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Identification and molecular characterization of highly divergent RNA viruses in cattle, Uganda.

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ABSTRACT

The risk for the emergence of novel viral zoonotic diseases in animals and humans in Uganda is high given its geographical location with high biodiversity. We aimed to identify and characterize viruses in 175 blood samples from cattle selected in Uganda using molecular approaches. We identified 8 viral species belonging to 4 families (*Flaviviridae*, *Peribunyaviridae*, *Reoviridae* and *Rhabdoviridae*) and 6 genera (*Hepacivirus*, *Pestivirus*, *Orthobunyavirus*, *Coltivirus*, *Dinornavirus* and *Ephemerovirus*). Four viruses were highly divergent and tentatively named Zikole virus (Family: *Flaviviridae*), Zeboroti virus (Family: *Reoviridae*), Zebtine virus (Family: *Rhabdoviridae*) and Kokolu virus (Family: *Rhabdoviridae*). In addition, Bovine *Hepacivirus*, Obodhiang virus, *Aedes pseudoscutellaris reovirus* and Schmallenberg virus were identified for the first time in Ugandan cattle. We report 8 viral species belonging to 4 viral families including divergent ones in the blood of cattle in Uganda. Hence, cattle may be reservoir hosts for likely emergence of novel viruses with pathogenic potential to cause zoonotic diseases in different species with serious public health implications.

1. Introduction

Since the mid-1900s, infectious diseases of livestock have been identified, not only as a major threat to animal health, welfare and production, but also to the overall global health security especially in developing countries (Cleaveland et al., 2017; Grace D et al., 2012). Moreover, more than 60% of documented emerging, and re-emerging human diseases are zoonotic in nature (Jones et al., 2008). That is, viruses that are endemic within animal populations spill over into humans through vectors and other transmission pathways and cause epidemics (Morse, 2004), or global pandemics as with SARS-CoV-2 (Lu et al., 2020)

Uganda is a known hotspot for the emergence of novel pathogens due to its conducive climatic conditions and high biodiversity that include a variety of wild and domestic animals, birds and insects acting as disease

vectors, hosts or reservoirs, and the attendant anthropogenic factors such as deforestation (Allen et al., 2017; Jones et al., 2008). Indeed, in just over 10 years, several new high consequence viral pathogens have been identified, including *Bundibugyo ebolavirus* (Family: *Filoviridae*) (Towner et al., 2008), *Sosuga pararubulavirus* (Family: *Paramyxoviridae*) (Albariño et al., 2014), Bukakata orbivirus (*Chobar George virus*; Family: *Reoviridae*) (Fagre et al., 2019) and Ntwetwe virus (Family: *Peribunyaviridae*) (Edridge et al., 2019). Other viruses of public health importance that were previously discovered in Uganda, especially during the middle of the 20th century, include *Zika virus*, *West Nile virus* and *O'nyong-nyong virus* (Dick et al., 1952; Smithburn et al., 1940; Williams et al., 1965).

Despite enhanced efforts to identify and manage potential disease-causing pathogens in Uganda through improved surveillance,

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increased public and health sector awareness, and expedited transportation of suspected samples to the testing laboratories (Borchert et al., 2014; Kemunto et al., 2018; Kiyaga et al., 2013; Shoemaker et al., 2018), the disease burden associated with undifferentiated illnesses of unknown origin remains high (Lamorde et al., 2018). As such, it is critical to implement broad investigations of potential disease-causing agents to include animal hosts and vectors to identify the potential sources of viral pathogens likely to cause diseases in both animals and humans. These investigations would align well with the WHO's Priority 'Disease X', which represents a currently unknown serious global health threat that can emerge in humans (Mehand et al., 2018). In our current study, we aimed at identifying and characterizing viruses from blood samples collected in cattle from selected districts of Uganda. Previous studies have alluded that Bovines are a major source of zoonoses (McDaniel et al., 2014). More specifically, cattle have been implicated as hosts for 2 human viruses. Firstly, the dated emergence of Human coronavirus OC-43 in 1890 coincides with a pandemic of human

respiratory disease in humans between 1889 – 1890, and it is believed that this was after the virus emerged following a cross species transmission event of Bovine coronavirus (Crookshank, 1897; Vijgen et al., 2005). Secondly, measles virus is said to have crossed to humans after it diverged from rinderpest virus, a historical Bovine virus, around the 11th to 12th Century (Furuse et al., 2010). Therefore, with the high consumption of meat and other dairy products, as the main source of food in Uganda (UBOS, 2017), it is prudent to understand the viral diversity in this animal reservoir to expand on our pandemic preparedness toolkit.

2. Materials and methods

2.1. Ethics statement

The authors confirm that the ethical policies of this journal, as noted on the author guidelines page, have been adhered to: sample collection

