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Dianke virus: A new mesonivirus species isolated from mosquitoes in Eastern Senegal

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ABSTRACT

An increasing number of insect-specific viruses are found around the world. Very recently, a new group of insect-specific viruses, the *Mesoniviridae* family, was discovered in Africa, Asia, North America and Australia. Here we report the first detection and isolation of a new virus belonging to *Mesonivirus* genus in Senegal, West Africa. The so-called Dianke virus was detected in 21 species of arthropods trapped in the eastern part of the country. Male individuals were also infected, supporting vertical transmission assertion of insect specific viruses. As described for other mesoniviruses, no viral replication was observed after inoculation of mammalian cells. Viral replication in mosquito cells was blocked at a temperature of 37 °C, highlighting the importance of thermal conditions in *Mesonivirus* host restriction. Similar to our study, where a diverse range of arthropod vectors were found infected by the new virus, several studies have detected mesonivirus infection in mosquitoes with concerns for human health. It has been shown that dual infections in mosquito can alter viral infectivity. Due to their extensive geographic distribution and host range, as well as their use as potential disease control agents in vector populations, more studies should be done for a better knowledge of arthropod-restricted viruses prevalence and diversity.

1. Introduction

Since the discovery of the first members of the *Mesoniviridae* family, order Nidovirales (Junglen et al., 2009; Zirkel et al., 2011; Nga et al., 2011), a growing number of new species has been found in different parts of the world (Africa, Asia, North America and Australia) (Lauber et al., 2012; Kuwata et al., 2013; Zirkel et al., 2013; Vasilakis et al., 2014; Warrilow et al., 2014; Hang et al., 2016). Mesoniviruses are enveloped, positive-sense, single stranded RNA ([+]ssRNA) viruses and constitute the only insect-associated genus of nidoviruses. The 20 kb nidovirus genome organization is generally ORF1a-ORF1b-ORF2a-ORF2b-ORF3a-ORF3b, except the *Alphamesonivirus1* species, which encodes a seventh open reading frame (ORF4) (Vasilakis et al., 2014). ORF1a and ORF1b are predicted to encode two polymerase polyproteins (pp) while the other ORFs encode structural proteins (Zirkel et al., 2011; Hang et al., 2016). Viral particles are enveloped, spherical in shape and 60–80 nm in diameter with club-shaped surface spikes (Zirkel et al., 2013). Like insect-specific flaviviruses (Hoshino et al., 2007; Huhtamo et al., 2009) and other mosquito-associated bunyaviruses (Marklewitz et al., 2013) and orbiviruses (Harrison et al., 2016), mesoniviruses are considered insect specific viruses (ISVs) as the totality of detection has come from mosquito pools and isolations only from insect cells lines, while no detection or replication has been reported in mammalian models (Nga et al., 2011; Zirkel et al., 2011;

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Vasilakis et al., 2014).

Here, we report the detection and isolation of a new closely related mesonivirus from mosquitoes trapped in eastern Senegal. Phylogenetic analyses of the complete genome of the strain showed a new circulating mesonivirus species in sympatric mosquitoes from eastern Senegal. The discovery of this new virus confirms that an increasing number of arthropod-specific viruses (ASVs) are discovered throughout the world with a potential impact on vectors and viruses of public health interest.

2. Material and methods

2.1. Study sites

The study consisted in mosquitoes trapping sessions done in the middle and at the end of the rainy season in 2012 and 2013 in several villages located along the two main roads of eastern Senegal, Africa. Permission to work within the different villages and household was provided by the CNERS (National Ethical Committee for Health Research) under the number 0000167 MSAS/DPRS/CNERS (see *CNERS_authorization* in supplemental material).

2.2. Mosquitoes trapping

In order to study the arboviruses circulation among domestic and peridomestic mosquitoes in eastern Senegal, the sampling protocol was as described by Diallo et al. (2012). Briefly, trapping was done in different human dwellings of villages of eastern Senegal. In each village, at least 10 houses were selected following a transect going from the peripheral borders to the center in order to cover all the ecological profiles. The different trapping sessions were performed during twilight, where most of arboviruses vectors such as *Aedes* species are active. Three methods were used: (1) human landing catches, the most appropriate method for determining the risk of human infection (Diallo et al., 2014), (2) CDC light traps with or without CO_2 bait, shown to have relatively high sampling efficiency (Okumu et al., 2008) but to be less specific to particular anthropophilic species, (3) indoor residual spraying for endophilic species collection. Adhesive trap catches were also performed for sandflies. After morphological identification, collected insects were pooled by species in tubes and stored at −80 °C until viral detection processing. Each pool was triturated in L-15 medium and RNA was extracted as described below.

2.3. Arboviruses screening

Each pool was triturated in Leibovitz-15 (L-15) medium (GibcoBRL, Grand Island, NY, USA) containing penicillin and streptomycin (Sigma, GmBh, Germany) and 10 % FBS (Gibco BRL, Grand Island, NY, USA) and centrifuged in order to collect the suspension.

RNA was extracted from these different suspensions using the QIAamp RNA Viral Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. RNA was eluted in 50 µl of AVE buffer and stored at −80 °C until use. For the arbovirus screening, conventional RT-PCR systems targeting different genera were used (table in supplement).

