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Nhumirim virus, a novel flavivirus isolated from mosquitoes from the Pantanal, Brazil

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Abstract

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The authors declare that they have no conflict of interest.

We describe the isolation of a novel flavivirus, isolated from a pool of mosquitoes identified as *Culex* (*Culex*) *chidesteri* collected in 2010 in the Pantanal region of west-central Brazil. The virus is herein designated Nhumirim virus (NHUV) after the name of the ranch from which the mosquito pool was collected. Flavivirus RNA was detected by real-time RT-PCR of homogenized mosquitoes and from the corresponding C6/36 culture supernatant. Based on full-genome sequencing, the virus isolate was genetically distinct from but most closely related to Barkedji virus (BJV), a newly described flavivirus from Senegal. Phylogenetic analysis demonstrated that NHUV grouped with mosquito-borne flaviviruses forming a clade with BJV. This clade may be genetically intermediate between the *Culex*-borne flaviviruses amplified by birds and the insect-only flaviviruses.

Introduction

The genus *Flavivirus* in the family *Flaviviridae* consists of enveloped, positive-sense single-stranded RNA viruses. Based on their host associations, flaviviruses have been grouped into insect-only viruses and vertebrate viruses that either have no known vector or are mosquito- or tick-vectored [1]. In Brazil, flaviviruses are involved in a large number of human disease cases comprised largely of dengue fever as well as sporadic local outbreaks of sylvatic yellow fever [2, 3]. Outbreaks caused by other flaviviruses, including Rocio virus (ROCV), as well as isolated human cases of infection with Saint Louis encephalitis virus (SLEV), Cacipacore virus (CPCV) and Ilheus virus (ILHV) have also been reported in the country [4–7]. Flaviviruses of eleven species have been isolated in Brazil, including Naranjal-like virus from a marsupial, Culex flavivirus from *Culex* mosquitoes, and a potential novel flavivirus recently isolated from ticks [8–10]. In 2011, West Nile virus (WNV) was reported in Brazil for the first time based solely on serological evidence in healthy horses sampled in 2009 and 2010 in the Pantanal wetland region of west-central Brazil [11]. In addition to WNV, neutralizing antibodies for eight flaviviruses including SLEV, ROCV, ILHV, CPCV, Bussuquara, dengue, yellow fever and Iguape viruses, were also recently detected in Pantanal equines. However, considering the high cross-reactivity observed among flaviviruses, only the circulation of WNV, CPCV, ILHV, SLEV and ROCV could be inferred in Pantanal equines [12]. Prevalence data for specific flaviviruses in the Pantanal has relied mainly on serologic surveys rather than direct isolation. Efforts to detect flavivirus infections in mosquitoes from the Pantanal region have previously resulted in the isolation of ILHV only [13].

Serological data provide only indirect evidence of the presence of a virus and can be difficult to interpret given the antigenic cross-reactivity among flaviviruses [14]. As a result, the interpretive value of serological studies can be limited, resulting in an incomplete appreciation of the breadth of circulating flaviviruses. Few studies in the Brazilian Pantanal have attempted arbovirus detection directly in arthropods, and these have focused primarily on mosquitoes [11, 13, 15]. However, modern molecular techniques have facilitated virus detection and characterization, and as a result, many new flaviviruses have been discovered recently worldwide [16–19]. Although ticks have not commonly been implicated as arbovirus vectors in Brazil, these arthropods have been reported to be the most important

vectors of disease-causing pathogens in domestic and wild animals [20], and their potential competence as arbovirus vectors in Brazil remains unknown.

To improve interpretation of serologic surveys, we sought to isolate and identify circulating mosquito-borne and tick-borne viruses, particularly flaviviruses, from the Brazilian Pantanal. Therefore, we collected and tested mosquitoes and ticks for the presence of flaviviruses using vertebrate and invertebrate tissue culture systems in concert with viral RNA detection. We present herein the isolation and full genomic RNA sequence of a novel flavivirus designated Nhumirim virus, for which we propose the abbreviation NHUV.