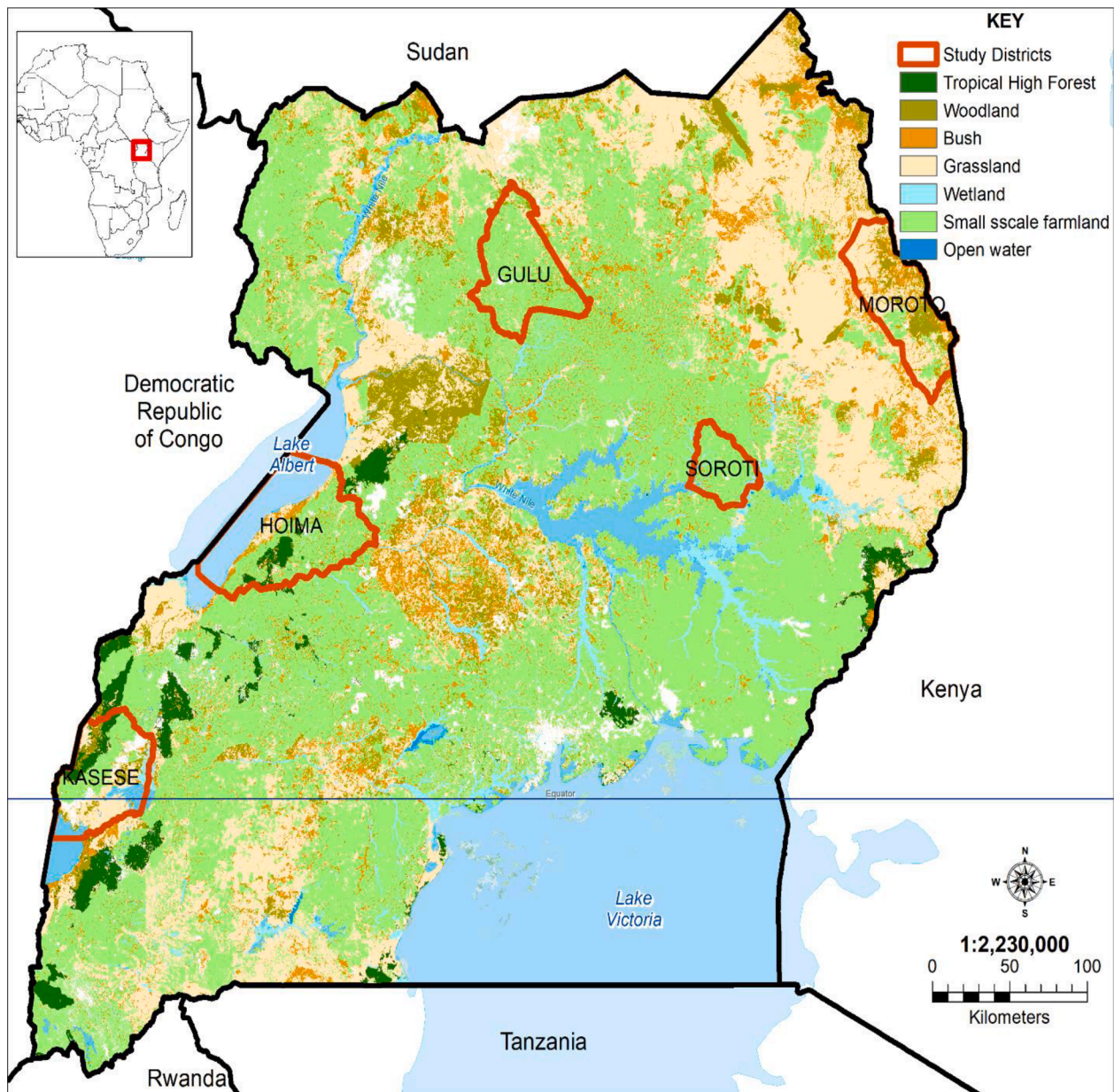


Fig. 1. Map of Uganda showing the sampling sites in this study.

Source: This map was created using open-source data in ArcGIS software, v10.2, Environmental Systems Research Institute, Inc., Redlands, CA, USA). Inset is a map of Africa with the enlarged region boxed in red.

was approved by the Institutional Animal Care and Use Committee (IACUC), School of Veterinary Medicine & Animal Resources (SVAR), Makerere University (Reference Number: SVARREC/03/2017) and the Uganda National Council for Science and Technology (UNCST) (Reference Number: UNCST A580). In addition, written consent was obtained from all animal owners, or their representative(s), following a detailed explanation of the study objectives.

2.2. Study sites

Uganda is an East African country located along the Equator, lying between 4°N and 1°S latitudes, and between 30°E and 35°E longitudes. Thus, many areas within the country have a typical tropical climate; experiencing relatively humid conditions (average of 70%), rainfall (700–1500 mm) and daily temperatures that are between 20–30 °C throughout the year (UBOS, 2017). Its natural vegetation is varied although it is mainly open wooded savannah grassland in most of the country, thus making livestock keeping a major agro-economic activity for the country. Recent data from the Ministry of Agriculture, Animal Industry and Fisheries (MAAIF) and Uganda Bureau of Statistics (UBOS), show that Uganda has over 14 million cattle, 16 million goats, 5 million sheep and 4 million pigs with every household owning at least one type of livestock (UBOS, 2017).

This study was conducted using blood samples from cattle that were sampled from 5 purposively selected districts of Uganda: Kasese (0°N, 30°E), Hoima (1°N, 31°E), Gulu (3°N, 32°E), Soroti (1°N, 33°E) and Moroto (2°N, 34°E) (Fig. 1 and Table 2). These districts were selected as they represent areas with distinct agroecological variations (Drichi et al., 2003; Wortmann and Eledu, 1999). Briefly, Kasese and Moroto districts lie at the extreme ends of the western and eastern borderlines, respectively, of the Ugandan Cattle Corridor – a large broad zone of semi-arid rangelands, where cattle-rearing is a preoccupation of the local communities. Gulu and Soroti districts lie within the swampy lowlands of the Lake Kyoga basin, while Hoima district is a medium altitude area with mixed agricultural practices.

2.3. Study samples

In this study, we used samples collected as part of a multidisciplinary baseline investigation on tickborne disease in cattle in Uganda (Balinandi et al., 2021; Malmberg and Hayer, 2019). A subset of 175 cattle samples was randomly selected from a total of 500 sampled animals, comprising 35 samples from each of the 5 study districts (Gulu, Soroti, Moroto, Hoima, and Kasese). In the field, information about each of the

sampled animal such as their age, sex, breed, health status, tick burden, grazing system, etc., was collected at the time of sample collection using a standardized biodata form. In addition, about 4ml of whole blood was drawn from the jugular or tail veins of restrained cattle into EDTA tubes (BD Vacutainer®, Franklin Lakes, NJ, USA). Blood was then immediately separated into small aliquots of whole blood and serum, which were immediately frozen in liquid Nitrogen before they were transported to Uganda Virus Research Institute (UVRI), Entebbe, Uganda, and stored at -80 °C until RNA extraction and family-wide conventional pan-PCR was undertaken.

2.4. pan-PCR

Total nucleic acids were extracted from whole blood samples using the MagMax™ 96 viral Isolation kit (Applied Biosystems, Vilnius, Lithuania), according to the manufacturer's instructions. Extracted RNA was transcribed into cDNA using Superscript III First-strand Synthesis Supermix (Invitrogen, CA, USA). We assayed the samples for 5 viral taxa: *Filoviridae*, *Flaviviridae*, *Alphavirus*, *Rhabdoviridae*, and *Orthobunyavirus*, using protocols previously developed and applied for the USAID/PREDICT II program (Moureau et al., 2007; PREDICT, 2014; Sanchez-Secco et al., 2001; Zhai et al., 2007) (Table 1). These viral taxa also represent the main groups of viruses from which most members of arboviruses are frequently identified (Hubálek et al., 2014; Woolhouse and Gowtage-Sequeria, 2005).

2.5. High-throughput sequencing

Ten serum samples (Table 2), corresponding to the whole blood samples that were positive for any viral family, including those that showed non-specific bands on gel electrophoresis, were preserved in TRIzol™ Reagent (Invitrogen, Thermo Scientific) and shipped on dry ice to the Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden, for high-throughput sequencing.

RNA was extracted using the GeneJet RNA purification kit (Thermo Scientific™), as per the manufacturer's instructions. The eluted serum RNA was further concentrated by RNeasy MinElute Cleanup Kit (Qiagen) before the RNA was randomly amplified with the Ovation RNA-Seq System v2 (Tecan) following the manufacturer's recommended protocols. The concentration and size distribution of generated double-stranded cDNA products were estimated by the TapeStation 4200 system (Agilent Technologies). Sequencing libraries were prepared from 50ng of DNA according to the manufacturer's preparation guide #112219, using the SMARTer ThruPLEX DNA-Seq kit (R400676,

Table 1
pan-PCR Primer Sequences and Protocols used for Virus Detection in Cattle, Uganda, 2017.

