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Detection and genetic characterization of *Mamastrovirus* 5 from Brazilian dogs

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ABSTRACT

Mamastrovirus 5 (MAstV5), belonging to the *Astroviridae* (AstV) family, previously known as canine astrovirus or astrovirus-like particles, has been reported in several countries to be associated with viral enteric disease in dogs since the 1980s. Astroviruses have been detected in fecal samples from a wide variety of mammals and birds that are associated with gastroenteritis and extra enteric manifestations. In the present study, RT-PCR was used to investigate the presence of MAstV5 in 269 dog fecal samples. MAstV5 was detected in 26% (71/269) of the samples. Interestingly, all MAstV5-positive samples derived from dogs displaying clinical signs suggestive of gastroenteritis, other enteric viruses were simultaneously detected (canine parvovirus, canine distemper virus, canine coronavirus, canine adenovirus and canine rotavirus). Based on genomic sequence analysis of MAstV5 a novel classification of the species into four genotypes, MAstV5a-MAstV5d, is proposed. Phylogenetic analyses based on the ORF2 amino acid sequences, samples described herein grouped into the putative genotype 'a' closed related with Chinese samples. Other studies are required to attempt the clinical and antigenic implications of these astrovirus genotypes in dogs.

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Introduction

Viruses belonging to the *Astroviridae* (AstV) family are spherical, non-enveloped, 28–30 nm in size, with a surface that forms a characteristic star-like structure.[1] The RNA genome of AstV ranges from 6.8 to 7.9-kb in size, polyadenylated at the 3′ end, and contains three ORFs designated as ORF1a, ORF1b and ORF2. ORF1a encodes a protease, ORF1b encodes an RNA-dependent RNA-polymerase,[2,3] while ORF2 encodes the viral capsid structural polyprotein that is required for virion assembly.[4]

The viral classification was previously based on the host and consisted of two genera, *Avastrovirus* and *Mamastrovirus*. However, recent characterization of novel astroviruses has taken in consideration that isolates from different animal species can be genetically similar, while genetically diverse viruses can be isolated from the same animal species.[2] Based on this analysis, the International Committee on Taxonomy of Viruses renamed canine astrovirus as *Mamastrovirus* 5 (MAstV5).[5]

Astroviruses have been detected in fecal samples from a wide variety of mammals and birds that are associated with gastroenteritis.[2] In children, AstVs are the second most common cause of gastroenteritis after rotaviruses.[2,6] Human AstVs can also cause significant disease in the elderly[7] and immune-compromised patients.[8,9] In addition to enteric manifestations, AstVs have been associated with fatal hepatitis in ducks,[10] interstitial nephritis in young chickens,[11] stunting and pre hatching mortality in duck and goose embryos,[12] as well as shaking mink syndrome[13]. Recently, an AstV was also hypothesized to be the causative agent of nonsuppurative encephalitis in cattle.[14]

Since the 1980s, astrovirus-like particles have been reported in dogs with and without diarrhea.[15–17] To date, canine astroviruses or astrovirus-like particles infecting dogs have been reported in several countries.[15–25] Despite the detection of MAstV5 in association with gastroenteritis in dogs, which suggests a possible role for MAstV5 as a canine enteric pathogen, the association of MAstV5 with clinical disease remains obscure in such reports. Here, we investigated the presence of MAstV5 using RT-PCR in fecal samples from dogs of different ages with and without diarrhea. The partial genomes of selected MAstV5 RNA-positive samples were also sequenced to perform a phylogenetic analysis comparing them with the MAstV5 sequences described in the literature as the cause of enteric disease.[18,20,23,26] Additionally, MAstV5 was proposed to be classified in four putative genotypes.

Materials and methods

Samples and nucleic acid extraction

A total of 269 dog fecal samples were collected between 2008 and 2014 in veterinary clinics and hospitals by convenience. These samples were obtained from eight Federal States of Brazil (Acre, Mato Grosso do Sul, Paraná, Rio Grande do Sul, Rio de Janeiro, Rondônia, Santa Catarina and São Paulo). The animal's age was recorded and ranked from puppy (equal or less than one-year-old) to adult dog (more than one-year-old); some samples from dogs of unknown age were included. Animals not presenting diarrhea at the time of sampling were considered asymptomatic and those presenting clinical signs of enteric disease diarrhea were classified as symptomatic. Samples were diluted to 20% (w/v) in phosphate buffered saline (PBS, pH 7.4) and stored at −80 °C for further analysis. Subsequently, viral DNA isolation from the supernatant was performed using a commercial kit (NewGene Preamp®, Simbios Biotecnologia, Brazil) based on guanidine isothiocyanate and silica.[27] Viral RNA was isolated using TRIzol® LS Reagent (Life Technologies™, USA) according to the manufacturer's instructions.

Oligonucleotides for MAstV5 detection and sequencing

An initial screening using RT-PCR to detect a larger number of *Mamastrovirus* species was achieved by amplifying 422 bp of the ORF1b fragment using oligonucleotides, as previously described.[28] For the specific detection of MAstV5, 92 nucleotide sequences of this species were retrieved from GenBank database (http://www.ncbi.nlm.nih.gov/nucleotide), and aligned using CLUSTAL W within Molecular Evolutionary Genetics Analysis version 6 (MEGA6).[29] The MAstV5 specific RT-PCR was designed with a primer pair targeting the region of ORF2 that amplified a 250 bp fragment selected using Primer3 software.[30] In addition, the 16S rRNA gene from *Escherichia coli* was amplified using the primer pair FC27 and R530 as an endogenous internal control in each fecal sample evaluated for the specific presence of MAstV5.[31] For partial genome amplification, sets of 12 pairs of sequencing primers were selected to amplify overlapping fragments of ORF1 (ORF1a and ORF1b) and capsid protein (ORF2) segment representing a consensus sequence of approximately 5000 nucleotides. The primer sequences are shown in Table 1.

Nested RT-PCR for MAstV5 detection

The cDNA was synthesized using SuperScript® III Reverse Transcriptase Kit (Life Technologies, USA) using the reverse primers in a total volume of 20 μL, following the manufacturer's instructions. The cDNA amplification was conducted in a final volume of 25 μL containing 1× PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP mix, 0.2 μM of each primer and 1 unit of Platinum® Taq DNA Polymerase (Life Technologies, USA). The first round of RT-PCR screening was carried out with an initial incubation at 94 °C for 3 min, 30 cycles of amplification consisting of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. The second round was performed in a final volume of 25 μL that contained 2 μL of the first reaction product and the thermocycler conditions were the same as those used for the first round.

The MAstV5-specific RT-PCR with specific and internal control primers was performed as a multiplex protocol. Cycling conditions were an initial cycle at 94 °C for 5 min, 25 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and polymerization at 72 °C for 1 min, which was followed by a final extension cycle at 72 °C for 7 min. To confirm the specific amplification of MAstV5, RT-PCR products

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