Materials and methods

Study area

In February and October 2009 and April and October 2010, adult mosquitoes and ticks were sampled at sites randomly selected within fourteen beef cattle ranches in the Nhecolândia sub-region of Pantanal, west-central Brazil (18°18′–19°15′S, 57°05′–55°24′W) (Fig. 1). The collections for this study were approved by the Animal Ethics Committee of Fundação Oswaldo Cruz of Ministry of Health of Brazil (License CEUA-Fiocruz LW-1/12, protocol P-74/10-5) in compliance with the requirements of Brazilian Law 11794/2008, which rules on the scientific use of animals. The collections were also approved by the Instituto Chico Mendes de Conservação da Biodiversidade of Ministry of Environment of Brazil (licenses ICMBio 18363-1/2009 and 18363-2/2010).

Mosquitoes and ticks

The methods used to collect and identify mosquitoes were described previously [13]. Briefly, adult mosquitoes were caught using CDC light traps, and they were also collected from horses, research team members, caimans and the walls of a local residence using manual aspirators. Mosquitoes were identified based on morphologic appearance according to taxonomic keys. Ticks were collected from local horses and transported alive to a field laboratory where they were grouped into pools of up to 10 individuals that were sorted with respect to specific host, species, age class, engorgement status, and gender and then stored in liquid nitrogen. Ticks were identified based on morphologic appearance according to a taxonomic key [21].

Virus isolation

Mosquito and tick pool homogenates were assayed for cytopathic effect (CPE) on C6/36 monolayers, and for plaque formation in Vero cells using standard protocols [22]. Briefly, pools from one to 50 mosquito specimens were triturated in a mixer mill in 1 mL of BA-1 diluent using a copper-coated steel ball bearing. Tick pools were triturated with a sterile mortar and pestle in 2 mL of BA-1 diluent. The mosquito and tick homogenates were then clarified by brief centrifugation. Inocula of 0.1 mL of supernatants were tested in duplicate in both C6/36 and Vero cell culture systems using 6-well polystyrene culture plates. The plates were incubated at 28 °C and 37 °C, respectively, for 1 h with discrete motion every 15 min to optimize virus adsorption and to avoid dehydration of the monolayers during the adsorption process. At the end of this period, 3 mL of maintenance medium was added to

the C6/36 cultures, and 3 mL of 0.5 % agarose in culture media was added to the Vero cultures. C6/36 monolayers were incubated at 28 °C in 5 %CO_2 for 10 days and observed daily for CPE. Plates with Vero monolayers were divided in two groups and then incubated at 37 °C at 5 % CO_2. The first group was incubated for two days, and the second group for five days, before being stained with neutral red in 3 mL of additional 0.5 % agarose culture medium overlay. Both groups were maintained at 37 °C in 5 % CO_2 for 10 days and observed daily for viral plaques.

Real-time RT-PCR screening

A SYBR Green® real-time RT-PCR method for the universal detection of flaviviruses was applied to all mosquito and tick samples to screen for flaviviral RNA [23]. First, RNA was extracted from the supernatants of triturated mosquitoes and ticks using a QIAamp Viral RNA Mini Kit (QIAGEN, Inc., Valencia, CA, USA) and then tested for flavivirus genus-specific viral RNA by real-time RT-PCR. The protocol utilized was based on the amplification of a 269- to 272-nucleotide region at the N-terminus of the NS5 gene [23]. Samples with amplicons that yielded a melting curve peak above 90 -d(RFU)/d(T) [RFU = relative fluorescence units] at a temperature above 75 °C were considered positive and tested further by DNA sequencing.

Amplicon sequencing and phylogenetic analysis

Extracted RNA from samples that were positive by real-time RT-PCR were reverse transcribed and subjected to PCR amplification and sequencing using the same forward and reverse primers used for the real-time RT-PCR protocol [23]. DNA amplifications and sequencing reactions were carried out in a DNA Engine® PTC-200 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the products were analyzed on an ABI 3130 Genetic Analyzer. Sequences were assembled using Lasergene 9 software (DNASTAR, Inc., Madison, WI, USA) and aligned to reference sequences available in the GenBank database using the BLASTn algorithm.

