) C-terminal extension and, like LPHV, KKOV, and YOGV feature, a short (28 aa) C-terminal extension that is rich in proline and basic residues (Figure 2). The alignment also displayed striking conservation of an unusually high number of large hydrophobic residues (W, Y, F) and of basic residues (K, R, H), many of which have been implicated in RNA binding.[19,38] There was also conservation of five of the six residues (R384, E387, K411, H453, and Q457) implicated in DNA binding at the CCHFV endonuclease active site, although conservative substitutions occurred in some of the viruses. Interestingly, a caspase-3 cleavage site identified at the tip of the CCHFV stalk domain[18] was not conserved; none of the sequences in this region of the other nairoviruses contain a motif that is known to be recognized by caspase-3.[40] In CCHFV, mutations that inhibit cleavage at this site have been shown to enhance viral polymerase activity and it has been proposed that the site is targeted by host cell defences.[18,41] Although the site is conserved in all CCHFV strains,[41] it is not evident why naturally occurring mutations would not be selected rapidly during infection, allowing the virus to evade the host response.

The M RNA segments were each found to encode large proteins with a predicted N-terminal signal peptide and multiple membrane-spanning domains, and displayed extensive but discontinuous homology with the M polyglycoprotein of CCHFV. As in CCHFV, the N-terminal region of each of the nairovirus polyproteins in our data set contains a mucin-like serine/threonine-rich domain with a large number of predicted O-linked and N-linked glycosylation sites that extend up to or beyond predicted (protease) cleavage sites (Figure 3). The number of predicted O-glycosylation sites ranged from 21 in YOGV to 148 in GOSV, with large variations in the length of the mucin-like domain contributing primarily to variations in size of the M polyproteins. The mucin-like domains displayed very low amino acid sequence identity, which was detectable only between closely related viruses in the data set. The hypervariable domain of the M polyproteins extends beyond the mucin-like domain into a region that corresponds to the gp38 domain of CCFHV, commencing at one or more furin-like or subtilisin kexin isozyme-1 (SKI-1) protease cleavage sites and terminating at a conserved SKI-1 cleavage site (R[R/K]LL↓) that marks the N-terminus of structural glycoprotein Gn (Figure 3). Although there is little overall sequence identity with CCHFV gp38 across the viruses in the data set, there is recognizable similarity in the C-terminal region of this domain and some conservation of a set of six to eight cysteine residues (Supplemental Figure 1). The Gn glycoproteins extend from the conserved SKI-1 cleavage site to signal peptide cleavage sites that follow the second transmembrane domain in each of the viruses. The Gn glycoproteins share 12 totally conserved cysteine residues and a conserved N-glycosylation site immediately following the second cysteine residue in the predicted ectodomain (Supplemental Figure 2). A second predicted N-glycosylation site immediately following the fifth cysteine residue in the ectodomain does not occur in ERVV, TFAV, or CCHFV. Unlike CCFHV, the Gc glycoproteins of each of the other nairoviruses in the data set commence at the predicted signal peptide cleavage site following the second transmembrane domain and terminate beyond the third transmembrane domain at the C-terminus of the M polyglycoprotein (Figure 3). The predicted Gc ectodomains of these class I transmembrane glycoproteins feature 28 conserved cysteine residues and two cysteine residues that were conserved in all viruses including CCHFV but are not

