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## 30 **Abstract**

31 Astroviruses have been described in several animals species frequently associated with diarrhoea,  
32 especially in young animals. In dogs, astrovirus-like particles have been observed sporadically and  
33 very little is known about their epidemiology and characteristics. In this paper, we describe the  
34 detection of astrovirus-like particles in symptomatic puppies. Furthermore, for the first time in this  
35 species, the presumptive identification made by electron microscopy was confirmed by genetic  
36 analysis of the viral RNA conducted directly on the clinical specimens. Genetic sequences of ORF2  
37 (2443 nt), encoding for the capsid protein, and partial sequence of ORF1b (346 nt), encoding for the  
38 viral polymerase, identified the viruses as member of the family *Astroviridae*. The phylogenetic  
39 analysis clearly clustered canine astroviruses in the genus *Mamastrovirus*. Relative closest  
40 similarities were revealed with a cluster comprising human, porcine and feline astroviruses, based  
41 on the ORF2 sequences available. Based on the species definition for astroviruses and on the data  
42 obtained in this study, we suggest a new species of astrovirus - canine astrovirus, CaAstV - to be  
43 included in the genus *Mamastrovirus*.

## 45 **Introduction**

46 Astroviruses are small single-stranded positive sense RNA viruses of approximately 28-30 nm in  
47 diameter belonging to the family *Astroviridae* (Fauquet et al., 2005). These round star-shaped  
48 viruses have been detected in many species of birds (genus *Avastrovirus*) and mammals (genus  
49 *Mamastrovirus*), including humans. Generally, astrovirus infections in animals are associated with  
50 enteric diseases, with mild to severe signs such as diarrhoea, vomiting, abdominal pain, sometimes  
51 associated with fever and immunodepression (Moser et al., 2005). In some cases, astroviruses can  
52 cause extra-enteric infections, as is the case of the avian nephritis virus (AVN), known to cause  
53 interstitial nephritis and growth retardation of young chickens (Imada et al., 2000) and the duck  
54 astrovirus (DAstV), that cause severe hepatitis and mortality rates up to 50% in ducklings (Gough et

55 al., 1984). Some astroviruses do not cause a detectable disease: the BAstV (bovine astrovirus) is  
56 asymptomatic or induces only slight changes in faeces of experimentally infected calves (Bridger et  
57 al., 1984) and the FAstV (feline astrovirus) cause disease only occasionally (Marshall et al., 1984b).  
58 In other instances, the association of astroviruses with diseases is simply unknown, as the case of  
59 the recently discovered astroviruses in insectivorous bats (Chu et al., 2008). The most well  
60 characterized astroviruses are those that infect humans (HAstVs). They are classified in 8 different  
61 serotypes, HAstV-1 to HAstV-8 (Walter et al., 2003) and HAstV-1 appears to be the most common,  
62 in particular among children, elderly and immunocompromised persons (Moser et al., 2005, Walter  
63 et al., 2003).

64 To date, astrovirus-like particles have been reported only three times in dogs (Marshall et al.,  
65 1984a, Vieler et al., 1995, Williams 1980). However, the previous studies observations were based  
66 on electron microscopy only and their presence in canine faeces had never been confirmed by  
67 genetic or antigenic analysis. It is noteworthy that electron microscopy often leads to wrong  
68 classification of small viral particles since they share similar morphological and physiochemical  
69 features (Guy et al., 2004). In the above mentioned reports, astroviruses were detected in  
70 symptomatic puppies, associated with other enteric viruses such as parvoviruses, rotaviruses and  
71 coronaviruses.

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

## 74 **Materials and methods**

### 75 *Sample collection*

76 Faecal samples were collected individually in sterile tubes from one group of 11 diarrhoeic puppies  
77 and immediately submitted to the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe, Italy)  
78 for suspected viral enteritis. In addition, to exclude the presence of other pathogens, bacteriological

79 and parasitological tests were requested and performed according to standard procedures (Capelli et  
80 al., 2006, Quinn et al., 1994).

