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Short communication

Genetic characterization of a new astrovirus detected in dogs suffering from diarrhoea

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ABSTRACT

Astroviruses have been described in several animals species frequently associated with diarrhoea, especially in young animals. In dogs, astrovirus-like particles have been observed sporadically and very little is known about their epidemiology and characteristics. In this paper, we describe the detection of astrovirus-like particles in symptomatic puppies. Furthermore, for the first time in this species, the presumptive identification made by electron microscopy was confirmed by genetic analysis of the viral RNA conducted directly on the clinical specimens. Genetic sequences of ORF2 (2443 nt), encoding for the capsid protein, and partial sequence of ORF1b (346 nt), encoding for the viral polymerase, identified the viruses as member of the family *Astroviridae*. The phylogenetic analysis clearly clustered canine astroviruses in the genus Mamastrovirus. Relative closest similarities were revealed with a cluster comprising human, porcine and feline astroviruses, based on the ORF2 sequences available. Based on the species definition for astroviruses and on the data obtained in this study, we suggest a new species of astrovirus – canine astrovirus, CaAstV – to be included in the genus Mamastrovirus.

1. Introduction

Astroviruses are small single-stranded positive sense RNA viruses of approximately 28–30 nm in diameter belonging to the family *Astroviridae* (Fauquet et al., 2005). These round star-shaped viruses have been detected in many species of birds (genus Avastrovirus) and mammals (genus Mamastrovirus), including humans. Generally, astrovirus infections in animals are associated with enteric diseases, with mild to severe signs such as diarrhoea, vomiting, abdominal pain, sometimes associated with fever and immunodepression (Moser and Schultz-Cherry, 2005). In some cases, astroviruses can cause extra-enteric infections, as is the case of the avian nephritis virus (AVN), known to cause interstitial nephritis and growth retardation of young chickens (Imada et al., 2000) and the duck astrovirus (DAstV), that cause severe hepatitis and mortality rates up to 50% in ducklings (Gough et al., 1984). Some astroviruses do not cause a detectable disease: the bovine astrovirus (BAstV) is asymptomatic or induces only slight changes in faeces of experimentally infected calves (Bridger et al., 1984) and the feline astrovirus (FAstV) cause disease only occasionally (Marshall et al., 1984b). In other instances, the association of astroviruses with diseases is simply unknown, as the case of the recently discovered astroviruses in insectivorous bats (Chu et al., 2008). The most well characterized

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astroviruses are those that infect humans (HAstVs). They are classified in eight different serotypes, HAstV-1 to HAstV-8 (Walter and Mitchell, 2003) and HAstV-1 appears to be the most common, in particular among children, elderly and immunocompromised persons (Moser and Schultz-Cherry, 2005; Walter and Mitchell, 2003).

To date, astrovirus-like particles have been reported only three times in dogs (Marshall et al., 1984a; Vieler and Herbst, 1995; Williams, 1980). However, the previous studies observations were based on electron microscopy only and their presence in canine faeces had never been confirmed by genetic or antigenic analysis. It is noteworthy that electron microscopy often leads to wrong classification of small viral particles since they share similar morphological and physiochemical features (Guy et al., 2004). In the above-mentioned reports, astroviruses were detected in symptomatic puppies, associated with other enteric viruses such as parvoviruses, rotaviruses and coronaviruses.

In this paper we report the first case of confirmatory PCR and genetic characterization of canine astrovirus particles identified by EM from ill puppies.

2. Materials and methods

2.1. Sample collection

Faecal samples were collected individually in sterile tubes from one group of 11 diarrhoeic puppies and immediately submitted to the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe, Italy) for suspected viral enteritis. In addition, to exclude the presence of other pathogens, bacteriological and parasitological tests were requested and performed according to standard procedures (Capelli et al., 2006; Quinn et al., 1994).