Virus Taxon	Protocol ¹	Target	Size of Positive Gel Band (bp)	1 st Round PCR Primer Sequences ²	2 nd Round PCR Primer Sequences ³	Reference
Family: <i>Filoviridae</i>	P-005	L gene	~630	F: TITTYTCHVTICAAAICAYTGGG R: ACCATCATRITRCTIGGAAKGCCTT	F: TITTYTCHVTICAAAICAYTGGG R: GCYTCISMIAHGTGTGIACATT	Modified (Zhai et al., 2007)
Family: <i>Flaviviridae</i>	P-006	NS5 gene	~270	F: TGYRBTAYAAACATGATGGG R: GTGTCCCAICNGCNGTRTC	- -	(Moureau et al., 2007)
Family: <i>Rhabdoviridae</i>	P-010	L gene	~260	F: CCADMCBTTTTGYCKYARRCCTTC R: AAGGYAGRTTTTTYKCDYTRATG	F: CCADMCBTTTTGYCKYARRCCTTC R: AARTGGAAYAAYCAYCARMG	Unpublished ³
Genus: <i>Alphavirus</i>	P-014	NSP4 gene	~195	F: GAYGCITAYYTIGAYATGGTIGAIGG R: KYTCYTCIGTRTYTTIGTICIGG	F: GIAAYTYGAAAYTIACICARATG R: GCRAAIARIGCIGCIGTYTIGGICC	(Sanchez-Secco et al., 2001)
Genus: <i>Orthobunyavirus</i>	P-016	L gene	~500	F: GARATHAATGCWGAYATGTCHAAAT R: TAIGTYTYTTCATRTDGCYTG	F: AAATGGAGTGCARGATGT R: TAIGTYTYTTCATRTDGCYTG	Unpublished ³

¹USAID/PREDICT II Surveillance protocol, version 2016.

²Sequences are in 5' to 3' direction.

³Assay was developed and validated at the University of California, Davis, USA.

Table 2
Characteristics of pan-PCR Positive Cattle, Uganda, 2017.

Animal ID#	Sample Collection Area		Animal Characteristics				Results of pan-PCR
	District	GPS Location	Age ^a	Sex	Breed	Health	
G04B	Gulu	N 03.03243, E 032.30449	2	M	Ankole	Good	Positive (Rhabdovirus)
G11A	Gulu	N 03.01411, E 032.28941	6	F	Ankole	Good	Positive (<i>Orthobunyavirus</i>)
H11A	Hoima	N 01.64420, E 031.29474	5	F	Ankole	Good	Indeterminate (<i>Orthobunyavirus</i>)
H26B	Hoima	N 01.32115, E 031.23425	2	F	Zebu	Good	Indeterminate (<i>Orthobunyavirus</i>)
K09B	Kasese	S 00.10048, E 029.82413	8	F	Ankole	Good	Indeterminate (<i>Orthobunyavirus</i>)
K16B	Kasese	S 00.04747, E 029.82864	2	F	Ankole	Good	Indeterminate (<i>Orthobunyavirus</i>)
K28A	Kasese	N 00.29223, E 030.16044	1	M	Ankole	Good	Positive (<i>Orthobunyavirus</i>)
M15B	Moroto	N 02.53587, E 034.53321	1	F	Zebu	Good	Positive (Rhabdovirus)
S37B	Soroti	N 01.92280, E 033.49659	1	M	Zebu	Good	Indeterminate (<i>Orthobunyavirus</i>)
S41B	Soroti	N 01.93135, E 033.49284	1	M	Zebu	Good	Positive (Rhabdovirus)

^aEstimated age in years.

Takara-Clontech). Briefly, DNA was fragmented using a Covairs E220 system, aiming at 350–400 bp fragments. The ends of the fragments were end-repaired, and stem-loop adapters were ligated to the 5' ends of the fragments. The 3' end of the stem-loop was subsequently extended to close the nick. Finally, the fragments were amplified, and unique index sequences were introduced using 8 cycles of PCR, followed by purification using AMPure XP beads (Beckman Coulter). The quality of the library was evaluated using the Agilent 2200 TapeStation system and a D1000 ScreenTape-kit. The adapter-ligated fragments were quantified by qPCR using the Library quantification kit for Illumina (KAPA Biosystems/Roche) on a CFX384Touch instrument (BioRad), prior to cluster generation and sequencing.

Thereafter, a 300 pM pool of the sequencing libraries in equimolar ratio was subjected to cluster generation and paired-end sequencing, with 150 bp read length in an SP flow cell and the NovaSeq6000 system (Illumina Inc.) using the v1 chemistry, according to the manufacturer's protocols. Base-calling was done on the instrument using RTA 3.3.4 and the resulting .bcl files were demultiplexed and converted to FASTQ

format with tools provided by Illumina Inc., allowing for one mismatch in the index sequence. Additional statistics on sequence quality were compiled with an in-house script from the Fastq-files, RTA and CASAVA output files. Sequencing was performed by the SNP&SEQ Technology Platform, part of the SciLifeLab, in Uppsala, Sweden. Sequence libraries have been deposited in SRA under the study PRJEB46076 with Accession Numbers: ERR6496566, ERR6496567, ERR6496875, ERR6496876, ERR6496901, ERR6496964, ERR6497048, ERR6497050, ERR6497350 and ERR6497480.

2.6. Bioinformatics

A flow chart summarizing the bioinformatics pathway we used to analyze our data under this study is shown in Fig. 2. Briefly, quality control and trimming of the raw sequencing reads was performed using FASTP (Lipman and Pearson, 1985). The good quality reads were then aligned to the cattle genome (*Bos taurus*.ARS-UCD1.2.; www.ncbi.nlm.nih.gov/assembly/GCF_002263795.1/) using Bowtie2 (Langmead and

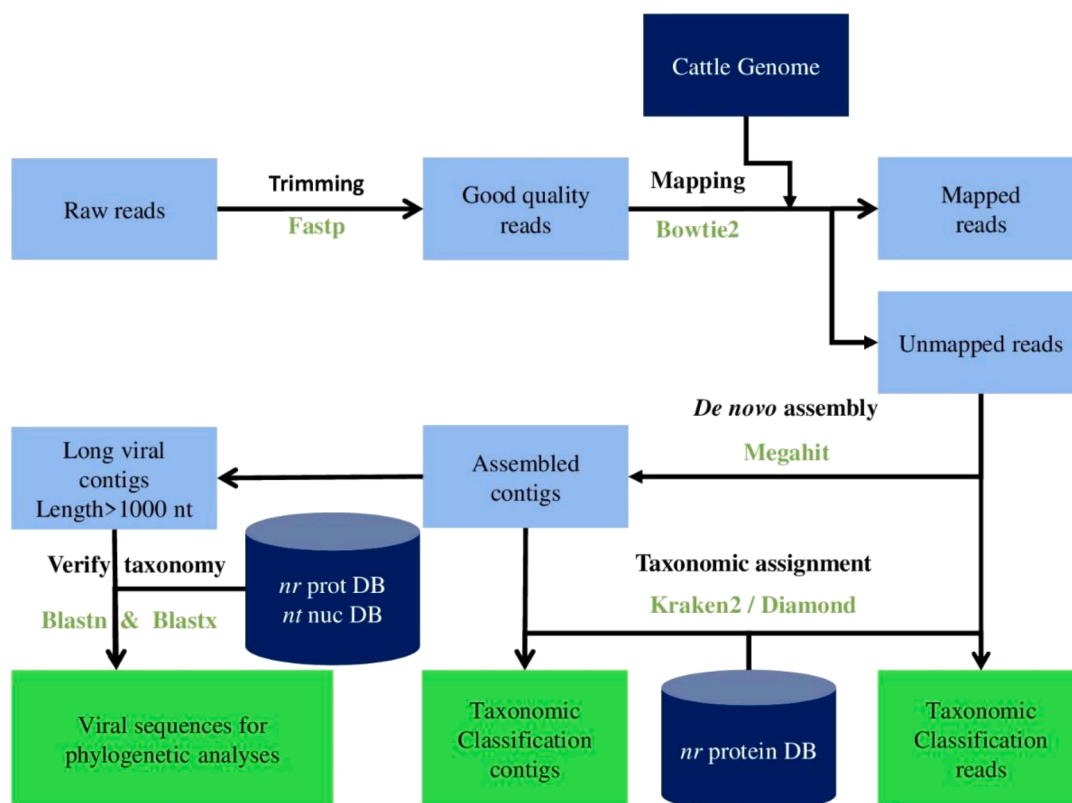


Fig. 2. Flow chart representing the bioinformatics analysis pathway.

