JVI Accepts, published online ahead of print on 25 September 2013
J. Virol. doi:10.1128/JVI.01862-13

Discovery of a unique novel clade of mosquito-associated bunyaviruses

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Running title: Novel cluster of mosquito-associated bunyaviruses

Word count in abstract: 250

Word count in text: 5795

Figures: 8

Tables: 3

Abstract

Bunyaviruses are the largest known family of RNA viruses, infecting vertebrates, insects and plants. Here we isolated three novel bunyaviruses from mosquitoes sampled in Côte d'Ivoire, Ghana and Uganda. The viruses define a highly diversified monophyletic sister clade to all members of the genus *Orthobunyavirus* and are virtually equidistant to orthobunyaviruses and tospoviruses. Maximal amino acid identities between homologous putative proteins of the novel group and orthobunyaviruses ranged between 12-25%. The type isolates tentatively named Herbert virus (HEBV), Taï virus (TAIV) and Kibale virus (KIBV) comprised genomes with L, M, and S segments of about 7.4 kb, 2.7 kb, and 1.1 kb, respectively. HEBV, TAIV, and KIBV encode the shortest bunyavirus M segments known and did not seem to encode NSs and NSm proteins but contained an elongated L segment with a ca. 500 nt insertion that shows no identity to other bunyaviruses. The viruses replicated to high titers in insect cells but did not replicate in vertebrate cells. The enveloped virions were 90-110 nm in diameter and budded at cellular membranes with morphological features typical of the Golgi complex. Viral RNA recovered from infected cells showed 5´-terminal non-templated sequences of 9-22 nt, suggestive of cap snatching during mRNA synthesis as described for other bunyaviruses. Northern blotting identified RNA species of full and reduced lengths, suggested upon analogy with other bunyaviruses to constitute antigenomic sense vRNAs and transcript mRNAs, respectively. Functional studies will be necessary to determine if this group of viruses constitutes a novel genus in the bunyavirus family.

Introduction

The family *Bunyaviridae* is among the largest and most diversified families of RNA viruses, comprising more than 350 serologically distinct viruses (70). Ninety-six viruses have been formally classified as distinct species by the International Committee on Taxonomy of Viruses (ICTV), and full genome sequences are yet to be determined for the majority of isolates (70). The family comprises five genera whose members can cause pathogenic infections in vertebrates (genera *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus*) and plants (genus *Tospovirus*). Several bunyaviruses are considered emerging and re-emerging pathogens due to their recent invasion of new habitats and increasing incidence in humans or livestock, such as the Crimean-Congo hemorrhagic fever virus (CCHFV), Rift Valley fever virus (RVFV), Sin Nombre virus (SNV), severe fever with thrombocytopenia syndrome virus, and Schmallenberg virus (SBV) (4, 8, 27, 85, 97, 103). Orthobunyaviruses, phleboviruses and nairoviruses are transmitted to their vertebrate hosts by mosquitoes, midges, phlebotomine sandflies, and ticks. The genus *Hantavirus* is unique in that its members have no arthropod vectors but are transmitted by aerosolized rodent excreta (92).

Bunyaviruses share general features such as their overall virion morphology or their ability to replicate in the cytoplasm and bud into the Golgi cisternae (56, 57, 65, 66, 77). Criteria to classify bunyaviruses into genera can be derived from more specific properties such as genome organization, coding strategies, as well as phylogenetic relationships (70). Members of each genus are further subdivided by serology into serogroups and antigenic complexes. Phylogenetic relationships are generally in good agreement with antigenic classification, justifying the use of sequence information as the major criterion for classification of bunyavirus genera (70).

Branching inconsistencies within genera have become evident by comparing phylogenetic relationships based on different genes, revealing a potential for bunyaviruses to undergo intra-generic genome segment reassortment (16, 101, 102).

The enveloped, spherical bunyavirus virions are ca. 100 nm in diameter and contain segmented, single-stranded, negative-sense RNA genomes implementing negative- or ambisense coding strategies (79). The small (S) segment encodes the nucleocapsid (N) protein. The medium (M) segment codes two glycoproteins (Gn and Gc) and the large (L) segment encodes the RNA-dependent RNA polymerase (RdRp). The S and M segments of the genera *Orthobunyavirus*, *Phlebovirus* and *Tospovirus* encode two additional nonstructural proteins, NSs and NSm, respectively. Orthobunyaviruses encode their N and NSs proteins in overlapping ORFs translated from one same mRNA that is complementary to the corresponding virion RNA segment (28). Phleboviruses and tospoviruses use an ambisense coding strategy and translate their NSs from a subgenomic (sg) mRNA, which has the same polarity as the virion-sense RNA (vRNA) (44). Recently it was shown that some hantaviruses also code for an NSs protein in an ORF overlapping the N ORF, with expression enabled by ribosomal leaky scanning (45, 95). Interestingly, accessory proteins are not consistently represented throughout genera, as M segments of tick-transmitted phleboviruses do not encode NSm proteins (68, 75, 103) and viruses in the Anopheles A, Anopheles B and Tete virus serogroups within the genus *Orthobunyavirus* do not encode NSs proteins (62). Bunyavirus NSs proteins either inhibit the cellular interferon response in their vertebrate hosts or suppress the RNA interference (RNAi) mechanism in their plant hosts (12, 15, 88). Nairoviruses are special regarding their strategy to counteract the antiviral host response as they code for an ovarian tumor (OTU) domain within their L protein that is suggested to

suppress the host-cell inflammatory and antiviral response and thus plays a role as pathogenicity factor (30, 41, 52).

Bunyaviruses are distributed worldwide but appear to have higher diversity and prevalence in tropical and sub-tropical regions (79). The investigation of bunyaviruses in such regions can yield novel insight into phylogeny and diversity. For instance, the Gouléako virus (GOLV, previously GOUV; the abbreviation was changed as GOUV was already used for Gou virus, a hantavirus isolated from *Rattus rattus* in China (71)) recently discovered in mosquitoes is almost equidistant phylogenetically to the five established genera, but closest to the genus *Phlebovirus* (60). Gouléako virus appears to be restricted to arthropod hosts, while all other known phleboviruses can also infect specific vertebrate hosts (60), suggesting that Gouléako virus represents a new taxonomic entity, potentially a new genus.

During a pilot study on mosquito-associated viruses in Côte d'Ivoire, a short RT-PCR fragment of a putative RdRp gene with distant relationship to bunyaviruses was encountered (49, 60). The virus was tentatively named Herbert virus (HEBV; strain F23/CI/2004). Here, we provide a full characterization of the virus isolated in cell culture, as well as related viruses isolated from mosquitoes in Côte d'Ivoire, Ghana and Uganda.

Materials and methods

Mosquito collection and species identification. Mosquitoes were trapped from February to June 2004 in Taï National Park, Côte d'Ivoire (49), and from February to June 2008 in Kibale National Park, Uganda. Habitat types included primary and secondary tropical forest, agricultural plantations, villages, and research camps within primary rainforest. Furthermore, mosquitoes were collected at the botanical garden and at the residential area at Kwame Nkrumah University of Science and Technology (KNUST) in Kumasi, Ghana. Mosquitoes were trapped with CDC miniature light and gravid traps (John W. Hock Company, USA) and with BG sentinel traps (Biogents, Regensburg, Germany). Traps were baited with Octenol, worn socks, Limburger cheese, or simple syrup (1 liter of water mixed with 100 g sugar). Species were identified by morphological criteria (26, 31, 33, 50).