2.2. Electron microscopy

Faeces were 10-fold diluted in phosphate buffered saline (PBS), repeatedly frozen and thawed and clarified by a two-step centrifugation (2500 × *g* at 8 °C for 30 min and 7000 × *g* at 8 °C for 30 min). An aliquot of 85 μL of the supernatant was ultracentrifuged for 15 min in a Beckman Airfuge, using an A-100 rotor, at 20 psi (125,000 × *g*). The grids were stained using a 2% sodium phosphotungstate solution in distilled water (pH 6.8) for at least 3 min. The dried grids were observed using a TEM Philips operating at 80 kV, at a magnification of 19,000–45,000. Morphometric measurements were performed on astrovirus-like positive samples. A minimum of 20 viral particles were measured at a magnification of 36,000 and statistically analysed with Soft-imaging software analySIS 2.1 (GmbH© 1996).

2.3. Virus isolation

Virus isolation was attempted for all the samples submitted in MDCK and VERO cells. Samples were diluted 1:10 in PBS solution with antibiotics (10,000 U/mL penycillin, 10 mg/mL streptomycin, 0.25 mg/mL gentamycin and 5000 U/mL mycostatin), then clarified by centrifugation and filtered (0.22 μm disposable filter). Samples were inoculated into confluent cell monolayers with and without trypsin 20 μg/mL (Trypsin Invitrogen, cat. no. 15090.046) (Lee and Kurtz, 1981). The inoculum was adsorbed for 1 h at 37 °C, then removed and Eagle's Minimum Essential Medium was added. Cell cultures were observed daily for cytopathic effect (CPE). Up to five blind passages were performed for cell cultures being considered negative. Presence of virus particles in cell supernatants at the end of each passage was assessed by EM.

Human astrovirus serotype 8 was propagated in CaCo-2 cells and used as positive control for subsequent RT-PCR assays.

2.4. RT-PCR design, sequencing and phylogenetic analysis

RNA from the faecal samples was isolated with NucliSens® easyMAG™ (BioMérieux) according to the manufacturer's instructions.

Since several astroviruses contain a very conserved RNA motif, called stem-loop-2-like motif (s2m), in the 3′ non-coding region (Jonassen et al., 1998), RT-PCR was performed using a forward primer located within s2m towards a reverse primer mostly targeting the viral poly-(A) tail AV12 (5′-TTT TTT TTT TTT TTT TTT GC-3′), which amplifies the 3′-end of the genome if s2m is part of it, about 40–200 nt (Jonassen, 2008; Jonassen et al., 2001). cDNA synthesis was performed using SuperScript III Rnase H⁻ Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol, with 0.125 μM AV12 primer. The RT reaction was performed in a thermocycler (MJ Research) at 50 °C for 30 min, followed by an inactivation step at 70 °C for 15 min. 2,5 μL cDNA were used in a 25 μL PCR reaction and amplified by using HotStar Taq DNA polymerase (Qiagen). The primers used were s2m-core (0.5 μM): 5′-CCG AGT A(C/G)G ATC AGG G-3′ and AV12 (0.5 μM). The concentration of Mg^{2+} in the reaction was 1.5 mM. The amplification programme consisted of an initial 15 min step at 95 °C, followed by 40 cycles with 94 °C for 40 s, 55 °C for 20 s and 72 °C for 40 s. A final elongation step at 72 °C for 5 min was performed, followed by chilling to 8 °C. RNA isolated from human astrovirus serotype 8 was used as a positive control in the RT-PCR set-up. Negative controls consisted of RNase/DNase-free water.

All PCR products were purified by using ExoSAP-IT® (GE Healthcare Bio-Sciences) according to the manufacturer's instructions. Sequencing was performed by using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit v1.1 (Applied Biosystems) according to manufacturer's instructions, and analysed on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