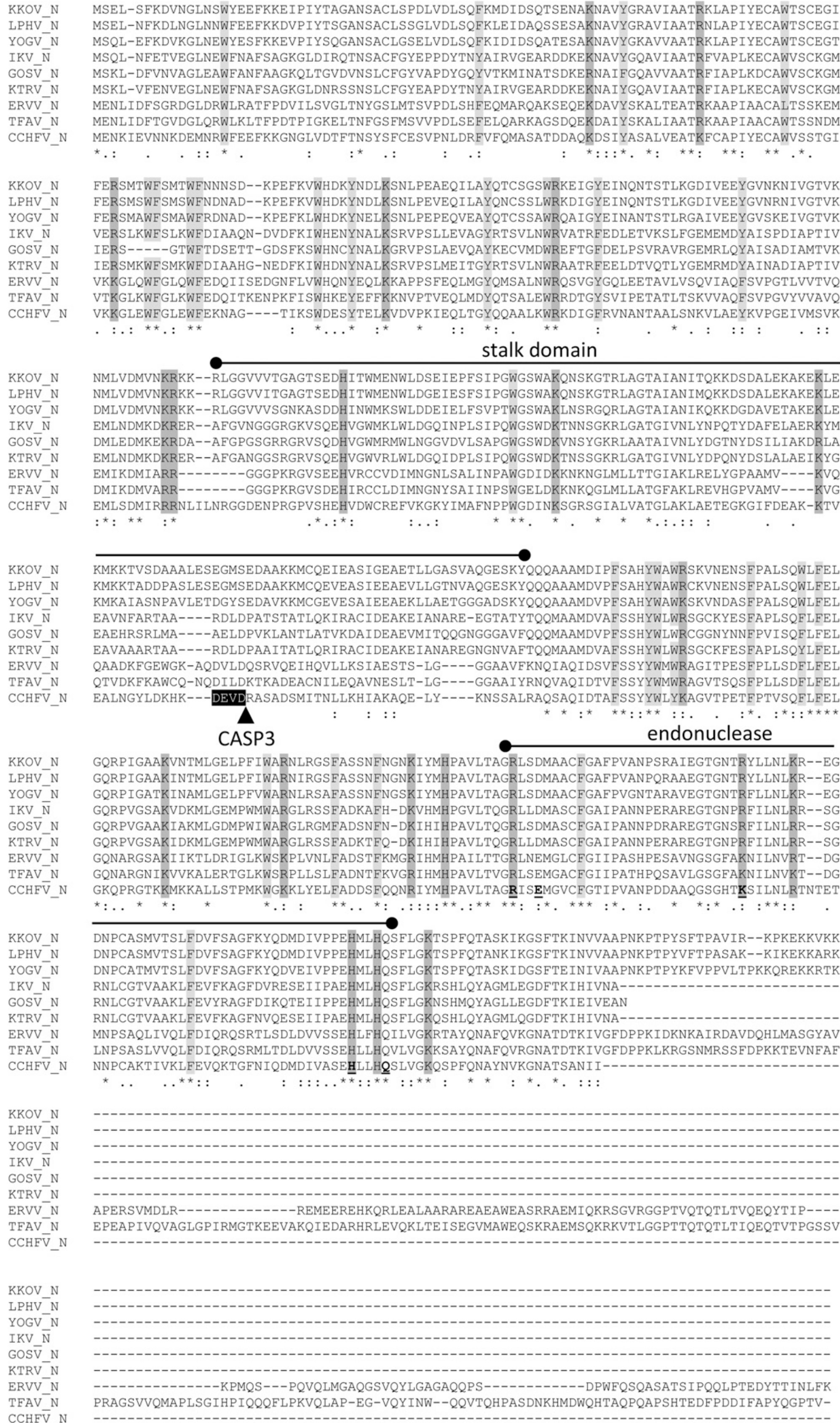

FIGURE 2. ClustalX alignment of the deduced amino acid sequences of the nucleoproteins of nine nairoviruses. Conserved basic (K, R, H) and large hydrophobic (W, Y, F) residues are shaded. The stalk domain and endonuclease domains are marked and the caspase three cleavage site identified only in Crimean-Congo hemorrhagic fever virus (CCHFV) is shown. Identical (*), strongly conserved (:), and weakly conserved (.) residues as assigned in the Gronnet Pam250 matrix are indicated below the alignment.

TABLE 2
Amino acid sequence identities (%) of nairovirus N proteins as determined by p-distance estimations in MEGA 6

	KKOV	YOGV	LPHV	IKV	KTRV	GOSV	ERVV	TFAV	CCHFV
KKOV									
YOGV	80.0								
LPHV	91.5	79.3							
IKV	46.1	46.9	45.4						
KTRV	46.9	46.5	46.3	85.9					
GOSV	46.5	47.1	47.1	63.3	66.3				
ERVV	33.0	32.2	33.3	35.0	33.9	32.2			
TFAV	36.2	35.8	36.2	33.3	33.0	33.0	70.6		
CCHFV	37.1	36.5	37.3	35.8	36.7	35.2	44.3	44.8	

CCHFV = Crimean–Congo hemorrhagic fever virus; ERVV = Erve virus; GOSV = Gossas virus; IKV = Issyk-Kul virus; KKOV = Kasokero virus; KTRV = Keterah virus; LPHV = Leopards Hill virus; TFAV = Thiafora virus; YOGV = Yogue virus.
The aligned regions of each protein up to the end of the CCHFV N protein were compared.

