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Detection and characterization of canine astroviruses

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Astroviruses (AstVs) have been identified only occasionally in dogs. A canine AstV, strain Bari/08/ITA, was detected from a pup with gastroenteric signs and the virus was isolated in cell culture and characterized molecularly. In the full-length capsid protein, the virus displayed genetic similarities (83.5 % aa identity) to another canine AstV strain, although a high rate of variation occurred in the hypervariable domain, which is related to AstV antigenic specificity. Specific antibodies were detected in the convalescent dog, indicating seroconversion, and in 59 % of a collection of dog serum samples. Using primers specific for canine AstV, designed to detect a conserved region of ORF1b, canine AstVs were detected in 24.5 % of young pups with gastroenteritis, either alone or in mixed infections with other canine pathogens. In contrast, AstVs were detected in only 9.3 % of asymptomatic pups. These findings indicate that canine AstVs are common in dogs and may suggest a possible role as canine enteric pathogens.

INTRODUCTION

The family *Astroviridae* includes human and animal small round viruses (SRVs) with a peculiar star-like surface when observed by electron microscopy (EM). Astroviruses (AstVs) are non-enveloped and their genome is composed of a plus-sense ssRNA of 6.4–7.3 kb, containing three ORFs and a 3′ poly(A) tail (Méndez & Arias, 2007). Two ORFs, located at the 5′ end of the genome (ORF1a and ORF1b), encode non-structural proteins, whilst ORF2, located at the 3′ end, encodes the capsid protein (Méndez & Arias, 2007). AstVs were first identified by EM in 1975 in Scotland in stools of infants hospitalized with diarrhoea (Madeley & Cosgrove, 1975). Subsequently, similar SRVs were identified from several mammalian and avian species (Bridger 1980; Englund *et al.*, 2002; Hoshino *et al.*, 1981; Imada *et al.*, 2000; Kjeldsberg & Hem, 1985; McNulty *et al.*, 1980; Snodgrass & Gray, 1977; Todd *et al.*, 2009; Tzipori *et al.*, 1981; Woode & Bridger, 1978), including bats (Chu *et al.*, 2008) and aquatic mammals (Rivera *et al.*, 2010). AstV infection is associated with gastroenteritis in most animal species, and humans AstVs are regarded as the second or third most common cause of viral diarrhoea in children (Méndez & Arias, 2007). AstVs have also been associated with extra-intestinal diseases, such as nephritis in chicken (Imada *et al.*, 2000), hepatitis in ducks (Todd *et al.*, 2009) and shaking syndrome in minks (Blomström *et al.* 2010).

SRVs have been detected only occasionally in dogs by EM. In some cases, due to their morphological similarities (about 25–35 nm in size, round shape and absence of envelope), this definition has been used to refer to AstV-, calicivirus- or picornavirus-like particles. AstV-like particles were first detected in beagle pups with diarrhoeal disease in the USA in 1980 in a mixed infection with canine parvovirus type 2 (CPV-2) and canine coronaviruses (CCoVs) (Williams, 1980). AstV-like particles were also detected in 3/157 normal faecal samples (but not in 29 diarrhoeal samples) in a survey in Australia in 1984 (Marshall *et al.*, 1984). In a large survey in Germany, SRVs were identified in 41/4044 faecal samples (1.0 %) of diarrhoeal dogs (Vieler & Herbst, 1995). More recently, AstVs have been identified in dogs with enteric signs and have been characterized molecularly, suggesting that the detected viruses may represent a distinct AstV species (Toffan *et al.*, 2009). Here, we report the identification of a canine AstV, strain Bari/08/ITA, in a pup with gastroenteric signs that tested negative for all other common viral canine pathogens. The virus was isolated in Madin–Darby canine kidney (MDCK) cell cultures and was characterized

The GenBank/EMBL/DDBJ accession number for the 3.2 kb region at the 3′ end of the genome of canine astrovirus strain Bari/08/ITA determined in this study is HM045005.

molecularly from the 3′ end of ORF1b to the poly(A) tail. Specific antibodies were detected in the convalescent dog, indicating seroconversion, and also in a collection of dog serum samples available in our laboratories. Primers specific for canine AstV were designed to detect a conserved region of ORF1b and used to screen a collection of faecal samples, providing firm evidence that viruses genetically/antigenically similar to strain Bari/08/ITA may circulate in dogs.

RESULTS

Characterization of the virus isolate as AstV

AstV RNA was detected in a 90-day-old mixed-breed pup with watery diarrhoea. The pup was hospitalized 2 days after the onset of the gastroenteric disease, due to severe dehydration. After 2 days of illness, the dog recovered completely. The faeces of the pup tested negative for all known common canine viral pathogens, including CPV-2, CCoVs, distemper virus (CDV), canine adenoviruses (CAV-1 and CAV-2), reovirus, rotavirus and calicivirus. AstV RNA was detected for 10 days after hospitalization. The faecal samples collected on days 2–6 post-hospitalization (p.h.) were pooled and inoculated onto MDCK cells. After the third serial passage, a clear cytopathic effect was observed, characterized by enlargement and/or detachment of cells and the appearance of fine granules in the cytoplasm (Fig. 1). Viral antigens were observed by immunofluorescence assay in the cells as fine granules dispersed in the cytoplasm, aggregating in a perinuclear position (Fig. 2). The immunofluorescence assay was positive using the convalescent serum of the pup infected by AstV Bari/08/ITA but negative using either monospecific serum or mAbs for common canine virus pathogens (parvovirus, coronavirus, CDV, rotavirus and reovirus). The isolate, strain Bari/08/ITA, was confirmed to be AstV using AstV-specific primers, and tested negative using primers and probes specific for common canine pathogens. In addition, upon EM observation, SRV particles were identified in the stools of the pup and in the third passage in MDCK cells.