Virus isolation, purification and growth. Virus isolation from mosquitoes collected in Côte d'Ivoire was done in C6/36 (derived from *Ae. albopictus* larvae) (42) and Vero E6 (*Ceropithecus aethiops* kidney) cells as described previously (48, 49). Female mosquitoes from Uganda and Ghana were homogenized individually in 500 µl of L-15 medium without additives using 3-5 ceramic beads and a TissueLyser instrument (Qiagen, Hilden, Germany). Trapped male mosquitoes were pooled (1-20 specimens) according to trapping location and genus and homogenized in 1 ml of L15 medium. Suspensions were cleared from debris by centrifugation at 2500 rpm for 10 minutes at 4°C. Pools of female mosquitoes were generated using 100 µl of supernatant of 10 homogenized mosquito suspensions and used for virus isolation as described (48). Virus stocks of the fourth passage of HEBV (isolate C60/CI/2004) and KIBV (isolate P07/UG/2008) were generated. Virus titers were determined by $TCID_{50}$ titration and virus positive wells were identified by real-time PCR. For virus growth kinetics, C6/36

and U4.4 (derived from *Ae. albopictus* larvae (83)) cells were infected at multiplicities of infection (MOI) of 0.1 and 0.01 in duplicate, respectively, as described in (104). Aliquots of infectious cell culture supernatant were harvested every 24 h for periods of five days and viral genome copies were quantified by real-time RT-PCR (HEBV-F 5'-AGAATGCTTTGTCAGTGG, HEBV-R 5'- AGCAGCAACTTATAAAACAAATC, HEBV-TM 5'-6-FAM-TTCTCCGCTAATAAAA-MGB; KIBV-F 5'-TAATTTGAATGGTGAGCCTTTTTCT, KIBV-R 5'-GCTGTCTGAATACCGGATAATCTTG, KIBV-TM 5'-6-FAM-ATTCCCTGTCATTGGAGCTTGCTCTTTCTT-TQ2).

Infection of vertebrate cells. Green monkey kidney cells (Vero E6), baby hamster kidney cells (BHK-J), mouse embryo fibroblasts (MEF) from BALB/c MDA5 -/- knock-out mice, MEF from BALB/c RIG-I -/- knock-out mice, mouse fibroblasts (L929) and porcine-stable equine kidney cells (PSEK) were infected with HEBV (4^{th} passage of isolate F23/CI/2004) with MOIs of 10, 1, 0.5, and 0.1, and incubated at 33°C and 37°C, respectively. Cell culture supernatants were passaged in fresh cells every 7 days in 1/10 dilutions for five consecutive passages. Supernatants from identical cell culture types infected with different MOIs were pooled and all passages were subjected to real-time RT-PCR for screening.

RT-PCR screening. RNA was extracted from homogenized female and male mosquito pools or from individually homogenized female mosquitoes using 140 µl of the supernatant and the Viral RNA Kit (Qiagen, Hilden) and cDNA was synthesized using SuperScriptII according to manufacturers' instructions (Invitrogen, Karlsruhe, Germany). Pools were screened by real-time RT-PCR or by nested RT-PCR using the primer pairs HEBV-F1 5' ATGCTGAYATGTCIAAGTGGTSTGC and HEBV R1 5'

179 TGATTGTCATCGSTRTGIACYA for the first round and the primer pairs HEBV-F2 5'
180 ATGCTGAYATGTCIAAGTGGTSTGC and HEBV-R2 5'
181 TCAARTTVCCTTGGAKCCART for the nested PCR.
182
183 **Electron microscopy.** For electron microscopy analyses, viral particles were purified
184 through a 36% sucrose cushion and the pellet was resuspended in phosphate
185 buffered saline (48, 72). Viral particles were fixed with 2% paraformaldehyde and
186 analyzed by transmission electron microscopy after staining with 1% uranyl acetate
187 (5, 38). For ultra-thin sections, infected cells were fixed with 2.5% glutaraldehyde,
188 enclosed in low-melting agar, embedded in resin and evaluated by transmission EM
189 after ultrathin sectioning (48).
190
191 **Genome sequencing.** Viral genome fragments from infectious cell culture
192 supernatant of HEBV were generated by random-primed RT-PCR optimized for the
193 detection of encapsidated nucleic acids (so-called "particle-associated nucleic acid
194 PCR" (48, 60)). Briefly, RNA was extracted from ultracentrifuged virus pellets using
195 the viral RNA Kit (Qiagen, Hilden, Germany) and double strand cDNA was
196 synthesized with random hexamers linked to a defined primer sequence tail using the
197 double strand cDNA kit (Promega, Madison, USA). Amplification was performed
198 using oligonucleotides that bind to the sequence tail and cloned into the pCR2.1
199 TOPO vector (Invitrogen, Karlsruhe, Germany). Colonies were analyzed by PCR and
200 inserts >500 nucleotides (nt) were sequenced using dye terminator chemistry
201 (Applied Biosystems, Darmstadt, Germany). Primer sequences were trimmed and
202 sequences were assembled using SeqMan II (LaserGene, DNA Star). Consensus
203 sequences were compared on nt and translated amino acid (aa) level to the
204 GenBank database applying BLASTn and BLASTx algorithms

(http://www.ncbi.nlm.Nih.gov/Genbank). Fragment-specific primers and generic orthobunyavirus oligonucleotides were used for amplification of sequence gaps. The 3' and 5' genome termini were confirmed by RACE-PCR (Roche, Mannheim, Germany). The complete genome was re-sequenced for confirmation on both strands by long-range PCR and primer walking techniques. Full genome sequencing of KIBV was performed by using fragment-specific primers and primers based on the HEBV genome. Full genome sequences of HEBV isolates F33/CI/2004, F45/CI/2005, and F53/CI/2004; as well as from TAIV isolate F47/CI/2004, were generated by deep sequencing on 454 Junior (Roche) and Ion Torrent™ (Invitrogen) platforms in Bonn. Reads were identified by reference mapping to HEBV F23/CI/2004, as well as BLAST comparisons against a local amino acid sequence library containing translations of ORFs detected in HEBV and KIBV genomes.

Genome and phylogenetic analyses. Nucleotide and amino acid sequences were compared with other sequences by BLASTn and BLASTx against GenBank (http://www.ncbi.nlm.Nih.gov/Genbank) and protein motifs were identified by web-based comparison to the Pfam database (http://www.pfam.janelia.org). Identification of cleavage sites of the signal peptide was accomplished using signalP-NN (http://www.cbs.dtu.dk/services/SignalP). Prediction of the hydropathy profile was performed by TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0) and N-linked glycosylation sites were identified using NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc). For phylogenetic analyses, aa sequences of the N, Gn, Gc and RdRp genes were aligned with representative sequences of other bunyaviruses in Geneious using MAFFT (51). Phylogenetic analyses were conducted by the maximum likelihood (ML) algorithm with the BLOSUM62 substitution matrix in a general time-reversible evolutionary model assuming no

systematic rate variation across alignment sites, with confidence testing based on 1000 bootstrap iterations in PhyML (35). Sequence alignments used for phylogenies, including all bunyavirus genera, were 587 aa, 140 aa, 622 aa, and 364 aa in lengths for the N, Gn, Gc, and RdRp proteins, respectively, from which least conserved columns were removed before analysis. Phylogenetic analyses including HEBV, TAIV, and KIBV, all available orthobunyavirus, and tospovirus sequences were based on 3228 aa, 485 aa, 520 aa, and 331 aa for the RdRp, Gn, Gc, and N proteins, respectively.

mRNA analyses. C6/36 cells infected with HEBV and KIBV were harvested 24 hpi. RNA was extracted using the RNA Extraction Kit (Qiagen, Hilden) and analyzed by 5' RACE (Invitrogen, Karlsruhe) or by Northern blotting as described previously (104, 105). DIG-labeled probes for HEBV and KIBV were generated using primer pairs HEBV-N-F 5'- TCATCTTATACAGGAGTTCAAAGAAGCGC and HEBV-N-R 5'-ACATGACTAAACAAGTGTGAGCCTGG, KIBV-N-F 5'-TGGCTTTAAATGGGACCCGGC and KIBV-N-R 5'-GCTAAACAAGTGAGCACCTGGGG, KIBV-X1 5'-CAAGAAGGGCATTGATCTGGTTGTC and KIBV-X2 5'-GCACAGGCACACATCCCCTG, respectively.

Protein analyses. Proteins were analyzed as described previously (105). Briefly, viral particles were purified by gradient ultracentrifugation on a continuous gradient of 1 to 2 M sucrose in 0.01 M Tris-HCl 4 mM Na-EDTA at 35,000 rpm (SW40 rotor; Beckman) for 22h at 4°C. Fractions (0.4 ml each) were tested by real-time PCR and two fractions with the highest amount of genome copies were concentrated trough a 36% sucrose cushion at 35,000 rpm (SW40 rotor; Beckman) for 2h at 4°C. The virus

pellet was resuspended in 150 µl PBS buffer overnight at 4°C. Proteins were lysed in 4XNuPage LDS Sample Buffer at 70°C for 10 minutes and separated by SDS-PAGE on a NuPage Novex 4-12% Bis Tris gel with NuPage MES SDS Running Buffer (Invitrogen, Darmstadt, Germany). Bands were analyzed by limited tryptic digestion and mass spectrometry using a Matrix-assisted laser desorption / ionization with a time-of-flight mass spectrometer (MALDI-TOF). RdRp and Gc proteins were additionally analyzed by liquid chromatography mass spectrometry (LC-MS).