Table 3
Viruses identified by Metagenomics from pan-PCR Positive Cattle, Uganda, 2017.¹⁻⁴

Animal ID	Contig Characteristics			Blast results				Virus information		
	Number of contigs.	Length (bp)	Coding complete? ¹	BlastN Top Hit	BlastN Pident (%)	BlastX Top Hit	BlastX Pident (%)	Virus Taxonomy (Family, Genus)	Virus ² (Name given from this study)	GenBank Accession Numbers
H11A	3	1,140 – 3,019	No	KP265947 Bovine <i>hepacivirus</i> isolate GHC55 polyprotein gene	85.9	CAG9163317 polyprotein [Bovine <i>hepacivirus</i>]	97.2	<i>Flaviviridae</i> , <i>Hepacivirus</i>	<i>Hepacivirus N</i>	CAJZAA010000001-CAJZAA010000003
G04B	1	8645	Yes	KP265946 Bovine <i>hepacivirus</i> isolate GHC52 polyprotein gene	87.2	CAG9163317 polyprotein [Bovine <i>hepacivirus</i>]	97.2	<i>Flaviviridae</i> , <i>Hepacivirus</i>	<i>Hepacivirus N</i>	OU592967
K16B	2	5,421 – 6,972	Yes	MH282908 Bat <i>pestivirus</i> BtSk-PestV-1/GX2017 polyprotein gene	74.5	AYV99177 polyprotein [Bat <i>pestivirus</i> BtSk-PestV-1/GX2017]	85.06	<i>Flaviviridae</i> , <i>Pestivirus</i>	Divergent <i>Pestivirus</i> (Zikole virus)	OU592965
K28A	4	1,218 – 3,074	No	HE795105 Shamonda virus Ib An 5550 RdRp gene segment L	91.1	YP_006590076 RNA-dependent RNA polymerase [Shamonda <i>orthobunyavirus</i>]	97.6	<i>Peribunyaviridae</i> , <i>Orthobunyavirus</i>	<i>Schmallenberg orthobunyavirus</i>	CAJZAJ010000001-CAJZAJ010000004
S37B	1	2273	No	NC_055248 Kundal virus strain MCL-13-T-316 segment 1	84.5	YP_010086008 RNA-dependent RNA polymerase [Kundal virus]	94.4	<i>Reoviridae</i> , <i>Coltivirus</i>	Divergent <i>Coltivirus</i> (Zeboroti virus)	OU592974
H26B	2	1,031 – 1,410	No, not all segments recovered	DQ087277 Aedes pseudoscutellaris reovirus segment 2	99.2	YP_443936 VP2 [Aedes pseudoscutellaris reovirus]	100	<i>Reoviridae</i> , <i>Dinovernavirus</i>	<i>Aedes pseudoscutellaris reovirus</i>	CAJZAK010000001-CAJZAK010000002
K09B	4	1,013 – 1,167	No, not all segments recovered	DQ087282 Aedes pseudoscutellaris reovirus segment 7	98.2	CAG9163330 VP7 [Aedes pseudoscutellaris reovirus]	99.2	<i>Reoviridae</i> , <i>Dinovernavirus</i>	<i>Aedes pseudoscutellaris reovirus</i>	CAJZAC010000001-CAJZAC010000004
M15B	1	14618	Yes	HM856902 Obodhiang virus Sudan	96.4	YP_006200965 L protein [Obodhiang virus]	98.3	<i>Rhabdoviridae</i> , <i>Ephemerovirus</i>	<i>Obodhiang ephemerovirus</i>	OU592970
G04B	1	14630	Yes	MH507505 Puchong virus isolate P5-350 Malaysia	78.3	QEA08648 polymerase [Puchong virus]	90.5	<i>Rhabdoviridae</i> , <i>Ephemerovirus</i>	Divergent <i>Puchong ephemerovirus</i> (Kokolu virus) ³	OU592964
S41B	3	1,400 – 12,917	No	KM085029 Koolpinyah virus isolate DPP819	78.0	YP_006202628 L gene product [Kotonkan virus]	86.7	<i>Rhabdoviridae</i> , <i>Ephemerovirus</i>	Divergent <i>Kotonkan Ephemerovirus</i> (Zebtine virus) ⁴	CAJZAB010000001-CAJZAB010000003

¹If contig coding was complete (“Yes”) or partial (“No”).

²In cases where viruses fell into known species, we have provided the species name in italics. If the virus was considered divergent (<90% amino acid similarity) we have provided a proposed virus name in parentheses.

³Kokolu virus is unlikely to be a novel ephemerovirus based on the ICTV cutoff criteria for Rhabdoviridae, however it is divergent at the amino acid level, so we have proposed a virus name, and indicated that it may fall within the same virus species as Puchong ephemerovirus.

⁴Zebtine virus is unlikely to be a novel ephemerovirus based on the ICTV cutoff criteria for Rhabdoviridae (amino acid % of 85), however it is divergent at the amino acid level so we have proposed a virus name, and indicated that it may fall within the same virus species as Kotonkan ephemerovirus.

Salzberg, 2012) to eliminate reads from the host. The remaining reads were *de novo* assembled using MEGAHIT (Li et al., 2016). The obtained assembled contigs were taxonomically classified using two methods: KRAKEN 2 (Wood et al., 2019) and DIAMOND (Buchfink et al., 2015), both using the NCBI non-redundant protein database *nr* (release of April 2019) (Deng et al., 2006). This analysis was also performed at reads level, on the reads remaining unmapped after the alignment to the cattle genome.

The longest assembled contigs (length > 1000 nt) that were classified as viral were retrieved for further analyses. BLASTN and BLASTX (Altschul et al., 1997; Chen et al., 2015) were performed to confirm the closest homologues and to check the alignments with viral sequences from the database. For some identified viruses for which only partial genomes were obtained by *de novo* assembly, all reads from the dataset of interest were mapped on the reference genome of the closest virus found in the taxonomic classification step. Prokka (Seemann, 2014) was also used to predict and annotate open reading frames from these longest contigs. Viral sequences generated in this study have been deposited in GenBank with Accession numbers as shown in Table 3

2.7. Phylogenetic analysis

Reference sequences were downloaded from RefSeq (www.ncbi.nlm.nih.gov/refseq/) and in addition to the top blast hits, amino acid or nucleotide sequences, were aligned using MAFFT (Katoh and Standley, 2013) in Geneious Prime (www.geneious.com/), and gaps trimmed using trimAL (Capella-Gutiérrez et al., 2009). Phylogenetic trees were constructed using IQ-TREE (Nguyen et al., 2015) with the best model of amino acid or nucleotide substitution and 1000 bootstraps, presented as a value of 1.