Sequence and phylogenetic analysis of the isolate Bari/08/ITA

A 3142 nt sequence was determined, spanning the 3′ end of ORF1b, the full-length ORF2 and the 3′ non-coding region to the poly(A) tail. The 3′ end of ORF1b comprised 716 nt, encoding a 237 aa polypeptide fragment at the C terminus of the polymerase complex. In this 237 aa fragment, the highest identity was 79.6 % to California sea lion AstV type 2 (CSL-2). However, identity was 100 % with shorter fragments (111 aa) of AstV strains detected from dogs (Toffan *et al.*, 2009). There was an 8 nt overlap between the termination codon of ORF1b and the initiation codon of ORF2. The highly conserved nucleotide stretch upstream of

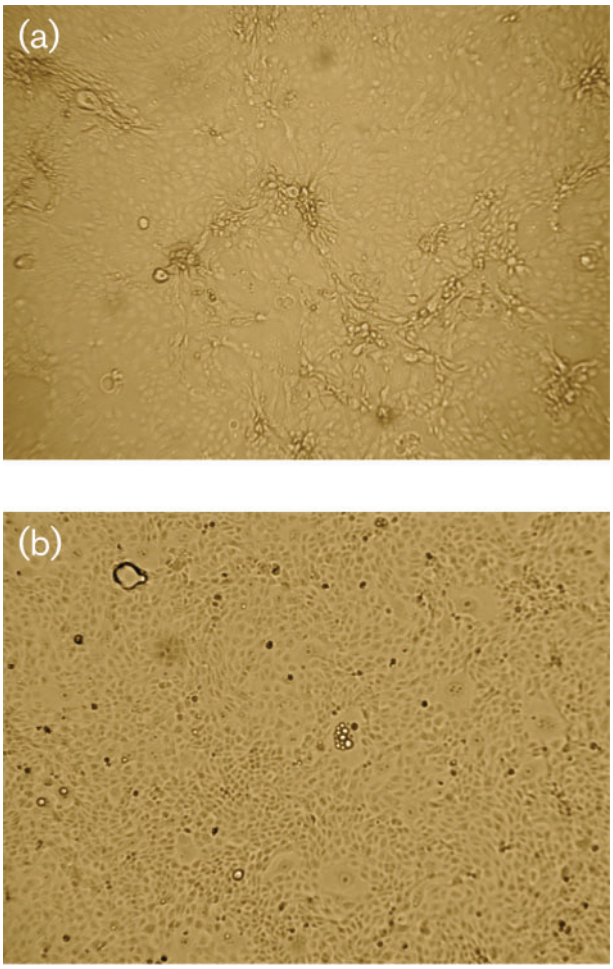

Fig. 1. (a) Cytopathic effect induced by canine AstV replication in MDCK cells infected for 24 h at the third passage. (b) Mock-infected MDCK cells are shown for comparison.

ORF2, 5′-ATTTGGAGNGGNGGACCNAAN$_{5–8}$ATGNC-3′, believed to be part of a promoter region for synthesis of subgenomic RNA (Walter *et al.*, 2001), was nearly completely conserved in the sequence of strain Bari/08/ITA. ORF2 was 2325 nt and encoded a capsid protein of 774 aa, with a predicted molecular mass of 84.7 kDa. The non-coding region was 87 nt in length. By pairwise comparison, the highest identity (83.5 %) in the capsid protein was found to a canine AstV, strain Italy/05. Amino acid identity with other mammalian AstVs ranged from 20.1 to 35.6 %, whilst amino acid identity to avian AstVs was 13.7–17.3 % (Table 1). The 6 aa C terminus of the VP1 (SRGHAE) was highly conserved with several mammalian AstVs. This motif is within a highly conserved nucleotide stretch, s2m, overlapping the termination codon of ORF2 and is also shared by some coronaviruses and picornaviruses (Jonassen *et al.*, 1998). By phylogenetic analysis, the strain clustered with the canine strain Italy/05, intermingled with AstVs detected from aquatic mammals in the genogroup *Mamastrovirus* (Fig. 3).

