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2 **in the Hunan province, China**

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ABSTRACT

Astroviruses (AstV) can infect a variety of hosts including mammalian and avian species and are commonly associated with enteric infections. Recently mammalian AstVs have been linked to extra-intestinal manifestations, including neurologic disorders in humans, cattle and minks, demonstrating a zoonotic potential. Until now, five porcine AstV (PAstV) genotypes have been identified, with PAstV1, PAstV2, PAstV3 and PAstV5 implicated in cross-species transmission. The knowledge on PAstV epidemiology in China is still limited. In this study, two duplex differential RT-PCRs were developed to investigate the distribution and prevalence of PAstV1, PAstV2, PAstV4 and PAstV5. Two-hundred-eighteen samples were collected from 33 farms and pigs with known diarrhea status in nine regions of the Hunan province in China. Specifically, 126 small intestines, 51 fecal swabs, 20 lungs, 19 spleens and two kidneys were obtained. PAstVs were detected in all nine regions and in 81.8% (27/33) of the investigated pig farms. The overall PAstV prevalence was 46.3% (101/218), with PAstV5 as the predominant type with a positive rate of 24.8% (54/218). The prevalence of PAstV4, PAstV1 and PAstV2 was 16.1% (35/218), 14.7% (32/218) and 10.1% (22/218), respectively. Besides being present in intestines and fecal swabs, PAstV RNA was also detected in lungs, spleens and kidneys. Sequencing revealed a high genetic divergence within each genotype and a higher positive rate of PAstV5 was associated with pigs with diarrhea compared to pigs without diarrhea. This study discovered PAstV4 circulating in China for the first time, and revealed PAstV5 as the dominant genotype in pig herds in the Hunan provincein China.

Keywords: Porcine astrovirus; Prevalence; Phylogenetic analysis; China; Pigs.

Introduction

Astroviruses (AstV), which belong to family *Astroviridae,* are small, non-enveloped, positive-sense single-stranded RNA viruses, with genomes of 6.4 to 7.7 kb in length, which are characterized by five- or six-pointed star-like surfaces [3]. Two genera have been defined, namely *Avastrovirus* infecting birds and *Mamastrovirus* infecting mammals [3]. Infection with *Avastrovirus* species often involves intestinal or extra-intestinal manifestations (e.g. damage to the liver, kidney, or the immune system) while infection with *Mamastrovirus* is predominantly associated with gastroenteritis [3]. Recently, member of the *Mamastrovirus* genus have been associated with extra-intestinal manifestations [7, 9, 20, 24, 25], including encephalitis in cattle [16, 26, 27] and a shaking syndrome in mink [1]. These findings may indicate that the clinical significance of AstVs in mammals is increasing.

Porcine astrovirus (PAstV) was first identified by electron microscopy in 1980 [4] and was isolated in 1990 [30]. Since then, PAstVs have been identified worldwide, including Africa, Asia, North America, and South America [10, 14, 33, 35]. Five genetically distinct genotypes (PAstV1 to PAstV5) have been defined with different prevalence and were identified in pig regardless of clinical signs [13, 28, 33]. It has been suggested that PAstV1, PAstV2, PAstV3 and PAstV5 may have crossed host species previously [21, 33]. In China, the knowledge for PAstV is still limited, with only four studies reporting the identification and distribution of PAstV. Specifically, a PAstV sequence belonging to PAstV2 (HQ647383) has been identified in domestic pigs with diarrhea in 2009 [12], a PAstV1 (GQ914773) strain was described in healthy domestic pigs in 2008 [29], and a PAstV2 (KP747573) sequence and a PAstV5 (KP747574) sequence were obtained from healthy domestic piglets [15]. Very

3

69   recently, several PAstV2 and PAstV5 sequences were identified in domestic pigs or wild

70   boars in the Sichuan province, China [8]. However, generally, the PAstV genetic diversity

71   and prevalence of different PAstV genotypes in China is still not well understood.

72   In the present study, we report the discovery of PAstV4 in China and the prevalence of

73   PAstV1, PAstV2, PAstV4 and PAstV5 in domestic pigs from Hunan province, China.

74

75   **Materials and methods**

76   **Sample collection**

77   Two-hundred-and-eighteen independent samples were randomly collected from

78   January to August of 2014 and included 126 small intestines, 51 fecal swabs, 20 lung tissues,

79   19 spleens and two kidneys. The majority of the samples were from diagnostic case

80   submissions to the College of Veterinary Medicine of Hunan Agricultural University.