Virus isolation attempts were also performed from 150 µl of suspensions of each specimen that were inoculated separately onto monolayers *Aedes albopictus* C6/36 cells in 25-cm^2 tissue-culture flasks. After incubation at 28 °C for a maximum of 7 days post infection (pi), supernatant was collected and an aliquot of 200 µL was used for 4 serial passages, or until the observation of a cytopathic effect (CPE). Supernatants were also tested for several arboviruses by conventional reverse transcription (RT)-PCRs (Table S1 in supplement). Immunofluorescence assay (IFA) was performed as previously described to assess cell infection (Digoutte et al., 1992).

2.4. Next generation sequencing (NGS)

Supernatants obtained from some CPE-inducing samples but without virus identification by IFA or RT-PCR were processed for NGS. Library construction, sequence and genomic analysis were performed as described previously (Vasilakis et al., 2014). Briefly, viral RNA was fragmented using fragmentation buffer (Illumina 15016648) and the Illumina TruSeq RNA Sample Preparation kit was used for strand synthesis, adapter ligation and library amplification under conditions prescribed by the manufacturer (Illumina). Samples were tracked using the "index tags" incorporated into the adapters as defined by the manufacturer. Cluster formation of the library DNA templates was performed using the TruSeq PE Cluster Kit v3 (Illumina) and the Illumina cBot workstation using conditions recommended by the manufacturer. Paired end 50 base sequencing by synthesis was performed using TruSeq SBS kit v3 (Illumina) on an Illumina HiSeq 1000 using protocols defined by the manufacturer. Sequences were assembled into contigs with the SeqMan and NextGen suites of the DNAStar Lasergene 7 program (Bioinformatics Pioneer DNAStar, Inc., Madison, WI) and their relationships to other viruses were determined by a BLAST search. The open reading frames were identified using "SnapGene software (from GSL Biotech; available at snapgene.com)". The MUSCLE algorithm was used for multiple alignments and maximum likelihood (ML) tree constructions were performed in MEGA software version 7 (Kumar et al., 2016). Calculation of the pairwise evolutionary distances (PED) between the highly conserved protein domains of ORF1ab (3CLpro, RdRp and ZnHel1) of the mesoniviruses was performed using the Jones-Taylor-Thornton matrix based-model in MEGA 7 with complete deletion gap and 1000 bootstrap replicates.

2.5. Transmission electron microscopy

Ultrathin sections were prepared for ultrastructural observation as previously described (Vasilakis et al., 2014). Briefly, after fixation of infected cells for at least 1 h in a mixture of 2.5 % formaldehyde and 0.1 % glutaraldehyde in 0.05 M cacodylate buffer pH 7.3, the monolayers were washed in 0.1 M cacodylate buffer and cells were scraped off. The pellets were post-fixed in 1 % OsO_4 in 0.1 M cacodylate buffer pH 7.3 for 1 h, washed with distilled water and *en bloc* stained with 2 % aqueous uranyl acetate for 20 min at 60 °C. The pellets were dehydrated in ethanol, processed through propylene oxide and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on Leica EM UC7 ultramicrotome (Leica Microsystems, Buffalo Grove, IL), stained with lead citrate and examined in a Philips 201 transmission electron microscope at 60 kV.

2.6. Conventional pan-Mesonivirus RT-PCR

Confirmation of NGS results was done using conventional RT-PCRs. For cDNA synthesis, 10 µl of RNA extract was mixed with 1 µl of random primers (pdN6) following the manufacturer's instructions (2 pmol) and the mixture was heated at 95 °C for 2 min. Reverse transcription was performed in 20 µl mixture containing 2.5 U RNasin (Promega, Madison, USA), 1 µl of deoxynucleotide triphosphate (dNTP) (10 mM each DNTP), 5 U of AMV reverse transcriptase (Promega, Madison, USA) and incubated at 42 °C for 1 h. A pan *Mesonivirus* nested PCR system was then performed as previously described (Zirkel et al., 2013) using primer pair MeniV-F1/MeniV-R1 followed by MeniV-F2/MeniV-R2 mixed with 10X buffer, 5 µl of dNTPs 10 mM, 3 µl of $MgCl_2$, and 0.5 µl of Taq polymerase (Promega, Madison, USA).

2.7. Development of a pan Mesonivirus qRT-PCR

Multiple alignments of 11 mesonivirus sequences previously available in Genbank (Table 1) was made using MEGA 7 (Kumar et al., 2016). Based on a conserved region of 167bp between ORF3b and the

Table 1
Sequences used for primers and probe design of the pan *Mesonivirus* qRT-PCR assay.

Strains	Collection place	Collection date	Accession number
Bontang virus strain JKT7774	Indonesia	1981	KC807166.1
Karang Sari virus strain JKT10701	Indonesia	1981	KC807171.1
Ngewotan virus strain JKT9982	Indonesia	1981	KC807170.1
Kamphang Phet virus strain KP84-0156	Thailand	1984	KC807172.1
Cavally virus isolate C79	Cote d'Ivoire	2004	HM746600.1
Hana virus strain A4/CI/2004	Cote d'Ivoire	2004	JQ957872.1
Houston virus strain V3872	USA: Houston	2004	KC807175.1
Nse virus strain F24/CI/2004	Cote d'Ivoire	2004	JQ957874.1
Dak Nong virus	Viet Nam	2007	AB753015.2
Nam Dinh virus isolate NDiV-NJ8-09	China	2009	KF771866.1
Casuarina virus isolate 0071	Australia	2010	KJ125489.1