The sequences obtained from the samples that contained s2m, were most similar to human astroviruses, and new RT and PCR were therefore performed with a forward consensus primer designed in the YGDD-motif of the polymerase gene of astrovirus, Astro-YGDD (5′-TTA TGG AGA TGA (C/T)(A/C)G GCT-3′), towards a reverse primer s2m-rev (5′-CCC TCG ATC CTA CTC GG-3′). The expected size of the PCR products was about 2800 nt. cDNA synthesis was performed using 0.125 μM of a specific primer designed partially in the poly(A)-tail (5′-TTT TTT TTT TTG CCT AAA CTA-3′), and performed as

described above with some modifications to allow for long-range cDNA synthesis: the amount of SuperScript™ III RT enzyme was raised to 400 U/reaction, and the RT reaction was performed at 55 °C for 60 min, followed by an inactivation step at 70 °C for 15 min. 2.5 μL cDNA was used in a 25 μL PCR reaction and amplified by using BD Advantage 2 Polymerase (BD Biosciences Clontech). The primers used were Astro-YGDD (1.0 μM) and s2m-rev (0.5 μM). The amplification programme consisted of an initial 1 min step at 95 °C, followed by 35 cycles with 95 °C for 30 s, 60 °C for 20 s and 68 °C for 5 min. A final elongation step at 68 °C for 5 min was performed, followed by chilling to 8 °C. The PCR products obtained from this amplification were sequenced and, based on these preliminary results, new canine astrovirus specific forward primer could be designed about 60 nt downstream the YGDD-motif, HundAstroF2800 (5′-GAT GTT TTT GGA ATG TGG GT-3′), to allow for a more specific amplification. A new dog long-range cDNA was then successfully amplified using this primer (0.5 μM) as a forward primer towards s2m-rev in a similar amplification as described above using BD Advantage 2 Poymerase, but the annealing temperature was decreased to 55 °C. The new-amplified PCR products were then sequenced, and new primers were designed and used for sequencing of this PCR product in a primer-walking strategy.

Software used for sequence analysis and phylogeny were Sequencher version 4.1.4 (Gene Codes Corporation; http://www.genecodes.com), FASTA similarity search and CLUSTALW Multiple Sequence Alignment Program (http://www.ebi.ac.uk), and MEGA version 3.1 (Kumar et al., 2004, http://megasoftware.net). Topology of the phylogenetic trees obtained was then compared to the topology of the trees generated with Bayesian methods. In detail, the selection of the most appropriate model of molecular evolution was obtained using the Akaike information criterion implemented in the computer program ModelTest vers. 3.7. Subsequently, Bayesian methods implemented with the computer program MrBayes vers. 3.1.1 were applied to generate the dendrograms and to assess statistical supports for the branches. ORF analyses, and protein domain predictions were performed using JustBio (http://www.justbio.com), PSORT II prediction (http://psort.nibb.ac.jp/form2.html), TMHMM server version 2 (http://www.cbs.dtu.dk/services/TMHMM/) and HMMTOP (http://www.enzim.hu/hmmtop/index.html).

3. Results

3.1. Case history and laboratory results

In June 2005 eleven 2–3-month-old puppies were imported to Italy from an East European country for commercial purposes. In the country of origin, the puppies underwent antibiotic (spyramicin for 8 days), antihelminthic (levamisole) and multi-vitamin treatment. Few days after the end of the quarantine period they started to show depression, decreased feed consumption and diarrhoea. A viral enteritis was suspected and samples were collected singularly and submitted for laboratory examinations. All puppies recovered spontaneously after 5–7 days of illness.

Table 1
Summary of viruses detected in faecal samples of puppies EM positive for astrovirus by different diagnostic methods.

Sample	Virus observed by EM[a]	Virus isolation	RT-PCR for Astrovirus[b]
3	Astrovirus-like particles	None	Positive
6	Parvovirus-like and Astrovirus-like particles	None	Positive
8	Astrovirus-like particles	Rotavirus[c]	Positive
11	Astrovirus-like particles	None	Positive

[a] Observation by electron microscopy performed directly on faecal samples.
[b] RT-PCR analysis performed on faecal samples with AV12 and s2m-core primers.
[c] Identification made by EM on MDCK cell with cytopathic effect after two blind passages.

