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Short Communication

Kolente virus, a rhabdovirus species isolated from ticks and bats in the Republic of Guinea

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Kolente virus (KOLEV) is a rhabdovirus originally isolated from ticks and a bat in Guinea, West Africa, in 1985. Although tests at the time of isolation suggested that KOLEV is a novel rhabdovirus, it has remained largely uncharacterized. We assembled the complete genome sequence of the prototype strain DakAr K7292, which was found to encode the five canonical rhabdovirus structural proteins (N, P, M, G and L) with alternative ORFs (>180 nt) in the P and L genes. Serologically, KOLEV exhibited a weak antigenic relationship with Barur and Fukuoka viruses in the Kern Canyon group. Phylogenetic analysis revealed that KOLEV represents a distinct and divergent lineage that shows no clear relationship to any rhabdovirus except Oita virus, although with limited phylogenetic resolution. In summary, KOLEV represents a novel species in the family *Rhabdoviridae*.

Members of the family *Rhabdoviridae* have a diverse host range that includes vertebrates, invertebrates and plants, and transmission often requires arthropod vectors such as mosquitoes, fleas, sandflies, lice and ticks (Bourhy *et al.*, 2005; Dietzgen *et al.*, 2012; Tesh *et al.*, 1972). Whilst most rhabdoviruses have been assigned to nine genera (*Vesiculovirus*, *Lyssavirus*, *Sigmavirus*, *Tibrovirus*, *Perhavirus*, *Ephemerovirus*, *Novirhabdovirus*, *Cytorhabdovirus* and *Nucleorhabdovirus*) (Dietzgen *et al.*, 2012), several recently identified members have not been assigned to any genus (Allison *et al.*, 2011; Gubala *et al.*, 2008, 2010, 2011; Kondo *et al.*, 2006; Kuzmin *et al.*, 2006; Longdon *et al.*, 2010; Quan *et al.*, 2010b; Tao *et al.*, 2008). Here, we demonstrate that Kolente virus (KOLEV; strain DakAr K 7292) is a probable novel rhabdovirus species with a distinctive morphology and genome organization. The virus strain used in this study was isolated from a pool of *Amblyomma* (*Theileriella*) *variegatum* ticks and from a roundleaf bat (*Hipposideros jonesi*) collected during an arbovirus survey in the Kindia region (10°3′N, 12°52′W) of western Guinea in 1985 (Butenko, 1996; Konstantinov *et al.*, 2006). These collections were part of a longitudinal survey conducted by the USSR-Guinea Virological and Microbiological Laboratory from 1978 to 1991. Initial studies at the Pasteur Institute in Dakar suggested that KOLEV is a rhabdovirus (Butenko, 1996).

The GenBank/EMBL/DDBJ accession number for the sequence of Kolente virus is KC984953.

Three supplementary figures and one table are available with the online version of this paper.

We first demonstrated that monolayer cultures of baby hamster kidney (BHK-21) cells inoculated with KOLEV developed a marked cytopathic effect in 5 days. Subsequently, newborn mice (1–2 days old) inoculated intracranially with KOLEV showed signs of illness (loss of balance, paralysis and lethargy) 4–5 days after inoculation. Intraperitoneal inoculation of adult outbred mice (10–12 weeks old) with KOLEV did not cause visible illness, but the animals developed virus-specific antibodies. Mice (ICR strain) were obtained from Harlan Sprague–Dawley, and all animal work was conducted under an Institutional Animal Care and Use Committee-approved protocol at the University of Texas Medical Branch (UTMB).

In ultrathin sections of KOLEV-infected BHK-21 cells, virions exhibited bullet-like morphology (~75 × 100–160 nm). Virions were observed mostly at the cytoplasmic membrane, where they were seen budding (Fig. 1a); aggregates of tightly packed virions were also observed in the intracellular spaces (Fig. 1b). Sections were processed as described previously (Vasilakis *et al.*, 2013) and cut on a Leica EM UC7 ultramicrotome (Leica Microsystems), stained with lead citrate and examined in a Philips 201 transmission electron microscope at 60 kV.