Nucleotide sequence accession numbers. The complete genome sequences of HEBV, TAIV, and KIBV were assigned GenBank accession numbers JQ659256-JQ659258 and KF590572-KF590586. Further sequence fragments from HEBV, TAIV, and KIBV strains over 200 nt were assigned to GenBank accession numbers KF590587- KF590623.

Results

Detection of a novel cluster of mosquito-associated bunyaviruses

In order to investigate the distribution of HEBV and to detect related viruses, we tested pooled female mosquitoes collected in Taï National Park, Côte d'Ivoire (432 pools consisting of 4,839 mosquitoes), Kibale National Park, Uganda (81 pools consisting of 807 mosquitoes) and in Kumasi, Ghana (62 pools consisting of 1,230 mosquitoes) by RT-PCR. HEBV was detected in 39 mosquito pools originating from Côte d'Ivoire and in six mosquito pools originating from Ghana showing nucleotide distances of 94.6 to 98.3% and 94.9 to 99.2% to HEBV (strain F23/CI/2004) within their RdRp genes, respectively (**Table 1**). Individual mosquitoes from positive pools originating from Ghana were tested for infection with HEBV, resulting in a prevalence of 1.1% (14/1,230). Mosquitoes from positive pools from Côte d'Ivoire could not be tested individually as in this case mosquito pools had been homogenized and no individual mosquitoes were available. Two further distinct viruses with distant relationship on nt level to HEBV (72.6 to 72.9%) were obtained from two pools originating from Côte d'Ivoire and from two pools originating from Uganda. On aa level, these viruses had distant relationships to orthobunyaviruses of the Simbu serogroup according to initial BLAST comparisons. The viruses were tentatively named Taï virus (TAIV) and Kibale virus (KIBV). Testing of individual mosquitoes from positive pools from Uganda indicated a prevalence of 0.4% (3/807). Mosquito species and sampling locations are summarized in **Table 1**.

Virus isolation, growth and morphology

HEBV was successfully isolated from 28 pools of mosquitoes in C6/36 cells. TAIV and KIBV were each isolated from two different mosquito pools, respectively. RT-PCR studies showed that both TAIV-containing cell cultures were co-infected with

296 mesoniviruses (105) and these could not be removed from cell cultures by repeated
297 rounds of end-point purification. As plaque purification was not possible due to
298 absence of CPE (see below), TAIV supernatants were not further purified for the
299 purposes of this study and growth curve studies were done only for HEBV and KIBV,
300 for which pure supernatants were available.
301
302 HEBV (isolate C60/CI/2004) and KIBV (isolate P07/UG/2008) reached titers of 3.2 x
303 10^9 $TCID_{50}$ /ml and 3.2 x 10^7 $TCID_{50}$ /ml in infected C6/36 cells, respectively. Growth
304 of HEBV and KIBV was compared in C6/36 and U4.4 cells (**Figure 1A**). For both
305 viruses a 10 to 100 fold higher replication in C6/36 cells than in U4.4 cells was
306 observed by two to three days post infection (dpi). Notably, no CPE was observed for
307 both viruses in U4.4 cells and only weak changes in morphology were detected in
308 C6/36 cells.
309
310 In order to get insight in the putative host tropism, growth of HEBV (isolate
311 F23/CI/2004) was investigated in six different vertebrate cell lines. No CPE was
312 observed and no virus replication was measured by real-time RT-PCR over five blind
313 passages in any of those vertebrate cells (**Figure 1B-C**). Additionally KIBV was
314 inoculated at an MOI of 10 in Vero cells. No virus replication was detected by 7 dpi by
315 real-time RT-PCR.
316
317 In order to assess the potential for transovarial or transveneral transmission, we
318 further tested 269 pools of 1,716 male mosquitoes trapped during the survey in Côte
319 d'Ivoire, 39 pools of 386 male mosquitoes trapped in Ghana, and 11 male
320 mosquitoes trapped in Uganda for infection with HEBV, TAIV or KIBV. No virus was
321 detected by RT-PCR in any of the male mosquitoes.

Virus morphology during maturation was studied in ultra-thin sections of C6/36 cells infected with HEBV (isolate F23/CI/2004). Two types of spherical viral particles 50-60 nm in diameter, of high or low electron density, respectively, were observed in structures resembling Golgi vesicles (**Figure 2A-B**). These were termed intracellular annular viruses (IAV) and intracellular dense viruses (IDV) in agreement with terminology used in studies on Bunyamwera virus (77). Budding or maturation of viral particles at the Golgi membrane was observed into Golgi vesicles filled with IAV and IDV (**Figure 2A-B**). Mature spherical, enveloped virions of about 90-110 nm in diameter were detected in virus pellets generated by ultracentrifugation of cell culture supernatants infected with HEBV (**Figure 2C**).

Genome sequencing and phylogenetic analyses

The entire genomes of four different HEBV (isolates F23/CI/2004, F33/CI/2004, F45/CI/2004, and F53/CI/2004), one TAIV (isolate F47/CI/2004), and one KIBV (isolate P05/UG/2008) were sequenced. All genomes were found to comprise three segments (**Figure 3**). Seven reverse complementary terminal nt were found to be conserved between HEBV, TAIV, and KIBV (**Table 2**). These were identical to terminal sequences in members of the genus *Orthobunyavirus*, where, however, those conserved sequences are 10 nt in length. The three genomes differed in length of their untranslated regions (UTR) of S and M segments (**Figure 3**). Pairwise nt identities among all HEBV genomes ranged between 96.1 and 99.7%. Nucleotide and aa identities of S, M, and L segment ORFs of HEBV, TAIV, and KIBV were >61% (**Table 3**).

No significant similarity was found between the S, M, and L segment ORFs and ORFs of any other viruses using nucleotide BLAST. Low but significant levels of identity (ranging from 12-25%) with N protein-, glycoprotein-, and RdRp protein sequences of orthobunyaviruses (closest related virus was Oropouche virus) were identified by BLASTx using the deduced aa sequences of these ORFs (**Table 3**).

Phylogentic trees were inferred based on the deduced aa sequences of the RdRp, Gn, Gc and N genes. Analyses on all genes including representative sequences of established bunyavirus genera yielded congruent topologies. HEBV, TAIV, and KIBV formed a novel independent monophyletic clade that shared the most recent common ancestor (MRCA) with the genus *Orthobunyavirus* in all genes (**Figure 4**). HEBV, TAIV, and KIBV sequences were almost equidistant to all members of the genera *Orthobunyavirus* and *Tospovirus*.

For a more detailed assessment, additional phylogenetic analyses were done including only the novel viruses as well as all orthobunyaviruses and tospoviruses, so as to avoid losses of sequence information due to indels (**Figure 4**, small pictograms). To investigate whether the novel viruses might fall in the intra-genetic distance range of orthobunyaviruses or tospoviruses, pairwise identity rates for viruses the most divergent from each other of both genera was investigated. The three novel viruses showed a similar distance to each pair indicating a similar distance to all members of both genera (**Table 3**). HEBV, TAIV, and KIBV showed a mean distance of 71-79% to orthobunyavirues and of 81-86% to tospoviruses in all genes, similar to the distance between orthobunyaviruses and tospoviruses (81-86%).