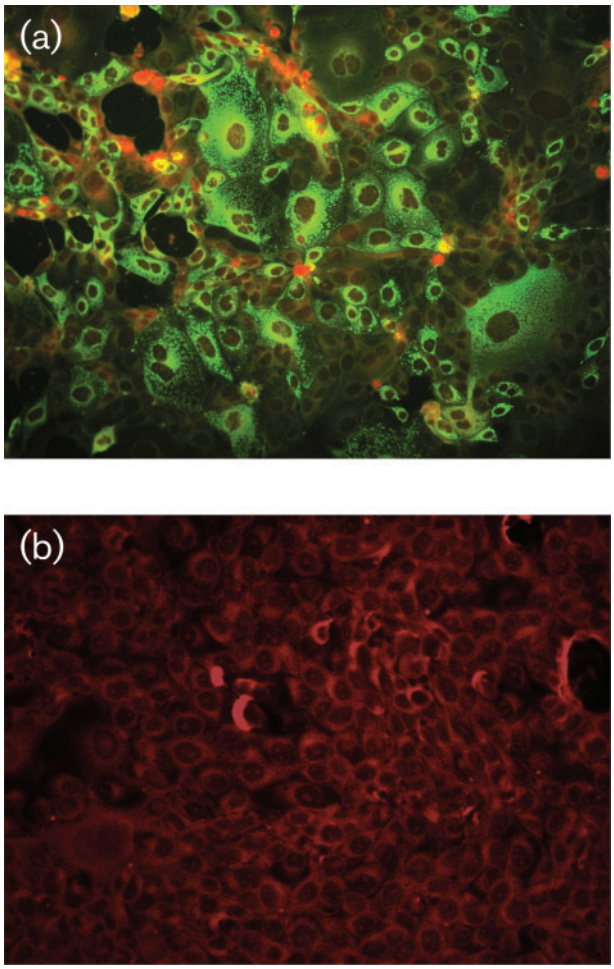

Fig. 2. (a) Indirect immunofluorescence on MDCK cells infected for 24 h using the convalescent serum of the AstV-infected pup. (b) Indirect immunofluorescence using a monospecific antiserum for CPV-2 is shown for comparison.

Screening for canine enteric and non-enteric viral pathogens and for AstV

A total of 110 stool samples from animals with gastroenteric disease were screened for common canine viral pathogens and for AstV. By RT-PCR, AstV RNA was detected in 27/110 samples (24.5 %), either alone (10/110, 9.1 %) or in mixed infections with CPV-2 (5/110, 4.5 %) and CCoVs (8/110, 7.3 %) or with CPV-2 plus CCoVs (4/110, 3.6 %) (Table 2).

In addition, a total of 75 rectal swabs collected from asymptomatic animals were screened by RT-PCR with the AstV-specific primers 625F-1 and 626R-1 (see Methods) and canine AstV RNA was detected in 7/75 swabs (9.3 %). Sequence analysis of the amplicons confirmed the specific nature of the bands (data not shown). The differences in AstV prevalence between symptomatic and asymptomatic animals were found to be statistically significant ($P<0.05$).

Table 1. Amino acid identity in the capsid protein between the canine viruses Italy/05 and Bari/08/ITA, and avian and mammalian AstVs representative of the various AstV species/types

Values were calculated based on the full-length capsid protein alignment, without removing gaps.

AstV	Italy/05	Bari/08/ITA
Human type 1	34.1	33.6
Human type 2	33.2	34.0
Human type 3	33.9	33.9
Human type 4	34.7	34.4
Human type 5	34.2	34.8
Human type 6	34.9	35.2
Human type 7	34.3	34.4
Human type 8	34.5	35.0
Human VA1	21.9	22.0
Human VA2	20.3	20.7
Human MLB1	22.2	21.9
Mink	22.7	23.0
Pig	35.2	35.0
CSL-1	20.3	20.1
CSL-2	35.1	35.6
Bottlenose dolphin	29.8	29.9
Bat type 1	20.7	20.3
Bat LC03	20.4	20.6
Bat LD38	20.4	20.6
Duck	16.9	16.3
Turkey type 1	15.1	15.2
Turkey type 2	16.7	17.1
Turkey type 3	17.3	17.3
Avian nephritis virus type 1	13.6	13.7
Dog Italy/05	100.0	83.5
Dog Bari/08/ITA	83.5	100.0

Detection of antibodies specific for strain Bari/08/ITA in dog sera

Antibodies specific for virus Bari/08/ITA were detected in 32/54 (59.3 %) serum samples by immunofluorescence. The majority (14/22, 63.6 %) of the serum samples testing negative were from pups aged <3 months, whilst only 3/32 (9.4 %) of the positive sera were from dogs aged <3 months. This age-related pattern was found to be statistically significant ($P<0.001$). Specific antibodies were identified in the convalescent serum of the AstV-infected dog but not in the serum collected at the time of hospitalization.

DISCUSSION

SRVs have been detected by EM only occasionally in dogs with and without enteric signs but the exact nature of these viruses was not established (Marshall *et al.*, 1984; Schaffer *et al.*, 1985; Vieler & Herbst, 1995; Williams, 1980). Caliciviruses characterized as either feline or canine

Fig. 3. Phylogenetic tree based on the full-length amino acid sequence of the capsid protein of AstVs of avian and mammalian origin. The tree was determined using the neighbour-joining method without any distance correction. The GenBank accession numbers of the AstV strains used are listed in Table 3. Strain Bari/08/ITA identified in this study is indicated by a filled circle. ANV-1, Avian nephritis virus type 1. Bar, number of amino acid substitutions per 100 sites.

vesiviruses (Evermann *et al.*, 1985; Mochizuki *et al.*, 1993; San Gabriel *et al.*, 1997) and noroviruses (Martella *et al.*, 2008, 2009) have been identified in the stools of dogs with enteric signs and therefore caliciviruses must be considered when SRVs are observed by EM. Recently, SRVs characterized molecularly as AstVs were detected in the stools of pups with enteritis, imported from Eastern Europe (Toffan *et al.*, 2009), in conjunction with CPV-2- and CCoV-like particles.