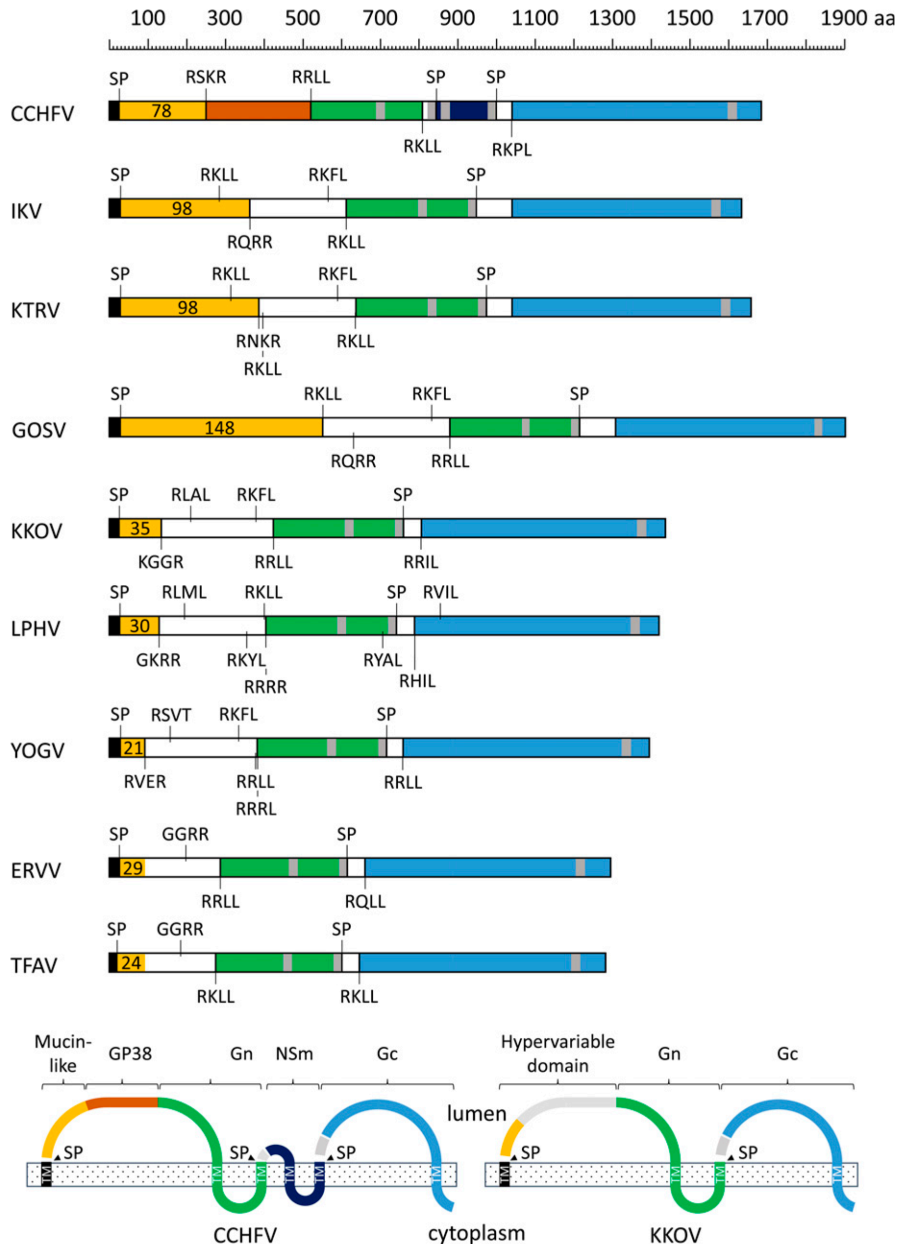

FIGURE 3. Schematic illustration of the structures and predicted membrane topologies of the polyglycoproteins encoded in the M segments of nine nairoviruses. Regions corresponding to the mucin-like domain (orange), gp38 (rust), Gn (green), NSm (dark blue), and Gc (sky blue) are shaded. Predicted signal peptidase cleavage sites (SP) and potential furin-like and SK-1 cleavage sites are shown. The number of predicted O-linked glycosylation sites in the mucin-like domain are also shown.

