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Short communication

Genomic characterization of a novel pegivirus species from free-ranging bottlenose dolphins (*Tursiops truncatus*) in the Indian River Lagoon, Florida

Thaís C.S. Rodrigues[a], Kuttichantran Subramaniam[a], Stephen D. McCulloch[b], Juli D. Goldstein[b], Adam M. Schaefer[c], Patricia A. Fair[d], John S. Reif[e], Gregory D. Bossart[f,g], Thomas B. Waltzek[a,*]

[a] University of Florida, 2187 Mowry Road, 32611, Gainesville, FL, USA
[b] Protect Wild Dolphins Alliance, 2046 Treasure Coast Plaza, 32960, Vero Beach, FL, USA
[c] Florida Atlantic University, 5600 US 1, North, 34946, Fort Pierce, FL, USA
[d] Medical University of South Carolina, 179 Ashley Ave, 29425, Charleston, SC, USA
[e] Colorado State University, 102 Administration Bldg, 80523, Fort Collins, CO, USA
[f] Georgia Aquarium, 225 Baker Street, 30313, Atlanta, GA, USA
[g] University of Miami, PO Box 016960 (R-46), 33101, Miami, FL, USA

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ABSTRACT

We report the discovery of the first cetacean pegivirus (family *Flaviviridae*) using a next-generation sequencing approach. One of two infected bottlenose dolphins had elevated activities of liver enzymes, which may suggest hepatocellular injury. Further research is needed to determine the epidemiology and pathogenicity of dolphin pegivirus.

Members of the family *Flaviviridae* include significant human and veterinary pathogens such as dengue, yellow fever, West Nile, Zika, Japanese encephalitis, hepatitis C, and swine fever viruses (Simmonds et al., 2017). Within the family, the genus *Pegivirus* was recently created to include related viruses that infect humans, non-human primates, bats, horses, rodents, and pigs (Smith et al., 2016). Herein, we provide the first report of a pegivirus sequenced from a cetacean using a next-generation sequencing approach.

Twenty adult bottlenose dolphins (*Tursiops truncatus*), 7 females and 13 males, were captured in the Indian River Lagoon (IRL), Florida, in 2015 for health assessment under the U.S. NMFS permit no. 14352 (Bossart et al., 2017). A clinical examination was performed on each animal including phlebotomy for serum biochemistry analysis and virus discovery (Fair et al., 2016). 500 µL of each serum sample were randomly assigned to one of four pools. Virus particles within each pool were pelleted by ultracentrifugation at 100,000 × g at 4 °C for 1 h in a Beckman Type 50.2 Ti rotor. The resulting pellets were resuspended in 3 ml resuspension buffer (RSB; 10 mM Tris − HCl, pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$). To remove adventitiously associated cellular DNA, concentrated virions (3 ml) were treated with DNAse (200 µg mL–1, Sigma) in the presence of 10 mM $MgCl_2$ for 1 h at 37 °C. The reaction was stopped by adding EDTA to a final concentration of 50 mM. RNA was extracted from each of the four suspensions using a QIAamp Viral RNA Mini Kit with carrier RNA added according to manufacturer's instructions (Qiagen, Valencia, USA). Separate cDNA libraries were generated for each of the four RNA extracts using a NEBNext Ultra RNA Library Prep Kit (Illumina, San Diego, USA) and sequenced on an Illumina MiSeq sequencer. *De novo* assembly of paired-end reads was performed in SPAdes 3.5.0 (Bankevich et al., 2012). BLASTX searches of the resulting contigs against a custom virus database, created from virus protein sequences retrieved from the UniProt Knowledgebase (https://www.uniprot.org/uniprot/) using CLC Genomics Workbench V7 revealed the near complete genome (9649 bp) of a novel virus with greatest maximum scores to porcine pegivirus (PPgV; GenBank accession no. AVI26314) and Theiler's disease associated virus (TDAV; GenBank accession no. AGH70217).