In order to obtain a longer sequence for confirmation of the presence of a unique flavivirus, viral RNA was extracted from harvested C6/36 supernatants using a QIAamp Viral RNA Mini Kit, and one-step RT-PCR was performed using Superscript III (Invitrogen, Life Technologies, Carlsbad, CA) and previously described panflavivirus NS5 primers FU1(F) and CFD3(R) [24]. An amplicon of the expected size (approximately 1 kb by agarose gel electrophoresis) was purified using a QIAprep Spin Miniprep Kit (QIAGEN) and sequenced on an ABI 3100 instrument using internal primers described previously [24]. Sequences were assembled using Geneious 6.0 (Biomatters, Auckland, NZ) and subjected to an NCBI BLASTn analysis to identify the viruses with the highest level of nucleotide sequence identity.

Deep sequencing and *de novo* viral genome assembly

Total RNA was extracted from flavivirus-positive C6/36 supernatant using RNAzol®RT (Molecular Research Center, Inc., Cincinnati, OH, USA) and a Direct-zol™ kit (Zymo Reseach, Irvine, CA, USA) according to the manufacturer's instructions. RNA-Seq libraries were constructed by incorporating Illumina-compatible sequencing adaptors using SMART

(switch mechanism at 5′ end of RNA transcript) cDNA technology; the libraries were uniquely barcoded to multiplex the samples and sequenced on a Mi-Seq system (Illumina Inc., San Diego, CA, USA) as described previously [25]. Each RNA-Seq library generated 1–5 million 150-base-pair oligonucleotides, and raw sequences were demultiplexed using the Mi-Seq computational software.

Raw demultiplexed Illumina data were processed with Virominer, a custom PERL language pipeline that orchestrates standard bioinformatic tools in a manner that optimizes speed and sensitivity. First, Virominer uses Cutadapt [26] to trim end adaptor sequences that may have been generated during the library preparation, and FLASH (fast length adjustment of short reads to improve genome assemblies) to join overlapping read pairs [27]. Next, Bowtie2 [28] is used to filter out a large proportion of the sequences by aligning them against host indexes and removing them from downstream analysis. The pipeline then runs additional rounds of Bowtie2 alignments against sequence indices compiled for microbial organisms (known viruses, parasites, bacteria, and archaea). All oligonucleotide sequences that matched the viral index, plus all sequences that failed to hit any index, were then used as input for *de novo* assembly of contigs using Trinity [29]. The pipeline then automatically annotates all contigs over 500 bp by aligning the nucleotide sequences to the NCBI nucleotide database (nt) with blastn and then aligning the remaining unidentified sequences to the NCBI protein database (nr) with blastx. All sequence contigs with significant BLAST alignments are then summarized taxonomically using Krona [30]. The Virominer pipeline annotates each contig with the top hit BLAST result and makes it readily available via the Krona interface. The Virominer pipeline is available via Github at https://github.com/odoublewen/virominer.git.

Results

A total of 69 ticks collected from 28 equines and one caiman from ten ranches were grouped in 41 pools for virus isolation and real-time RT-PCR. The following species were identified: *Amblyomma cajennense* (n = 21), *Anocentor nitens* (n = 44), *Rhipicephalus microplus* (n = 3), *Amblyomma* sp. (n = 1). All pools tested negative for viruses and/or viral RNA by virus isolation and real-time RT-PCR.

A total of 2950 adult female mosquitoes were collected from five ranches, representing at least 16 species [13]. Nhumirim virus was detected only in a single pool of 43 non-engorged female mosquitoes identified morphologically as *Culex* (*Culex*) *chidesteri*. These mosquitoes were captured on April 17, 2010, from a CDC light trap placed overnight 1.5 m above the ground at the edge of a dry natural lake bed and adjacent to an artificial pond (18°59′40.5″S, 56°37′22.3″W) located roughly 160 km from the city of Corumbá, in the state of Mato Grosso do Sul (Fig. 1). A total of 175 specimens of *Cx. chidesteri* were captured, 146 (83.4 %) by outdoor CDC light trap and 29 (16.6 %) by human landing collections [13]. The pool was apparently negative for CPE on C6/36 cells and for plaques on Vero cultures, but positive for flaviviral RNA by real-time RT-PCR, with peak dissociation measured as 295 -d(RFU)/d(T) at 81.5 °C. A 218-nucleotide sequence initially obtained by Sanger sequencing demonstrated 82 % identity with Barkedji virus (BJV) isolate ArD86177 (GenBank: EU078325.1). A larger fragment of the NS5 gene (1,061

nucleotides) that was obtained subsequently confirmed BJV as the most similar flavivirus, with 74 % identity.