Genome organization of the novel bunyaviruses

373 HEBV, TAIV and KIBV S segments comprised an ORF of 225 aa to 226 aa in
374 complementary sense RNA (cRNA) that putatively encoded a 25 kDa to 27 kDa
375 protein, presumably the N protein (**Figure 3**). No ORF was present near the N
376 terminus of the N ORF, where an NSs protein of ca. 11 kDa is typically located in all
377 members of the genus *Orthobunyavirus*. However, additional ORFs of 42 to 63 aa in
378 cRNA sense were identified within the putative N ORF of HEBV, TAIV, and KIBV
379 (**Figure 3**). No similarities to other sequences in GenBank were detected for the
380 smaller ORFs.
381
382 The M segments of HEBV, TAIV, and KIBV were the shortest bunyavirus M
383 segments reported so far, about 1.2-1.7 kb shorter than the average size of
384 orthobunyavirus M segments (**Table 2**). The segments contained a single ORF
385 ranging between 830 aa and 838 aa in length that putatively encoded in cRNA sense
386 the glycoprotein precursor (GPC) polyprotein that is posttranslationally cleaved into
387 the two envelope glycoproteins Gn and Gc (**Figure 3**). The GPC polyproteins in
388 HEBV and TAIV had two possible in-frame translation initiation codons ($_{47}$AUG,
389 $_{53}$AUG and $_{32}$AUG, $_{53}$AUG, respectively). For KIBV GPC only one translation initiation
390 codon at $_{47}$AUG was found. Signal peptidase cleavage sites, putative transmembrane
391 domains (TMD) and potential N-linked glycosylation sites of HEBV, TAIV and KIBV
392 are summarized in **Figure 3**. Alignment of the putative GPC ORFs of HEBV, TAIV,
393 and KIBV to the pfam database and with orthobunyavirus glycoproteins suggested
394 the Gc proteins of the novel viruses to be truncated by 482 aa at their N-termini
395 compared to those of orthobunyaviruses and suggesting Gn and Gc to have
396 molecular weights of 35 kDa and 56 kDa (**Figure 3**). In contrast to
397 orthobunyaviruses, no coding regions for putative NSm proteins were identified in all
398 three viruses. Putative Gn zinc binding (29) and Gc fusion-peptide domains (69) were

399 identified in the predicted Gn and Gc genes of HEBV, TAIV, and KIBV, respectively
400 (**Figure 5**).
401
402 The L segments of the novel viruses were about 500 nt longer than L segments of
403 orthobunyaviruses due to the insertion of a unique and conserved region from aa
404 position $_{905}$LYI to $_{1,064}$GLY (**Figure 3**). No significant similarity to other sequences in
405 GenBank, including those of other bunyaviruses, was identified. A putative
406 endonuclease domain was identified at the N terminus of the L protein in HEBV,
407 TAIV, and KIBV (36, 74) (**Figure 5**). HEBV, TAIV, and KIBV were almost identical in
408 the motifs of the third conserved region of the RdRp and exhibited the invariant
409 residues found for bunyaviral RdRp motifs, but clearly differed from members of any
410 of the other established genera (**Figure 5**) (1, 32).
411
412 **Transcription mechanism**
413 To investigate if the novel bunyaviruses contain non-templated sequences at their 5'
414 ends, total RNA was analyzed from infected cells in 5´-RACE RT-PCRs with reverse
415 primers placed on all genome segments of HEBV and KIBV. Non-virally templated
416 sequences of 9 to 16 nt and of 10 to 22 nt were detected at the 5' ends of all HEBV
417 and KIBV segments, respectively, indicating viral mRNA 5´-ends to be formed
418 following the typical mechanism for bunyaviruses (**Figure 6**) (9, 46, 82).
419
420 Bunyaviruses generate three different types of RNA for replication and transcription
421 including negative sense genomic RNA (vRNA), positive sense replicative cRNA, and
422 mRNA species that contain 5'-methylated capped non-viral (primer) sequences and
423 truncations at their 3' ends compared to the vRNA and cRNA (28). We did an
424 preliminary analysis of transcription of the S segment of HEBV and KIBV by Northern

Blot. Two bands were detected for HEBV and KIBV, respectively (**Figure 7**). The larger bands likely corresponded to vRNA and cRNA occurring during viral replication and the smaller bands were likely to represent viral mRNA transcription products. No shorter RNA transcripts such as expected in case of transcription from hypothetical downstream promoters were detected (refer to placement of Northern blot probes as shown in **Figure 3**).

Major structural proteins

To identify the major structural proteins, HEBV particles were purified by gradient ultracentrifugation and viral proteins were separated by SDS-PAGE before staining with Coomassie brilliant blue. Four distinct proteins of about 280 kDa, 60 kDa, 36 kDa, and 27 kDa were identified (**Figure 8**). MALDI-TOF mass spectroscopy confirmed two bands to correspond to Gn and N proteins, respectively (**Figure 8**). The RdRp and Gc proteins were identified by LC-MS because MALDI-TOF yielded no conclusive results for these proteins (**Figure 8**). While migrations of the L and N proteins corresponded well with their predicted molecular weights, the bands corresponding to Gc and Gn proteins migrated at higher molecular mass equivalents than predicted upon their amino acid sequences, which would be compatible with N-linked glycosylation at the sites identified above (**Figure 3**).

Discussion

In this study, we discovered and characterized three novel bunyaviruses detected in mosquitoes from Côte d'Ivoire, Ghana, and Uganda. The data showed that HEBV, TAIV, and KIBV represent three novel bunyaviruses that do not group with any of the established bunyavirus genera. Although formal classification criteria for bunyavirus genera are not established, inferred tree topologies showed that the novel viruses form a novel phylogenetic sister group to orthobunyaviruses. Phylogenetic distances and comparisons of sequence similarity suggested these viruses to be sufficiently related with each other to classify them in one genus. In contrast, they were collectively about as distant from the established bunyavirus genera as the latter were from each other. This suggests that the novel viruses might form a separate genus. In order to generate auxiliary classification criteria we investigated host range, viral growth and morphology, genome organization, as well as features of genome replication and gene expression.

HEBV, TAIV, and KIBV were detected in mosquitoes of three different genera (mainly in *Cx. nebulosus, Cx. quinquefasciatus,* and *Cx. simpliforceps*) and replicated well in RNA interference (RNAi) competent U4.4 (2, 63) and in C6/36 cells that have impaired Dicer 2-based RNAi responses (14, 81, 96) indicating no growth restrictions in insect cells with an intact antiviral RNAi system. The growth phenotype in insect cells involving no or very little CPE and the inability to replicate in a large range of vertebrate cells was unexpected. Insect-restricted viruses normally cause clear CPE in insect cells. Absence of CPE in insect cells is rather typical for viruses that can additionally infect vertebrate hosts (70), which in turn could not be confirmed here by cell culture experiments. Notably, for the maintenance of insect-restricted viruses in nature, insect cycles involving horizontal (transveneral) and vertical (transovarial)

transmission are necessary. For instance, transovarial and transveneral transmission to up to 30% of arthropod offspring has been described for bunyaviruses (80, 91, 93). Some viruses can be maintained in overwintering vectors or in time periods with low density of amplifying hosts (61, 89). In contrast, in this study we have gained no evidence for infection of any of the novel viruses in male mosquitoes, which is a hallmark of transovarial or transveneral transmission. Further infection studies on a larger range of vertebrate cell lines, as well as ecological investigations of insects and potential amplificatory vertebrate hosts will be necessary to clarify whether the novel viruses constitute arboviruses. Critically, proof of their insect restriction would constitute a criterion to delineate the novel viruses from the genus *Orthobunyavirus*, a classical group of arboviruses employing vertebrate-based amplification.

Species withinin the genus *Orthobunyavirus* are classically defined by serological criteria (70). The genetic distance between established orthobunyavirus serogroups ranges between 27-53% based on glycoprotein and nucleocapsid protein amino acids. Serogroups will not serologically cross-react with each other (17, 24, 76, 78). Because the amino acid distance between the novel viruses and any orthobunyavirus ranged from 88-89%, and similar distances existed between orthobunya- and tospoviruses, we could not expect the new viruses to yield any meaningful cross-reactivities using any animal serum directed against orthobunya- or tospoviruses. Serological cross-comparisons were therefore not attempted.

