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Identification and whole genome characterization of novel anelloviruses in masked palm civets (*Paguma larvata*): Segregation into four distinct clades☆

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ABSTRACT

The members of the family *Anelloviridae* are small and single-stranded DNA viruses with marked diversity in sequence and length, which ubiquitously infect many vertebrates, including mammals, birds and reptiles. The anelloviruses isolated from mammals are currently classified into 11 assigned and four proposed genera; some anelloviruses remain unassigned. The present study was conducted to identify anelloviruses in wild-caught masked palm civets (*Paguma larvata*) in Japan using a rolling-circle amplification method. Thirteen novel anellovirus strains were identified from 8 of 10 masked palm civets and their entire genomic sequences (2039–2535 nucleotides) were determined; they were classifiable into four distinct clades. Comparative analyses of all reported anelloviruses for which the entire or near-entire genomic sequences have been determined, including the 13 strains obtained in the present study, revealed that anelloviruses can provisionally be classified into 20 clades, which may correspond to 20 genera (including 11 assigned and four proposed genera) by a > 70% amino acid sequence difference in open reading frame 1 (ORF1). This study suggested that novel anelloviruses of marked diversity are circulating in animals worldwide, and that the rolling-circle amplification method would be useful for identifying novel anelloviruses and other viruses with a circular DNA genome.

1. Introduction

In 1997, *Torque teno virus* (TTV) was discovered in a Japanese patient with post-transfusion hepatitis of unknown etiology (Nishizawa et al., 1997). TTV is currently classified within the *Anelloviridae* family (Biagini et al., 2004), which is a non-enveloped, circular and single-stranded DNA virus (Miyata et al., 1999; Mushahwar et al., 1999; Okamoto et al., 1999). Since the initial discovery, many anellovirus species—which are highly prevalent—have been isolated in humans and non-human primates, including chimpanzees (Okamoto et al., 2000a), which are ubiquitously infected (Leary et al., 1999; Maggi et al., 1999; Itoh et al., 1999). In 2000 and 2007, *Torque teno mini virus* (TTMV) and *Torque teno midi virus* (TTMDV), which belong to two new distinct genera with a smaller genome size and a lower identity, respectively, were found in humans and non-human primates (Takahashi et al., 2000a; Ninomiya et al., 2007).

Anelloviruses have also been detected in wild and domestic animals, including lower primates (cotton-top tamarins, douroucouli, Japanese macaques and lemurs), tupaias, wild boar, pine martens, badgers, sea lions, bats, seals, rodents, giant pandas, opossums, pigeons, sea turtles, livestock (pigs, sheep, cows, camels, horses, and chickens) and companion animals (dogs and cats). The entire or near-full genome sequences of these anelloviruses have been determined (Okamoto, 2009; Manzin et al., 2015; de Souza et al., 2018). Their genomes were obtained by an inverse PCR with primers that are conserved among anellovirus species (Okamoto et al., 2001; Hino, 2002; Okamoto et al., 2002; Zhang et al., 2017b), by the rolling-circle amplification method (Biagini et al., 2007; Macera et al., 2011; Cornelissen-Keijsers et al., 2012; Nishiyama et al., 2014) or by next-generation sequencing (Ng et al., 2009a,b; Ng et al., 2011b; Van den Brand et al., 2012; Li et al., 2015; Fahsbender et al., 2017; Zhang et al., 2017a,b; Amatya et al., 2017; de Souza et al., 2018).

☆ The nucleotide sequence data reported herein have been assigned DDBJ/EMBL/GenBank accession numbers LC387536-LC387548.

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It has been shown that anelloviruses have high diversity in their nucleotide sequence (56% cutoff value for anellovirus genera in the nucleotide divergence of the entire ORF1) (ICTV 9th Report, 2011) and distinct size (2.1–3.9 kb), while the genomic organization and motif sequences are conserved (Okamoto et al., 2001, 2002; Ng et al., 2009b; Okamoto et al., 2009). Two-thirds to three-quarters of the genome encodes four main ORF-proteins (ORF1–4). The largest ORF (ORF1) encodes the capsid protein (Okamoto et al., 2002); ORF2 with a conserved motif "WX7HX3CX1CX5H" (Hijikata et al., 1999; Tanaka et al., 2001; Peng et al., 2002) is believed to be responsible for cellular and/or viral protein regulation and processing during natural infection (Zheng et al., 2007); and ORF3 with a serine-rich tract at the C-terminus (Tanaka et al., 2001), is phosphorylated, similarly to the non-structural protein 5 A of hepatitis C virus, which suppresses the antiviral action of interferon (Asabe et al., 2001). The function of ORF4 is unknown, and absent in the anelloviruses of pigs, tupaias, dogs and cats (Okamoto, 2009). One quarter to one third of the genome is the untranslated region (UTR) with a GC-rich tract (Kamada et al., 2004), in which the region around TATA-box, Sp1- and Cap-sequence are conserved (Okamoto et al., 2002).

The masked palm civet or gem-faced civet (*Paguma larvata*) is a civet species that is distributed in the Indian Subcontinent and Southeast Asia (Duckworth et al., 2016). It is also found in Taiwan and Japan (Inoue et al., 2012). There is currently no available information on anelloviruses in masked palm civets. Thus, in the present study, we applied the rolling-circle amplification method to investigate the presence of anelloviruses and to determine and characterize their entire genomic sequences in wild-caught masked palm civets in Japan.

2. Materials and methods

2.1. Samples

Serum samples were collected from 10 masked palm civets that were captured in Nikko, Tochigi Prefecture, which is located approximately 150 km north of Tokyo, Japan, from May to July in 2015 with permission from the Industrial Environment Division of Nikko City (Nikko-Shi-Shirei-Fuji-San no. 4–18). Eight of the masked palm civets (Pag-1–Pag-8) were adults and apparently healthy, while the remaining two (Pag-9 and Pag-10) were cubs and had skin scabies (Table 1). Serum samples were kept at −80 °C until testing.