The complete coding sequence of NHUV, determined by deep sequencing, was found to contain a single open reading frame of 10,891 nucleotides in length (GenBank accession no. KJ210048) encoding a polyprotein of 3,445 amino acids. The genomic sequence of NHUV was unique when compared to other flaviviruses (Fig. 2), sharing only 63 % identity with the two BJV strains available in the GenBank database. The NCBI file with the highest identity was the BJV isolate 363/11 (GenBank accession no. KC496020.1), which had an e-value of 0.0, a maximum score of 3261, coverage of 93 %, and identity of 68 %.

Discussion

Considering Brazil's enormous biodiversity distributed in distinct biomes including Atlantic Rain Forest, Amazon, Cerrado, Pampas, Coastal Environments and Pantanal, the circulation of previously undescribed mosquito-borne flaviviruses in Brazil was expected. The Brazilian Amazon alone houses the world's largest variety of known arboviruses [31]. We have reported the discovery of a novel flavivirus isolated from mosquitoes identified as *Cx. chidesteri*, a mosquito species previously reported in the region [15]. The virus is designated Nhumirim virus, according to the location where it was discovered. Nhumirim means "small field" in the Tupi/Guarani native Brazilian language. The detection of a novel flavivirus in the Pantanal region highlights the need for caution in interpreting results of flaviviral serosurveys. Discerning the circulation of an unknown flavivirus in the Pantanal is particularly important, since undiagnosed diseases and sometimes outbreaks of neurological disorders are sporadically observed in local horses. For example, serologic evidence from a recent outbreak of neurologic disease among local equines indicated that the etiologic agent may have been a flavivirus antigenically related to SLEV [12]. In spite of serologic evidence of recent circulation of several mosquito-borne flaviviruses in the Pantanal region [12], only ILHV has been isolated there recently [13].

All of the tick species collected in the present study have been described previously in the Pantanal region, and homogenates from these ticks have tested negative for flaviviruses [13, 15]. Despite the awareness of tick-borne viruses as an important public-health concern, mainly in Eurasia, isolations of tick-borne viruses in Brazil are rare, with just a couple of reports of virus detection in ticks in the country. In 1997, a flavivirus was isolated from *Amblyomma cajennense* specimens collected from a morbid capybara in the state of São Paulo, Southeast Brazil, but this virus has not been characterized further [32]. In 2006, a potential novel flavivirus was isolated from *Rhipicephalus microplus* collected in dairy cattle, also from São Paulo [10].

Mosquito-borne flaviviruses can be differentiated epidemiologically into two groups. One group (including WNV and SLEV) causes neurological disease in humans and is associated with *Culex* mosquitoes as vectors and birds as amplifying hosts. The second group (including dengue and yellow fever viruses) causes hemorrhagic disease in humans and is associated with Aedini mosquitoes as vectors and primates as amplifying hosts [33]. However, this pattern can vary according to ecological and viral features. For example,

ROCV, the causative agent of the largest Brazilian epidemic of arboviral encephalitis, is believed to be transmitted by Aedini mosquitoes [34]. In 2004 and 2005, an unusual outbreak of acute encephalomyelitis was associated with dengue virus in northern Brazil, and between 2005 and 2006, SLEV was linked to hemorrhagic disease in the southeast region of the country [5, 35].

Culex flaviviruses and unclassified insect flaviviruses that have no recognized association with vertebrates have been isolated from a variety of mosquito species [36–40]. These insect flaviviruses do not appear to infect vertebrate cells and have not been associated with human or animal disease [1].