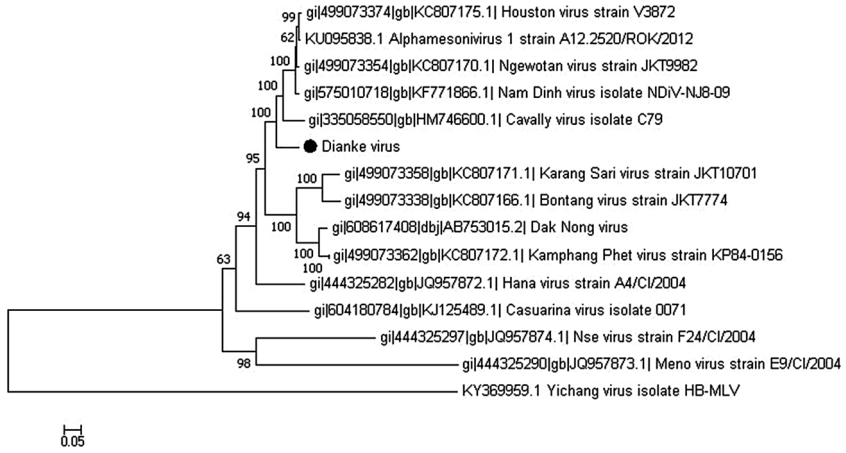

Fig. 1. Maximum likelihood tree of the full-length genome of Dianke virus and other mesoniviruses. Scale bar indicate nucleotide substitutions/site.

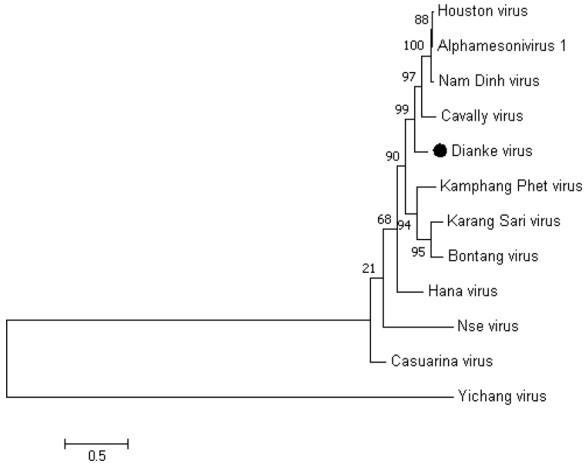

Fig. 2. Maximum-likelihood tree obtained from amino acid analysis of concatenated conserved protein domains of ORF1ab: 3CLpro-RdRp-HEL1. (Position on Dianke virus genome: 3CLpro: 4167nt-5072nt; RdRp: 9023nt-10387nt; HEL1: 11825nt-12853nt.) Scale bar indicate amino acid substitutions/site.

3′poly (A) tail, the primers and probe were then designed using Primer3 software (Untergasser et al., 2012). RT-qPCRs were performed in an ABI Prism 7500 SDS Real-Time cycler (Applied Biosystems, Foster City, USA) using Quantitect One-Step RT-PCR kit (Qiagen, Hilden, Germany) according to manufacturer's recommendations with 5 µl of RNA template.

2.8. Mesonivirus in vitro infection assays

Viral stock was prepared from C6/36-passage 2 and the same concentration was inoculated into four different cell lines: mosquito C6/36 cells, *Cercopithecus aethiops* Vero cells, *Homo sapiens* Hep G2 and human rhabdomyosarcoma RD cells. Each infection was performed in 25-cm^{2}tissue-culture flasks for incubation at 28 °C, 33 °C or 37 °C. Monitoring was done until strong CPE observation or at day 3, day 7 and day 10 p.i. Supernatant were then collected and the viral RNA quantification was done using the pan *Mesonivirus* qRT-PCR.

Continuous cell lines were originally provided by the ATCC (American Type Culture Collection, Manassas, Va.) and were cultured in Leibovitz-15 (L-15) medium (GibcoBRL, Grand Island, NY, USA) supplemented with 10 % FBS (Gibco BRL, Grand Island, NY, USA), penicillin-streptomycin (Sigma, GmBh, Germany). For C6/36 cells culture, Leibovitz-15 (L-15) medium was also supplemented with additional 10 % of Bacto™ Tryptose Phosphate Broth (Becton, USA).

The experiment was performed in duplicate for each cell line and temperature condition.

3. Results

3.1. Mesonivirus isolation from mosquitoes trapped in Eastern Senegal

During the arbovirus screening, CPE was observed 4 days p.i on C6/36 cells inoculated with extract from seven (7) mosquito pool homogenates from Sabodala, one (1) specimen from Fadiga, six (6) from Kedougou town and two (2) others in Dianke Makhan, with negative results both by PCR and IFA targeting numerous arboviruses (Table S1 in supplement). One of the C6/36 cell supernatants was randomly chosen for Next Generation Sequencing (NGS) as described above, yielding 20,118 bp apart from the extreme 3′-poly (A) tail - termini. The sequence shared 90 % nucleotide identity with some previously described mesoniviruses. This result was confirmed using a conventional pan *Mesonivirus* RT-nested PCR, which was positive both for