Four (ID nos. 3, 6, 8 and 11) out of 11 samples collected and analysed by EM revealed the presence of astrovirus-like particles. In one sample, parvovirus-like particles were also observed (Table 1). Astrovirus-like particles appeared as small round, non-enveloped viruses of 27–30 nm in diameter with distinctive features due to small surface projections with a five to six point star-like appearance. Aggregation of viral particles was a common finding in the samples examined. The difference in size (27–30 nm vs. 18–20 nm) permitted to easily discriminate between astrovirus and parvovirus (Fig. 1). In 6/7 of the remaining samples, no virus particles were revealed by EM. In one sample, parvovirus-like and coronavirus-like particles were detected.

Astrovirus-positive samples by EM were submitted for virus isolation. Growth of astrovirus-like particles was not detected by EM in the cell cultures used in this study. In one sample (no. 8) rotavirus was isolated and identified by

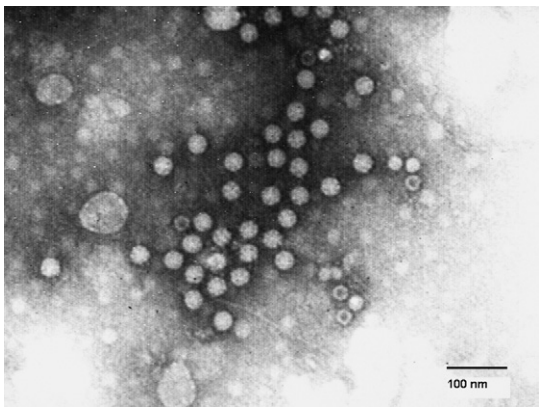

Fig. 1. Electron microscopy picture of astrovirus (larger particles of 18–20 nm) and parvovirus (smaller particles of 27–30 nm) observed directly in faecal sample of puppy no. 6 (36,000 Kx). The characteristic "star-like appearance" of astrovirus particles is evident compared to the round shaped morphology of parvovirus. Negative staining was obtained with 2% sodium phosphotungstate solution.

EM after two passages in cell cultures displaying CPE (Table 1).

Bacteriological cultures were not conclusive and analysis yielded negative results specifically for *Salmonella* spp., *Yersinia* spp. and *Campylobacter* spp. Parasitological examination revealed infestation by *Toxocara canis* in sample no. 11.

3.2. RT-PCR and sequencing

Amplification products using s2m-AV12 primers were obtained only for the four samples found positive for astrovirus-like particles by EM and their identity was confirmed by sequencing. Long-range RT-PCR products were sequenced as well and, by the adoption of the primer-walking strategy, sequences encompassing the C-terminal part of the viral polymerase as well as the entire capsid gene were generated for 2 out of 4 samples (namely, sample nos. 3 and 8), and sequence encompassing almost the entire capsid gene (2487 nt) was generated for an additional sample (no. 6). Sequences have been deposited in GenBank database with accession numbers from FM213330 to FM213332. The phylogenetic dendrograms for the partial ORF 2 (Fig. 2) and for ORF 1b were obtained through the alignment of 2443 and 346 nt, respectively. Based on the capsid gene sequences, similarity of 94.4% at nucleotide level was revealed among the astrovirus sequences in sample 3 and 8. Higher similarity (98.3%) was detected for the partial sequence related to ORF 1b. Sequences clustered in the clade of the Mamastroviruses, including viruses isolated or sequenced from mammalian hosts, but clearly grouped in a lineage separated from the astroviruses previously sequenced from feline, porcine, ovine, bat, mink and human hosts (Fig. 2). Among the available sequences, the closest similarity for the capsid gene was found with HAstV7 (22%). Similar findings and tree topology (data not shown) were observed for the analysis of the partial ORF1b sequences, with a closest similarity found with HAstV1 (59.4%). However no sequence information is available for the feline or porcine astroviruses in the part of the polymerase sequenced for the present work.

The dog astrovirus was found to have an in-frame start codon for capsid precursor protein 180 nt upstream of the start codon homologous to other Mamastrovirus genomes. This leads to an overlapping reading frame for the C-terminal part of the polymerase and the N-part of the capsid precursor of 62 amino acids (Fig. 3). Amino acid sequence analysis of this N-terminal additional peptide predicted a transmembrane domain (data not shown).