NHUV did not cause a clear CPE in C6/36 (*Aedes albopictus*) cells or plaques in Vero cells. However, these limited experiments with two cell lines do not exclude the possibility that NHUV might be capable of replicating in these and in other cells. Other flaviviruses closely related to NHUV, such as BJV virus, similarly lack a recognized association with vertebrates. BJV was first discovered in the Barkedji area of Senegal in 2007 (Dupressoir A and others, unpublished data) and then detected in *Cx. perexiguus* mosquitoes from Israel in 2011 [41]. These two BJV strains are most closely related genetically to Nounane virus (NOUV), a flavivirus isolated from *Uranotaenia* mosquitoes in Cote d'Ivoire in 2004 [18]. NOUV forms a clade with Nanay virus (NANV), a recently discovered flavivirus isolated from *Culex* (*Melanoconion*) *ocossa* mosquitoes in Peru [40]. Both NOUV and NANV failed to replicate in a variety of mammalian cells or in intracerebrally inoculated newborn mice. However, NANV and NOUV are clearly distinct genetically from the insect-only flaviviruses. It remains unknown whether these viruses, as well as BJV and NHUV, are maintained by a vertebrate reservoir or by vertical transmission in mosquitoes. The marginal genetic clustering of NHUV and BJV with other flaviviruses suggests that they may be genetically intermediate between the *Culex*-borne flaviviruses amplified by birds and the insect-only flaviviruses. Based on the positioning in the phylogenetic tree, it appears likely that NHUV and its close genetic relatives may have recently lost the ability to replicate in certain vertebrate cell lines.

NHUV was detected in non-engorged female mosquitoes identified as *Cx. chidesteri* in the Pantanal, but the vector competence of this mosquito species for NHUV remains unknown. In fact, very little is known about *Cx. chidesteri* biology in the region. In 2007, in a preliminary investigation of mosquitoes in the same sub-region of the present study, only two specimens of this species were identified among 3684 specimens of mosquitoes captured. Because both specimens were males, the specific identification could be confirmed by morphologic appearance of the genitalia [15]. In the present study, only females were captured, and identification was based on morphologic appearance according to taxonomic keys [42, 43]. More studies are needed, including wider entomological investigations in the Pantanal, to confirm *Cx. chidesteri* as potential NHUV vector in the region. *Cx. chidesteri* is a catholic species that feeds on a variety of vertebrates, particularly birds [44–46]. This species has been also found in urban areas and is known by its endophilic behavior in Brazil. However, it seems they are usually more attracted by the artificial lights of housing rather than the humans themselves [44, 46]. In our 2010 mosquito collections, many more *Cx.*

chidesteri were attracted to the lights of CDC traps than were collected landing on team members [13].

Conclusion

We describe the discovery of a novel flavivirus, herein named NHUV, from a pool of mosquitoes identified as *Culex* (*Culex*) *chidesteri*, collected in April 2010 in the Pantanal region of west-central Brazil, a region known for its tremendous biodiversity. The virus was isolated in a mosquito cell line without causing cytopathic effects and did not form plaques in a simian cell line. Its RNA genome was sequenced and found to be most closely related to those of other mosquito-borne viruses with no known vertebrate associations. However, these viruses are genetically distinct from the insect-only flaviviruses. NHUV is the sixth flavivirus to be reported from the Pantanal. The variety of flaviviruses in the region highlights the need for highly specific serological methods to be applied in order to avoid the detection of cross-reacting neutralizing antibodies, which could lead to a misinterpretation of flavivirus serosurvey results in the region.

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Fig. 1.