A single open reading frame encoding a putative multi-functional polyprotein was identified using CLC Genomics Workbench V7. Two structural and seven non-structural proteins, as previously described for pegiviruses (Simmonds et al., 2017), were predicted by Hmmer searches against the PFam database (HmmerWeb version 2.22.0) and by comparison to the polyproteins of TDAV and PPgV (Fig. 1; Table 1). The amino acid (aa) sequences of the NS3 protein of 28 pegiviruses were retrieved from the National Center for Biotechnology Information

* Corresponding author at: Department of Infectious Diseases and Immunology, College of Veterinary Medicine, University of Florida, 2187 Mowry Road, 32611, Gainesville, FL, USA.
E-mail address: tbwaltzek@ufl.edu (T.B. Waltzek).

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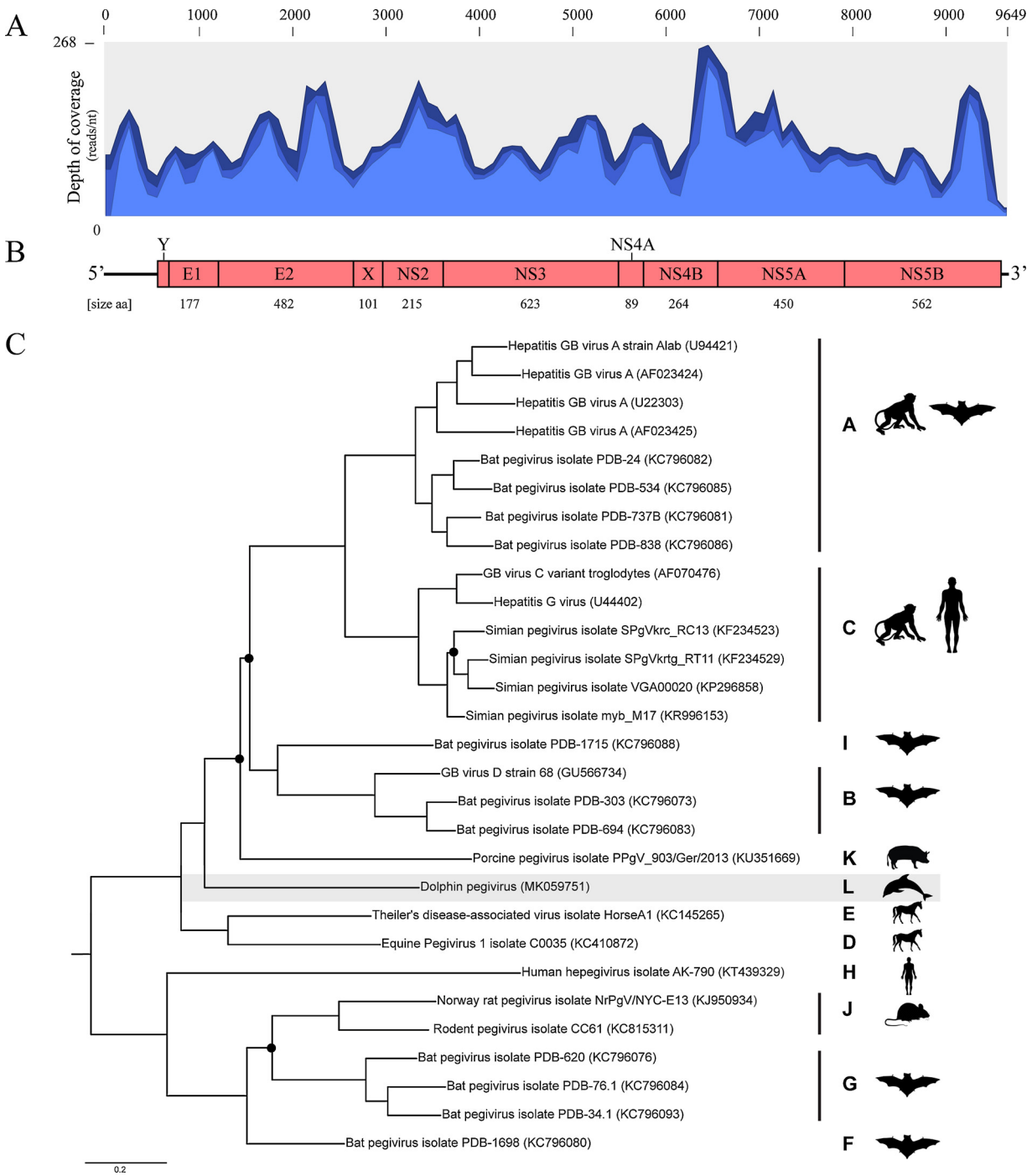