81   Samples were included based on availability at the time of sample collection resulting in

82   limited numbers of lung, spleen and kidney samples compared to small intestines and fecal

83   swabs. To avoid possible cross-contaminations, samples were collected with sterile

84   instruments and whenever possible on different days. The samples came from 33 pig farms

85   located in nine regions/cities of the Hunan province, China, including Changsha, Hengyang,

86   Yiyang, Changde, Xiangtan, Chenzhou, Loudi, Zhuzhou and Yueyang. Among the samples,

87   98 were from pigs with a history of diarrhea, and the remaining 120 samples were from pigs

88   without clinical signs of diarrhea. The age of the pigs sampled ranged from 7 to 60 days. The

89   samples were shipped on ice and kept in a -80 ºC freezer until use.

90

91   **Sample processing and viral RNA extraction**

92   After re-suspending the fecal swabs in 1 ml sterile PBS, they were centrifuged at 4000

93   rpm for 10 min, and 0.2 ml of the suspension was transferred into a 2 ml Eppendorf tube and

94   used for viral RNA extraction. For tissue samples, approximately 0.1 gram of each the sample

95   was placed into a 2 ml Eppendorf tube containing four 1.5 mm steel balls and were grinding

96   using the Mixer Mill MM 400 (Retsch, Germany) for 1 min. Then 1 ml TRIzol reagent

97   (Invitrogen^{TM}) was added into the 2 ml tubes and RNA was extracted according to the

98   manufacturer's instructions. The viral cDNA was synthesized by using the Transcriptor First

99   Strand cDNA Synthesis Kit (Roche^{TM}) and random primers as described by the manufacturer.

100 The obtained viral cDNA were stored at -80 °C freezer.

101

102 **Development of two duplex differential RT-PCR assays for detecting PAstVs**

103 Based on the alignments of the available genome sequences of PAstV, four pairs of

104 primers were designed within conserved regions aiming to amplify PAstV1 and PAstV2 and

105 PAstV4 and PAstV5 (Table 1). As the prevalence of PAstV3 has been reported to be very low,

106 only one genome of PAstV3 (JX556691) is available in GenBank, and as the genetic

107 heterogeneity within PAstV genotypes is large [33], the prevalence of PAstV3 was not

108 investigated in the present study. Initially, single-plex RT-PCR assays were designed and

109 tested and once optimized two duplex differential RT-PCRs with the same primer pairs

110 targeting PAstV1-2 and PAstV4-5 were developed. The PCR assays were carried out in a 30

111 μl total reaction volume containing15 μl PCR mix (TIANGEN, Beijing, China), 0.5 μl of 10

112 mM of each of the primers, 2 μl cDNA, and nuclease-free H$_2$O. The amplification reactions

113 were performed under the following conditions: 2 min at 94°C, 35 cycles of 30 s at 94°C, 20 s

114   at 57°C and 30 s at 72°C, followed by 72 °C for 7 min. The PCR products were then checked

115   on a 0.8 % agarose gel under UV light, and the samples with positive results were recorded.

116   The sensitivity of the two duplex differential RT-PCR was evaluated by serial dilution

117   of the cDNA used in the single-plex PCR. The specificity of the primers was confirmed by

118   BLAST analysis, and in addition, positive controls for other swine RNA viruses including

119   porcine transmissible gastroenteritis virus (TGEV), porcine reproductive and respiratory

120   syndrome virus (PRRSV), porcine epidemic diarrhea virus (PEDV) and classical swine fever

121   virus (CSFV) were tested with the two duplex differential RT-PCR assays.

122

123   **DNA sequencing and sequence analysis**

124   Four pairs of primers, located in open reading frame (ORF) 1b or ORF2, were

125   designed to amplify the partial genome sequences of the four PAstV genotypes. The two pairs

126   of primers used for PAstV1 and PAstV2 were the same as the single and duplex differential

127   RT-PCR reactions (Table 2). The PCR products were checked under UV light, gel purified by

128   QIAquick Gel Extraction Kit (QIAGEN Inc.), and the purified products were sequenced

129   directly using the forward and reverse primers. The obtained sequences were analyzed by

130   DNAMAN 7.0 (Lynnon Corporation) and the phylogenetic analysis was performed by the

131   Neighbor-Joining method with the p-distance model through MEGA 6.0 [31].

132

133   *Statistical analysis*

134   Differences in prevalence were investigated by chi-square tests using the JMP®

135   Pro12.0.1 software (SAS, Cary, NC, USA). A *P* value less than 0.05 was considered

6

136   significant.