Complement fixation (CF) tests were conducted to compare KOLEV with nine other selected vertebrate rhabdoviruses (Table 1a). All viruses were obtained from the World Reference Center for Emerging Viruses and Arboviruses, located at UTMB. CF tests were conducted according to a microtechnique described previously (Beaty *et al.*, 1989; Tesh *et al.*, 1983) using 2 full units of guinea pig complement. Titres were recorded as the highest dilutions giving 3+ or 4+ fixation of complement on a scale of 0–4+. Antigens used in CF tests were prepared from infected newborn mouse brains by sucrose/acetone extraction (Clarke & Casals, 1958). Specific hyperimmune mouse ascitic fluids were prepared against each of the ten rhabdoviruses (Table 1a), as described previously (Vasilakis *et al.*, 2013). KOLEV showed a distant antigenic relationship with Barur virus (BARV) and Fukuoka virus (FUKAV), but not with the other seven viruses tested (Table 1b). BARV and FUKAV are assigned to the Kern Canyon serogroup of animal rhabdoviruses, along with Kern Canyon virus (KCV) and Nkolbisson virus (NKOV) (Tordo *et al.*, 2005). Nine of the ten viruses included in the CF tests showed varying degrees of relatedness to each other, and several viruses including BARV and FUKAV, NKOV and Gossas virus (GOSV), as well as Le Dantec virus (LDV) and Keuraliba virus (KEUV), are very closely related antigenically (Table 1b). Similar results have been reported previously (Calisher *et al.*, 1989). Oita virus (OITAV) is the only virus among this group that is completely unrelated antigenically.

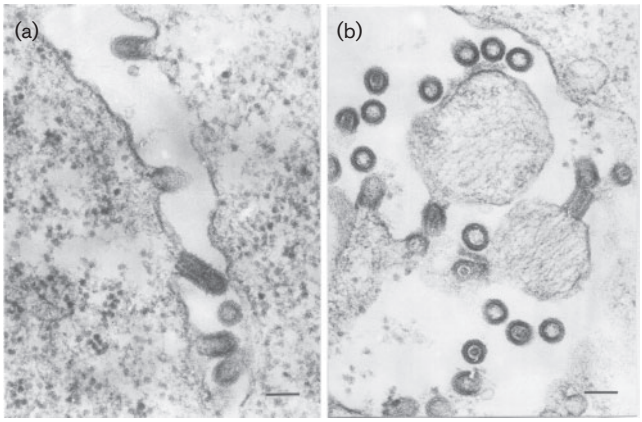


Fig. 1. Ultrastructure of KOLEV in BHK-21 cells. (a) Electron micrograph showing virions forming at the cell surface. (b) Cross-section of virions at the cell surface and in an intracellular space. Bars, 100 nm.

To characterize KOLEV further, we obtained its complete genome sequence by 50 bp paired-end sequencing on a HiSeq2000 Illumina platform. Briefly, a confluent monolayer of BHK-21 cells (in a T25 flask) was infected with KOLEV and harvested 5 days later when an extensive cytopathic effect was present. Preparation and processing of viral RNA for sequencing was as described previously (Vasilakis *et al.*, 2013). About 14 % of the sequence reads in the sample assembled into the viral contig, resulting in ~330 000 reads mapped out of ~2.3 million total.

The 11 120 nt negative-sense ssRNA KOLEV genome comprised a 61 nt 3′ leader sequence, a 75 nt partially complementary 5′ trailer sequence and five coding regions bounded by highly conserved sequence motifs [GUUGCUC and G(U/C)ACUUUUUUU] similar, respectively, to the transcription initiation (TI) and transcription termination/polyadenylation (TTP) sequences of other rhabdoviruses (Fig. 2c). Long ORFs encoded the five canonical rhabdovirus structural proteins (3′N-P-M-G-L-5′). Alternative ORFs in the P gene (P′; 195 nt) and L gene (L′; 225 nt) encoded potential proteins of >60 aa. The TI and TTP sequences of each gene were separated by short (1– 4 nt) intergenic sequences, except for the P and M genes, which overlapped by 24 nt.