TABLE 3

Amino acid sequence identities (%) of nairovirus Gn proteins as determined by p-distance estimations in MEGA 6

	KKOV	YOGV	LPHV	IKV	KTRV	GOSV	ERVV	TFAV	CCHFV
KKOV									
YOGV	61.1								
LPHV	67.4	59.6							
IKV	38.2	39.5	38.6						
KTRV	38.6	40.4	37.9	85.6					
GOSV	41.7	40.4	39.5	67.4	68.7				
ERVV	27.3	26.3	25.7	28.2	28.2	30.1			
TFAV	27.9	28.2	29.5	29.8	29.2	28.5	72.4		
CCHFV	26.6	26.0	26.0	28.5	28.5	27.9	37.9	36.7	

CCHFV = Crimean–Congo hemorrhagic fever virus; ERVV = Erve virus; GOSV = Gossas virus; IKV = Issyk-Kul virus; KKOV = Kasokero virus; KTRV = Keterah virus; LPHV = Leopards Hill virus; TFAV = Thiafora virus; YOGV = Yogue virus.

The sequences were assumed to extend from the conserved protease site (R[R/K]LL) to the signal peptidase cleavage site following the second-last transmembrane domain in the M polyprotein.

present in ERVV and TFAV. Interestingly, CCFHV, TFAV, and ERVV also have a single (unpaired) cysteine residue that may form an intermolecular disulfide bridge (Supplemental Figure 3). The Gc glycoproteins each have two to three predicted N-glycosylation sites, none of which is totally conserved and multiple short regions (three to five amino acids) of highly conserved sequence. As in CCHFV, predicted subtilisin-like protease cleavage sites in the N-terminal region of each of the viruses in the data set may result in processing of Gc to remove a short polypeptide (45–53 amino acids) containing four conserved cysteine residues. As has been reported previously for LPHV and ERVV,[30] the M polyglycoproteins of TFAV, YOGV, KKOV, GOSV, KTRV, and IKV lack the NSm protein of CCHFV due to the absence of an additional double-membrane-spanning region between Gn and Gc.

The L RNA segment of each of the viruses encodes the large multifunctional RNA-dependent RNA polymerase (RdRp) that shares extensive sequence homology with the L proteins of other bunyaviruses. The virus sequences in the data set contained each of the conserved domains (regions 1–4) identified in other bunyaviruses,[13] as well as the ovarian tumor (OTU)-like protease domain near the N-terminus (Supplemental Figure 4) that, although detected in various other RNA viruses, is unique to the nairovirus among the *Bunyaviridae*.[11,42] In CCHFV, this OTU-like cysteine protease has been shown to inhibit NF-κB-dependent host innate immune pathways by de-conjugating ubiquitin and the interferon-stimulated gene 15 (ISG15) product from cellular target proteins.[43] Like other nairoviruses, the L proteins of the viruses in our data set are significantly larger than those of other bunyaviruses due to an extended N-terminal region containing this domain, an extended C-terminal region and a long extension between conserved regions 2 and 3.[8]

Phylogenetic analysis of the nucleotide and amino acid sequences of a highly conserved region of the RdRp revealed that the viruses sequenced in this study cluster into three well-supported clades composed of 1) KKOV, YOGV, and LPHV; 2) KTRV, IKV, GOSV, and Uzun Agach virus (UAV); and 3) TFAV and ERVV (Figure 1). Amino acid sequence identities determined from Clustal X alignments of the N, Gn, Gc, and L protein sequences confirmed these relationships with amino acid sequence identities ≥ 59.6% among viruses assigned to each clade (excluding UAV for which only 441 bp of the L gene have been sequenced). In contrast, amino acid sequence identities between viruses representing different clades range from 25.7% to 56.7% (Tables 2–5). Complement-fixation tests conducted using the available antigens and mouse immune ascitic fluids from KKOV, YOGV, KTRV, IKV, and TFAV also supported the assignment of the viruses to these groups (Table 6). As reported previously,[24] KKOV is closely related antigenically to YOGV and together these viruses can be considered to form the Kasokero serogroup of which LPHV is also a likely member. Similarly, IKV and KTRV can be considered to form the Issyk-Kul serogroup. The two available strains of IKV (LEIV-315K/Kyrgyzstan/1970 and LEZ 86-1784/Germany/1986) were indistinguishable in the CF test. TFAV was distinct from the other viruses tested here but has previously been shown to be closely related antigenically to ERVV with which it forms the Thiafora serogroup.[23]