Fig. 1. Genomic organization and genetic analysis of the dolphin pegivirus (DPgV). (A) Coverage map of next-generation sequencing data to the assembled DPgV genome. Different shades of blue from top to bottom shows the maximum, average, and minimum coverage values as calculated using a window size of 10 bp. (B) Genomic arrangement of DPgV depicting the predicted individual viral proteins within the polyprotein and their aa sizes. Y, protein of unknown function; E, envelope glycoprotein; X, additional glycoprotein; NS, nonstructural protein. (C) Phylogram depicting the relationship of DPgV, proposed to be formally named Pegivirus L, to the other species within genus *Pegivirus* based on the aa sequence alignment of the NS3 protein. The Maximum Likelihood tree was generated using 1000 bootstraps and branch lengths are based on the number of inferred substitutions, as indicated by the scale. Nodes with bootstrap values < 80% are indicated by black circles. Common names of the viruses are listed, followed by GenBank accession numbers in parenthesis and the letter that represents the formal name of each *Pegivirus* species. The reported hosts for each species are represented by a silhouette in front of the species letter.

(NCBI) GenBank database and aligned using the Multiple Alignment using Fast Fourier Transform (MAFFT) server (https://mafft.cbrc.jp/alignment/software/) with default parameters. Maximum Likelihood phylogenetic analysis performed using the IQ-TREE server (http://iqtree.cibiv.univie.ac.at/) with 1000 non-parametric bootstraps supported the virus as a unique branch between porcine and equine pegiviruses (Fig. 1C).

Genetic analysis was conducted using the Sequence Demarcation Tool v.1.2 (Muhire et al., 2014) with the MAFFT alignment option implemented. The NS3 aa sequence identity of this novel dolphin virus to other pegiviruses ranged from 40.6 to 54.6% (highest similarity to TDAV), which is lower than the 69% threshold proposed for species demarcation (Smith et al., 2016) (Fig. 2). Names attributed to pegivirus species follow alphabetical order according to their chronological

Table 1
Description and position of predicted individual viral proteins in the dolphin pegivirus genome. E, envelope glycoprotein; NS, nonstructural protein.

Protein	Description	Nucleotide position
Y	Unknown function	575..688
E1	Envelope glycoprotein	689..1219
E2	Envelope glycoprotein	1220..2665
X	Additional glycoprotein	2666..2968
NS2	Membrane-associated protease	2969..3613
NS3	Helicase and protease activities	3614..5482
NS4A	Cofactor for NS3	5483..5749
NS4B	Replication complex formation	5750..6541
NS5A	Phosphorylation-dependent modulation of RNA replication	6542..7891
NS5B	RNA-dependent RNA polymerase	7892..9577

discovery (Simmonds et al., 2017). Thus, we propose this novel species, dolphin pegivirus (DPgV; GenBank accession no. MK059751), to be formally named Pegivirus L, pending acceptance by the International Committee on Taxonomy of Viruses.