Location of Nhumirim virus isolation. A black dot indicates the location of the Nhumirim ranch in the Pantanal wetland region (shaded gray) where Nhumirim virus was isolated from *Culex* (*Culex*) *chidesteri* mosquitoes. MT, State of Mato Grosso; MS, State of Mato Grosso do Sul

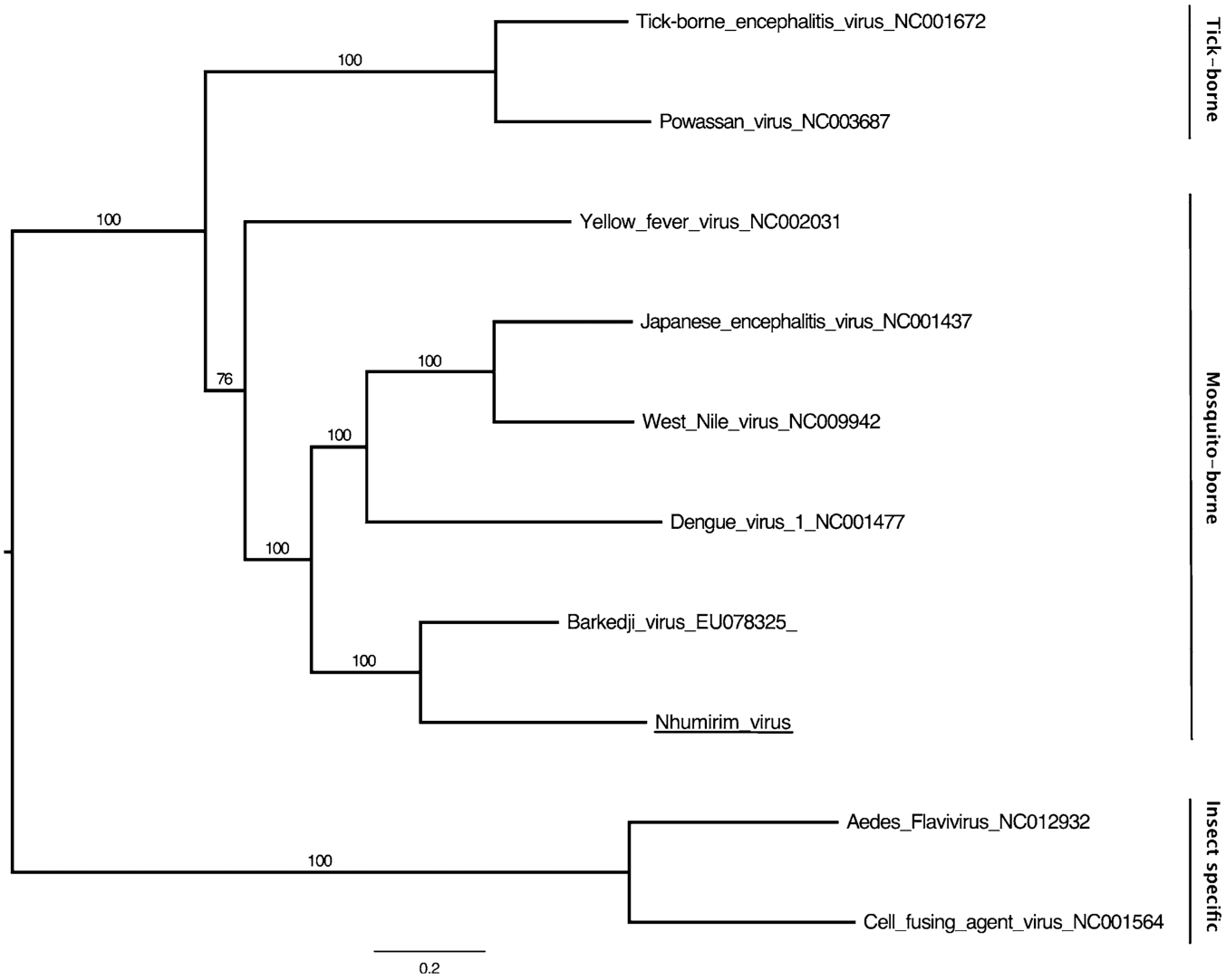

Fig. 2.

Phylogenetic relationships of full coding sequence of Nhumirim virus. Phylogenies were constructed with RAxML 7.06 and MUSCLE on the Cipres Science Gateway. One thousand replicates of bootstrap resampling were utilized. Bootstrap percentage values are indicated on the branches. Labels include virus names and accession numbers. The scale bar indicates branch length, expressed as the expected number of substitutions per site