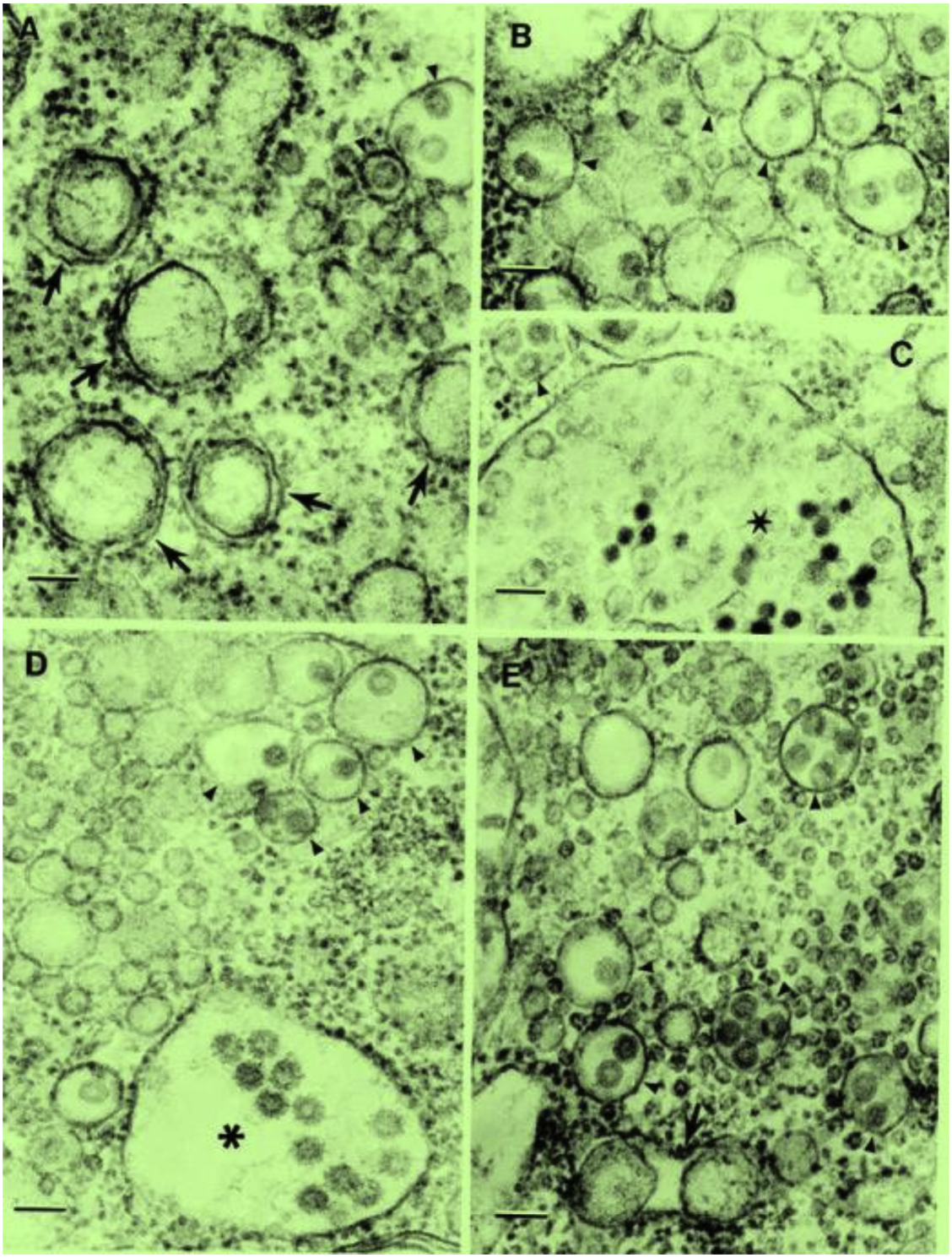

Fig. 3. Ultrastructure of Dianke virus in C6/36 cells.
Bars in all pictures = 100 nm.
A- Virus particles with smooth surface 55–60 nm in diameter inside cytoplasmic vacuoles (arrowheads). Arrows indicate vesicles 120–250 nm in diameter inside vacuoles, presumably of granular endoplasmic reticulum origin.
B- Virus particles with smooth surface 55–60 nm in diameter inside multiple cytoplasmic vacuoles (arrowheads).
C- Three particles with smooth surface 55–60 nm in diameter inside small cytoplasmic vacuole (arrowhead) and smaller (40–45 nm in diameter) enveloped particles with dark core inside huge vacuole (star).
D- Two types of virus particles inside vacuoles: smooth surfaced 55–60 nm in diameter inside small smooth surfaced vacuole s (arrowheads) and particles ~ 65 nm in diameter with spikes ~5 nm long inside an expanded cistern of granular endoplasmic reticulum (asterisk).
E- Smooth surfaced virus particles 55–60 nm in diameter inside smooth surfaced cytoplasmic vacuoles (arrowheads) and vesicles 140–160 nm in diameter inside a cistern of granular endoplasmic reticulum (arrow). Cytoplasm contains multiple vesicles 25–30 nm in diameter.

Table 2
Nucleotide sequences of primers and probe used for qPCR pan *Mesonivirus* assay (based on Cavally virus isolate C79 strain, HM746600.1).