Table 2. Results of screening for common canine pathogens and for AstV in symptomatic dogs (n=110)

Virus	*n* (%)
CPV-2	37 (33.6)
CCoV	15 (13.6)
CDV	1 (0.9)
CPV-2 + CCoV	8 (7.3)
CPV-2 + CCoV + CAV	1 (0.9)
AstV	10 (9.1)
AstV + CPV	5 (4.5)
AstV + CCoV	8 (7.3)
AstV + CCoV + CPV	4 (3.6)
CPV-2 (total)	55 (50.0)
CCoV (total)	36 (32.7)
AstV (total)	27 (24.5)

In this study, a canine AstV, strain Bari/08/ITA, was identified by RT-PCR in a young dog that tested negative for all known common canine viral pathogens. Upon sequence analysis, the virus was characterized as a member of the genus *Mamastrovirus* and displayed highest identity in the polymerase complex (in a short 111 aa overlap; 100 % aa identity) and in the full-length capsid protein (83.5 % aa identity) to the canine strain Italy/05 (Toffan *et al.*, 2009). The genetic diversity between strain Italy/05 and Bari/08/ITA appeared markedly higher than that observed between human AstVs of the same serotype. Human AstVs are classified into eight serotypes based on the antigenic relationships determined by immunofluorescence, ELISA, neutralization assays and immunoelectron microscopy (Koopmans *et al.*, 1998; Kurtz & Lee, 1984). The amino acid identity in the full-length capsid protein between strains of the same serotype is usually >94 %, whilst strains of different serotypes may display amino acid identities as low as 69–73 % (Méndez-Toss *et al.*, 2000). By

analysing the capsid protein of human AstVs, a high degree of conservation can be observed in the N-terminal portion (aa 1–415) of the capsid protein, whilst downstream of these conserved 415 residues, considerable variability is seen among strains of different serotypes (Méndez & Arias, 2007). Between strains Italy/05 and Bari/08/ITA, a high degree of variation occurred between aa 422 and 668 (50 % aa identity), whilst high conservation was found between aa 1 and 422 (98.3 %) and in the C-terminal residues downstream of aa 668 (95.7 %). The hypervariable C-terminal region downstream of aa 415 is believed to form the spikes of the virion and to interact with the cell receptors (Krishna, 2005), as neutralizing mAbs have been mapped to this variable domain (Bass & Upadhyayula, 1997; Sanchez-Fauquier *et al.*, 1994). Accordingly, although genetically similar, the antigenic relationships between the two canine viruses are not predictable and should be assessed by cross-neutralization assays.

Strain Bari/08/ITA was successfully adapted to grow in a canine cell line (MDCK) after three serial passages, and virus replication induced a clear cytopathic effect. Viral antigens were visualized in the cytoplasm of infected cells by immunofluorescence, and SRV particles were observed in the MDCK lysates by EM. Adaptation of AstVs to replication in cell culture is fastidious and, with the exception of avian AstVs, appears to be trypsin dependent (Méndez & Arias, 2007). In addition, AstVs tend to replicate almost exclusively in cells derived from their natural host (Méndez & Arias, 2007). Accordingly, the use of canine cells was an important requisite for adaptation to replication of strain Bari/08/ITA because dogs are the homologous host for these viruses.

Using the isolate Bari/08/ITA as antigen, specific antibodies were identified in the convalescent dog and in 59.0 % of the tested canine sera. AstV-specific antibodies were detected almost exclusively in dogs of >3 months, whilst most dogs aged <3 months were seronegative. This is consistent with the fact that, at 2–3 months of age, pups become susceptible to infectious agents, as maternally derived immunity tends to wane (Pollock & Carmichael, 1982).

Following screening of a collection of faecal samples obtained from young dogs (aged 1–6 months) with gastroenteritis, canine AstV was found in 24.5 % of the pups, either alone or in mixed infections with CPV-2 and CCoVs. This prevalence rate was unexpectedly high. By contrast, the prevalence of canine AstV in asymptomatic young dogs was only 9.3 %. Thus, canine AstV prevalence differed significantly ($P<0.05$) between the two groups of animals. The majority of AstV-infected pups were also infected by other canine pathogens such as CPV-2 or CCoV, whilst single infections by AstV were detected only in 9.1 % of the symptomatic pups. These findings are not unexpected, as mixed infections are not infrequent, especially in animal communities such as kennels or shelters. Experimental infections are necessary to elucidate the pathogenic role of canine AstVs and to understand whether they can act by themselves as the primary causative agent of gastrointestinal disease, or whether they trigger mechanisms of synergism in co-infections, as observed between coronaviruses and parvoviruses (Appel, 1988; Pratelli *et al.*, 1999).

It was remarkable that the prevalence rates of canine AstV revealed by RT-PCR in this study were markedly higher than those described in previous investigations. In a large EM-based survey in Germany, SRVs (including in this definition AstVs, picornaviruses and caliciviruses) were identified only in 41/4044 (1.0 %) faeces of dogs with enteritis (Vieler & Herbst, 1995). These discrepancies are probably the result of the high sensitivity of RT-PCR techniques. By coupling the virological and serological findings, it is possible to depict a conceivable scenario in which, by the age of 3–4 months, most dogs have come in contact with canine AstVs. Larger age-stratified serological investigations are necessary in order to assess precisely the patterns of seroprevalence in dogs. However, these pilot epidemiological investigations appear to suggest that these viruses are ubiquitous in canine populations.