The s2m feature of the canine astrovirus was somewhat different from all other described s2m, having a longer stem-part, but all conserved residues involved in the structure of this motif were still conserved (Robertson et al., 2005).

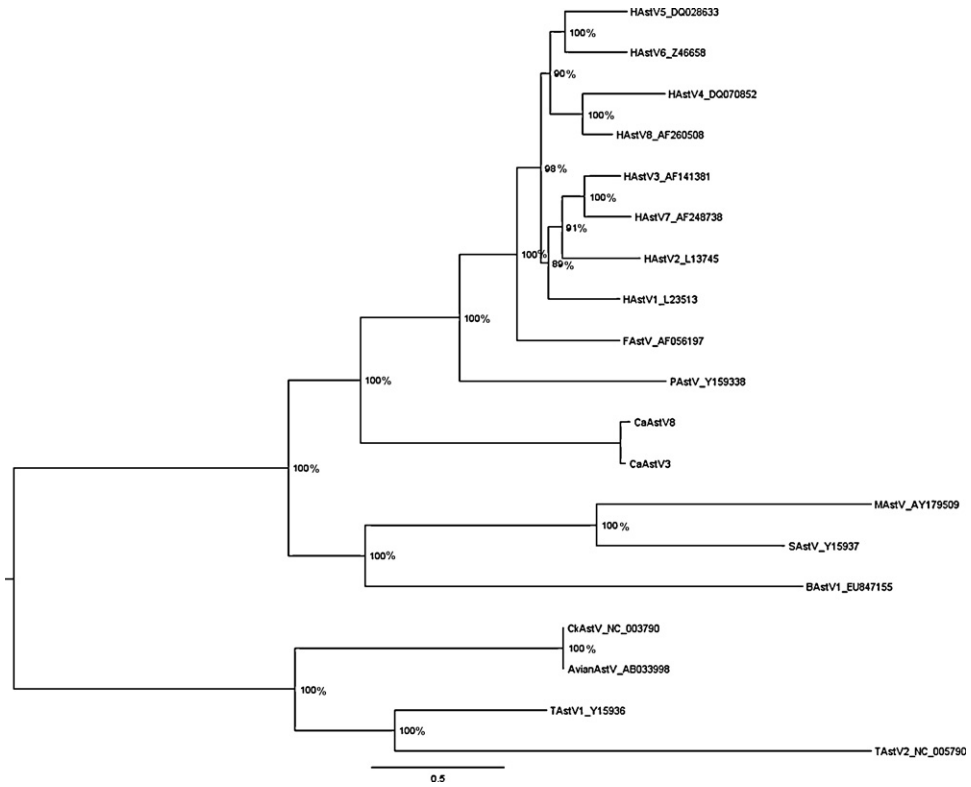

Fig. 2. Phylogenetic tree constructed by Bayesian analysis of the nucleotide sequences of ORF2. Posterior probabilities of the clades are indicated at the nodes. In this figure, bat astrovirus sequence is identified as BAstV; sheep astrovirus as SAstV; mink astrovirus as MAstV; feline astrovirus as FAstV; human astroviruses as HAstV; porcine astrovirus as PAstV; canine astroviruses as CaAstV; turkey astrovirus type I and II as TAstV1 and TAstV2, respectively. GenBank accession numbers are indicated.