Examination of the host associations of each clade revealed some structure by both host and vector across the tree (Figure 1). Clade I viruses were each isolated from bats of the suborder Yingochiroptera (Pteropodidae and Hipposideridae)

TABLE 4

Amino acid sequence identities (%) of nairovirus Gc proteins as determined by p-distance estimations in MEGA 6

	KKOV	YOGV	LPHV	IKV	KTRV	GOSV	ERVV	TFAV	CCHFV
KKOV									
YOGV	74.8								
LPHV	79.7	74.5							
IKV	54.8	53.6	54.6						
KTRV	54.8	52.6	55.0	83.6					
GOSV	56.0	53.9	55.0	70.6	71.5				
ERVV	39.3	38.9	38.2	34.8	36.4	35.4			
TFAV	38.2	38.9	37.5	37.3	37.3	36.2	77.6		
CCHFV	36.7	38.1	36.7	36.4	37.2	34.7	47.5	45.4	

CCHFV = Crimean–Congo hemorrhagic fever virus; ERVV = Erve virus; GOSV = Gossas virus; IKV = Issyk-Kul virus; KKOV = Kasokero virus; KTRV = Keterah virus; LPHV = Leopards Hill virus; TFAV = Thiafora virus; YOGV = Yogue virus.

The sequences were assumed to extend from the signal peptidase cleavage site following the second-last transmembrane domain to the C terminus of the M polyprotein.

TABLE 5
Amino acid sequence identities (%) of nairovirus L proteins as determined by p-distance estimations in MEGA 6

	KKOV	YOGV	LPHV	IKV	KTRV	GOSV	ERVV	TFAV	CCHFV
KKOV									
YOGV	78.3								
LPHV	85.1	77.6							
IKV	56.8	56.4	56.5						
KTRV	56.8	56.3	56.5	90.4					
GOSV	56.8	56.7	56.5	80.0	80.0				
ERVV	39.3	39.1	39.2	40.9	40.6	41.0			
TFAV	40.1	39.8	39.8	40.6	40.5	40.9	72.6		
CCHFV	43.1	43.1	43.2	43.4	43.2	43.5	49.2	49.7	

CCHFV = Crimean–Congo hemorrhagic fever virus; ERVV = Erve virus; GOSV = Gossas virus; IKV = Issyk-Kul virus; KKOV = Kasokero virus; KTRV = Keterah virus; LPHV = Leopards Hill virus; TFAV = Thiafora virus; YOGV = Yogue virus.

in Africa; clade II viruses were each isolated from bats of the suborder Yangochiroptera (Vespertilionidae and Molossidae) in Asia and Africa; and clade III viruses were each isolated from shrews (Soricidae) in Africa and Europe.

DISCUSSION

The risks to public health associated with new and emerging viral diseases are well recognized. Most newly emerging viruses are zoonotic and many are transmitted by arthropod vectors.[44] The identification and characterization of the myriad of viruses infecting animal reservoirs and vectors will help mitigate disease risks by increasing awareness of potential threats and improve preparedness for future emergence events. New and poorly characterized viruses that may be of particular interest are those that are closely related to and share a similar ecology with known emerging zoonotic pathogens. Here, we report the molecular characterization and phylogenetic assignment of six bunyaviruses (TFAV, YOGV, KKOV, GOSV, KTRV, and IKV) that exist, together with CCHFV, in the genus *Nairovirus*.

The most striking feature of the genome organization of each of these nairoviruses is the structural arrangement of the M segment-encoded polyglycoprotein. Each contains domains corresponding to the CCHFV mucin-like protein, gp38, Gn, and Gc. However, as reported recently for LPHV and ERVV,[30] a double-membrane spanning domain between Gn and Gc, corresponding to the CCHFV NSm protein, was absent from TFAV, YOGV, KKOV, GOSV, KTRV, and IKV. The function of NSm is presently unknown but homologues also occur in the M polyglycoprotein sequences of NSDV, Dugbe virus, Kupe virus, and Hazara virus that cluster phylogenetically with CCHFV. Interestingly, although HAZV is the most closely related virus to CCHFV, both phylogenetically and serologically, it is nonpathogenic in humans and uniquely features a 43 amino acid deletion in the cytoplasmic domain NSm.[45] Triple-membrane-spanning NSm proteins are encoded in the M segments of orthobunyaviruses and have been implicated as scaffold proteins involved in virus assembly and morphogenesis.[46–48] Although also located between the Gn and Gc glycoproteins, they share no detectable sequence identity with nairovirus double-membrane-spanning NSm proteins.

