Short Communications

Molecular characterisation of calicivirus and astrovirus in puppies with enteritis

T. X. Castro, R. C. N. Cubel Garcia, E. M. Costa, R. M. Leal, M. da P. T. Xavier, J. P. G. Leite

CANINE parvovirus (CPV) and canine coronavirus (CCoV) are considered to be the main pathogens responsible for acute gastroenteritis in young dogs Greene and Decaro 2012). Recently, canine caliciviruses (CaCV), genus *Norovirus* and canine astroviruses (CaAstV) have been associated with outbreaks of enteritis in puppies that were either singly infected or coinfecting with CCoV and/or CPV Martella and others 2008, Mesquita and others 2010, Ntafis and others 2010, Zhu and others 2011, Grellet and others 2012, Martella and others 2012).

Previous reports have shown that CPV and/or CCoV are responsible for approximately 48 per cent of the enteritis cases in puppies in the state of Rio de Janeiro, Brazil Castro and others 2012). However, cases involving CaCV or CaAstV infections have not been described yet in South America. The purpose of this study was to perform the molecular characterisation of CaCV in faecal samples from puppies showing clinical signs of gastroenteritis using RT-PCR and sequence analysis. In addition, the unexpected detection of CaAstV in puppies with enteritis was reported.

Faecal samples collected between February 2005 and May 2012 from 232 client-owned puppies with diarrhoea, that were less than one year of age, from private animal hospitals in the city of Rio de Janeiro, Brazil, were analysed. This trial was licensed by the Ethics Committee of Animal Research-PROPP/ UFF-CEPA/ NAL, No. 0223/12.

For genome detection, nucleic acids were extracted from 10 per cent faecal suspensions in 0.01 M Tris/HCl/Ca²⁺ using the PureLink Spin Column-Based Kit (Invitrogen). Reverse transcription was performed using random primers (Invitrogen) and the Superscript III enzyme (Invitrogen) according to the manufacturer's instructions. PCR was performed on cDNA isolated from each sample using the primers p289H/p290H, which target the highly conserved motifs of the RNA-dependent RNA polymerase (RdRp) of calicivirus (ORF-1b) Farkas and others 2004).

All the PCR amplicons were purified using the QIAquick PCR purification Kit (Qiagen, California, USA) and sequenced in both directions using the Big Dye Terminator kit (Applied Biosystems, California, USA). Nucleotide (nt) similarity with sequences deposited in the GenBank database was assessed using the BLAST tool <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. Sequence analysis were performed within the MEGA V.4.0 software programme Tamura and others 2007). The FindModel programme was used to identify the optimal evolutionary model that best fit the sequence dataset Posada and Crandall 2001).

Faecal samples were also screened for the presence of CPV, CCoV and Group A Rotavirus (RV-A) by either PCR or RT-PCR and sequencing according to the protocols previously described Pratelli and others 2000, Buonavoglia and others 2001, Gómez and others 2011).

For 17 of the 232 samples, amplicons with 300 pb were generated, submitted for sequence analysis. In one sample (RJ900/08), CaCV infection was confirmed, and this strain shared higher nt identity with the *Vesivirus* genus (81 per cent) than with the canine (43–51 per cent) and human noroviruses (27–35 per cent) (Fig 1). This sample also tested positive for CCoV-1.

In another sample (RJ905/08), the comparison of the sequences obtained with strains deposited in GenBank, using the BLAST tool, indicated that this fragment displayed 87.5 per cent nt identity with the CaAstV strain Bari/08/ITA (HM045005). The remaining 15 sequences showed non-specific results based on a BLAST search of the GenBank nt database.

To confirm CaAstV detection in the sample RJ905/08 and to investigate the 15 non-specific results, a new cDNA synthesis and PCR was carried out using the One-Step RT-PCR System (Invitrogen) according to the manufacturer's instructions. Two sets of primers targeting the RdRp (ORF1b) Chu and others 2008, Martella and others 2012) and the capsid region (ORF2) Grellet and others 2012) of CaAstV were used.

According to sequence analysis of the capsid and RdRp regions, CaAstV infection was confirmed in 6/16 samples. Two puppies from the same litter were infected with CaAstV alone. One puppy was coinfecting with both CaAstV and CCoV-I. Two puppies were coinfecting with CaAstV, CCoV-I and CPV-2a. Lastly, one puppy was coinfecting with CaAstV, CCoV-I, CCoV-II and CPV-2a.

Four CaAstV capsid sequences presented enough quality for phylogenetic analysis. These strains displayed 77.1–95.8 per cent nt identity to other CaAstV strains previously deposited from China, France and Italy. The phylogenetic tree based on the CaAstV capsid region is shown in Fig 2.

This is the first report of CaCV and CaAstV infection in dogs from South America. The RdRp region, which is the most conserved region of the genome, has been widely used for the molecular detection of these viruses Vinjé and others 2003, Ludert and others 2004). The nt sequences in this region display a genetic similarity even in distinct and genetically different RNA virus families De Benedictis and others 2011), and this fact could explain the cross-reactivity of the primer set used for CaCV detection. This cross-reactivity was previously reported by Ludert and others (2004), in whose study an RV-A RdRp gene segment was detected after RT-PCR was performed using the same primer pair for norovirus investigation in children with enteritis. This study was the first to report the cross-reactivity of the primer set p289H–p290H with an astrovirus, and further extensive molecular and epidemiological studies are needed to confirm the validity of this finding for other RNA virus families.

Recent molecular investigations indicate that CaAstVs are indeed widespread in the dog population either alone or in coinfections with other viral canine pathogens Martella and others 2011). In this study, the majority of CaAstV-infected pups were also infected with CPV and/or CCoV, but single CaAstV infection was reported in two puppies (RJ1109/12 and RJ1110/12) from the same litter. This

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FIG 1: Hasegawa-Kishino-Yano plus gamma phylogenetic tree based on the partial RNA-dependent RNA polymerase (RdRp) gene of calicivirus strains. Brazilian canine calicivirus RJ900/08 strain (Genbank accession number JN705937) is highlighted. Bootstrap values based on 2000 generated trees are displayed at nodes (values >50 per cent are shown)

FIG 2: Kimura 2-parameter plus gamma phylogenetic tree based on the 634 bp fragment of the capsid gene of astrovirus strains. The four Brazilian canine astrovirus strains (Genbank accession number KC470086-KC470089) are highlighted. Bootstrap values based on 2000 generated trees are displayed at nodes (values >50 per cent are shown)

fact suggests that CaAstVs may play a role as enteric pathogens in young dogs.

The *Vesivirus* genus has been occasionally described in dogs presenting with enteric clinical symptoms Mochizuki and others 2002). Other caliciviruses, such as canine norovirus and canine sapovirus, were also detected in dogs in Europe Martella and others 2009) and the USA (Linlin and others 2011). Further studies in the Brazilian canine population are necessary to monitor the arrival and circulation of other genus of calicivirus.

In conclusion, the results of this study suggest that puppies with acute gastroenteritis should be screened for astroviruses and caliciviruses as well as the established pathogens CPV and CCoV.

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