In conclusion, in this study, we have reported for the first time the isolation of a canine AstV in a canine cell line. Serological and virological evidence was also collected demonstrating that: (i) AstVs are common in young dogs with gastroenteritis, either alone or in mixed infections with other canine enteric pathogens, such as CPV-2 and CoCVs; (ii) AstVs are less common in asymptomatic young dogs; and (iii) antibodies to a canine AstV may be detected in dogs at high prevalence but not in young pups. Experimental infections and implementation of the diagnostic algorithms for canine enteric pathogens with AstV-specific assays will be pivotal to dissect the role of these viruses in dogs.

METHODS

History of the animal and virological examinations. A 90-day-old mixed-breed pup developed a gastroenteric disease with watery diarrhoea. The pup was hospitalized 2 days after the onset of the gastroenteric disease, due to severe dehydration. After 2 days of illness, the dog recovered completely. At the time of hospitalization, the faeces were collected and screened for the presence of common canine viral pathogens by either gel-based PCR or quantitative PCR and RT-PCR. DNA and RNA extracts were prepared using DNeasy and QIAamp viral RNA kits (Qiagen). The faeces of the pup tested negative for all known common canine viral pathogens, including CPV-2, CCoVs, CDV, CAV-1 and CAV-2, reovirus, rotavirus and calicivirus. Unexpectedly, the stools tested positive to AstV using a broadly reactive primer pair, targeted to the ORF1b region (Chu *et al.*, 2008). After the detection of astroviral RNA in the faeces of the dog, the animal was kept under observation. Faecal samples were collected daily on days 2–30 p.h. to monitor virus shedding. Astroviral RNA was detected until day 10 p.h. In addition, serum samples were obtained from the dog at hospitalization and on days 14 and 28 p.h.

Cultivation in canine cell lines. Faecal samples collected on days 2–6 p.h. were pooled and then used in attempts to adapt the virus to

growth in tissue culture cells. Evidence was obtained for viral growth in MDCK cells grown in Dulbecco's modified Eagle's medium supplemented with 10 μg trypsin ml^{-1} (type IX; Sigma), after the third serial passage. The cytopathic effect was characterized by enlargement and/or detachment of cells and the appearance of fine granules in the cytoplasm. Viral antigen in the infected cells was revealed by indirect immunofluorescence using the convalescent serum (day 28 p.h.) of the dog and an FITC-conjugated anti-dog serum (Sigma-Aldrich). Immunofluorescence using monospecific sera or mAbs for common canine virus pathogens (CPV-1 and -2, CCoV-2, CDV, rotavirus and reovirus) was also carried out. Using PCR or RT-PCR, the isolate was recognized by AstV-specific primers but not by primers and probes specific for common canine pathogens.

Electron microscopy observation. Faecal samples collected on days 2–6 p.h. were pooled and processed for EM observation. In addition, the third virus passage in MDCK cells was analysed. Briefly, faecal samples were diluted 1 : 10 in distilled water, vortexed and centrifuged for 20 min at 4000 ***g*** and again for 10 min at 9300 ***g*** for clarification. The supernatant and cell lysate were then ultracentrifuged (Beckman Airfuge) for 15 min at 82 000 ***g***. After negative staining with 2 % sodium phosphotungstate (pH 6.8), samples were examined using a Philips CM10 electron microscope.

Sequence and phylogenetic analysis of the astrovirus strain. Amplicons obtained with the AstV ORF1b-specific primers (Chu *et al.*, 2008) were excised from the gel and purified using a QIAquick gel extraction kit (Qiagen). The fragment was then subjected to direct sequencing using BigDye Terminator Cycle chemistry and a 3730 DNA Analyser (Applied Biosystems). The programs BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and FASTA (http://www.ebi.ac.uk/Tools/sss/fasta/) with default values were used to find homologous hits.

To determine the sequence and genomic organization of the AstV strain, a 3.2 kb region at the 3′ end of the genome was amplified by RT-PCR, as described by Wang *et al.* (2005). cDNA was synthesized using a SuperScript III First-Strand cDNA Synthesis kit (Invitrogen) with primer VN_3T_{20} (5′-GAGTGACCGCGGCCGCT$_{20}$-3′). PCR was then performed with TaKaRa La *Taq* polymerase (TaKaRa Bio Europe) with forward primer 5′-GARTTYGATTGGRCKCGKTA-YGA-3′ (Chu *et al.*, 2008) and VN_3T_{20}. Finally, the amplicon was purified and cloned using a TOPO XL Cloning kit (Invitrogen). Additional primers also were designed to determine the complete 3.2 kb sequence by an overlapping strategy.

Sequence editing and multiple alignments were performed with BioEdit software package version 2.1 (Hall, 1999). Phylogenetic analysis (neighbour-joining and the unweighted pair group method with arithmetic mean) with bootstrap analysis (1000 replicates) and no distance correction was conducted by using the MEGA software package version 4.0 (Tamura *et al.*, 2007).

Analysis of the capsid protein of strain Bari/08/ITA. Pairwise identity in the full-length capsid protein of strain Bari/08/ITA to a selection of AstV strains was determined using multiple alignments generated with BioEdit software package (Hall, 1999). The values were calculated by the uncorrected distance method using a 31-sequence alignment without removing the gaps, including sequences of human and animal AstVs. The strains and sequences used are listed in Table 3.