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138   **GenBank accession numbers**

139   The nucleotide sequences obtained in the present study were deposited in GenBank

140   under the following accession numbers: KY047664 - KY047762

141

142   **Results**

143   **Assay validation**

144   The sensitivity of the duplex differential RT-PCR assays was comparable to the

145   single-plex RT-PCR assays, and the specificity of both duplex-RT-PCR assays and the four

146   single-plex RT-PCR assays was 100% as viral RNA of TGEV, PRRSV, PEDV and CSFV

147   was not detected (data not shown). Examples of the bands obtained with the two duplex

148   differential RT-PCRs separated by agar gel electrophoreses are showed in Fig. 1.

149

150   **Prevalence of PAstVs**

151   PAstV was detected on 27 of the 33 (81.8%) farms investigated, and all of the nine

152   regions in the Hunan province were positive, with an overall PAstV positive rate of 46.3%

153   (101/218) (Table 3). All of the four investigated PAstV genotypes were discovered in the

154   Hunan province, with PAstV5 having the highest prevalence of 24.8% (54/218), followed by

155   PAstV4 with a prevalence of 16.1% (35/218). PAstV1 and PAstV2 showed low positive rates

156   of 14.7% (32/218) and 10.1% (22/218), respectively.

157   When the PAstV prevalence was compared in pigs with or without enteric signs, it was

158   was 57.1% (56/98) in pigs with diarrhea compared to 37.5% (45/120) in pigs without diarrhea

159   which was significantly ($P < 0.05$) different (Table 4). Furthermore, when genotype

160   prevalence rates were compared, PAstV5 had a significantly ($P < 0.05$) higher infection rate in

161   pigs with enteric signs compared to pigs without diarrhea, while clinical status of a pig did not

162   affect the prevalence of PAstV1, PAstV2 and PAstV4 (Table 4). The sample types with the

163   highest infection rate were small intestines (52.4%) and fecal swabs (49%) (Table 5). PAstV

164   was also detected in lungs, spleens and kidneys with a considerable high rate (Table 5), which

165   may indicate that PAstVs could have a wide tissue tropism not limited to the intestine.

166   However, due to the limited sample size for extra-enteric sample types, results need to be

167   interpreted with caution and additional studies to further confirm the obtained results are

168   needed. None of the lung tissues, spleen tissues and kidneys used in this study was obtained

169   from pigs with a history of diarrhea.

170

171   **Molecular and phylogenetic analysis**

172   Sequencing was successful on 16 PAsV1 PCR products, 12 PAsTV2 PCR products, 32

173   PAstV4 PCR products and 39 PAstV5 PCR products, and the p-distances between the

174   sequences within the same genotypes were calculated. For PAstV1, the obtained 16 sequences

175   corresponded to five geographic regions/cities, with the identities between them ranging from

176   90.4% to 100%. All showed a closer relationship to the GenBank PAstV1 sequence

177   (KF787112) from the Guanxi province, China, with an average homology of 91.4%.

178   Moreover, in the phylogenetic analysis, the present 16 PAstV1 sequences clustered into a

179   single clade, and clustered together with PAstV1 (KF787112) from Guanxi province, China

180   (Fig. 1). Based on the phylogenetic analysis, PAstV1 could be classified into two subclades,

8

181   PAstV1-1 and PAstV1-2, and the sequences obtained in this study clustered all within

182   PAstV1-2. PAstV1-1 includes old PAstV1 sequences identified before 2008 from Japan,

183   Canada and China. Moreover, from the present analysis, PAstV1 showed a close relationship

184   with AstVs recovered from cats, California sea lions, and the classic human AstV (Fig. 2).

185   For PAstV2, the present 12 sequences corresponded to four different regions and

186   showed identities of 98.2%-100% between each other, with an average of 82.1% identity to

187   other available PAstV2 sequences in GenBank. Phylogenetic analysis indicated that the

188   present PAstV2 sequences from the Hunan province clustered into a monophyletic group (Fig.