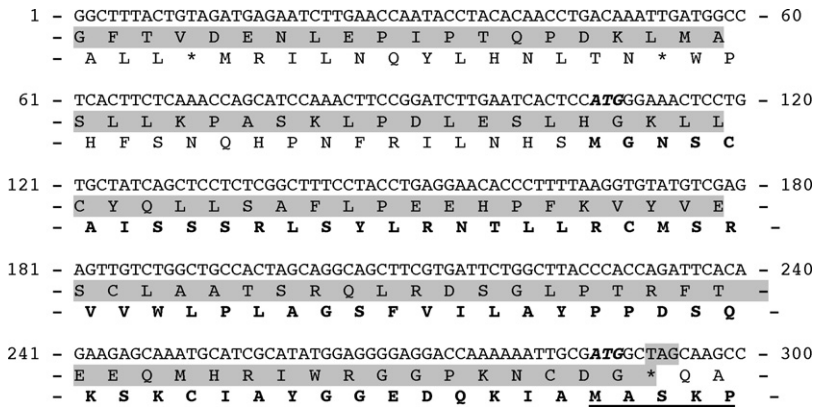

Fig. 3. Part of the CaAstV sequence in the junction between the ORF1b and ORF2 genes. Both nucleotide sequences and the amino acid sequences for the C-terminal part of the ORF1b (with grey shade), and for the putative capsid precursor protein (in bold) are shown, displaying the two possible start codons for the putative capsid precursor in bold italics. The start of the ORF2 protein homologous to other Mamastroviruses is underlined. The stop codon of the ORF1b is shaded grey.

4. Discussion and conclusions

Description of virus particles resembling astroviruses in canine faeces is sporadic and consisting only in three reports from Australia, Germany and USA (Marshall et al., 1984a; Vieler and Herbst, 1995; Williams, 1980). Astroviruses have been previously observed in dogs with diarrhoea, but Marshall et al. (1984a,b) reported astrovirus-like particles also in asymptomatic puppies. Based on the anamnestic records (i.e. previous antibiotic and antihelminthic treatments) and laboratory results (i.e. presence of co-infections with parvovirus, rotavirus and *T. canis*) presented in this paper, it was not possible to associate the presence of astroviruses with the enteric disease. However, based on the results presented herein, their role in the aetiology of canine enteric diseases should be considered and investigate further.

Unfortunately, the isolation attempts in continuous cell lines failed, confirming the difficulties to *in vitro* replicate astroviruses reported in the literature (Chu et al., 2008, Moser and Schultz-Cherry, 2005). However, RT-PCR and sequencing could confirm the EM identification in 4/4 cases, allowing the correct identification and the genetic characterization of astroviruses in dogs.

In some mammalian species, the detection of astroviruses in clinical samples, such as faeces, is considered a common finding. In humans, these viruses are considered one of the most common causes of diarrhoea in children (Walter and Mitchell, 2003). Very recently, astrovirus detection rates up to 100% have been reported in insectivorous bats in Hong Kong (Chu et al., 2008). On the contrary, in other species such as dogs or cats, their detection appears very sporadic. Whether this is due to a true lower prevalence or to the fact that they are simply under-detected remains questionable and further analysis should be conducted to increase our understanding of the epidemiology of this infection in domestic carnivores.

The canine astrovirus displayed some unique genomic features, including a possible upstream start for the capsid precursor protein. None of the possible start codons for the capsid precursor were in an optimal context for translation initiation as proposed by Kozak (1989), as they both lack a purine at position-3, but the most upstream codon did have a context most resembling the consensus vertebrate sequence around start codons GCCRCCAUGG (Kozak, 1989). The canine astrovirus genome, however, displayed the very conserved region right upstream of the Mamastrovirus-homologous start codon, that could be involved in subgenomic RNA transcription (Jonassen et al., 2003), and it is therefore not clear if a subgenomic RNA spanning the first initiation codon is synthesised. However, the fact that a functional domain could be predicted for the additional N-terminal peptide could suggest that longer transcripts actually are translated. The role of the transmembrane domain and membrane localisation of the resulting protein would still need to be elucidated.

The phylogenetic analysis clearly clustered canine astroviruses in the genus Mamastrovirus, in a well defined and distinct branch of the phylogenetic tree. The relatively closest similarities were revealed with a cluster comprising human, porcine and feline astroviruses, based on the ORF2 sequences available.

Based on the species definition for astroviruses (Fauquet et al., 2005) and on the data obtained in this study, we suggest a new species of astrovirus (canine astrovirus, CaAstV) to be included in the genus Mamastrovirus.

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