Collection of faecal samples from animals and extraction of DNA and RNA. A total of 110 stool samples from dogs with signs of mild to severe gastroenteritis were screened. The samples were a subset of a collection obtained between January and December 2007 from young dogs (aged 1–6 months) of various origins (animals housed in shelters and/or pet shops and hospitalized animals). In addition, a total of 75 rectal swabs collected from overtly healthy pups and/or young dogs (aged 2–6 months) were analysed. DNA and RNA extracts were prepared using DNAeasy and QIAamp viral RNA kits (Qiagen).

Table 3. List of AstV strains included in the present study

Only strains for which the complete sequence of the ORF2 (encoding the capsid protein) was available were included in the study. Strain designation of the viruses is reported as in the original studies and/or as found in GenBank.

Species of origin	Type	Strain	GenBank accession no.
Human	Type 1	Dresden	AY720892
Human	Type 2	Oxford	L06802
Human	Type 3	WH1859	DQ630763
Human	Type 4	Goiania/GO/12/95/Brazil	DQ070852
Human	Type 5	Goiania/GO/12/94/Brazil	DQ028633
Human	Type 6	192-BJ07-CHN	GQ495608
Human	Type 7	Oxford	AF248738
Human	Type 8	Yuc-8	AF260508
Human	VA1	VA1	FJ973620
Human	VA2	VA2/human/Stl/WD0680/2009	GQ502193
Human	MLB1	MLB1	FJ222451
Human	HMO-A	NI-295	NC_013443
Human	HMO-B	NI-196	GQ415661
Human	HMO-C	NE-3010	GQ415662
Ovine			Y15937
Mink			AY179509
Porcine		Tokushima83-74	AB037272
Feline		Bristol	AF056197
Dog		Italy/05	FM213332
California sea lion	Type 1	CSL1	FJ890351
California sea lion	Type 2	CSL2	FJ890352
Bottlenose dolphin	Type 1	Bd1	FJ890355
Bat	Type 1	AFCD337	EU847155
Bat	LC03	Hp/Guangxi/LC03/2007	FJ571074
Bat	LD38	Tm/Guangxi/LD38/2007	FJ571065
Duck		C-NGB	NC_012437
Turkey	Type 1		Y15936
Turkey	Type 2		NC_005790
Turkey	Type 3	2001	AY769616
Chicken	ANV-1	G-4260	AB033998

Screening for canine enteric and non-enteric viral pathogens. All faecal samples were screened for the presence of common canine viral pathogens either by gel-based PCR or by quantitative PCR and RT-PCR. The samples were screened for CPV-2 (Decaro *et al.*, 2005), enteric CCoVs (Decaro *et al.*, 2004) and CDV (Elia *et al.*, 2006) by qPCR or qRT-PCR using a LightCycler instrument (i-Cycler iQTM Real-Time Detection; Bio-Rad Laboratories) and IQ Supermix (Bio-Rad Laboratories) after reverse transcription of RNA with Moloney murine leukemia virus reverse transcriptase (Applied Biosystem) for RNA viruses. Screening for CAV-1 and CAV-2 (Hu *et al.*, 2001),

canine respiratory coronavirus (Decaro *et al.*, 2008), rotavirus (Gentsch *et al.*, 1992) and norovirus (Vennema *et al.*, 2002) was accomplished by conventional PCR or RT-PCR in a Gene Amp PCR System 9700 (Applied Biosystem), using TaKaRa La *Taq* polymerase for DNA viruses and a Superscript III One-Step RT-PCR kit (Invitrogen) for RNA viruses.

Screening for astroviruses by RT-PCR. The RNA extracts were screened using a primer pair specific for canine AstVs. Primers 625F-1 (5′-GTACTATACCRTCTGATTTAATT-3′) and 626R-1 (5′-AGACC-AARGTGTCATAGTTCAG-3′) were designed using the ORF1b sequence to selectively amplify a fragment of 300 bp. Statistical analyses were conducted using the R program version 2.8.1 (http://www.r-project.org/index.html). A χ^2 test, with Yates's correction for continuity, was used to evaluate the differences in AstV prevalence between symptomatic and asymptomatic animals.

Serological investigations. Detection of specific antibodies for strain Bari/08/ITA in the canine serum samples was performed using an indirect immunofluorescence assay. A total of 54 serum samples, obtained from dogs aged 2 months to 7 years, were screened. The virus Bari/08/ITA was used as antigen. Confluent MDCK cell monolayers were infected and gently harvested in the presence of advanced cytopathic effect (affecting at least 70–80 % of the cells). Infected cells were distributed onto multispot glass slides and dried at 37 °C. The slides were fixed in 100 % cold acetone at room temperature for 30 min and stored at −80 °C until used. Ten microlitres of each serum sample, diluted 1 : 50, was layered onto the cells and incubated at 37 °C for 30 min. After three washes with PBS, antiserum to dog IgG conjugated with FITC (Sigma-Aldrich) was added and the slides were incubated at 37 °C for 30 min. After three washes with PBS, the slides were observed under a fluorescent microscope and antibody titres were calculated as the highest serum dilutions still giving cytoplasmic fluorescence in the antigen preparations. Statistical analyses were conducted using the R program. A χ^2 test, with Yates's correction for continuity, was used to evaluate the differences between age groups ($\leqslant$3 months).