Based on BLAST results and phylogenetics, viral contigs were not included in the analysis if they were likely contaminants. For example, detecting viruses in clades dominated by arthropod viruses were not included. Furthermore, short contigs that blasted only against highly conserved 3' ends of viruses likely represented misassignment. Finally,

we cross-referenced our data with well-known lists of viral contaminants (Asplund et al., 2019). For all virus taxa, trees were constructed using the ORF containing the RNA-dependant RNA polymerase (RdRp). For the flaviviruses, we also included NS3 non-structural polyprotein, as this previously has also been found suitable (Atif et al., 2016; Rice, 1996).

We considered viruses to be divergent if they had a < 90% RdRp protein identity, < 80% genome identity (Shi et al., 2018; Shi et al., 2016), candidature for novel species delineation was considered in accordance with cut-off thresholds specified by the International Committee on the Taxonomy of Viruses (ICTV) for each viral family (Lefkowitz et al., 2018).

3. Results

3.1. pan-PCR screening of viruses in blood samples from Ugandan cattle

To understand the degree of viral diversity and presence of specific viruses we screened 175 blood samples obtained from cattle in Uganda using a pan-PCR approach. The cattle ranged in age from < 1–12 years (Median, 3.5; Mean, 3.7), and the breeds were Zebu (64.7%), Ankole (27.7%) and others (7.6%). At the time of sample collection, sampled cattle had rectal temperatures ranging from 36.0–40.6 °C, and an average of 68 ticks per animal (Range: 0–450). However, the cattle from which we detected viruses were mainly young ones with an average age of 2.9 years (Range = 1–8), 60% of which were of the Ankole breed. These cattle had a normal rectal temperature (Range: 37.3–38.2 °C) and a low to moderate tick burden (Range: 2–132 ticks).

Using a pan PCR approach, we found that 2.9% (5/175) of the samples were positive for either of 2 viral taxa. These included *Orthobunyaviruses* ($n = 2$) from samples collected in Kasese and Gulu districts, and rhabdoviruses ($n = 3$) from samples collected in Gulu, Soroti and Moroto districts. In addition, we detected non-specific (indeterminate) electrophoretic bands in 5 samples that were obtained from animals in the districts of Hoima ($n = 2$), Kasese ($n = 2$) and Soroti ($n = 1$), particularly for *Orthobunyaviruses*.

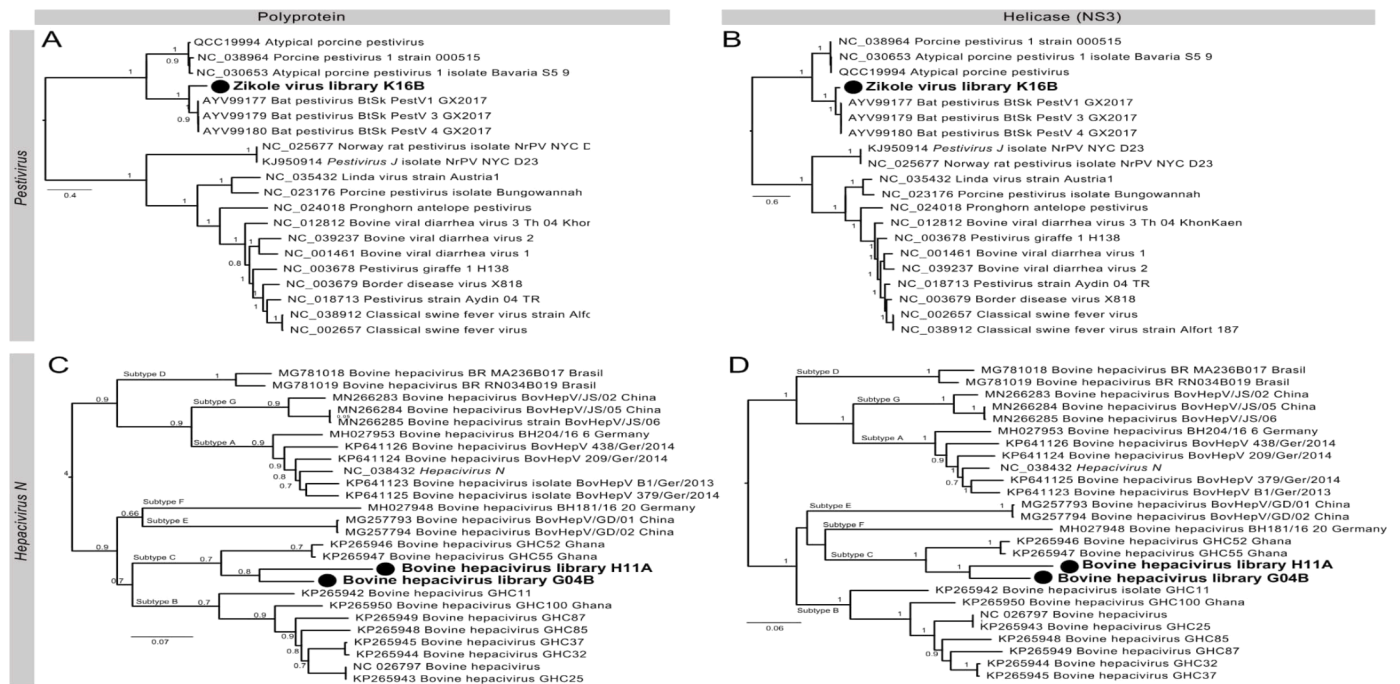


Fig. 3. Phylogeny of select taxa of the *Flaviviridae*. Amino acid phylogeny (A, B) and nucleotide phylogeny (C, D) of *Pestivirus* (Zikole virus) and *Hepacivirus N* (Bovine Hepacivirus). Phylogenies in A, C are of the polyprotein and B, D are of the NS3 Helicase. For *Pestivirus*, the tree was midpoint rooted for clarity only, and for the Bovine Hepacivirus, the tree was rooted as per Lu et al. (2019), including subtype information. Virus names presented here respect the virus names presented in GenBank and Ref Seq. In cases where we use the ICTV ratified species name, it appears in italics. Scale bar indicates number of substitutions per site. Sequences in this study are denoted by a filled circle.