2.2. Extraction of nucleic acids and amplification by RCA

Nucleic acids were extracted from 100 μl of serum using SMITEST® EX R&D (Medical & Biological Laboratories, Nagoya, Japan) and were dissolved in 10 μl of Ultrapure distilled water (Thermo Fisher Scientific Inc., Waltham, MA), which was subjected to RCA according to a previously described method (Dean et al., 2001; Niel et al., 2005; Rosario et al., 2012) with slight modifications. Briefly, 5 μl of nucleic acid extract was denatured at 95 °C for 3 min, cooled on ice and added to 20 μl of reaction buffer (50 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM $[NH_4]_2SO_4$, and 4 mM dithiothreitol) containing 5 U of phi29 DNA polymerase (New England BioLabs, Ipswich, MA), 25 μM random primers (Thermo Fisher Scientific Inc.), dNTPs (1 mM each; TaKaRa Bio, Shiga, Japan) and 100 μg/ml of bovine serum albumin (BSA) (New England BioLabs). The amplification reaction was performed at 30 °C for 18 h, followed by treatment at 65 °C for 10 min to inactivate the phi29 DNA polymerase. One microliter of amplification product was digested with 5 U of *Eco*RI or *Pst*I restriction endonuclease (New England BioLabs) at 37 °C for 60 min. The restriction digests were subjected to electrophoresis on 1% Seakem GTG agarose gel (Lonza, Rockland, ME) and visualized by UV light exposure after ethidium bromide staining.

Table 1
Serum samples collected from wild-caught masked palm civets (*Paguma larvata*) in Japan.

No.	Date captured	Sex	Body weight (kg)	Isolated Pl-TTV strain(s)
Pag-1	2015.05.01	M	3.6	Pl-TTV1-1, Pl-TTV1-2
Pag-2	2015.05.06	M	2.8	Pl-TTV2
Pag-3	2015.05.07	M	2.7	Pl-TTV3
Pag-4	2015.05.17	M	3.2	
Pag-5	2015.05.17	M	4.0	Pl-TTV5-1, Pl-TTV5-2
Pag-6	2015.05.21	M	3.2	Pl-TTV6
Pag-7	2015.05.21	M	3.2	Pl-TTV7
Pag-8	2015.07.01	F	2.5	
Pag-9	2015.07.08	F	1.0	Pl-TTV9-1, Pl-TTV9-2
Pag-10	2015.07.11	M	1.0	Pl-TTV10-1, Pl-TTV10-2, Pl-TTV10-3

2.3. Cloning of amplified products

Restriction endonuclease, *Eco*RI or *Pst*I-digested fragments with a molecular size of 0.8–3 kilobase pairs (kbp) were purified using a FastGene gel extraction kit (Nippon Genetics, Tokyo, Japan). The purified DNAs were inserted into a plasmid vector pBlueScript II SK (Merck Millipore, Temecula, CA) that had been linearized with *Eco*RI or *Pst*I and treated with bacterial alkaline phosphatase (BAP; TaKaRa Bio). Chemically competent *Escherichia coli* DH5α (Quick competent DH5α; TOYOBO, Osaka, Japan) was transformed with ligated DNA and incubated at 37 °C overnight for blue/white colony screening on LB/agar plates containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (TaKaRa Bio) and ampicillin. Recombinant plasmid DNAs were purified from 3 ml of culture medium of each transformant using a FastGene plasmid mini kit (Nippon Genetics).

2.4. PCR amplification

Conventional or inversed PCRs were performed using *TaKaRa Ex Taq* or *LA Taq* polymerase in GC buffer I (TaKaRa Bio) with specific primers to amplify anellovirus DNA in serum samples and to confirm the nucleotide sequences surrounding the restriction cleavage site (*Eco*RI or *Pst*I) used for cloning into a plasmid DNA.

2.5. DNA sequencing and sequence analysis

Nucleotide sequences of cloned DNAs were determined using a BigDye Terminator v3.1 Cycle Sequencing kit on an ABI PRISM 3130*xl* Genetic Analyzer according to the manufacturer's protocol (Thermo Fisher Scientific Inc.). The sequencing primers used for colony screening were universal and reverse M13 primers (PhM13 Forward primer: CGACGTTGTAAAACGACGGCCAGT; PhM13 Reverse Primer: CAGGAAACAGCTATGAC) and the primers used for genome walking were specifically designed. Upon sequencing of the GC-rich region, GC-Rich Buffer A or B from an AccuPrime GC-Rich DNA Polymerase kit (Thermo Fisher Scientific Inc.) was added to the sequencing reaction mixture. A sequence analysis was performed using the GENETYX software program (version 10.1.5; Genetyx Corp., Tokyo, Japan). Multiple sequence alignments were created using the MUSCLE software program (version 3.8) (Edgar, 2004), and the alignment was confirmed by visual inspection. Phylogenetic trees were constructed by the maximum-likelihood (ML) method based on the entire genomic sequence, entire nucleotide and amino acid sequence of ORF1, which was encoded on the anellovirus genome, and nucleotide sequence of UTR with 1000 bootstrapping replicates using MEGA version 7.0.20 (Tamura et al., 2013). One hundred sequences of anellovirus strains (used as reference strains), which were retrievable from the DDBJ/EMBL/GenBank databases as of May 2018, and chicken anemia virus (CAV; accession no. M55918), which was used as an outgroup, were included in the trees.

The prediction of splicing acceptor and donor sites in the *orf3* gene

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