A nested reverse transcription PCR assay targeting the NS3 protein (Table 2) was designed to screen all 20 samples individually for DPgV RNA and determine its prevalence in the study population. DPgV RNA was detected in the serum samples collected from a female (93D) and a male (92 L) dolphin (2/20; 10%). Sanger sequencing of the purified PCR products resulted in sequences identical to the DPgV sequence generated by NGS. Although no clinical signs were reported for the DPgV positive dolphins (n = 2), animal 93D had moderately elevated serum activities of aspartate aminotransferase (748U/L) and alanine aminotransferase (181U/L), and a mildly increased gamma-glutamyltransferase (36U/L), as compared to DPgV-negative dolphins (n = 18) from this study and reference values for healthy bottlenose dolphins in the IRL (Goldstein et al., 2006), which may suggest hepatocellular injury (Table 3).

Pegiviruses have been reported to be associated with cases of hepatitis (Simons et al., 1995), non-Hodgkin's lymphoma (Chang et al., 2014), and encephalitis (Bukowska-Ośko et al., 2018) in humans; however, the role of these viruses in disease has not been established. In addition, TDAV was first identified in the serum of horses with acute liver insufficiency (Chandriani et al., 2013), but recently an equine parvovirus (equine parvovirus-hepatitis) has been reported as the possible causative agent of Theiler's disease (Divers et al., 2018; Lu et al., 2018). The pathogenicity of DPgV is unknown, but the possibility of liver involvement in an infected dolphin highlights the importance of further investigation into the potential role of DPgV in disease in

Table 2
Primers targeting the dolphin pegivirus NS3 protein used in the nested reverse transcription PCR assay to screen Indian River Lagoon bottlenose dolphin serum samples.

Name	Sequence, 5'→ 3'
DPgV Forward outer	TTTCTGGCTCAACCGGCTA
DPgV Reverse outer	TGGTTGGGTTCAAGGTCA
DPgV Forward inner	GTGTGGGTCGGGTGTTGA
DPgV Reverse inner	GTCACCAGAGTATCCCGTTG

Table 3
Serum biochemistry values for the bottlenose dolphins (*Tursiops truncatus*) 93D and 92 L, positive for dolphin pegivirus (DPgV), compared to the values of the DPgV negative dolphins in this study and to the reference ranges for healthy bottlenose dolphins in the Indian River Lagoon. AST, aspartate aminotransferase; ALT, alanine aminotransferase; AP, alkaline phosphatase; GGT, gamma-glutamyltransferase; TBilirubin, total bilirubin; TProtein, total protein; A/G, albumin to globulin ratio; BUN, blood urea nitrogen; Creat, creatinine; CPK, creatinine protein kinase; BUN/Creat, blood urea nitrogen to creatinine ratio.

Variable	Unit of measure	93D	92 L	DPgV negative dolphins (Range)	Reference[a] (Range)
AST	U/L	748	165	162.72–246.83	163.93–347.85
ALT	U/L	181	14	24.95–47.71	21.03–67.81
AP	U/L	345	161	98.27–695.06	122.81–421.97
GGT	U/L	36	26	23.82–27.73	23.55–31.35
TBilirubin	mg/dL	0	0	0–0.11	0.06–0.12
Cholesterol	mg/dL	156	139	110.19–168.98	114.75–165.71
Triglycerides	mg/dL	56	64	54.66–95.45	50.61–115.45
Iron	µg/dL	149	67	59.24–154.43	63.78–133.92
TProtein	g/dL	7.5	7.1	6.7–8.09	6.87–8.03
Albumin	g/dL	5	4.3	4.29–4.88	4.16–4.82
Globulin	g/dL	2.5	2.8	1.99–3.63	2.28–3.64
A/G	g/dL	2	1.5	1.16–2.43	1.25–1.93
Glucose	mg/dL	94	89	79.13–108.75	81.73–106.01
BUN	mg/dL	75	74	46.3–71.58	56.81–75.77
Creat	mg/dL	1	1.1	0.82–1.61	0.82–1.36
CPK	U/L	157	27	53.05–270.61	111–193
BUN/Creat	mg/dL	75	67.27	32.05–73.97	45.79–84.21
Amylase	U/L	3	3	1.18–2.39	0.69–1.55

[a] Goldstein et al. (2006).