The KOLEV N ORF encoded a 426 aa polypeptide (47.8 kDa) that shared 54.5 % sequence identity with the OITAV N protein, including a core sequence of 155 aa of which 123 (79.4 %) were identical (Fig. S1, available in JGV Online). Although only a partial N gene sequence was available, KOLEV shared significant amino acid sequence identity with the N protein of Mount Elgon bat virus (MEBV), which was isolated from horseshoe bats (*Rhinolophus hildebrandtii*) in Kenya in 1964 (Metselaar *et al.*, 1969). In the same core region of the N protein, KOLEV and MEBV shared 129 (83.2 %) of 155 aa (Fig. S1).

The KOLEV P ORF encoded a 296 aa acidic polypeptide (32.8 kDa). Although similar in size and net charge, BLASTP and HHMER searches and pairwise alignments failed to

Table 1. Serology of KOLEV

(a) Vertebrate rhabdoviruses used in serological studies.

Virus name (abbreviation)	Strain	Source of isolate	Genus	Country	Collection date
Barur virus (BARV)	I 6235	Rodent	*Rattus wroughtoni*	India	1962
Fukuoka virus (FUKAV)	FUK 11	Midge	*Culicoides punctatus*	Japan	1982
Gossas virus (GOSV)	DAK AN D 401	Bat	*Tadarida* sp.	Senegal	1964
Kern Canyon virus (KCV)	USA M 206	Bat	*Myotis yumanensis*	USA	1956
Keuraliba virus (KEUV)	DAK AN D 5314	Rodent	*Tatera kempii*	Senegal	1968
Kolente virus (KOLEV)	DakAr K7292	Tick	*Amblyomma variegatum*	Guinea	1985
Le Dantec virus (LDV)	DAK HD 763	Human		Senegal	1965
Mount Elgon bat virus (MEBV)	BP 846	Bat	*Rhinolophus hildebrandtii*	Kenya	1964
Nkolbisson virus (NKOV)	YM 31-65	Mosquito	*Eretmapodites leucopus*	Cameroon	1965
Oita virus (OITAV)	296-1972	Bat	*Rhinolophus cornutus*	Japan	1972

(b) Results of CF tests with Kolente and other selected rhabdoviruses.

Antigen	Antibody*									
	KOLEV	KCV	BARV	FUKV	NKOV	GOSV	LDV	KEUV	OITAV	MEBV
KOLEV	512–128	0	0	0	0	0	0	0	0	0
KCV	0	128–512	0	0	0	8/⩾8	0	0	0	0
BARV	8/⩾8	0	256/⩾512	512/⩾512	16/⩾8	16/⩾8	0	0	0	0
FUKV	8–8	0	128/⩾512	1024/⩾512	16/⩾8	16/⩾8	0	0	0	0
NKOV	0	0	0	0	1024/⩾512	1024/⩾512	0	0	0	0
GOSV	0	0	8/⩾8	0	1024/⩾512	1024/⩾512	0	0	0	0
LDV	0	0	0	0	0	0	1024/128	16/32	0	0
KEUV	0	0	0	0	0	8/⩾8	256/⩾128	512/⩾128	0	0
OITAV	0	0	0	0	0	0	0	0	1024/⩾8	0
MEBV	0	0	0	16 ⩾8	0	0	0	0	0	⩾1024/⩾8

*Antibody titers are expressed as the reciprocal of the highest positive antibody dilution; 0 represents a titre of <8.