	Sequences 5'-3'	Nucleotide position
Meso F (Forward primer)	CATGGACDNAACACAACAGCAG	19877-19898
Meso P (Probe)	FAM-AGGYGTACTGAAYTCYRAGGAGACG—BHQ1	19940-19964
Meso R (Reverse primer)	AATGYGTCTCTCRCAAYGTA	20022-20041

FAM: 6-carboxyfluorescein; BHQ1: 6-black hole quencher 1; D: A or G or T; N: A or C or G or T; Y: C or T; R: A or G.

Table 3
Specificity test for pan *Mesonivirus* qRT-PCR assay.

Virus	Strains	Mesonivirus qRT-PCR	Pan Mesonivirus (RT)-PCR	ct value
Chikungunya	S27 African prototype (AF369024.2)	Negative	Negative	–
Zika	ArD 165,522 (KF383090.1)	Negative	Negative	–
West Nile	Eg101 (AF260968.1)	Negative	Negative	–
Usutu	SAAR-1776 (AY453412.1)	Negative	Negative	–
Rift valley fever	MP-12 (DQ380154.1)	Negative	Negative	–
Yellow fever	17 D (X03700.1)	Negative	Negative	–
Dengue 2	Dak Ar 141,070 (EF105390.1)	Negative	Negative	–
Wesselsbron	ArB 4177	Negative	Negative	–
Bagaza	ArB 209 (AY632545.2)	Negative	Negative	–
Barkedji	ArD86177 (EU078325.1)	Negative	Negative	–
Mesonivirus	*Culex* species (original)	Positive	Negative	34.1
Mesonivirus	*Aedes* species (original)	Positive	Negative	33.5
Mesonivirus	*Culex* species (C6/36 passage)	Positive	Positive	24.3
Mesonivirus	*Aedes* species (C6/36 passage)	Positive	Positive	24.87

corresponding supernatants and original samples.

3.2. Phylogeny and genome analysis

Comparative analysis of the genome showed that the strain is clearly distinct from the other mesoniviruses previously characterized, with 10 % nucleotide sequence difference compared to Houston and Nam Dinh viruses, and forming a separate cluster (Fig. 1). This observation was confirmed by a parallel analysis using a neighbor-Joining approach for which one no discrepancy was noted, as shown in figure S1 in supplemental material. The classical mesonivirus genome organization was found, with the typical ORFs and the putative ribosomal frame-shift (RFS) element in ORF1a and ORF1b, the 3C-like serine protease (3CLP), RNA-dependent RNA polymerase (RdRp), zinc-binding (Z), helicase (Hel), exoribonuclease (ExoN), N7-methyltransferase (NMT) and 2′-O-methyltransferase (OMT) domains. Block insertions observed after the first 1300 nt of the ORF1a of some Asian strains (Vasilakis et al., 2014) were absent in the Senegal sequence. Protein sequence identity of ORF 1, ORF 2 and ORF 3 were 91 %, 94 % and 91 %, respectively (Table 4). Pairwise evolutionary distance (PED) is the reference species demarcation criterion in the *Mesonivirus* genus (Lauber et al., 2012). Using the conserved protein domains of ORF1ab ($3CL^{pro}$, RdRp and Hel) (Vasilakis et al., 2014), we calculated PED between the virus and some previously described mesoniviruses selected from the blast output using a protein alignment. We considered the same consensus threshold of 0.037 as minimum distance for the demarcation of nidovirus species based on the concatenated protein domains (Lauber et al., 2012). In accordance with a previous study (Warrilow et al., 2014), a PED value of 0.031 was obtained between CavV and NDiV isolates (Table 5), which constitutes the species *Alphamesonivirus 1* as previously described (Lauber et al., 2012). The Senegal isolate was closest to *Alphamesonivirus 1* with distances of 0.042, 0.044 and 0.053 separating it from Houston virus (HOUV), Nam Dinh virus (NDiV) and Cavally virus (CavV), respectively. The highest distance was observed with Nse (NseV), Casuarina (CASV) and Hana viruses (HAV) with PED values of 0.243, 0.123 and 0.117, respectively (Table 5). These results are reflected by the tree obtained with the different sequences involved in the maximum likelihood analysis (Fig. 2), as well as a neighbor-joining approach (figure S2 in supplemental material). Our isolates represent a new species in the *Mesonivirus* genus. We proposed Dianke virus (DKV) (Accession number: MN622133) as a name for this virus, referring to one of the sampling localities.

3.3. Dianke virus morphological characterization

In ultrathin sections of DKV-infected C6/36 cells, three types of virus particles were observed: enveloped spherical particles 55–60 nm in diameter with smooth surface located within smooth membrane-limited vacuoles containing from 1 to 5 particles (Fig. 3A-E); enveloped spherical particles ~65 nm in diameter with short (~5 nm) surface projections located mostly within enlarged cisterns of granular endoplasmic reticulum (Fig. 3D) and smaller spherical enveloped particles with dense core (40–45 nm in diameter) within large vacuoles (Fig. 3C). Characteristically, viruses produced the same kind of CPE – large vesicles 120–250 nm in diameter located mostly individually (Fig.3A) or in pairs (Fig. 3E) inside tight vacuoles, presumably of granular endoplasmic reticulum origin because some of them had ribosomes at their outer surface. Morphologically these vesicles resembled smooth membrane structures (SMS) regularly observed with replicating flaviviruses.