Various pathogenicity and tropism-related functions of orthobunyavirus and phlebovirus NSs proteins have been found in mammalian cells, including the suppression of host protein synthesis (6, 15, 37, 58), the inhibition of host cell antiviral interferon response (6, 12, 53, 59, 86, 94, 98), as well as the inhibition of

497 RNA polymerase II-mediated transcription (43, 58, 90). The inability of the novel
498 bunyaviruses to replicate in vertebrate cells might be due to the putative absence of
499 an NSs protein. Putative NSs proteins similar in sequence or position to those in
500 orthobunyaviruses, tospoviruses and phleboviruses were not identified in HEBV,
501 TAIV, and KIBV. The smaller ORFs located in the C terminal half of the N ORF of the
502 novel bunyaviruses may only encode proteins of 5-7 kDa, which is significantly
503 smaller than NSs proteins of other bunyaviruses. Moreover, no mRNAs
504 corresponding in size to the smaller ORFs were detected by Northern blot.
505 Interestingly, viruses of the Anopheles A, Anopheles B and Tete serogroups were
506 able to replicate in newborn mice and Vero cells albeit these viruses were shown not
507 to encode NSs proteins, and were not able to counteract the antiviral interferon
508 response (62). Another group of viruses within the genus *Orthobunyavirus*, the
509 Wyeomyia group viruses, have truncated NSs sequences that may not code for
510 functional proteins (19). However, antibodies were detected in humans and the
511 viruses are associated with febrile illness (1, 25, 84). Whether the inability of HEBV,
512 TAIV, and KIBV to replicate in vertebrate cells is due to the absence of an NSs
513 protein or is encoded within another genome region needs further in depth studies.
514
515 The only other known non-structural protein in bunyaviruses, the NSm protein, that
516 was shown to play a role in the pathogenesis of Rift Valley fever virus (7), was also
517 not present in the three novel viruses. The NSm protein is encoded within
518 orthobunyaviruses between the Gn and Gc proteins. The three proteins are
519 expressed as polyprotein from the M segment ORF and posttranslationally cleaved.
520 So far no orthobunyavirus (or tospovirus) without an NSm protein was reported,
521 providing an additional indication to the uniqueness of the novel viruses as a
522 separate taxonomic entity.

There is little information on the role of NSs and NSm proteins in mosquitoes. It has been shown that the BUNV NSs protein is essential for replication in U4.4 and Ae cells and required for replication and spread in *Ae. aegypti* mosquitoes (87). In contrast, no specific function of the La Crosse virus NSs protein and of the Rift Valley fever virus NSs protein was found in mosquito cells and mosquitoes, respectively (11, 23, 64). However, the NSm protein seems to be essential for replication of Rift Valley fever virus in mosquitoes (23). The Rift Valley fever virus NSm was also found to inhibit apoptosis in mammalian cells (100). In contrast, viruses of the California serogroup (genus *Orthobunyavirus*) seem to induce apoptosis triggered by the NSs protein (22), a function homologous to Reaper, a *Drosophila* protein that induces apoptosis (34, 40). Interestingly, sequence similarities to the Trp/GH3 motif of Reaper and the corresponding Reaper-like regions in the NSs of California serogroup viruses were identified in the L protein of HEBV, TAIV and KIBV ($_{283}$WRILESKLLET$_{293}$, $_{283}$WKDLETKLTST$_{293}$ and $_{283}$WKMLEEKLEK$_{293}$, respectively, conserved sequences among Reaper and HEBV, TAIV and KIBV are underlined). The Trp/ GH3 motif is conserved among Reaper and two other Drosophila proteins, Grim and Sickle, which have crucial functions in programmed cell death (20, 21, 99). Whether this Trp/GH3-like motif in HEBV, TAIV and KIBV may have homologous functions, needs to be studied.

Absence of any NS protein ORFs conserved across the clade comprising tospoviruses, orthobunyaviruses, and the novel viruses suggests that the most recent common ancestor of all of those viruses would not have encoded any of these genes. Rather, the different coding strategies for NS proteins suggest independent acquisitions during the formation of generic viral lineages. In particular, NSs and

549 NSm proteins might have been acquired during the evolution of orthobunyaviruses in
550 the course of acquiring replicative capability in vertebrate hosts.
551
552 A unique insertion of about 500 nt was identified in the RdRp gene of HEBV, TAIV
553 and KIBV. This additional region not found in any other bunyaviruses might represent
554 a putative accessory protein domain. Presence of an accessory domain in the L
555 protein is not unprecedented. For example, CCHFV L protein contains an OTU-like
556 cysteine protease that is suggested to suppress the host-cell inflammatory and
557 antiviral response (30). The L protein of orthobunyaviruses, tospoviruses,
558 hantaviruses and nairoviruses contain an N-terminal endonuclease domain (36, 39,
559 74). However, no sequence similarities of the unique region in HEBV, TAIV and KIBV
560 to any other viral proteins were found. We further specifically searched for GW/WG
561 motifs found to be conserved within viral RNA silencing suppressor proteins encoded
562 by many insect-restricted viruses (10). No such motifs were detected in all translated
563 HEBV, TAIV, and KIBV ORFs. Whether HEBV, TAIV, and KIBV express any
564 accessory proteins at all will therefore require further experimental studies.
565
566 While the ORFs were well conserved among HEBV, TAIV and KIBV, the high
567 variability of the UTRs and the extended length of up to 569 nt in the TAIV M
568 segment 5' UTR was surprising. The UTRs have many different functions and play a
569 role during replication, transcription, encapsidation, and packaging of the viral
570 genome (3, 54, 55, 67). 3' and 5' UTR lengths of the three genome segments are
571 generally well conserved among different orthobunyaviruses with M and L segment 3'
572 and 5' UTRs of about 50 to 100 nt and S segment 3' and 5' UTRs of about 80 to 200
573 nt. It will be interesting to study the functions of these highly different URTs.
574 Interestingly, the terminal nucleotides of the UTRs are strictly conserved among

bunyaviruses of the same genus, serving as a criterion for genus classification (70). HEBV, TAIV, and KIBV contained unique terminal nucleotides that were truncated compared to orthobunyaviruses, precluding their grouping in the genus *Orthobunyavirus* and providing further support that the viruses constitute a separate taxonomic entity.

Segmented negative-strand RNA viruses of the families *Orthomyxoviridae*, *Bunyaviridae*, and *Arenaviridae* use capped RNA primers that are cleaved from the 5' termini of host cell mRNAs, in order to initiate their transcription (9, 18, 32, 47, 73, 82). The lengths of reported capped primers vary from 10 to 20 nt (9, 18, 32, 47, 73, 82). We found non-templated sequences of 9 to 16 nt and 10 to 22 nt at the 5' termini of HEBV and KIBV mRNA's, respectively. Primer sequences containing a 3' U residue were found preferentially, suggesting that the 3' U might be able to undergo base pairing with the terminal 5' A residue of the vRNA during transcription initiation. This would be in good agreement with previous observations in orthobunyaviruses and hantaviruses, where capped primers preferentially terminate at G residues, potentially facilitating RNA primer binding to the terminal 5' C residue (32). Like observed for orthobunyaviruses, a number of primer sequences contained 3' GU or 3' AGU residues (13).

Analyses of RNA products in infected cells indicated that HEBV and KIBV generate truncated mRNAs, similar to what has been described for other bunyaviruses such as Snowshoe hare virus, an orthobunyavirus whose S segment mRNA is about 85 nt shorter than the vRNA species (28).

600 Taken together, our findings suggest that HEBV, TAIV and KIBV cannot be assigned
601 to any existing bunyaviruse genera, while they share common features with each
602 other sufficient to classify them as one genus. Although they are somewhat more
603 closely related to orthobunyaviruses than to other bunyavirus genera, their genome
604 organization and phylogenetic relationships separate them from other genera.
605 Further studies particularly on host restriction and antigenic properties will be
606 necessary to support their putative classification in a separate novel genus.
607

608 **Acknowledgements**

609 We thank the Uganda Wildlife Authority and Uganda National Council of Science and
610 Technology, the Ivorian Ministry of Environment and Forest and the Ministry of
611 Research, as well as the directorship of the Taï National Park and Makerere
612 University Biological Field Station for permission to conduct this research. We thank
613 Clement Nyikiriza and Charles Kyalisima for assistance in the field. We are grateful
614 to Fabian Leendertz for assistance with fieldwork research design and logistic
615 support. We are grateful to Annika Branting and Priyal de Zoysa for performing
616 Illumina Next Generation Sequencing. Funding for fieldwork in Uganda was provided
617 by the Emory University Global Health Institute. The project was supported by the
618 National Institutes of Health (AI057158 Northeast Biodefense Center-Lipkin), the
619 United States Department of Defense, and the United States Agency for International
620 Development (USAID) Emerging Pandemic Threats (EPT) Program, PREDICT
621 project, under the terms of cooperative agreement GHN-A-OO-09-00010-00, as well
622 as by the European Union DG Research, through the program ANTIGONE (grant
623 agreement no. 278976) and by the Deutsche Forschungsgemeinschaft (grant
624 agreement no. DR 772/3-1).