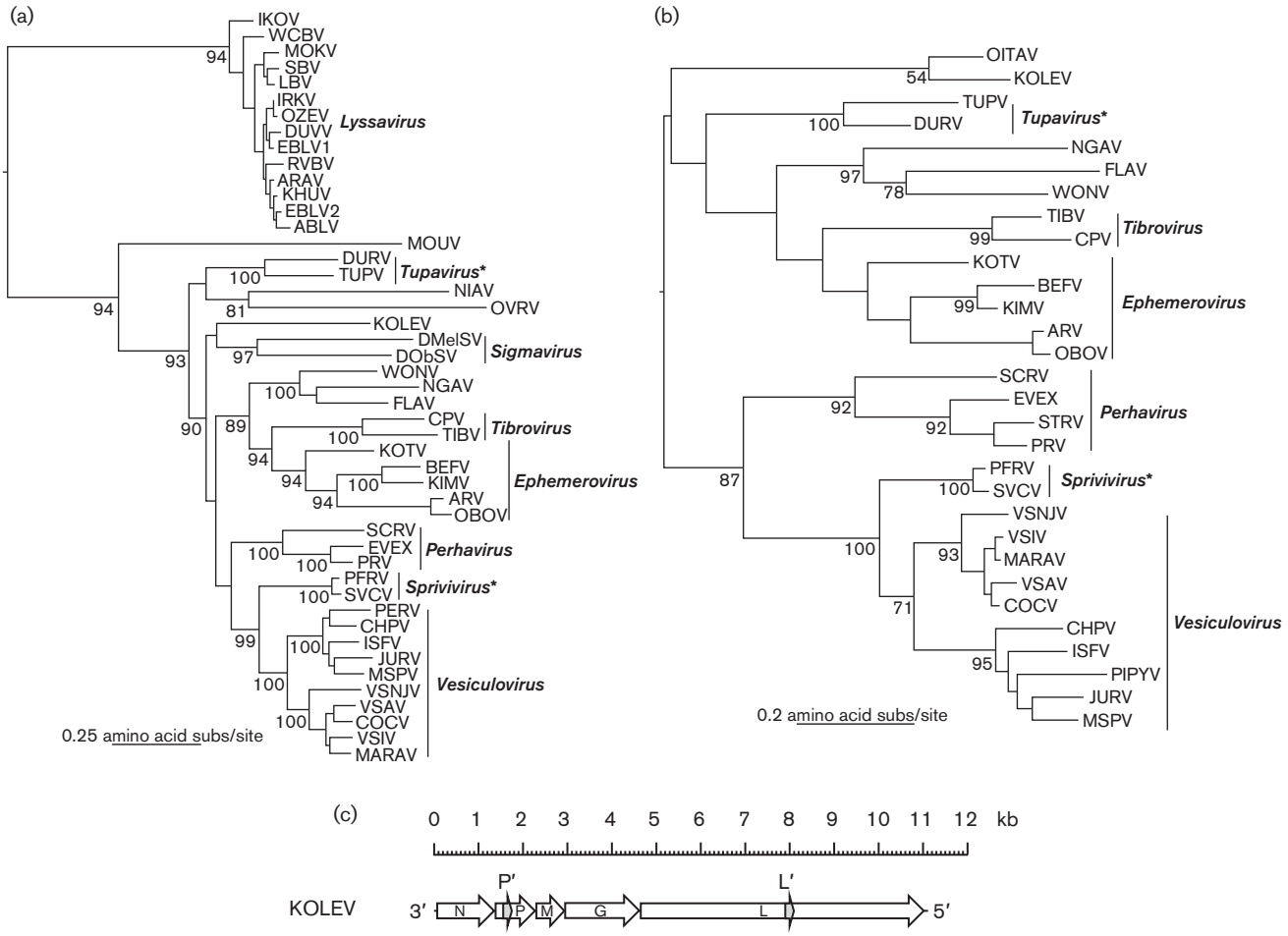
demonstrate significant amino acid sequence identity with the P proteins of other rhabdoviruses.

The KOLEV M ORF encoded a 207 aa polypeptide (23.8 kDa) that shared identifiable sequence similarity with the M proteins of other rhabdoviruses. The highest sequence similarity was observed with the M proteins of rhabdoviruses in the genus *Ephemerovirus* (Fig. S2). Interestingly, the late budding domain (L-domain) motif, which occurs in many rhabdoviruses and other enveloped viruses (Freed, 2002), appeared in KOLEV as PPxS rather than PPxY, which is characteristic of vesiculoviruses and ephemeroviruses (Jayakar *et al.*, 2000).