3.4. Pan mesonivirus qRT-PCR validation

A pan *Mesonivirus* qRT-PCR was designed using an alignment of partial sequences of eleven known mesonivirus strains (see above). The TaqMan probe was flanked by a 6-carboxyfluorescein (FAM) fluorescent reporter dye at 5′end and a black hole quencher 1 (BHQ-1) at the 3′-end. Primers and probe characteristics are shown in Table 2. A specificity test was performed with several viruses found in mosquito pools from Senegal (Table 3). The pan-*Mesonivirus* qRT-PCR was validated by a specificity test including some strains obtained during this study (Table 3) and showed good results with amplification only for mesonivirus samples. The qRT-PCR was also more sensitive than conventional RT-PCR.

In order to obtain a pan *Mesonivirus* qRT-PCR standard curve, the linear dynamic range of the qPCR assay was initially assessed using 8-$\log_{10}$ serial dilutions of a synthetic standard RNA. The assay was shown

Table 4
Blast results summary of Dianke virus open reading frames (ORFs): HOUV: Houston virus; NGEV: Ngewotan virus; NDiv: Nam Dinh virus; CAVV: Cavally virus; HAV: Hana virus; DkNV: Dak Nong virus; KSV: Karang Sari virus; KPV: Kamphang Phet; BV: Bontang virus; CASV: Casuarina virus; NseV: Nse virus; MeV: Meno virus.

ORF1						ORF2						ORF3					
Nucleotides			Protein			Nucleotides			Protein			Nucleotides			Protein		
	Id	Accession		Id	Accession		Id	Accession		Id	Accession		Id	Accession		Id	Accession
HOUV	88%	KC807178.1	NGEV	91%	AGL73185.1	HOUV	91%	KC807177.1	NGEV	94%	ASA47342.1	NGEV	92%	MF176279.1	NDiV	91%	AII17280.1
NGEV	88%	MF176279.1	NDiV	91%	YP_004767306.1	NGEV	91%	MF176279.1	NDiV	94%	AHG56128.1	NDiV	92%	KF522691.1	NGEV	91%	ASA47344.1
NDiV	88%	DQ458789.2	HOUV	91%	AGL73209.1	NDiV	91%	KF771866.1	HOUV	94%	AGL73201.1	HOUV	92%	KC807178.1	HOUV	91%	AGL73202.1
CAVV	87%	HM746600.1	CAVV	90%	YP_004598982.1	CAVV	90%	HM746600.1	CAVV	93%	AEH26445.1	CAVV	89%	HM746600.1	CAVV	86%	YP_004598983.1
HAV	80%	JQ957872.1	HAV	81%	YP_007697630.1	DkNV	85%	AB753015.2	DkNV	88%	BAN58308.1	KPV	89%	KC807174.1	DkNV	85%	BAN58309.1
DkNV	78%	AB753015.2	DkNV	78%	BAN58307.2	KSV	85%	KC807171.1	BV	87%	AGL73177.1	DkNV	88%	AB753015.2	KPV	85%	AGL73193.1
KSV	77%	KC807171.1	KPV	72%	AGL73197.1	KPV	85%	KC807174.1	KSV	88%	AGL73189.1	HAV	86%	JQ957872.1	HAV	82%	YP_007697631.1
KPV	77%	KC807173.1	CASV	71%	YP_009026379.1	BV	85%	KC807169.1	KPV	87%	AGL73192.1	BV	79%	KC807166.1	KSV	73%	AGL73190.1
BV	77%	KC807169.1	NseV	59%	YP_007697643.1	HAV	85%	JQ957872.1	CASV	85%	YP_009026378.1	CASV	79%	KJ125489.1	BV	72%	AGL73178.1
CASV	78%	KJ125489.1	MeV	50%	YP_007697637.1	CASV	82%	KJ125489.1	HAV	85%	YP_007697629.1				CASV	70%	YP_009026380.1
						MOUV	77%	KC768950.1	NseV	75%	YP_007697642.1				NseV	63%	YP_007697644.1
						NseV	76%	JQ957874.1	MOUV	76%	AGI52414.1				MeV	60%	YP_007697638.1

to be linear over the entire range of 10^8 to 10 genomic copies. The different end-point dilutions of standard RNA were tested in triplicate. A typical standard curve amplification plot and linear regression analysis of these data are shown in the figure S3 in supplemental material. This qRT-PCR system was used for a screening of mesoniviruses in other mosquito pools (MPs) caught in the same areas as the positive ones.

3.5. Evidence of DKV circulation in mosquitoes

After molecular screening for mesonivirus RNA (qRT-PCR), DKV was detected in 21 species of arthropods (mainly mosquitoes) trapped in the same areas. The most representative genus was *Aedes* with 7 species: *Ae. furcifer* (5MPs), *Ae. aegypti* (2), *Ae. cumminsii* (1), *Ae. fowleri* (1), *Ae. mcintoshi* (1), *Ae. minutus* (1), and *Ae. vittatus* (1), followed by *Culex* species such as *Cx. antennatus* (5), *Cx. decens* (3), *Cx. neavei* (3), *Cx. nebulosus* (1), *Cx. poicilipes* (1) and 1 pool of males *Cx. quinquefasciatus*. Species from the genus *Anopheles* were also found positive with *An. funestus* (2), *An. gambiae* (1), *An. pharoensis* (1) or *An. rufipes* (1). A mesonivirus was also detected from 8 pools of *Mansonia uniformis* (including 1 pool of males) and 2 pools from *Ma. africana*. The virus was also detected on 1 pool of Uranotaenia sp and another one of biting midges (ceratopogonids). A conventional, specific RT-PCR assay targeting a small part of the ORF1a was performed on all positive samples and the sequences obtained confirmed that the same virus strain was circulating in these different arthropods (99 % identity) in eastern Senegal (Fig. 4). The Genbank accession numbers of the different sequences generated during this study are available in the table S2 of the supplement.