Amino acid identity comparisons and phylogenetic analyses indicated that the viruses sequenced in the study fell into three groups comprising 1) KKOV, YOGV, and LPHV; 2) KTRV, IKV, and GOSV; and 3) TFAV and ERVV. Phylogenetic analyses also indicated that UAV, which was isolated in 1977 from a lesser mouse-eared bat (*M. blythii*) in Kazakhstan, falls within the KTRV group. Because of the paucity of biochemical data, nairovirus species are currently assigned according to serogroups.[9,10,23] Although serological analysis conducted here was limited by the unavailability of suitable antisera, consideration of cross-reactions in complement-fixation assays and phylogenetic analyses suggests that the groups comprising KKOV, YOGV, and LPHV may be assigned as a single species and that KTRV, IKV, GOSV, and UAV may also constitute a single species. TFAV and ERVV have previously been assigned as a single species based on complement fixation and indirect immunofluorescence data.[23] Cross-neutralization tests would be required to confirm these species assignments by current demarcation criteria.

We also observed that there may be a clade-specific association between nairoviruses and specific host taxa. GOSV, IKV, KTRV, and UAV were each isolated from bats of the suborder Yangochiroptera (or from ticks feeding on them), KKOV, YOGV, and LPHV were isolated from bats of the

TABLE 6
Complement-fixation tests to detect antigenic relationships between five nairoviruses

Antigen	Antibody					
	KKOV	YOGV	IKV	IKV	KTRV	TFAV
	–	–	LEIV 315K	LEZ 86-1784	–	–
KKOV	≥ 256*/≥ 8	≥ 256/≥ 8	0/–	0/–	0/–	0/–
YOGV	128/≥ 8	≥ 256/≥ 8	0/–	32/≥ 8	0/–	0/–
IKV LEZ 86-1784	0/–	0/–	≥ 256/≥ 8	≥ 256/≥ 8	≥ 256/≥ 8	0/–
KTRV	16/≥ 8	0/–	128/≥ 8	≥ 256/≥ 8	≥ 256/≥ 8	0/–
Veronal (1:5)	0	0	0	0	0	0

IKV = Issyk-Kul virus; KKOV = Kasokero virus; KTRV = Keterah virus; LEIV = IKV strain LEIV; LEZ = IKV strain LEZ; TFAV = Thiafora virus; YOGV = Yogue virus.
*Reciprocal of antiserum titer/reciprocal of antigen titer.

suborder Yingochiroptera,[49,50] and TFAV and ERVV were isolated from insectivores (shrews). This correlation extended across the nairovirus phylogeny (Figure 1) with various clades associated with either birds, bats, rodents, insectivores or ungulates. The available data also suggest that specific clades are associated with transmission by either hard ticks (Ixodidae) or soft ticks (Argasidae). For example, the viruses in the large clade comprising the CCHFV, NSDV, and Sakhalin serogroups have been isolated primarily from hard ticks.[51–56] Although there are two exceptional reports of the isolation of CCHFV from soft tick species, the circumstances suggest those reports should be viewed with caution[52,57] and there is experimental evidence that soft tick species are not competent vectors for CCHFV.[58,59] Tamdy virus and closely related Burana virus were also isolated from hard ticks.[60] However, with the sole exception of Chim virus,[61] viruses in the remaining clades have been isolated only from soft ticks.[20,62–66] As observed previously,[67] this may indicate a stable ecological association between nairoviruses and their hosts and vectors and suggests that constraints on transmission to vertebrates, including humans, may be limited primarily by vector-feeding preferences and the opportunity for exposure.

Several nairoviruses, including CCHFV, have been implicated in human disease. IKV has caused sporadic cases and epidemics of febrile illness in Central Asia,[68] ERVV has been associated with headache and other neurological symptoms in Europe,[69] and KKOV has been isolated from laboratory workers who developed a mild-to-severe clinical disease characterized by fever, nausea, myalgia, abdominal pain, and neurological symptoms.[24] Further investigations are required to determine the risks to public health from other viruses examined in this study.

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