### 81 *Electron microscopy*

82 Faeces were 10 fold diluted in phosphate buffered saline (PBS), repeatedly frozen and thawed and  
83 clarified by a two-step centrifugation (2,500 g at 8°C for 30 min and 7,000 g at 8°C for 30 min). An  
84 aliquot of 85 µl of the supernatant was ultracentrifuged for 15 min in a Beckman Airfuge, using an  
85 A-100 rotor, at 20 psi (125,000 g). The grids were stained using a 2% sodium phosphotungstate  
86 solution in distilled water (pH 6.8) for at least 3 minutes. The dried grids were observed using a  
87 TEM Philips operating at 80kV, at a magnification of 19,000-45,000. Morphometric measurements  
88 were performed on astrovirus-like positive samples. A minimum of 20 viral particles were measured  
89 at a magnification of 36,000 and statistically analysed with Soft-imaging software analySIS 2.1  
90 (GmbH© 1996).

### 91 *Virus isolation*

92 Virus isolation was attempted for all the samples submitted in MDCK and VERO cells. Samples  
93 were diluted 1:10 in PBS solution with antibiotics (10,000 U/mL penicillin, 10 mg/mL  
94 streptomycin, 0.25 mg/mL gentamycin and 5,000 U/mL mycostatin), then clarified by  
95 centrifugation and filtered (0.22 µm disposable filter). Samples were inoculated into confluent cell  
96 monolayers with and without trypsin 20 µg/ml (Trypsin Invitrogen, cat.n.15090.046) (Lee et al.,  
97 1981). The inoculum was adsorbed for 1 hour at 37°C, then removed, and Eagle's Minimum  
98 Essential Medium was added. Cell cultures were observed daily for cytopathic effect (CPE). Up to  
99 five blind passages were performed for cell cultures being considered negative. Presence of virus  
100 particles in cell supernatants at the end of each passage was assessed by EM.

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

### 103 *RT-PCR design, sequencing and phylogenetic analysis*

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

106 Since several astroviruses contain a very conserved RNA motif, called stem-loop-2-like motif  
107 (s2m), in the 3' non-coding region (Jonassen et al., 1998), RT-PCR was performed using a forward  
108 primer located within s2m towards a reverse primer mostly targeting the viral poly-(A) tail AV12  
109 (5'-TTT TTT TTT TTT TTT TTT GC-3'), which amplifies the 3'-end of the genome if s2m is part  
110 of it, about 40 - 200 nucleotides (Jonassen 2008, Jonassen et al., 2001). cDNA synthesis was  
111 performed using SuperScript III Rnase H<sup>-</sup> Reverse Transcriptase (Invitrogen) according to the  
112 manufacturer's protocol, with 0.125 μM AV12 primer. The RT reaction was performed in a  
113 thermocycler (MJ Research) at 50 °C for 30 min, followed by an inactivation step at 70 °C for 15  
114 min. 2,5 μl cDNA were used in a 25 μl PCR reaction and amplified by using HotStar Taq DNA  
115 polymerase (Qiagen). The primers used were s2m-core (0.5 μM): 5'-CCG AGT A(C/G)G ATC  
116 AGG G-3') and AV12 (0.5 μM). The concentration of Mg<sup>2+</sup> in the reaction was 1.5 mM. The  
117 amplification programme consisted of an initial 15 min step at 95 °C, followed by 40 cycles with 94  
118 °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  
119 performed, followed by chilling to 8 °C. RNA isolated from human astrovirus serotype 8 was used  
120 as a positive control in the RT-PCR set-up. Negative controls consisted of RNase/DNase-free water.  
121 All PCR-products were purified by using ExoSAP-IT® (GE Healthcare Bio-Sciences) according to  
122 the manufacturer's instructions. Sequencing was performed by using the ABI PRISM BigDye  
123 Terminator Cycle Sequencing Ready Reaction kit v1.1 (Applied Biosystems) according to  
124 manufacturer's instructions, and analysed on an ABI PRISM 3130xl Genetic Analyzer (Applied  
125 Biosystems).