3.6. Dianke virus: vertebrate and insect cells infections

The same concentration of DKV was inoculated both in mosquito and mammalian cells in order to access replication under different conditions of temperature. C6/36 cells exhibited CPE 2 days pi at 28 °C and 3 days p.i at 33 °C, while no CPE was observed at 37 °C (Fig. 5A). Moreover, a decrease of DKV RNA in C6/36 at 37 °C was observed until day 10. Mammalian cell monolayers were unaltered until day 10 pi with also a constant reduction of the viral quantity over time (Fig. 5B-C-D-E). However, this reduction was less important at 28 °C than the other thermal conditions with, for instance, a difference on the order of twice as pronounced for the Hep-G2 cells at day 10 pi.

4. Discussion

In this study, we reported the isolation of a new viral strain phylogenetically belonging to an insect-specific virus (ISV) group in Senegal. The isolates, obtained from different mosquito pools trapped in the Kedougou and Tambacounda areas (eastern Senegal), clearly belong to the *Mesoniviridae* family with 90 % nucleotide identity compared to other members. Genome organization showed typical mesonivirus features. The species demarcation based on previously employed criteria (Lauber et al., 2012) allowed us to conclude that this mesonivirus constitute a new species that we named Dianke virus (DKV), referring to one of the places where the strain have been found. Molecular screening undertaken in the same areas resulted in detection of the same virus in 43 mosquito pools from 21 species. These results are similar to previous observations where a broad host species range in mosquitoes suggested a worldwide mesoniviruses distribution (Vasilakis et al., 2014), while Colmant et al. (2017) described a species-specific host restriction for some insect-specific-flaviviruses.

Numerous papers indicate vertical transmission for some ISVs (Sang et al., 2003; Cook et al., 2006; Boiling et al., 2011; Haddow et al., 2013). In this study, DKV detection in some male mosquitoes supports this assertion.

Like our study where a large diversity of vectors was infected by the new strain, several studies have detected mesonivirus infection of

Table 5
Estimates of evolutionary distances between ORF1a conserved domain (3CL-RdRp-HEL) of Dianke virus and other mesoniviruses.

Nse virus										
Hana virus	0.249									
Casuarina virus	0.247	0.160								
Houston virus	0.238	0.112	0.108							
Kamphang Phet virus	0.236	0.121	0.123	0.080						
Karang Sari virus	0.250	0.122	0.133	0.082	0.044					
Bontang virus	0.252	0.128	0.137	0.092	0.051	0.039				
Nam Dinh virus	0.241	0.114	0.109	0.006	0.080	0.082	0.093			
Cavally virus	0.249	0.117	0.112	0.028	0.085	0.090	0.102	0.031		
Alphamesonivirus 1	0.239	0.114	0.106	0.002	0.082	0.084	0.094	0.008	0.028	
Dianke virus	**0.243**	**0.117**	**0.123**	**0.042**	**0.086**	**0.085**	**0.092**	**0.044**	**0.053**	**0.044**

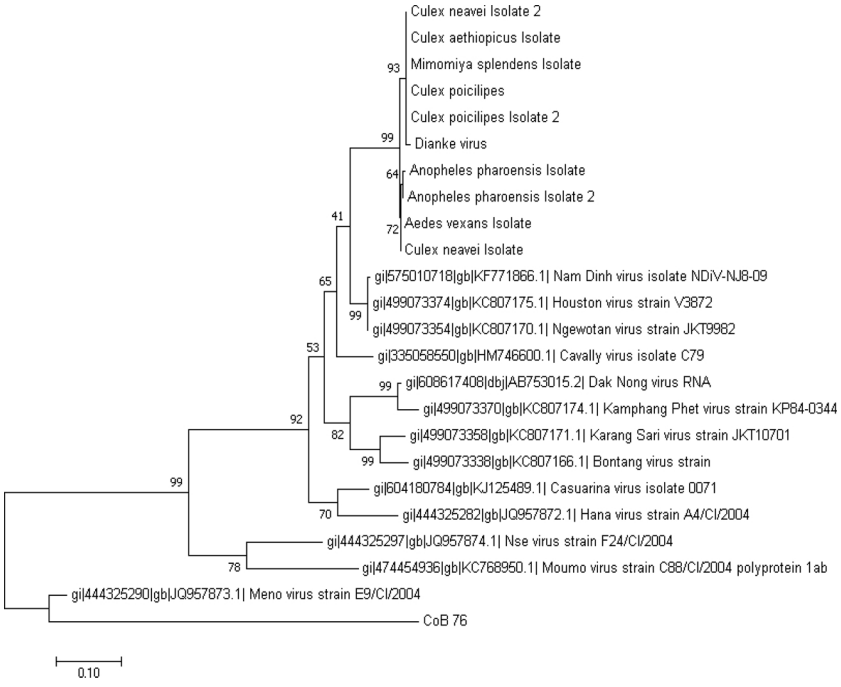

Fig. 4. Maximum likelihood tree of 303bp of the ORF1ab of Dianke virus, the mesoniviruses detected in mosquito pools from the same areas and others published ones. The tree is rooted with CoB76 (Bovine Coronavirus). The sequences from Dianke virus and mosquito isolates from Senegal form a common cluster (99%–100% nucleotide identity) different to the others strains.