625

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942 **Table 1:** Mosquito species infected with HEBV or KIBV.
943

Virus / strain	Mosquito species	# M	Sampling site	% Pairwise identity to HEBV F23/CI/2004
HEBV				
A11/CI/2004	*Cx. Eum. spp.*	20	Camp	94.8
A18/CI/2004	*Anopheles spp.*	1	Camp	96.3
A26/CI/2004	*Cx. nebulosus*	10	Camp	95.4
A27/CI/2004	N.d.	1	Camp	95.2
A28/CI/2004	*Cx. nebulosus*	22	Camp	95.7
A30/CI/2004	*Ur. mashonaensis*	6	Camp	95.8
A45/CI/2004	*Cx. telesilla*	11	Camp	95.8
A52/CI/2004	n.d.	8	Camp	96.3
A57/CI/2004	*Cx. spp.*	10	Camp	96.4
B40/CI/2004	n.d.	2	Pimary forest	96.1
B42/CI/2004	*Cx. spp.*	9	Pimary forest	95.9
C40/CI/2004	*Ur. mashonaensis*	20	Secondary forest	95.2
C43/CI/2004	*Cx. nebulosus*	17	Secondary forest	96.2
C45/CI/2004	*Cx. nebulosus*	16	Secondary forest	95.8
C57/CI/2004	*Cx. decens*	20	Secondary forest	95.9
C59/CI/2004	*Cx. decens*	20	Secondary forest	97.1
C60/CI/2004	*Cx. decens*	9	Secondary forest	97.1
C68/CI/2004	*Cx. spp.*	21	Secondary forest	96.2
C88/CI/2004	n.d.	20	Secondary forest	96.3
D24/CI/2004	*Cx. spp.*	23	Plantation	95.7
D28/CI/2004	*Anopheles spp.*	2	Plantation	95.4
D50/CI/2004	*Cx. nebulosus*	20	Plantation	96.5
D60/CI/2004	n.d.	15	Plantation	98.3
D61/CI/2004	n.d.	11	Plantation	94.6
D62/CI/2004	*Cx. spp.*	14	Plantation	96.2
F23/CI/2004	*Cx. nebulosus*	20	Village	
F25/CI/2004	*Cx. nebulosus*	21	Village	95.8
F26/CI/2004	*Cx. nebulosus*	50	Village	95.1
F27/CI/2004	*Cx. nebulosus*	40	Village	96.7
F28/CI/2004	*Cx. nebulosus*	20	Village	96.1
F30/CI/2004	*Cx. nebulosus*	20	Village	96.5
F32/CI/2004	*Cx. nebulosus*	15	Village	96.2
F33/CI/2004	*Cx. nebulosus*	12	Village	96.1
F43/CI/2004	*Cx. spp.*	1	Village	96.2
F45/CI/2004	*Cx. spp.*	26	Village	95.8
F47/CI/2004	*Culicidae spp.*	10	Village	95.7
F53/CI/2004	*Cx. quinquefasciatus*	8	Village	96.1
F54/CI/2004	*Cx. antenatus*	20	Village	96.3
F55/CI/2004	*Cx. antenatus*	9	Village	96.1
M257/P13/GH/2011	*Cx. quinquefasciatus*	1	Residential area	95.4
M538/P27/GH/2011	*Cx. nebulosus*	1	Botanical garden	96.7
M540/P27/GH/2011	*Cx. nebulosus*	1	Botanical garden	95.9
M566/P29/GH/2011	*Cx. nebulosus*	1	Botanical garden	100
M569/P29/GH/2011	*Cx. nebulosus*	1	Residential area	95.9
M572/P29/GH/2011	*Cx. nebulosus*	1	Residential area	96.3
M105/P06/GH/2011	*Cx. pipiens*	1	Residential area	96.6
M120/P06/GH/2011	*Cx. pipiens*	1	Residential area	96.6
M201/P11/GH/2011	*Cx. quinquefasciatus*	1	Residential area	95.4
M206/P11/GH/2011	*Cx. quinquefasciatus*	1	Residential area	97.1
M211/P11/GH/2011	*Cx. quinquefasciatus*	1	Botanical garden	96.2
M213/P11/GH/2011	*Cx. quinquefasciatus*	1	Residential area	97
M219/P11/GH/2011	*Cx. quinquefasciatus*	1	Residential area	97.1
M858/P43/GH/2011	*Cx. nebulosus*	1	Botanical garden	94.9

TAIV				
C48/CI/2004	*Cx. nebulosus*	nd	Secondary forest	75.8
F47/CI/2004	*Culicidae spp.*	10	Village	76.1
KIBV				
M15/P05/UG/2008	*Cx. simpliforceps*	1	Forest edge	72.7
M22/P05/UG/2008	*Cx. simpliforceps*	1	Forest edge	72.4
M202/P07/UG/2008	*Culex spp.*	1	Tea plantation	72.4

944 *, Pool; M, mosquito; nd, not determined; CI, Côte d'Ivoire; GH, Ghana; UG, Uganda

945

946 **Table 2:** Genome size and consensus terminal nucleotides of HEBV, TAIV and KIBV

947 compared to established genera of the family Bunyaviridae.

Genus/virus		Consensus terminal nucleotides	Genome size	Segment sizes (Accession #)		
				S	M	L
Hantavirus/ Hantaan virus		3' AUCAUCAUCUG- 5' UAGUAGUAUGC-	11845	1696 (M14626)	3616 (M14627)	6533 (X55901)
Nairovirus/ Dugbe virus		3' AGAGUUUCU- 5' UCUCAAAGA-	18855	1712 (M25150)	4888 (M94133)	12255 (U15018)
Tospovirus/ Tomato spotted wilt virus		3' UCUCGUUA- 5' AGAGCAAU-	16634	2916 (D00645)	4821 (S48091)	8897 (D10066)
Phlebovirus/ Rift Valley fever virus		3' UGUGUUUC- 5' ACACAAAG-	11979	1690 (X53771)	3885 (M11157)	6404 (X56464)
Unassigned/ Gouléako virus		3' UGUGU- 5' ACACA-	10633	1087 (HQ541736)	3188 (HQ541737)	6358 (HQ541738)
Orthobunyavirus/ Bunyamwera virus		3' **UCAUCAC**AUG- 5' **AGUAGUG**UGC-	12294	961 (D00353)	4458 (M11852)	6875 (X14383)
Unassigned/ Herbert virus	S	3' **UCAUCAC**ACG- 5' **AGUAGUG**CAC-	11202	1090	2684	7428
	M	3' **UCAUCAC**ACG- 5' **AGUAGUG**CAC-				
	L	3' **UCAUCAC**ACG- 5' **AGUAGUG**UGC-				
Kibale virus	S	3' **UCAUCAC**ACG- 5' **AGUAGUG**CAC-	11322	1212	2683	7427
	M	3' **UCAUCAC**ACG- 5' **AGUAGUG**CAC-				
	L	3' **UCAUCAC**ACG- 5' **AGUAGUG**CAC-				
Tai virus	S	3' **UCAUCAC**GUG- 5' **AGUAGUG**CAC-	11728	1156	3118	7454
	M	3' **UCAUCAC**GUG- 5' **AGUAGUG**CAC-				
	L	3' **UCAUCAC**GUG- 5' **AGUAGUG**UGC-				

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Table 3: Nucleotide and amino acid pairwise sequence identity values for HEBV, TAIV, KIBV, and OROV, as well as pairs of the most distantly related orthobunyaviruses and tospoviruses.