126 The sequences obtained from the samples that contained s2m, were most similar to human  
127 astroviruses, and new RT and PCR were therefore performed with a forward consensus primer  
128 designed in the YGDD-motif of the polymerase gene of astrovirus, Astro-YGDD (5'-TTA TGG

129 AGA TGA (C/T)(A/C)G GCT-3'), towards a reverse primer s2m-rev (5'-CCC TCG ATC CTA  
130 CTC GG-3'). The expected size of the PCR products was about 2800 nt. cDNA synthesis was  
131 performed using 0.125  $\mu$ M of a specific primer designed partially in the poly(A)-tail (5'-TTT TTT  
132 TTT TTG CCT AAA CTA-3'), and performed as described above with some modifications to  
133 allow for long-range cDNA synthesis: the amount of SuperScript™ III RT enzyme was raised to  
134 400 U/reaction, and the RT reaction was performed at 55 °C for 60 min, followed by an inactivation  
135 step at 70 °C for 15 min. 2,5  $\mu$ l cDNA was used in a 25  $\mu$ l PCR reaction and amplified by using BD  
136 Advantage 2 Polymerase (BD Biosciences Clontech). The primers used were Astro-YGDD (1.0  
137  $\mu$ M) and s2m-rev (0.5  $\mu$ M). The amplification programme consisted of an initial 1 min step at 95  
138 °C, followed by 35 cycles with 95 °C for 30 s, 60 °C for 20 s and 68 °C for 5 min. A final  
139 elongation step at 68 °C for 5 min was performed, followed by chilling to 8 °C. The PCR products  
140 obtained from this amplification were sequenced and, based on these preliminary results, new  
141 canine astrovirus specific forward primer could be designed about 60 nucleotides downstream the  
142 YGDD motif, HundAstroF2800 (5'-GAT GTT TTT GGA ATG TGG GT-3'), to allow for a more  
143 specific amplification. A new dog long-range cDNA was then successfully amplified using this  
144 primer (0.5  $\mu$ M) as a forward primer towards s2m-rev in a similar amplification as described above  
145 using BD Advantage 2 Polymerase, but the annealing temperature was decreased to 55 °C. The new-  
146 amplified PCR products were then sequenced, and new primers were designed and used for  
147 sequencing of this PCR product in a primer-walking strategy.

148 Software used for sequence analysis and phylogeny were Sequencher version 4.1.4 (Gene Codes  
149 Corporation; <http://www.genecodes.com>), FASTA similarity search and CLUSTALW Multiple  
150 Sequence Alignment Program (<http://www.ebi.ac.uk>), and MEGA version 3.1 (Kumar et al., 2004,  
151 <http://megasoftware.net>). Topology of the phylogenetic trees obtained was then compared to the  
152 topology of the trees generated with Bayesian methods. In detail, the selection of the most  
153 appropriate model of molecular evolution was obtained using the Akaike information criterion

154 implemented in the computer program ModelTest vers 3.7. Subsequently, Bayesian methods  
155 implemented with the computer program MrBayes vers. 3.1.1 were applied to generate the  
156 dendrograms and to assess statistical supports for the branches. ORF analyses, and protein domain  
157 predictions were performed using JustBio (<http://www.justbio.com>), PSORT II prediction  
158 (<http://psort.nibb.ac.jp/form2.html>), TMHMM server version 2  
159 (<http://www.cbs.dtu.dk/services/TMHMM/>) and HMMTOP  
160 (<http://www.enzim.hu/hmmtop/index.html>).

161

## 162 **Results**

### 163 *Case history and laboratory results*

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

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

178 Astrovirus-positive samples by EM were submitted for virus isolation. Growth of astrovirus-like  
179 particles was not detected by EM in the cell cultures used in this study. In one sample (no. 8)  
180 rotavirus was isolated and identified by EM after two passages in cell cultures displaying CPE  
181 (Table 1).

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

#### 185 *RT-PCR and sequencing*

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

203 is available for the feline or porcine astroviruses in the part of the polymerase sequenced for the  
204 present work.