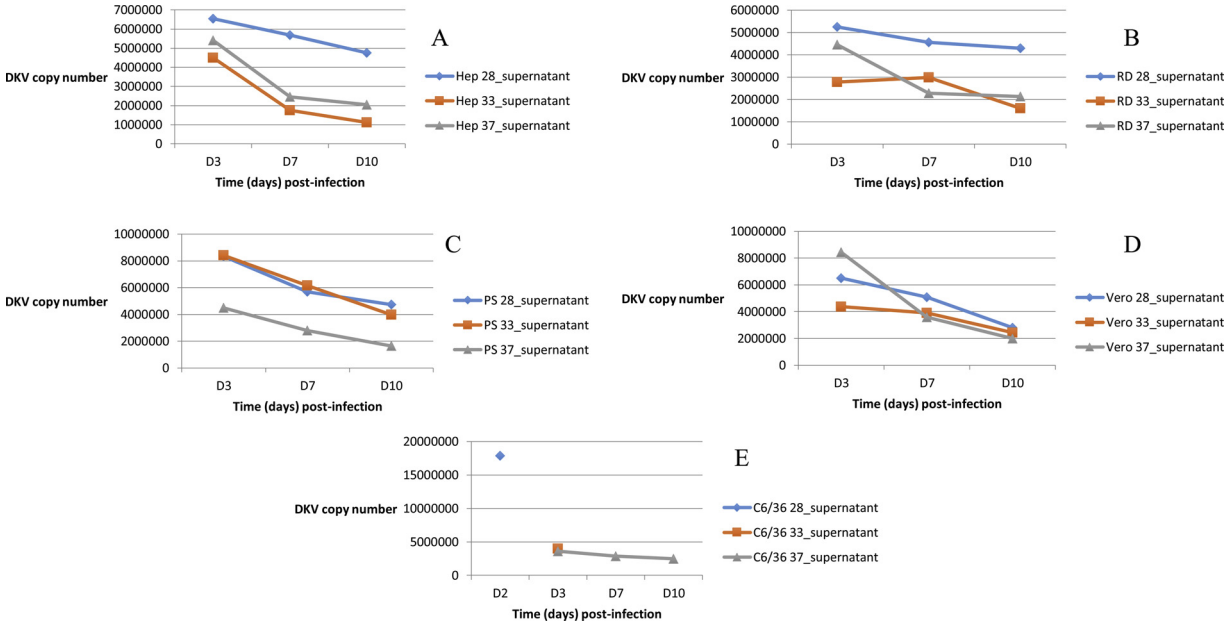

Fig. 5. Dianke virus (DKV) *in vitro* infections in different thermal conditions (blue: 28 °C, orange: 33 °C and grey (37 °C) on Hep-G2 cells (A), RD cells (B), PS cells (C), Vero cells (D) and C6/6 cells (E). The kinetics were prematurely stopped for C6/36 cells at 28 °C and 33 °C as a strong depletion was observed at day 2 and day 3 post-infection respectively.

mosquitoes with potentially significant health impacts (Zirkel et al., 2011; Nga et al., 2011; Vasilakis et al., 2014). Indeed, it has been shown that dual viral infections in mosquito can alter viral infectivity (Fujita et al., 2018) and ISVs are more and more considered as potential disease control agents in vector populations (Guzman et al., 2018; Öhlund et al., 2019). Virus phenotypic restriction could be more complex as Parry and Asgari (2018) highlighted that restriction of Dengue virus replication in *Ae.aegypti* mosquitoes could be hindered by interactions between a novel ISV and the endosymbiotic bacterium *Wolbachia pipientis*. In contrast, Zhang et al. (2017) pointed out that Cell fusing agent virus, another ISV, favor Dengue virus replication in *Ae.aegypti* cell lines. All together, the current studies highlight gaps in the understanding of interactions between ISVs and other pathogenic viruses. It has been shown that the host range restriction of insect-specific flaviviruses and alphaviruses occurs at different levels of viral replication cycle (Nasar et al., 2015; Junglen et al., 2017). Among intrinsic and extrinsic factors affecting viral replication and transmissibility, temperature was shown to be of an extreme importance (Samuel et al., 2016). The experiments undertaken in this study tends to confirm this assessment as DKV was unable to replicate in permissive mosquito C6/36 cells at 37 °C. However, the virus did not grow in different mammal cells at the mosquito temperature of 28 °C. The host restriction phenomenon for mesoniviruses and the other ISVs remain to be further studied.

An increasing number of arthropod-specific viruses (ASVs) are being discovered in hematophagous arthropods throughout the world (Calisher and Higgs, 2018). Due to their potential impact on the vectors fitness, more studies are needed to improve the knowledge about their prevalence and diversity.

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Disclosure of competing interest

No competing interests in this scientific work.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197802.

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