Gene	Percent nucleotide or amino acid sequence identity								
RdRp		**HEBV**	**TAIV**	**KIBV**	**OROV**	**SIMV**	**SORV**	**TZSV**	**BeNMV**
	HEBV		73.9	73.7	37.8	38.9	39.0	28.4	28.0
	TAIV	79.2		72.1	37.4	38.5	38.8	27.7	27.8
	KIBV	80.0	78.5		36.8	38.0	38.1	27.9	27.5
	OROV	24.7	24.6	24.8		60.8	56.2	27.8	27.4
	SIMV	24.7	24.6	24.6	58.2		55.7	27.3	27.3
	SORV	24.3	24.3	24.2	49.1	47.2		27.2	27.4
	TZSV	14.1	14.0	13.5	13.6	13.1	13.3		52.6
	BeNMV	13.5	14.0	13.0	14.2	12.6	12.4	41.4	
GPC		**HEBV**	**TAIV**	**KIBV**	**OROV**	**AKAV**	**TAHV**	**MYSV**	**BeNMV**
	HEBV		70.0	69.6	21.9	22.2	21.8	20.4	20.7
	TAIV	70.4		68.4	21.5	22.5	21.8	20.1	20.5
	KIBV	69.6	67.2		21.3	22.3	21.4	20.2	20.5
	OROV	12.3	12.6	12.0		43.2	42.6	26.5	26.5
	AKAV	12.0	11.5	11.7	24.7		45.0	23.6	24.3
	TAHV	10.7	11.4	11.8	31.8	29.3		23.1	22.5
	MYSV	11.6	12.5	12.4	9.8	10.9	10.3		39.3
	BeNMV	12.4	12.2	12.6	9.7	10.8	8.9	32.6	
N		**HEBV**	**TAIV**	**KIBV**	**OROV**	**BMAV**	**BORV**	**TZSV**	**INSV**
	HEBV		65.3	69.3	31.0	30.6	33.5	25.1	25.4
	TAIV	66.2		64.0	29.9	31.3	33.8	25.3	25.6
	KIBV	72.6	60.9		32.7	31.7	33.7	25.5	25.4
	OROV	19.8	20.2	20.2		38.2	37.3	24.4	27.1
	BMAV	16.5	17.4	15.9	32.1		39.7	26.9	24.3
	BORV	17.0	16.7	18.3	31.5	24.8		27.2	25.6
	TZSV	10.8	11.5	12.2	10.6	9.5	11.4		24.7
	INSV	13.2	12.0	12.7	14.6	11.7	11.5	24.9	

Top right, nucleotide sequence identity; bottom left, amino acid identity; AKAV, Akabane virus; BeNMV, Bean necrotic mosaic virus; BMAV, Batama virus; BORV, Boraceia virus; INSV, Impatiens necrotic spot virus; MYSV, Melon yellow spot virus; OROV, Oropouche virus; SIMV, Simbu virus; SORV, Sororoca virus; TAHV, Tahyna virus; TZSV, Tomato zonate spot virus.

958 **Figure legends**

959 **Fig. 1: Growth of HEBV and KIBV.** (A) C6/36 and U4.4 cells were infected with
960 HEBV and KIBV at MOIs of 0,1 and 0,01, respectively. Genome copies per milliliter in
961 cell culture supernatant were measured by RT-PCR for 5 days. (B) Vertebrate cells
962 were infected with HEBV at indicated MOI's and five blind passages at 37°C were
963 performed. Genome copies per milliliter in cell culture supernatant were measured by
964 RT-PCR at seven days post infection (solid bars) and of the fifth passage (dashed
965 bars). (C) Cells were infected and passaged as described under B but incubated at
966 33°C. Supernatants of same cell lines infected at different MOI's were pooled and
967 measured by RT-PCR.

968

969 **Fig. 2: Maturation and morphology of HEBV.** Ultra-thin sections of C6/36 cells
970 infected with HEBV (A-B) and negative stained ultracentrifuged virions of HEBV (C).
971 Budding arcs are indicated by black arrows, annular spherical particles by white
972 arrowheads and dense spherical particles by black arrowheads. Abbreviations of
973 cellular compartments are Nu, Nucleus, Mi, Mitochondria, and Go, Golgi apparatus.
974 Bar = 500nm (A) and 100 nm (B-C).

975

976 **Fig. 3: Schematic view of the genome organization of HEBV, TAIV and KIBV.**
977 Open reading frames are shown by light yellow boxes, mRNAs are indicated by black
978 arrows and non-template sequences at the 5'-terminus are symbolized by red boxes.
979 Predicted proteins are shown by light blue boxes. Northern blot probes are shown by
980 dark yellow boxes, putative transmembrane domains (hydrophobic regions) are
981 marked by green boxes, glycosylation sites by triangles, the unique region in the
982 RdRp gene by a light grey box, the endonuclease domain by a dark grey box, the
983 putative signal peptide by a blue, the Gn zinc finger motif by an orange, and the Gc

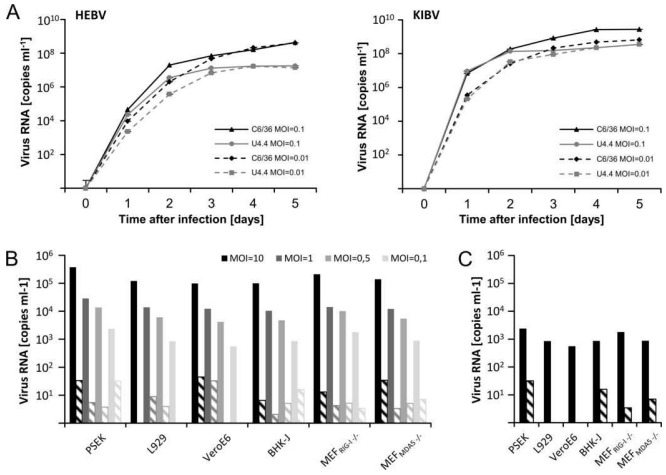
fusion peptide by a dashed box. Genome positions and predicted molecular protein masses are indicated.

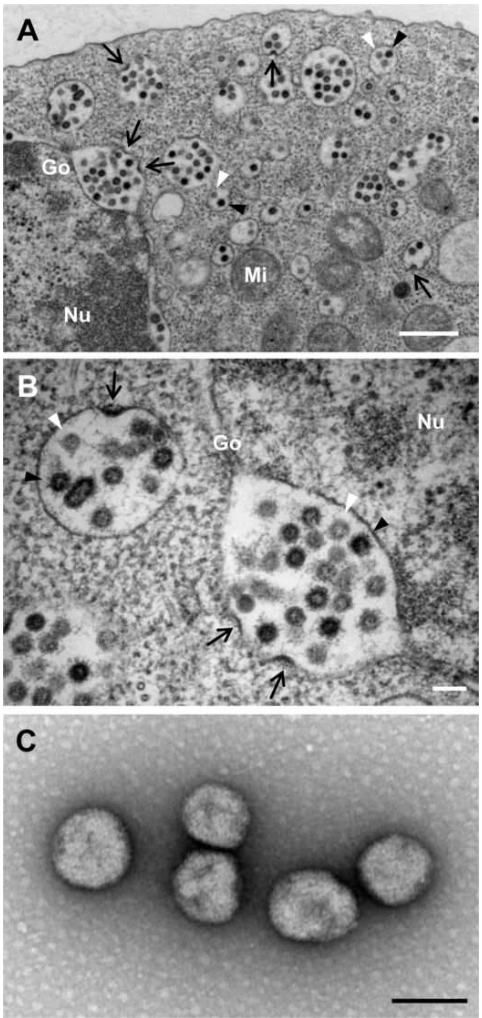
Fig. 4: Phylogenetic relationship of HEBV, TAIV and KIBV to representative members of the family *Bunyaviridae*. Phylogenies were investigated for the RdRp, Gn, Gc, and N proteins based on 364 aa, 140 aa, 622 aa, and 587 aa, respectively. Maximum likelihood (ML) analyses were performed on a gap free alignment guided by the BLOSUM62 substitution matrix and using MAFFT (E-INS-I algorithm). Confidence testing was performed by 1000 bootstrap replicates. Bars indicate evolutionary substitutions per position in the alignments. Smaller pictograms represent ML analyses of HEBV, TAIV, KIBV, all available orthobunyavirus, and tospovirus sequences based on 3228 aa, 485 aa, 520 aa, and 331 aa for the RdRp, Gn, Gc, and N proteins, respectively. Accession numbers for L, M and S segments are: AGUV, Aguacate virus, NC_015451, NC_015450, NC_015452; AINOV, Aino virus, NC_018465, NC_018459, NC_018460; AKAV, Akabane virus, NC_009894, NC_009895, NC_009896; AMBV, Anhembi virus, JN572062, JN572063, JN572064; ANDV, Andes virus, NC_003468, NC_003467, NC_003466; BeNMV, Bean necrotic mosaic virus, NC_018070, NC_018072, NC_018071; BUNV, Bunyamwera virus, NC_001925, NC_001926, NC_001927; CACV, Capsicum chlorosis virus, NC_008302, NC_008303, NC_008301; CDUV, Candiru virus, NC_015374, NC_015373, NC_015375; DOBV, Dobrava virus, NC_005235, NC_005234, NC_005233; GBNV, Groundnut bud necrosis virus, NC_003614, NC_003620, NC_003619; GOLV, Gouleako virus, HQ541738, HQ541737, HQ541736; GRSV-TCSV, Groundnut ringspot and Tomato chlorotic spot virus reassortant, NC_015469, NC_015468, NC_015467; HEBV, Herbert virus, JQ659256, JQ659257, JQ659258; HTNV, Hantaan virus, NC_005222, NC_005219, NC_005218; HVZ10, Hantavirus