The KOLEV G ORF encoded a 549 aa polypeptide with the structural characteristics of rhabdovirus G proteins, including predicted signal peptide and transmembrane domains. There were four predicted *N*-glycosylation sites that were each predicted to be glycosylated (NetNGlyc 1.0 Server), resulting in a fully processed G protein of ~72.2 kDa. The KOLEV G protein was most closely related to those of vesiculoviruses (BLASTP). Alignment with the vesicular stomatitis virus (VSV) Indiana G protein indicated that all 12 cysteine residues (C_I–C_{XII}) were present in the KOLEV G protein (Walker & Kongsuwan, 1999). Two additional cysteine residues in KOLEV G may form a seventh disulphide bridge in the lateral domain, upstream of the transmembrane domain (Fig. S3).

The KOLEV L ORF encoded a 242.2 kDa RNA-dependent RNA polymerase (L protein) containing the six conserved regions common to all rhabdoviruses (Poch *et al.*, 1990). In sequence alignments, the KOLEV L protein displayed 38.5–50.3 % amino acid sequence identity with the full-length L proteins of 46 available mammalian and insect rhabdoviruses (http://imed.med.ucm.es/Tools/sias.html).

Alternative ORFs in the P and L genes encoded putative small basic polypeptides of 7.7 kDa (pI 13.08) and 8.5 kDa (pI 11.47), respectively, that displayed no significant sequence similarity to any known protein. The putative P′ protein was similar in size and net charge to the C protein that is expressed from the VSV P gene (Spiropoulou & Nichol, 1993). The P′ protein was predicted to contain strong nuclear localization signals in both the N-terminal region (IARKSKRRRVNP) and the



Coding region	ORFs	Lengths (nt)	Transcriptional regulatory sequences	
			TI	TTP
1	N	1278	GUUGCUC	GUACUUUUUUU
2	P/P′	888/195	GUUGCUC	GCACUUUUUUU
3	M	621	GUUGCUC	GUACUUUUUUU
4	G	1647	GUUGCUC	GUACUUUUUUU
5	L/L′	6360/225	GUUGCUC	GUACUUUUUUU

Fig. 2. Phylogeny and organization of the KOLEV genome. (a, b) ML phylogenetic trees of rhabdoviruses based on (a) L protein and (b) N protein sequences. Bootstrap support values (>70 %) are shown for key nodes (except the grouping of KOLEV and OITAV). All horizontal branch lengths are drawn to a scale of amino acid substitutions/site. The L protein tree is rooted in the position observed in a broader analysis of members of the family *Rhabdoviridae* (i.e. including members of the genera *Cytorhabdovirus*, *Novirhabdovirus* and *Nucleorhabdovirus*; not shown), whilst the N protein tree is mid-point rooted. Asterisks indicate genera not yet formally approved by the International Committee on Taxonomy of Viruses. Definitions of virus abbreviations are listed in Table S1. (c) Schematic representation of the KOLEV genome organization. Block arrows indicate the location of long ORFs, including overlapping ORFs of >180 nt (grey shading). Shown below are the KOLEV coding regions, designated ORFs and putative transcription regulatory sequences.

C-terminal region (LWRKRRQGKL) and so may be involved in the regulation of host gene expression (cNLS Mapper Server) (Kosugi *et al.*, 2009). The putative L′ protein had a weak nuclear export signal in the C-terminal region (LAIEERSL) predicted by the NetNES 1.1 Server (la Cour *et al.*, 2004), but no other distinguishing characteristics. Only the P′ ORF had an initiation codon in a favourable Kozak context for translation.

Lastly, we generated a phylogenetic tree to reveal the relationships of KOLEV with other members of the diverse family *Rhabdoviridae*. Accordingly, the KOLEV L protein sequence was compared with those of 46 other

rhabdoviruses downloaded from GenBank (members of the genera *Cytorhabdovirus*, *Novirhabdovirus* and *Nucleorhabdovirus* were excluded because their excessive divergence reduced phylogenetic resolution within the other rhabdovirus taxa). The GenBank accession numbers for the genome sequences of the selected rhabdoviruses used in the phylogenetic analyses are listed in Table S1. All protein sequences were aligned using MUSCLE (Edgar, 2004), and ambiguously aligned regions were removed using Gblocks (Talavera & Castresana, 2007). This resulted in a sequence alignment of 1111 aa. Phylogenetic relationships were determined using the maximum-likelihood (ML) method available in PhyML 3.0 (Guindon *et al.*, 2010), employing the WAG+Γ model of amino acid substitution and subtree pruning and regrafting branch-swapping. The robustness of each node was determined using 1000 bootstrap replicates. An equivalent phylogenetic analysis was undertaken on a more limited number of N protein sequences. Because the N protein is substantially more divergent than the L protein, sequence alignment was only feasible among 30 species including KOLEV. Gblocks pruning after MUSCLE sequence alignment resulted in a dataset of 216 aa.