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

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

213

#### 214 **Discussion and conclusions**

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

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

228 In some mammalian species, the detection of astroviruses in clinical samples, such as faeces, is  
229 considered a common finding. In humans, these viruses are considered one of the most common  
230 causes of diarrhoea in children (Walter et al., 2003). Very recently, astrovirus detection rates up to  
231 100% have been reported in insectivorous bats in Hong Kong (Chu et al., 2008). On the contrary, in  
232 other species such as dogs or cats, their detection appears very sporadic. Whether this is due to a  
233 true lower prevalence or to the fact that they are simply under-detected remains questionable and  
234 further analysis should be conducted to increase our understanding of the epidemiology of this  
235 infection in domestic carnivores.

236 The canine astrovirus displayed some unique genomic features, including a possible upstream start  
237 for the capsid precursor protein. None of the possible start codons for the capsid precursor were in  
238 an optimal context for translation initiation as proposed by Kozak (1989), as they both lack a purine  
239 at position -3, but the most upstream codon did have a context most resembling the consensus  
240 vertebrate sequence around start codons GCCRCCAUGG (Kozak 1989). The canine astrovirus  
241 genome, however, displayed the very conserved region right upstream of the mamastrovirus-  
242 homologous start codon, that could be involved in subgenomic RNA transcription (Jonassen et al.,  
243 2003), and it is therefore not clear if a subgenomic RNA spanning the first initiation codon is  
244 synthesised. However, the fact that a functional domain could be predicted for the additional N-  
245 terminal peptide could suggest that longer transcripts actually are translated. The role of the  
246 transmembrane domain and membrane localisation of the resulting protein would still need to be  
247 elucidated.

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

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

255

## 256 **Acknowledgment**

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260

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262

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- 318

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

321

Sample	Virus observed by EM <sup>1</sup>	Virus isolation	RT-PCR for Astrovirus <sup>3</sup>
3	Astrovirus-like particles	None	Positive
6	Parvovirus-like and Astrovirus-like particles	None	Positive
8	Astrovirus-like particles	Rotavirus <sup>2</sup>	Positive
11	Astrovirus-like particles	None	Positive

322 Legend

323 <sup>1</sup>: Observation by electron microscopy performed directly on faecal samples

324 <sup>2</sup>: Identification made by EM on MDCK cell with cytopathic effect after two blind passages

325 <sup>3</sup>: RT-PCR analysis performed on faecal samples with AV12 and s2m-core primers

326

327

328

329 **Figure 1:** Electron microscopy picture of astrovirus (larger particles of 18-20 nm) and parvovirus  
330 (smaller particles of 27-30 nm) observed directly in faecal samples of puppies n. 6. (36,000 Kx).  
331 The characteristic “star-like appearance” of astrovirus particles is evident compared to the round  
332 shaped morphology of parvovirus. Negative staining was obtained with 2% sodium  
333 phosphotungstate solution.