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REFERENCES

Appel, M. J. (1988). Does canine coronavirus augment the effects of subsequent parvovirus infection? *Vet Med* **83**, 360–366.

Bass, D. M. & Upadhyayula, U. (1997). Characterization of human serotype 1 astrovirus-neutralizing epitopes. *J Virol* **71**, 8666–8671.

Blomström, A. L., Widén, F., Hammer, A. S., Belák, S. & Berg, M. (2010). Detection of a novel astrovirus in brain tissue of mink suffering from shaking mink syndrome by use of viral metagenomics. *J Clin Microbiol* **48**, 4392–4396.

Bridger, J. C. (1980). Detection by electron microscopy of caliciviruses, astroviruses and rotavirus-like particles in the faeces of piglets with diarrhoea. *Vet Rec* **107**, 532–533.

Chu, D. K., Poon, L. L., Guan, Y. & Peiris, J. S. (2008). Novel astroviruses in insectivorous bats. *J Virol* **82**, 9107–9114.

Decaro, N., Pratelli, A., Campolo, M., Elia, G., Martella, V., Tempesta, M. & Buonavoglia, C. (2004). Quantitation of canine coronavirus RNA in the faeces of dogs by TaqMan RT-PCR. *J Virol Methods* **119**, 145–150.

Decaro, N., Elia, G., Martella, V., Desario, C., Campolo, M., Trani, L. D., Tarsitano, E., Tempesta, M. & Buonavoglia, C. (2005). A real-time PCR assay for rapid detection and quantitation of canine parvovirus type 2 in the feces of dogs. *Vet Microbiol* **105**, 19–28.

Decaro, N., Elia, G., Campolo, M., Desario, C., Mari, V., Radogna, A., Colaianni, M. L., Cirone, F., Tempesta, M. & Buonavoglia, C. (2008). Detection of bovine coronavirus using a TaqMan-based real-time RT-PCR assay. *J Virol Methods* **151**, 167–171.

Elia, G., Decaro, N., Martella, V., Cirone, F., Lucente, M. S., Lorusso, E., Di Trani, L. & Buonavoglia, C. (2006). Detection of canine distemper virus in dogs by real-time RT-PCR. *J Virol Methods* **136**, 171–176.

Englund, L., Chriél, M., Dietz, H. H. & Hedlund, K. O. (2002). Astrovirus epidemiologically linked to pre-weaning diarrhoea in mink. *Vet Microbiol* **85**, 1–11.

Evermann, J. F., McKeirnan, A. J., Smith, A. W., Skilling, D. E. & Ott, R. L. (1985). Isolation and identification of caliciviruses from dogs with enteric infections. *Am J Vet Res* **46**, 218–220.

Gentsch, J. R., Glass, R. I., Woods, P., Gouvea, V., Gorziglia, M., Flores, J., Das, B. K. & Bhan, M. K. (1992). Identification of group A rotavirus gene 4 types by polymerase chain reaction. *J Clin Microbiol* **30**, 1365–1373.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.

Hoshino, Y., Zimmer, J. F., Moise, N. S. & Scott, F. W. (1981). Detection of astroviruses in feces of a cat with diarrhea. *Arch Virol* **70**, 373–376.

Hu, R. L., Huang, G., Qiu, W., Zhong, Z. H., Xia, X. Z. & Yin, Z. (2001). Detection and differentiation of CAV-1 and CAV-2 by polymerase chain reaction. *Vet Res Commun* **25**, 77–84.

Imada, T., Yamaguchi, S., Mase, M., Tsukamoto, K., Kubo, M. & Morooka, A. (2000). Avian nephritis virus (ANV) as a new member of the family *Astroviridae* and construction of infectious ANV cDNA. *J Virol* **74**, 8487–8493.

Jonassen, C. M., Jonassen, T. O. & Grinde, B. (1998). A common RNA motif in the 3′ end of the genomes of astroviruses, avian infectious bronchitis virus and an equine rhinovirus. *J Gen Virol* **79**, 715–718.

Kjeldsberg, E. & Hem, A. (1985). Detection of astroviruses in gut contents of nude and normal mice. *Arch Virol* **84**, 135–140.

Koopmans, M. P., Bijen, M. H., Monroe, S. S. & Vinjé, J. (1998). Age-stratified seroprevalence of neutralizing antibodies to astrovirus types 1 to 7 in humans in The Netherlands. *Clin Diagn Lab Immunol* **5**, 33–37.

Krishna, N. K. (2005). Identification of structural domains involved in astrovirus capsid biology. *Viral Immunol* **18**, 17–26.

Kurtz, J. B. & Lee, T. W. (1984). Human astrovirus serotypes. *Lancet* **324**, 1405.

Madeley, C. R. & Cosgrove, B. P. (1975). Letter: 28 nm particles in faeces in infantile gastroenteritis. *Lancet* **306**, 451–452.

Marshall, J. A., Healey, D. S., Studdert, M. J., Scott, P. C., Kennett, M. L., Ward, B. K. & Gust, I. D. (1984). Viruses and virus-like particles in the faeces of dogs with and without diarrhoea. *Aust Vet J* **61**, 33–38.