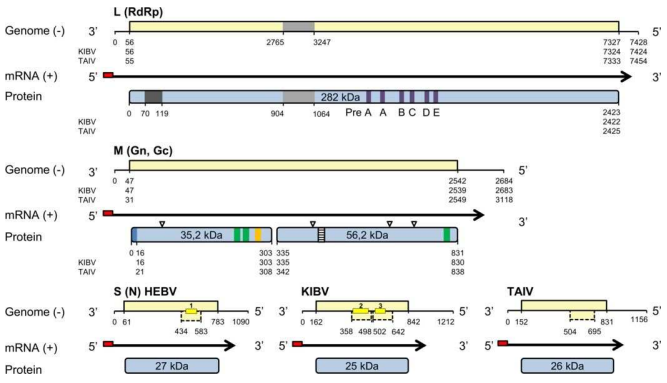
Z10 virus, NC_006435, NC_006437, NC_006433; INSV, Impatiens necrotic spot virus, NC_003625, NC_003616, NC_003624; KIBV, Kibale virus, KF590577, KF590576, KF590575; LACV, La Crosse virus, NC_004108, NC_004109, NC_004110; LEAV, Leanyer virus, HM627178, HM627176, HM627177; MCAV, Macaua virus, JN572068, JN572069, JN572070; MYSV, Melon yellow spot virus, NC_008306, NC_008307, NC_008300; OROV, Oropouche virus, NC_005776, NC_005775, NC_005777; PUUV, Puumala virus, NC_005225, NC_005223, NC_005224; RVFV, Rift Valley Fever virus, NC_014397, NC_014396, NC_014395; SATV, Sathuperi virus, NC_018461, NC_018466, NC_018462; SBV, Schmallenberg virus, JX853179, JX853180, JX853181; SEOV, Seoul virus, NC_005238, NC_005237, NC_005236; SFSV, Sandfly fever Sicilian virus, NC_015412, NC_015411, NC_015413; SFTSV, Severe Fever with Thrombocytopenia Syndrome virus, NC_018136, NC_018138, NC_018137; SHAV, Shamonda virus, NC_018463, NC_018467, NC_018464; SIMV, Simbuvirus, NC_018476, NC_018478, NC_018477; SNV, Sin Nombre virus, NC_005217, NC_005215, NC_005216; SORV, Sororoca virus, JN572071, JN572072, JN572073; TAIV, Taï virus, KF590574, KF590573, KF590572; TOSV, Tomato spotted wilt virus, NC_002052, NC_002050, NC_002051; TPMV, Thottapalayam virus, NC_010707, NC_010708, NC_010704; TSWV, Tomato spotted wilt virus, NC_002052, NC_002050, NC_002051; TULV, Tula virus, NC_005226, NC_005228, NC_005227; TZSV, Tomato zonate spot virus, NC_010491, NC_010490, NC_010489; UUKV, Uukuniemi virus, NC_005214, NC_005220, NC_005221; WSMOV, Watermelon silver mottle virus, NC_003832, NC_003841, NC_003843; WYOV, Wyeomyia virus, JN572080, JN572081, JN572082

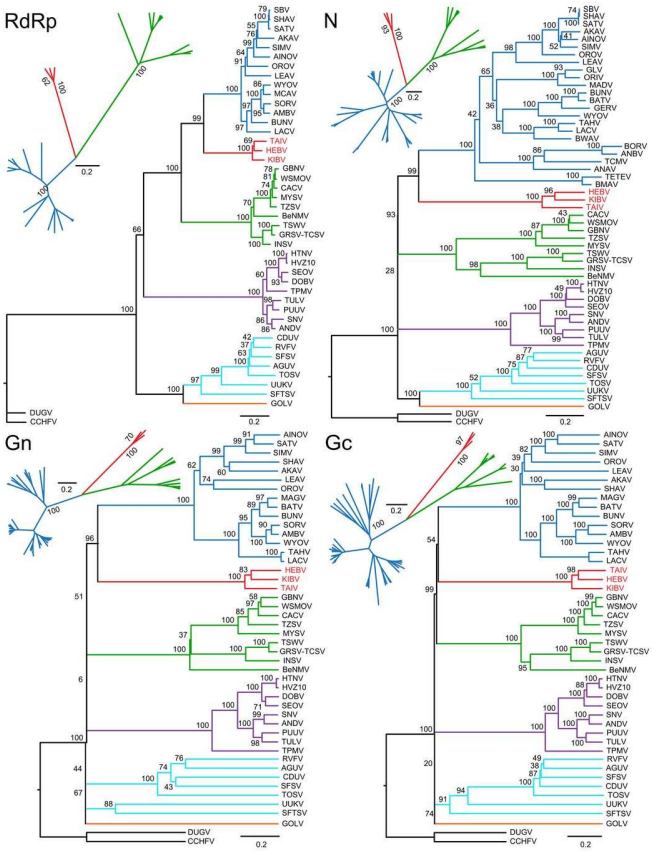
Fig. 5: Multiple sequence alignments of conserved domains of HEBV, TAIV and KIBV and other bunyaviruses. Alignments were performed using the E-INS-I

1036 algorithm in MAFFT and manually edited. Numbers represent genome positions.
1037 Amino acids with 100% identity are highlighted in black, with 75% identity in dark
1038 grey, and with 50% identity in light grey. Gn zinc finger motifs are highlighted in black
1039 and conserved basic residues in dark grey.
1040
1041 **Fig. 6: Non-templated sequences of mRNAs of HEBV and KIBV.** 5'-genome
1042 termini of L, M and S segment mRNAs of HEBV and KIBV. C6/36 cells were infected
1043 with HEBV and KIBV and total RNA was extracted 1 dpi, respectively. Genome
1044 termini were amplified by 5' RACE PCR, PCR products were cloned, and five random
1045 clones were analyzed. Non-template sequences (putative transcription primers
1046 obtained from host cell mRNAs) are marked by a gray box. Conserved genome
1047 termini of HEBV and KIBV are shown in bold.
1048
1049 **Fig. 7: S segment replication and transcription products analyzed by northern**
1050 **blotting.** Viral RNA was isolated from HEBV and KIBV infected C6/36 cells 2 dpi.
1051 RNA from non-infected C6/36 cells was used as a control. A DIG-labeled RNA was
1052 used as a size marker (M), with sizes given in nucleotides to the right. Positions of
1053 DIG-PCR-probes are shown in Fig. 3.
1054
1055 **Fig. 8: SDS-PAGE analysis of HEBV major structural proteins.** Particles were
1056 purified from cell culture supernatant of infected C6/36 cells by gradient
1057 ultracentrifugation. Proteins were stained with Coomassie blue R-250. Obtained
1058 MALDI-TOF data are shown below and LC-MS data above schematic view of
1059 proteins to the right.

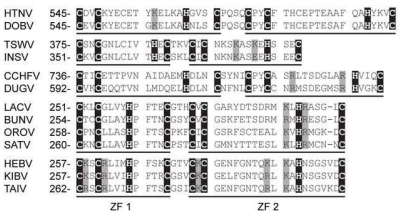




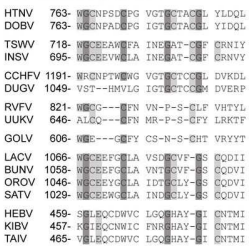




Gn zinc finger motif



Gc fusion peptide



Endonuclease