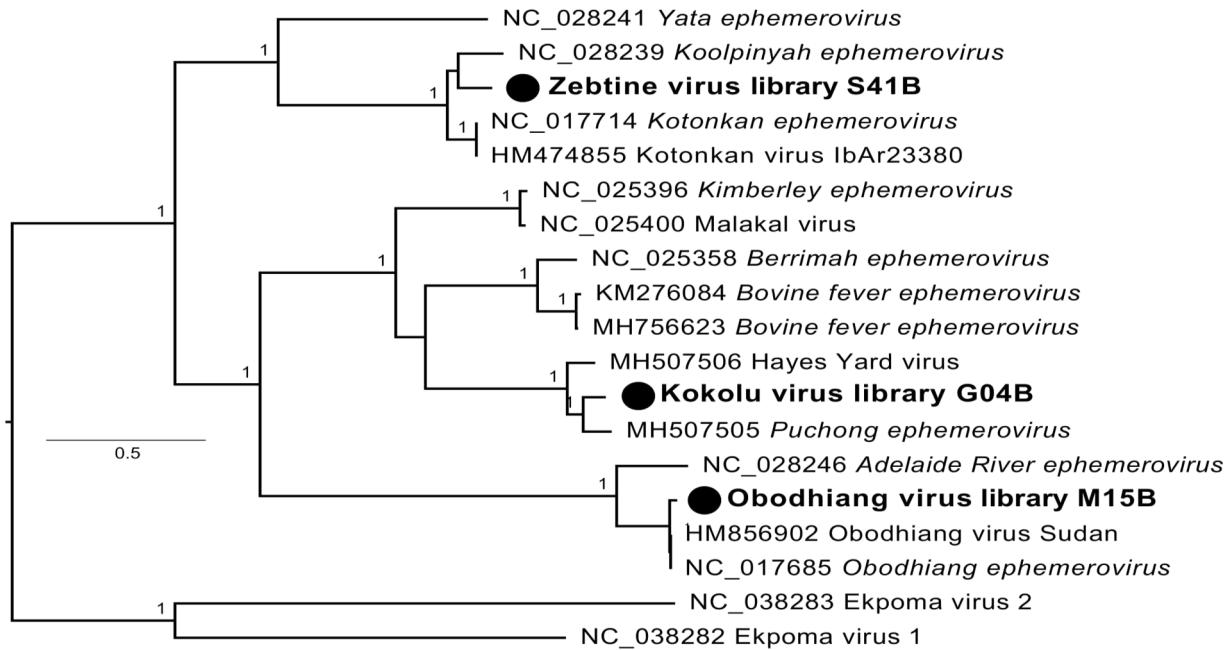


Fig. 4. Phylogeny of the RdRp (L) protein of genus *Ephemerovirus* (Family: Rhabdoviridae). Ekoma virus 1 and 2 (genus *Tibrovirus*) are set as the outgroup. Virus names presented here respect the virus names presented in GenBank and Ref Seq. Scale bar indicates number of amino acid substitutions per site. Sequences in this study are denoted by a filled circle.

3.2. Large diversity of viruses revealed using metagenomics

To better understand the viral diversity, we performed metagenomic analysis on serum samples corresponding to the 10 positive pan-PCR whole blood samples. Of these 10 serum samples, we found evidence

of at least one virus in 9 samples (Table 3). In one sample (G11A in Table 2) that had been collected from Gulu district, and was initially pan-PCR positive for *Orthobunyaviruses*, a 382bp contig had a 98.9% nucleotide similarity to *Crimean-Congo hemorrhagic fever orthonairovirus*. Upon further analysis, these short contigs also had high similarity to

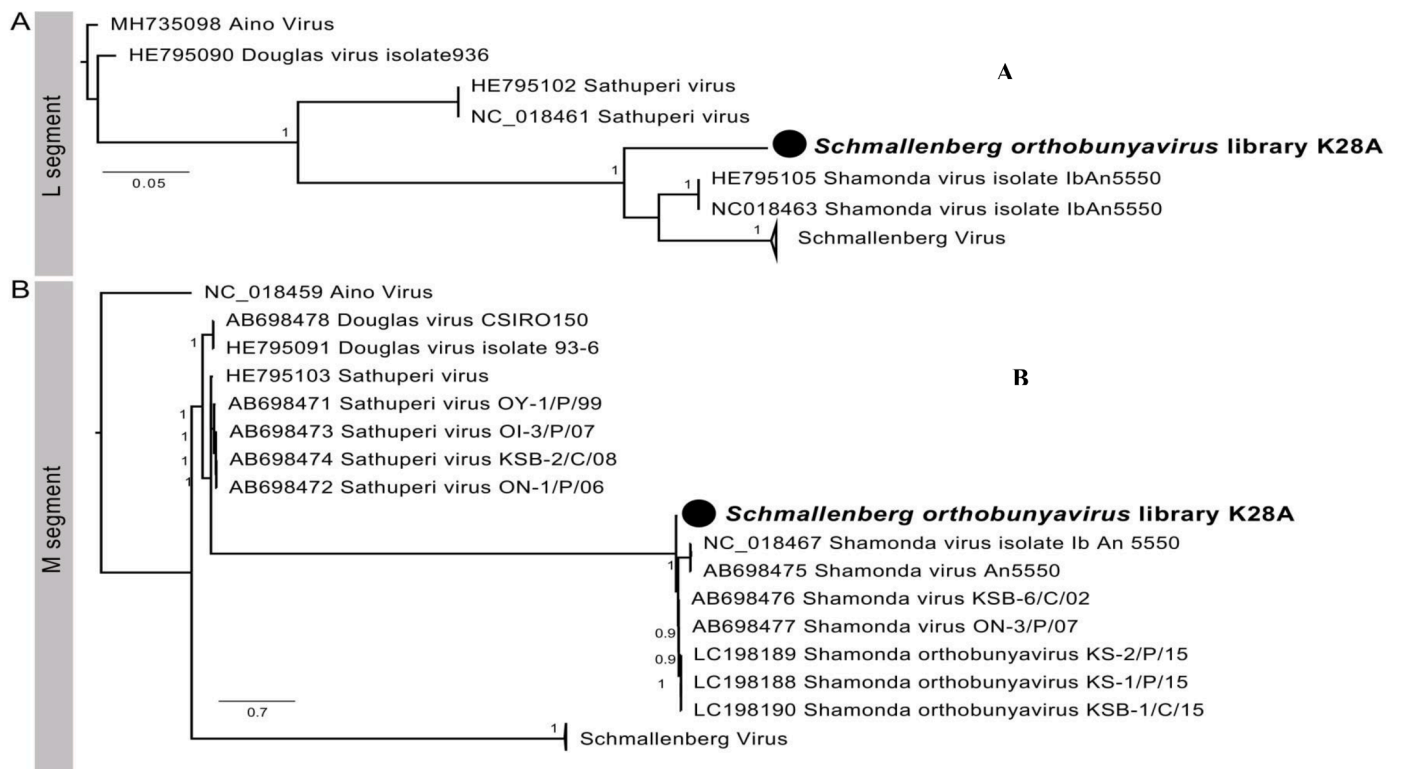


Fig. 5. Phylogeny of the (A) L segment and (B) M segment of *Schmallenberg orthobunyavirus* including Schmallenberg Virus, Shamonda Virus, Douglas Virus and Sathuperi virus. Virus names presented here respect the virus names presented in GenBank and Ref Seq. In cases were we use the ICTV ratified species name, it appears in italics. Scale bar indicates number of nucleotide substitutions per site. Sequences in this study are denoted by a filled circle.

