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Characterization of dog serum virome from Northeastern Brazil

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ABSTRACT

Domestic dogs share habitats with human, a fact that makes them a potential source of zoonotic viruses. Moreover, knowledge regarding possible bloodborne pathogens is important due to the increasing application of blood transfusion in dogs. In the present study, we evaluated the serum virome of 520 dogs using throughput sequencing (HTS). The serum samples were pooled and sequenced using an Illumina MiSeq platform. Our unbiased method identified prevalent canine pathogens as canine protoparvovirus 1 (canine parvovirus 2), undersearched agents as canine bocaparvovirus 1 (minute virus of canines) and canine circovirus, circular viruses closely related to viruses recently found in human samples, and new parvovirus and anelloviruses. The dog virome described in the present work furthers the knowledge concerning the viral population in domestic animals. The present data includes information regarding viral agents that are potentially transmitted through blood transfusion among dogs.

1. Introduction

Domestic dogs (*Canis lupus familiaris*) are the most popular pet worldwide and share extensive contact with humans. Dogs share their habitat with humans, other domestic animals, as well as wild animals, which renders them a potential risk factor for the transmission of zoonotic viruses, such as rabies virus (Lackay et al., 2008) and rotavirus (Tsugawa and Hoshino, 2008; Wu et al., 2012). Moreover, blood transfusions in veterinary medicine have become increasingly common and form an integral part of lifesaving and advanced treatment of the critically ill (Kisielewicz and Self, 2014; Langston et al., 2017). Although the screening for non-viral pathogens such as *Babesia* spp., *Leishmania* spp., *Ehrlichia* spp., *Anaplasma* spp., and *Brucella canis* is recommended (Reine, 2004; Wardrop et al., 2005),the knowledge and monitoring of viral agents through effective tools represents an important sanitary step.

Through virome analysis, it is possible to detect viruses whose propagation is difficult or impossible in cell culture and are not detectable by molecular detection tests since they contain no common gene, such as the ribosomal 16 S gene present in bacterial species (Delwart, 2007; Moreno et al., 2017). The enhanced availability and application of high throughput sequencing (HTS) technologies has facilitated the detection of known and unknown viruses (Goodwin et al., 2016; Kohl et al., 2015; Virgin, 2014). Moreover, metagenomic viral detection is “unbiased” as it uses non-specific primers to detect nucleic acid sequences (Toohey-Kurth et al., 2016; Virgin, 2014).

Previous studies concerning the dog virome addressed the viral components of the gastrointestinal flora (Li et al., 2011; Moreno et al., 2017) and characterized novel viruses as canine sapovirus and canine kobuvirus (Li et al., 2011). Despite the fact that serum virome was assessed with HTS in humans (Moustafa et al., 2017), cattle (Sadeghi et al., 2017; Toohey-Kurth et al., 2016; Wang et al., 2018), and horses (Li et al., 2015), no research has been conducted to investigate the dog serum virome. Thereby, the present study aimed to evaluate and characterize the serum virome using HTS of healthy dogs inhabiting Northeastern Brazil.

2. Materials and methods

2.1. Study design and sources of sera

The blood samples of 520 dogs were obtained between March 2015

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and March 2016 from five urban centers located in Paraíba state, Northeastern Brasil: João Pessoa (167 samples), Campina Grande (158 samples), Patos (90 samples), Sousa (37 samples), and Cajazeiras (68 samples). The blood samples were centrifuged at 2000 g for 10 min, and the serum was collected and stored at −80 °C prior to the analysis. The project was registered in the Ethics Committee on the Use of Animals (CEUA) of Universidade Federal de Campina Grande (UFCG) under protocol number #0041/280314.

2.2. Viral metagenomics and HTS

The 520 dog sera were assembled in one pool containing 100 µL of each serum sample. The total 52 mL were passed through a 0.22 µm filter and subsequently ultracentrifuged on a 25% sucrose cushion at ~100,000 × g for 3 h at 4 °C in a Sorvall AH629 rotor. The pellet containing the viral particles was incubated for 1.5 h with DNase and RNase enzymes (Thermo Fisher Scientific, Waltham, MA, USA) (Thurber et al., 2009). Subsequently, the viral RNA and viral DNA were isolated using TRIzol™ LS reagent (Thermo Fisher Scientific) and a standard phenol–chloroform protocol (Sambrook and Russel, 2001), respectively. The viral DNA was enriched through multiple displacement amplification (MDA), performed with Φ29 DNA polymerase (New England Biolabs, Ipswich, MA, USA) (Niel et al., 2005). Furthermore, the viral RNA was reverse-transcribed and enriched to dsDNA using TransPlex® Complete Whole Transcriptome Amplification (WTA) Kit (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's recommendations. The DNA products produced from these enrichment protocols (MDA products from viral DNA and WTA products from viral RNA) were pooled in equimolar amounts and purified using the PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Thermo Fisher Scientific). The quality and quantity of the DNA were assessed through spectrophotometry and fluorometry performed with NanoDrop™ (Thermo Fisher Scientific) and Qubit™ (Thermo Fisher Scientific) respectively. The viral libraries were further prepared with 50 ng of purified DNA, using the Nextera DNA Library Preparation Kit and sequenced using an Illumina MiSeq System using an Illumina v2 reagent kit (2 × 150 paired-end reads).