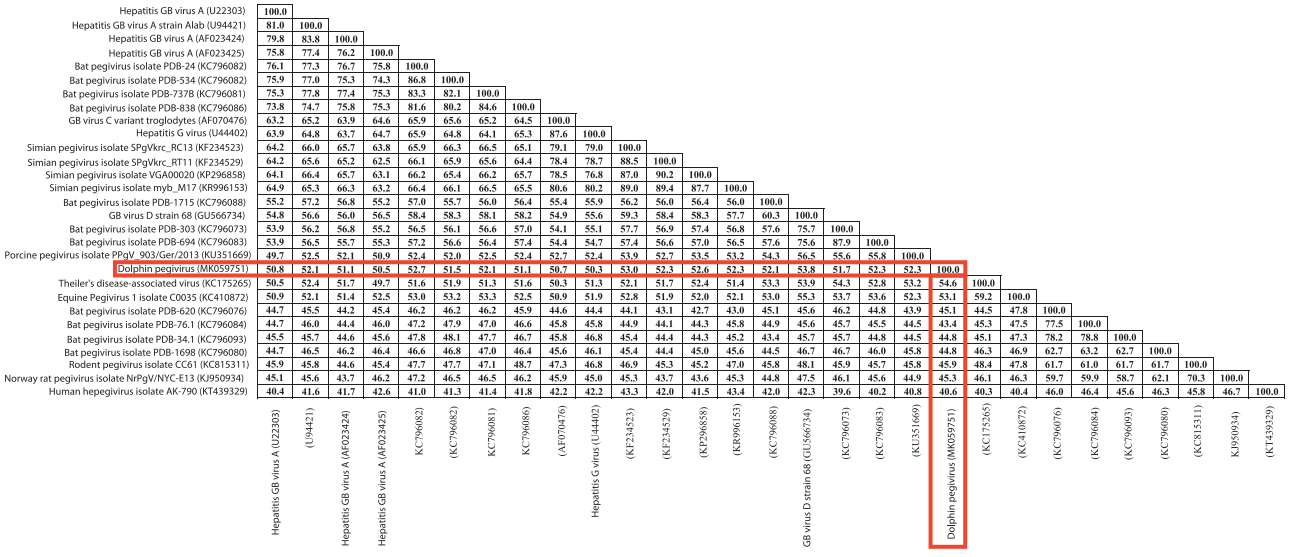

Fig. 2. Sequence identity matrix based on the NS3 amino acid alignment of the dolphin pegivirus (red box) compared to members of the 11 recognized pegivirus species.

bottlenose dolphins.

DPgV is the first virus within family *Flaviviridae* sequenced from a cetacean species, although the presence of anti-West Nile virus antibodies was reported in IRL bottlenose dolphins sampled in 2003 (Schaefer et al., 2009). The IRL is an ecologically and economically important ecosystem in Florida and our results underscore the importance of dolphins as sentinel species for the health of this marine ecosystem (East Central Florida Regional Planning Council, 2016). Further research is needed to understand possible impacts of DPgV infection on the health of IRL bottlenose dolphins.

Although little is known about the potential of pegiviruses to be transmitted between different hosts, at least one species is able to infect hosts from different mammalian orders, *Pegivirus A* infects monkeys and bats (Simmonds et al., 2017). Pegiviruses are blood-borne viruses and believed to be sexually transmitted (Berg et al., 2015; Simmonds et al., 2017). The route(s) of transmission and host range of DPgV remain to be determined. Further research is needed to better evaluate the transmission, prevalence, and pathogenicity of DPgV in cetaceans including IRL bottlenose dolphins.

Declarations of interest

None.

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