The ML tree of 47 rhabdovirus L protein sequences revealed that KOLEV falls as a distinct virus within the major group of insect, fish and animal rhabdoviruses, which includes members of the assigned genera *Sigmavirus*, *Vesiculovirus*, *Tibrovirus*, *Perhavirus* and *Ephemerovirus*, and the proposed genera *Tupavirus* and *Sprivivirus* (Fig. 2a). KOLEV was most closely related to members of the genus *Sigmavirus* although with very low bootstrap support (<50 %), such that its exact phylogenetic position could not be resolved using these data. To provide better resolution on the phylogenetic position of KOLEV, we inferred a second ML tree comprising 30 rhabdovirus N protein sequences, although of shorter alignment length (216 aa) (Fig. 2b). In this phylogeny, KOLEV was most closely related to OITAV, also isolated from insectivorous bats (Iwasaki *et al.*, 2004), and which has been assigned to the Kern Canyon group (Calisher *et al.*, 1989; Kuzmin *et al.*, 2006). However, this grouping also suffered from relatively poor bootstrap support (54 %), largely because there was insufficient sequence information to allow a proper determination of the phylogenetic position of KOLEV. The divergent nature of KOLEV strongly suggests that it represents a novel virus species.

Collectively, the data indicate that KOLEV is a novel rhabdovirus isolated from ticks and a roundleaf bat in the Republic of Guinea. However, little is known about its ecology, mode of transmission, host range or epidemiology. KOLEV has only been isolated twice, suggesting either that it is rare or that the paucity of serosurveillance data prevents accurate assessment of its full geographical range, prevalence and disease potential. Few rhabdoviruses have been isolated from ticks, but they include BARV (Kern Canyon group), Isfahan virus (genus *Vesiculovirus*) and sawgrass virus (currently unassigned) (Labuda & Nuttall, 2004). It is unknown whether KOLEV is transmitted by ticks or other haematophagous arthropods, or if it is maintained primarily by horizontal transmission among bats. Similarly, little is known of the biology of roundleaf bats, which have a limited distribution in West Africa. Leaf-nosed bats (*Hipposideros* spp.) have been the source of a wide range of viruses including Rift Valley fever virus (Boiro et al., 1987), severe acute respiratory syndrome-like coronaviruses (Quan *et al.*, 2010a), herpesviruses (Watanabe *et al.*, 2009), adenoviruses and rhabdovirus (Li *et al.*, 2010) and Shimoni bat lyssavirus (Kuzmin *et al.*, 2010). Further testing of human and animal sera from the same region would reveal the extent of exposure of humans, livestock and wildlife to KOLEV infection. CF tests (Table 1b; Calisher *et al.*, 1989) have indicated a distant antigenic relationship between KOLEV and nine of the ten other rhabdoviruses included in this study. Previous genetic analyses also suggested that some of these viruses may be related (Bourhy *et al.*, 2005; Dacheux *et al.*, 2010; Kuzmin *et al.*, 2006; reviewed by Gubala, 2012; Walker *et al.*, 2011). Nonetheless, the antigenic and genetic uniqueness of KOLEV underscores the diversity of members of the family *Rhabdoviridae* and highlights the necessity for further studies to characterize this diverse and complex family of viruses. The continuing development of faster and more accurate bioinformatics tools and the cost-effectiveness of genome sequencing platforms in tandem with established diagnostic assays (serology, phylogeny and electron microscopy) should bring taxonomic clarity to the relationships among members of this diverse virus family, as well as the possible association with human or animal diseases.

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