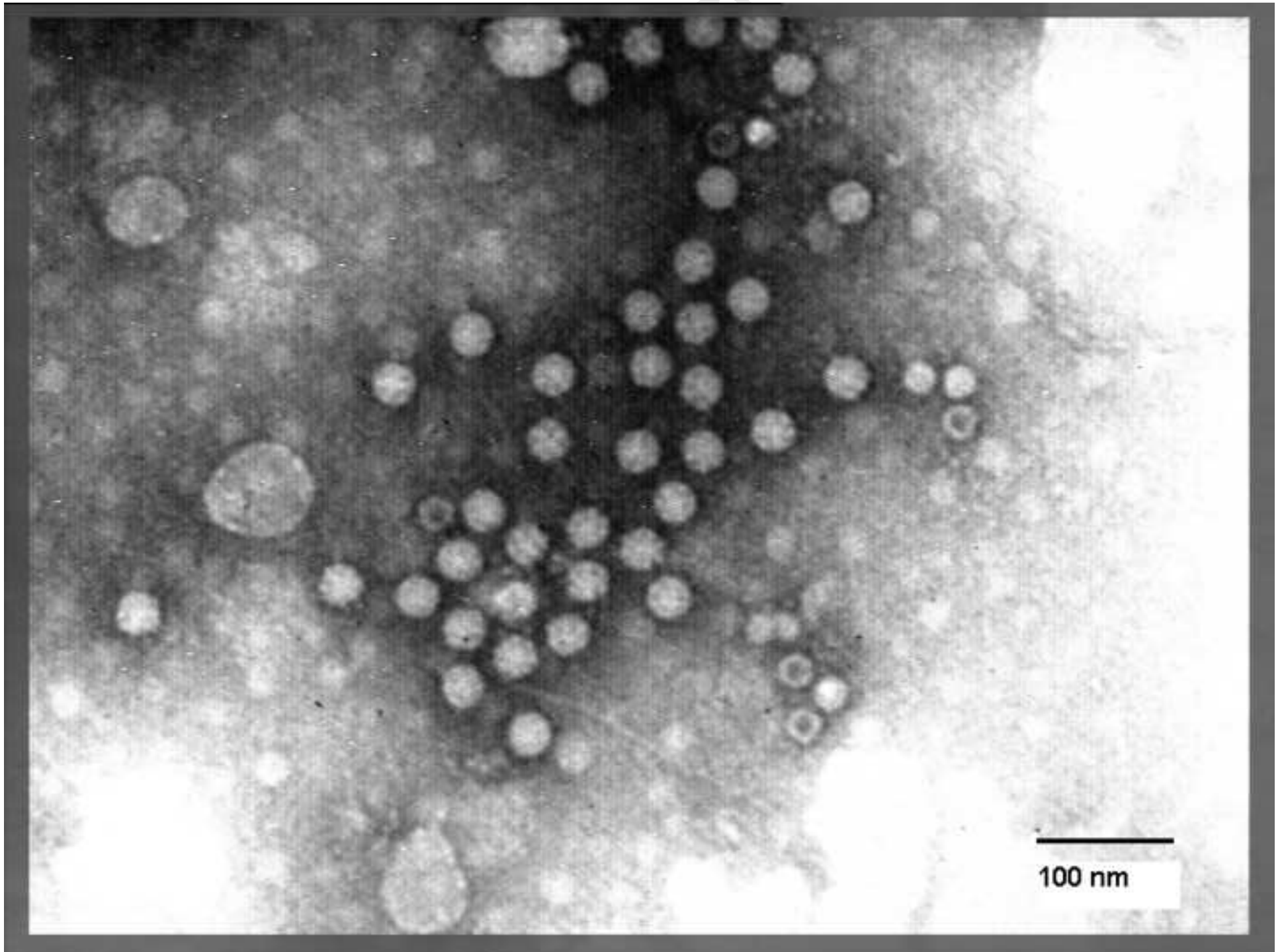
334

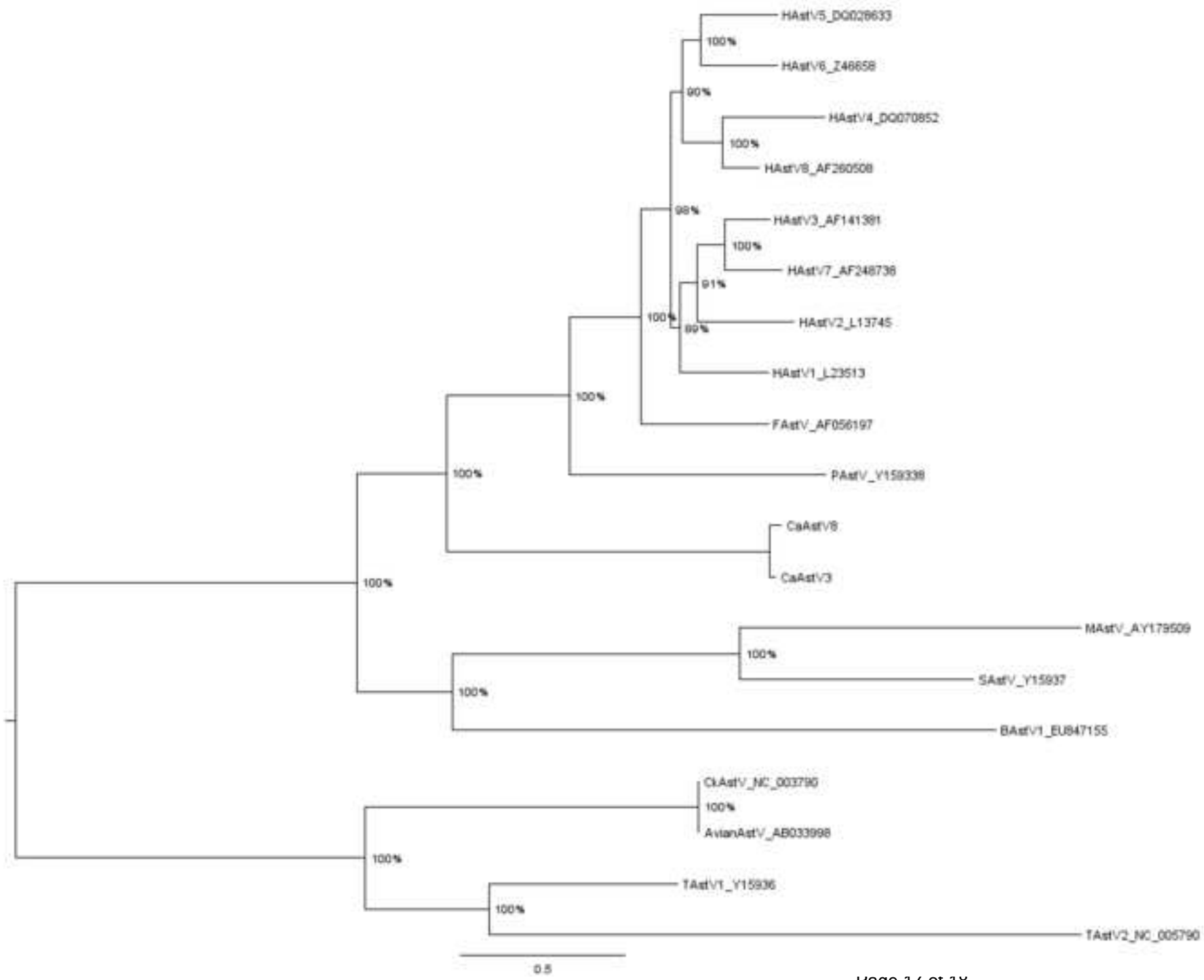
335 **Figure 2.** Phylogenetic tree constructed by Bayesian analysis of the nucleotide sequences of ORF2.  
336 Posterior probabilities of the clades are indicated at the nodes. In this figure, Bat Astrovirus  
337 sequence is identified as BAstV; Sheep Astrovirus as SAstV; Mink Astrovirus as MAstV; Feline  
338 Astrovirus FAstV, Human Astroviruses as HAstV; Porcine Astrovirus as PAstV; Canine  
339 Astroviruses as CaAstV; Turkey Astrovirus type I and II as TAstV1 and TAstV2, respectively.  
340 GenBank accession numbers are indicated.

341

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

347





1 - GGCTTTACTGTAGATGAGAATCTTGAACCAATACCTACACAACCTGACAAATTGATGGCC - 60  
- G F T V D E N L E P I P T Q P D K L M A  
- A L L \* M R I L N Q Y L H N L T N \* W P

61 - TCACTTCTCAAACCAGCATCCAAACTTCCGGATCTTGAATCACTCC**ATGGGAAACTCCTG** - 120  
- S L L K P A S K L P D L E S L H G K L L  
- H F S N Q H P N F R I L N H S **M G N S C**

121 - TGCTATCAGCTCCTCTCGGCTTTCCTACCTGAGGAACACCCTTTTAAGGTGTATGTCGAG - 180  
- C Y Q L L S A F L P E E H P F K V Y V E  
- **A I S S S R L S Y L R N T L L R C M S R** -

181 - AGTTGTCTGGCTGCCACTAGCAGGCAGCTTCGTGATTCTGGCTTACCCACCAGATTCA - 240  
- S C L A A T S R Q L R D S G L P T R F T -  
- **V V W L P L A G S F V I L A Y P P D S Q** -

241 - GAAGAGCAAATGCATCGCATATGGAGGGGAGGACCAAAAAATTGCG**ATGGCTAGCAAGCC** - 300  
- E E Q M H R I W R G G P K N C D G \* Q A -  
- **K S K C I A Y G G E D Q K I A M A S K P** -