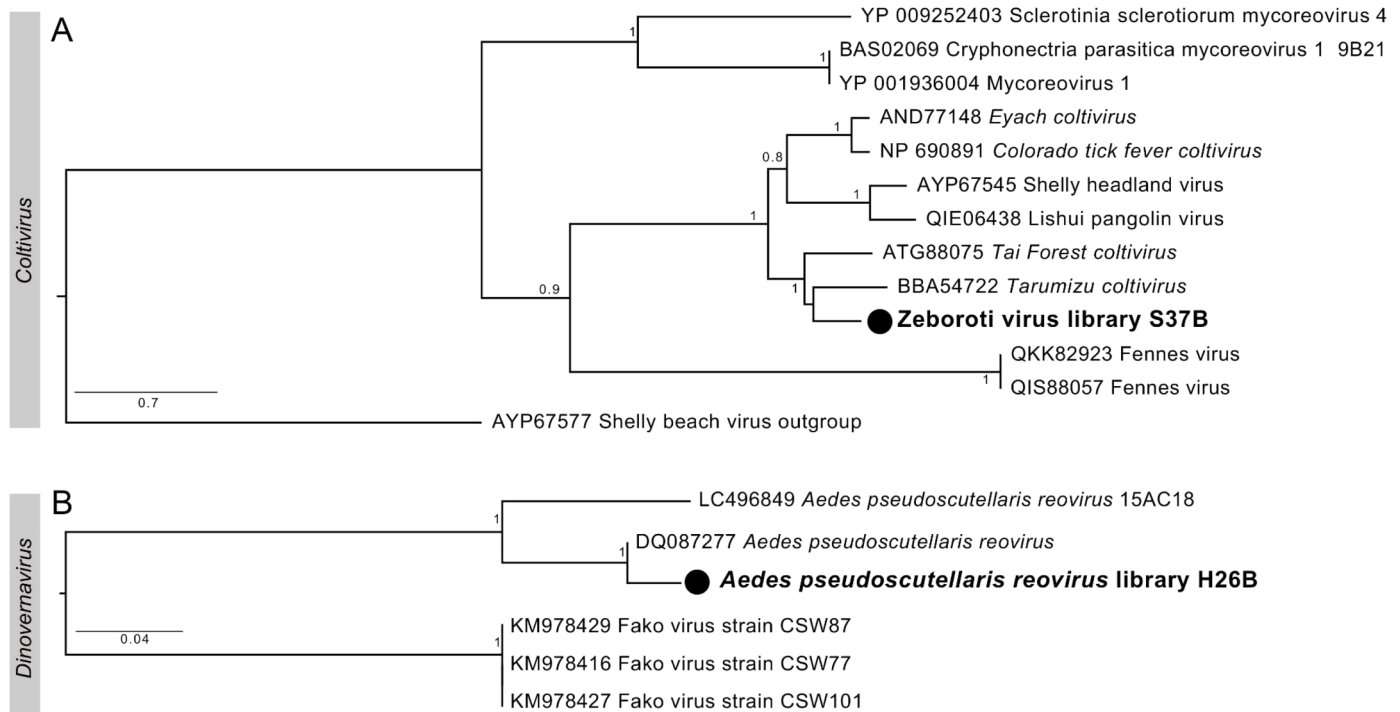


Fig. 6. Phylogeny of *Reoviridae*. (A). Segment 1 of *Coltivirus*. Shelly beach virus was set as the outgroup. Scale bar indicates number of amino acid substitutions per site. (B) Segment 2 of *Dinovernavirus* (*Reoviridae*). Virus names presented here respect the virus names presented in GenBank and Ref Seq. In cases where we use the ICTV ratified species name, it appears in italics. The tree was mid-point rooted for clarity only. Scale bar indicates number of nucleotide substitutions per site. Sequences in this study are denoted by a filled circle.

reverse genetic vectors (KJ648916, 98.91% identity, evaluate $1e-85$), and had low similarity and large gaps when aligned to other *Crimean-Congo hemorrhagic fever orthonaviruses*, and hence this contig was removed from further data analysis. From the remaining 9 samples, metagenomic sequencing identified a total of 8 viruses, belonging to 4 families and 6 genera (Table 3). Importantly, this metagenomic approach confirmed the presence of virus positive samples generated in the pan-PCR approach.

Despite the pan-PCR approach failing to identify flaviviruses in the samples, metagenomics identified viruses belonging to this family in 3 of the submitted samples. These samples had showed non-specific gel bands on conventional pan-PCR. The identified flavivirus species were Bovine *Hepacivirus* (Species *Hepacivirus N*, Genus *Hepacivirus*) in samples collected from Gulu and Hoima districts, and a divergent flavivirus most closely related to Bat *Pestivirus* (Genus *Pestivirus*) from Kasese district. This divergent flavivirus, which we have tentatively named Zikole virus, shares 74.5% nucleotide and 81% amino acid similarity to Bat *Pestivirus* (Fig. 3), and 90% nucleotide and 90% amino acid similarity to Atypical Porcine *Pestivirus* (*Pestivirus K*) across the entire polyprotein. Further, Zikole virus, shares 84.65% amino acid similarity to Bat *Pestivirus* and 66.61% amino acid similarity to Atypical Porcine *Pestivirus* (*Pestivirus K*) across the RdRp domain (NS5B). Phylogenetically, Zikole virus is highly divergent to the clades containing *Pestiviruses* from cattle, swine, and other ungulates considering both the polyprotein and NS3 (Helicase) domain (Fig. 3).

Sequencing revealed three members of *Rhabdoviridae* belonging to the genus *Ephemerovirus*. These 3 viruses were Obodhiang virus (Species *Obodhiang Ephemerovirus*) detected in a sample from Moroto district, and two divergent viral species – one related to *Puchong Ephemerovirus* from a sample collected in Gulu district that we have tentatively named Kokolu virus, and another related to *Kotonkan virus* (Species *Kotonkan Ephemerovirus*) in a sample from Soroti district that we have tentatively named Zebtine virus (Fig. 4). The Kokolu virus shares 78.3% identity at the nucleotide level and 90.5% at the amino acid level across the L gene

(containing the RdRp) with *Puchong Ephemerovirus*, while Zebtine virus shares 78.0% identity at the nucleotide level and 86.7% at the amino acid level, across the L gene with *Kotonkan Ephemerovirus*. As per the current ICTV guidelines for species demarcation, rhabdovirus species must have a minimum of 15% amino acid sequence divergence in the L gene (ICTV, 2017). As such, the Zebtine virus identified in this study, falls well outside that threshold, but the status of Kokolu virus is less clear given its high nucleotide divergence, despite its relatively low (9.5%) amino acid divergence. The strain of Obodhiang virus identified in this study represents the first detection in Uganda since its description in mosquitos collected from South Sudan in 1963 (Schmidt et al., 1965), substantially expanding its geographic range.

We also identified Schmallenberg virus (Species *Schmallenberg orthobunyavirus*, Genus *Orthobunyavirus*, Family *Peribunyaviridae*) from a sample collected from Kasese district, with a 91.1% nucleotide and 97.6% amino acid sequence identity in the RdRp gene (Fig. 4). Across the L segment, the virus revealed here is sister to the clade containing Shamonda and Schmallenberg viruses, although the M segment falls into the clade comprising Shamonda virus, and more distantly related to Schmallenberg virus (Fig. 5).

The final two virus species identified in this study belong to the Family *Reoviridae* (Fig. 6); the first, which we have tentatively called Zeboroti virus, is a divergent virus most closely related to Tarumizu tick virus (Species *Tarumizu Coltivirus*, Genus *Coltivirus*) in a sample from Soroti district, with 84.5% nucleotide and 94.4% amino acid identity. According to the ICTV species demarcation guidelines for this genus, viruses belong to the same species if they have >89% nucleotide identity in segment 12, or amino acid identities of > 55%, > 57% and > 60% in VP6, VP7 and VP12, respectively (Attoui et al., 2011). Unfortunately, we were only able to find genomic sequences belonging to segment 1 (out of the 12 putative segments), and therefore, ascertaining whether this virus is novel is challenging. Second, we identified *Aedes pseudoscutellaris reovirus* (Species *Aedes pseudoscutellaris reovirus*), the prototype-species for the genus *Dinovernavirus* of the *Reoviridae* family in two samples (one

from Kasese district and another from Hoima district) with 98.2% and 100% nucleotide and amino acid identity, respectively. This virus is not well characterized, and it is unclear whether this virus is an arbovirus, an insect-specific virus, or a contaminant.