Martella, V., Lorusso, E., Decaro, N., Elia, G., Radogna, A., D'Abramo, M., Desario, C., Cavalli, A., Corrente, M. & other authors (2008). Detection and molecular characterization of a canine norovirus. *Emerg Infect Dis* **14**, 1306–1308.

Martella, V., Decaro, N., Lorusso, E., Radogna, A., Moschidou, P., Amorisco, F., Lucente, M. S., Desario, C., Mari, V. & other authors (2009). Genetic heterogeneity and recombination in canine noroviruses. *J Virol* **83**, 11391–11396.

McNulty, M. S., Curran, W. L. & McFerran, J. B. (1980). Detection of astroviruses in turkey faeces by direct electron microscopy. *Vet Rec* **106**, 561.

Méndez, E. & Arias, C. (2007). Astroviruses. In *Fields Virology*, 5th edn, pp. 981–1000. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Willliams & Wilkins.

Méndez-Toss, M., Romero-Guido, P., Munguía, M. E., Méndez, E. & Arias, C. F. (2000). Molecular analysis of a serotype 8 human astrovirus genome. *J Gen Virol* **81**, 2891–2897.

Mochizuki, M., Kawanishi, A., Sakamoto, H., Tashiro, S., Fujimoto, R. & Ohwaki, M. (1993). A calicivirus isolated from a dog with fatal diarrhoea. *Vet Rec* **132**, 221–222.

Pollock, R. V. H. & Carmichael, L. E. (1982). Maternally derived immunity to canine parvovirus infection: transfer, decline, and interference with vaccination. *J Am Vet Med Assoc* **180**, 37–42.

Pratelli, A., Tempesta, M., Roperto, F. P., Sagazio, P., Carmichael, L. & Buonavoglia, C. (1999). Fatal coronavirus infection in puppies following canine parvovirus 2b infection. *J Vet Diagn Invest* **11**, 550–553.

Rivera, R., Nollens, H. H., Venn-Watson, S., Gulland, F. M. & Wellehan, J. F., Jr (2010). Characterization of phylogenetically diverse astroviruses of marine mammals. *J Gen Virol* **91**, 166–173.

Sanchez-Fauquier, A., Carrascosa, A. L., Carrascosa, J. L., Otero, A., Glass, R. I., Lopez, J. A., San Martin, C. & Melero, J. A. (1994). Characterization of a human astrovirus serotype 2 structural protein (VP26) that contains an epitope involved in virus neutralization. *Virology* **201**, 312–320.

San Gabriel, M. C., Tohya, Y., Sugimura, T., Shimizu, T., Ishiguro, S. & Mochizuki, M. (1997). Identification of canine calicivirus capsid protein and its immunoreactivity in Western blotting. *J Vet Med Sci* **59**, 97–101.

Schaffer, F. L., Soergel, M. E., Black, J. W., Skilling, D. E., Smith, A. W. & Cubitt, W. D. (1985). Characterization of a new calicivirus isolated from feces of a dog. *Arch Virol* **84**, 181–195.

Snodgrass, D. R. & Gray, E. W. (1977). Detection and transmission of 30 nm virus particles (astroviruses) in faeces of lambs with diarrhoea. *Arch Virol* **55**, 287–291.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Todd, D., Smyth, V. J., Ball, N. W., Donnelly, B. M., Wylie, M., Knowles, N. J. & Adair, B. M. (2009). Identification of chicken enterovirus-like viruses, duck hepatitis virus type 2 and duck hepatitis virus type 3 as astroviruses. *Avian Pathol* **38**, 21–30.

Toffan, A., Jonassen, C. M., De Battisti, C., Schiavon, E., Kofstad, T., Capua, I. & Cattoli, G. (2009). Genetic characterization of a new astrovirus detected in dogs suffering from diarrhoea. *Vet Microbiol* **139**, 147–152.

Tzipori, S., Menzies, J. D. & Gray, E. W. (1981). Detection of astrovirus in the faeces of red deer. *Vet Rec* **108**, 286.

Vennema, H., de Bruin, E. & Koopmans, M. (2002). Rational optimization of generic primers used for Norwalk-like virus detection by reverse transcriptase polymerase chain reaction. *J Clin Virol* **25**, 233–235.

Vieler, E. & Herbst, W. (1995). [Electron microscopic demonstration of viruses in feces of dogs with diarrhea]. *Tierarztl Prax* **23**, 66–69 (in German).

Walter, J. E., Briggs, J., Guerrero, M. L., Matson, D. O., Pickering, L. K., Ruiz-Palacios, G., Berke, T. & Mitchell, D. K. (2001). Molecular characterization of a novel recombinant strain of human astrovirus associated with gastroenteritis in children. *Arch Virol* **146**, 2357–2367.

Wang, Q. H., Han, M. G., Cheetham, S., Souza, M., Funk, J. A. & Saif, L. J. (2005). Porcine noroviruses related to human noroviruses. *Emerg Infect Dis* **11**, 1874–1881.

Williams, F. P., Jr (1980). Astrovirus-like, coronavirus-like, and parvovirus-like particles detected in the diarrheal stools of beagle pups. *Arch Virol* **66**, 215–226.

Woode, G. N. & Bridger, J. C. (1978). Isolation of small viruses resembling astroviruses and caliciviruses from acute enteritis of calves. *J Med Microbiol* **11**, 441–452.