2.3. Bioinformatic analysis

The quality of the sequences generated was evaluated using FastQC. Furthermore, the sequences with bases possessing a Phred quality score < 20 were trimmed with the aid of Geneious software (version 9.0.5). Subsequently, the paired-end sequence reads were *de novo* assembled into contigs with the CLC Genome Workbench version 6.0.1 (http://www.clcbio.com/products/clc-genomics-workbench). All assemblies were confirmed through mapping reads to contigs produced by the CLC Genome Workbench using Geneious software. Thereafter, the assembled contigs were examined for similarities with known sequences through the BLASTX software using Blast2GO (Gotz et al., 2008). Sequences with *E*-values ≤ 10^{-3} were classified as likely to have originated from eukaryotic viruses, bacteria, phages, or unknown sources, a conclusion reached based on the taxonomic origin of the sequence with the best *E*-value. Gene and protein comparisons were performed with BLASTN and BLASTP programs (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Sequences representative of viruses belonging to the families *Parvoviridae*, *Anelloviridae* and *Genomoviridae* were obtained from GenBank and aligned with the sequences identified in the present study with MUSCLE software (Edgar, 2004). Phylogenetic trees were constructed using MEGA6 (Tamura et al., 2013).

2.4. Real-time PCR

The 520 dog sera were screened individually for viral genomes previously obtained through HTS. Their DNA was isolated from sera using a standard phenol–chloroform protocol (Sambrook and Russel, 2001). Furthermore, primers targeting *Human associated gemykibivirus 2* (GmKV-2) and *Carnivore bocaparvovirus 1* (CBPV-1) were designed using the Primer 3 software available in the Geneious version 9.0.5 using the obtained contigs and representative strains available in GenBank. Primers previously described were utilized for *Carnivore protoparvovirus 1* (CPPV-1) (Kumar and Nandi, 2010) and *Canine circovirus* (CaCV) detection (Li et al., 2013). The sesavirus-related sequence obtained in the HTS was not assayed as it is impossible to design specific primers with small contigs (109 bp) that present a low identity with the sesavirus reference genome (GenBank accession number NC_026251.1). Moreover, the *Thetatorquevirus*-like sequences were also not assayed due to the number of different specimens detected by HTS. Table 1 summarizes the sequence, target, and product size of the primers utilized in the present study.

Table 1
Summary of sequences that matched with the animal virus present in the pooled dog serum sample and its frequency in the 520 serum samples.

Virus hits (BLASTX, E value < 1×10^{-3})	No. of hits	No. of reads	Contigs lengths	Frequency in the individual samples
CPPV-1	12	611	60–977	1.34% (07/520)
CBPV-1	6	7	131–643	0.38% (02/520)
Sesavirus	1	2	109	NA
CaCV	1	4	200	1.34% (07/520)
GmKV-2	6	168	54–670	5.38% (28/520)
Thetatorquevirus	31	395	69–475	NA

NA: Not analyzed.

Furthermore, real-time PCRs were designed with PowerUp SYBR™ Green Master Mix (Applied Biosystems, Foster City, CA, USA), with 2 pmol of each primer, and 2 µL of total DNA, q.s.p. 25 µL. The amplification was performed with the following cycling profile for HGmKV-2 and CPPV-1: 50 °C for 2 min and 95 °C for 3 min for initial denaturation and enzyme activation step, followed by 40 30 s cycles for denaturation at 95 °C, 30 s at 55 °C for annealing, and extension at 60 °C for 30 s. For CBPV-1 and CaCV, the following cycle conditions were applied: 50 °C for 2 min and 95 °C for 3 min for initial denaturation and enzyme activation step, followed by 40 cycles of 30 s for denaturation at 95 °C, and 1 min of annealing/extension at 60 °C. A threshold cycle (Ct) value of 40 indicates a viral nucleic acid quantity below detection level. In light of the fact that the real-time PCRs were not calibrated with known concentrations of target nucleic acids, the obtained Ct values only reflect relative viral loads in samples and cannot be utilized to compare viral genome loads across different samples. Moreover, all the real-time PCR products were stained with GelRed Loading Buffer (Quatro G Pesquisa e Desenvolvimento, Porto Alegre, RS, Brazil), electrophoresed in 2% agarose gels, and visualized under UV light for confirmation.

2.5. PCR and Sanger sequencing

All positive samples in real-time PCR were submitted for Sanger sequencing to confirm the specificity of the tests. The CPPV-1 positive samples obtained in real-time PCR were submitted to PCR using primers CPV 555F and CPV 555R that amplify a 583 bp of the VP2 gene (Buonavoglia et al., 2001) for CPPV-1 type definition.

The CBPV-1 whole genome were assembled using the viral reads obtained in HTS and through additional PCR protocols performed with previously described primers (Shan et al., 2010). The GmKV-2 whole genomes were obtained using the viral reads obtained in HTS and with additional PCR protocols executed with primers developed using the sequences obtained in HTS. Table A.1 summarizes the primers employed to obtain the CBPV-1 and GmKV-2 full genomes.

The PCR products were purified using the PureLink™ Quick PCR Purification Kit (Invitrogen, Carlsbad, CA, USA). Both DNA strands