4. Discussion

This study investigated RNA viruses in blood samples that were collected from cattle in 5 districts in Uganda. Overall, we identified 8 viral species, belonging to 4 distinct viral families (*Flaviviridae*, *Rhabdoviridae*, *Peribunyaviridae* and *Reoviridae*). Four of the identified viral species in this study were highly divergent (Zikole virus, Zeboroti virus, Kokolu virus and Zebtine virus), while the 4 other viruses (Bovine *Hepacivirus*, Schmallenberg virus, *Aedes pseudoscutellaris* reovirus, Obodhiang virus), are being reported for the first time in Uganda. Thus, our findings, perhaps, are an early indication that cattle in Uganda may be harbouring more viruses than previously known, in line with similar high levels of viral diversity in cattle reported elsewhere (Dacheux et al., 2019; Kato et al., 2016; Kwok et al., 2020; McDaniel et al., 2014).

Interestingly, a substantial proportion of the identified and described viruses in this study are transmitted by, or fall into clades, associated with arthropods. Specifically, the *Coltivirus* (Zeboroti virus) described here is most closely related to, and falls within a clade dominated by tick-borne viruses, some of which are found in many parts of North America and Europe, where they cause acute febrile human illnesses (Fujita et al., 2017; Geissler et al., 2014; Hubálek and Rudolf, 2012). Other viruses detected in this study were mainly those currently associated with mosquito and culicoid flies (Yanase et al., 2020). Ticks are a major vector for animal diseases in Uganda. And, we have previously reported a high tick burden, comprising of several tick species on cattle under this study (Balinandi et al., 2020). Taken together, the high tick burden with high diversity of tick species parasitizing cattle provide avenues for likely transmission of several infectious agents, some of which may be novel.

Indeed, from our study, we report 4 divergent viruses with unknown epidemiology in Uganda. Unfortunately, a major limitation of metagenomics is that, beyond detection, we are unable to determine the pathogenicity or medical-veterinary importance of the identified divergent viruses. However, their presence in cattle blood indicates potential for spillover into the environment through vectors, or other biological and mechanical mechanisms. Of particular interest, was the identification of a *Pestivirus* in our study, which we have tentatively named as Zikole virus. *Pestiviruses* are a group of reemerging viruses that have gained significant interest due to their wide geographical distribution, associated high socioeconomic losses when in livestock, a variety of syndromic manifestations in affected animals, failures in eradication programs, and a growing number of newly identified domestic and wildlife hosts (Blome et al., 2017; Postel et al., 2021). Currently, they are known to infect a variety of artiodactyls, including swine and ruminants, in which they cause mild to severe disease (Schweizer and Peterhans, 2014). The Zikole virus identified in this study, had a close phylogenetic relationship to Bat *Pestivirus*. While this may represent a mere common ancestry between these two viral species, recent studies have implicated bats as a major reservoir from which several known and unknown viral species have spilled over into other animal species (Wang and Anderson, 2019). From our study, the significance and impact of the identified Zikole virus on cattle, as well as human health remains unknown and a subject for further investigation. What is known, however, is that the common species of the genus *Pestivirus*, Bovine viral diarrhea virus type 1, now re-classified as *Pestivirus A* (Smith et al., 2017) causes serious disease in cattle (Liebler-Tenorio et al., 2006).

As much as Bovine *Hepacivirus*, Obodhiang virus and Schmallenberg virus, are being reported for the first time in Uganda, they are known to be widely spread elsewhere in African Bovines (Corman et al., 2015; Sibhat et al., 2018), where their clinical manifestations can range from

absent overt disease to abortions, stillbirths and/or congenital malformations (Baechlein et al., 2015; Peperkamp et al., 2015). In this study, all virus-positive animals displayed normal rectal temperatures (Table 2), perhaps, indicating their tolerance to these viral infections (Ayles and Schneider, 2012). Nevertheless, a detailed characterization of these viruses is still crucial in the context of risk analysis, design of public health surveillance systems, and outbreak investigation and response. Of concern is the fact that many, if not all, of the identified viruses in the present study still lack effective vaccines or antiviral treatments. They are of unknown epidemiology, including their circulation in nature, range of potential natural reservoirs and immunological responses in relation to clinical infections, whether in man or in animals. Thus, the main limitation of this study emanated from its cross-sectional nature, which hindered us from obtaining detailed information regarding these epidemiological perspectives.

Authors statement

We collected and analysed blood samples from cattle selected from five districts in Uganda as part of multidisciplinary project to improve cattle health through increased understanding of the microbial community in cattle and ticks. We used both molecular methods and throughput sequencing and identified 8 viral species belonging to 4 families: *Flaviviridae*, *Peribunyaviridae*, *Reoviridae* and *Rhabdoviridae*. Four viruses were highly divergent and we tentatively named them Zikole virus (Family: *Flaviviridae*), Zeboroti virus (Family: *Reoviridae*), Zebtine virus (Family: *Rhabdoviridae*) and Kokolu virus (Family: *Rhabdoviridae*). Our results show that cattle in Uganda harbour a diverse viral microbial community acquired through various vectors mainly ticks and mosquitoes. Some of the identified viruses may be pathogenic and zoonotic with potential to cause disease in the cattle host and/or reservoir host for likely transmission of diseases to other species and humans. We recommend extended surveillance of microbial community in cattle and other livestock species and identify microbial communities that may be of productive, health and zoonotic concerns to inform appropriate intervention measures

CRedit authorship contribution statement

Stephen Balinandi: Investigation, Methodology, Visualization, Writing – original draft. **Juliette Hayer:** Methodology, Data curation, Formal analysis, Writing – review & editing. **Harindranath Cholleti:** Methodology, Data curation, Investigation, Writing – review & editing. **Michelle Wille:** Methodology, Data curation, Formal analysis, Visualization, Writing – review & editing. **Julius J. Lutwama:** Investigation, Supervision, Writing – review & editing. **Maja Malmberg:** Conceptualization, Funding acquisition, Investigation, Methodology, Data curation, Formal analysis, Resources, Supervision, Project administration, Writing – review & editing. **Lawrence Mugisha:** Conceptualization, Investigation, Supervision, Project administration, Resources, Methodology, Visualization, Formal analysis, Writing – review & editing, Validation, Writing – original draft.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Ethical statement

The authors confirm that they read and adhered to journal's ethical policies as stated in the authors guidelines. The required ethical clearances to collect ticks and blood samples from cattle were obtained from the animal ethical committee and a research permit issued by the Uganda National Council of Science and Technology (UNCST).

Data availability statement

Data supporting all components of this study is available on request from the corresponding author. Sequence data was submitted to GeneBank and can be accessed using accession numbers provided in the body of the text.

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