189   3). Interestingly, AstV sequences recovered from porcupines (KJ571486) and roe deer

190   (HM447046) clustered also with the PAstV2 group, possible indicating a recent cross-species

191   transmission.

192   The 32 PAstV4 sequences were from seven regions of Hunan province with identities

193   of 93.4% -100% between each other. In the phylogenetic analysis, the present sequences were

194   clustered into two subgroups, designated as Hunan-1 and Hunan-2 groups (Fig. 4). The

195   Hunan-1 group showed a close relationship with PAstV4 sequences from Spain, while the

196   Hunan-2 group showed a closer relationship with some Italian PAstV4 strains (Fig. 4).

197   PAstV4 sequences in GenBank have a global distribution and have been identified in Europe,

198   Asia and North America (Fig. 4). Of note, except the PAstV4 sequences identified in the

199   present study, no other PAstV4 sequence have been reported from China to date, although

200   PAstV4 have high prevalence rates in other countries.

201   PAstV5 was detected in all of the nine investigated regions, with genetic identities of

202   92.7% to 100% between each other, and an average identity of 97.5% within the genotype.

203   Moreover, PAstV5 from the Hunan province formed a monophyletic branch in the

204   phylogenetic analysis, and showed a closer relationship to PAstV5 from US pigs than to a

205   PAstV5 sequence (KP747574) previously identified in China (Fig. 5).

206

Discussion

208   The present PAstV overall positive rate of 46.3% (101/218) in domestic pigs from the

209   Chinese Hunan province is much higher than the 17.5 % (21/120) reported recently from the

210   Chinese Sichuan province [8], 19.4% (25/129) from South Korea [14], 20.8% (25/120) from

211   Germany [18], and comparable to 34.2%, (67/196) from the Czech Republic [11], while it is

212   lower than the prevalence rate of 79.2% (76/96) from Canada [17], 89% (81/91) from Croatia

213   [6], 67.4%(163/242) from Italy [19], 100% from both Spain (83/83) and Australia (136/136)

214   [35], 85.7% (36/42) from Hungary [35], and 64% (326/509) from the USA [33]. Moreover, in

215   previous studies, PAstV2 or PAstV4 were reported to be the predominant genotypes in

216   domestic pigs [6, 14, 17, 19, 33, 35], while in the present investigation, PAstV5 was firstly

217   revealed to be the predominant genotype (24.8%,54/218) followed by PAstV4 (16.1%,

218   35/218). This is also different compared to previous investigations in the Sichuan province,

219   China, which indicated prevalence rates of 10% (12/120) for PAstV2 and 7.5% (9/120) for

220   PAstV5 [8]. The different results may be due to differences in the efficacy of different

221   primers used; moreover, PAstV has a large genetic divergence between genotypes but also

222   within each genotype, which makes it is difficult to design universal primers (including

223   degenerate primers) suitable for all know or unknown PAstV strains. On the other hand, the

224   distribution and prevalence of PAstV genotypes may be different among regions or countries.

225   Under experimental conditions using caesarian-derived, colostrum-deprived 4-day-old

226   pigs, PAstV1 infection could be associated with mild diarrhea [30]. However, any association

227   of the other four PAstV genotypes with clinic manifestations in pigs remains undetermined. It

228   has been reported that diarrheic pigs had a higher viral load of PAstV4 in nursery and

229   growing-finishing groups [35]. In the present study, PAstVs and more specifically PAstV5

230   had a significantly higher prevalence in pigs with diarrhea compared to pigs without diarrhea.

231   This is in contrast to most other studies which reported no significant association of PAstV

232   infection and diarrhea [5, 17, 19, 28]. Further virus isolation and pathogenicity studies are

233   needed to clarify the importance of this group of viruses in pigs.

234   Many mammalian AstV have been detected in extra-enteric tissues. Murine AstVs has

235   been detected in the spleen, liver, kidney and mesenteric lymph nodes [22, 34]. Novel bovine

236   and mink AstVs have been discovered in brain tissues [1, 16, 32], and novel human AstVs

237   have been discovered in brain tissues, serum samples, cerebrospinal fluid and urine [24, 25].

238   PAstV RNA could be detected in the blood of healthy pigs [5] and in nasal swabs from pigs

239   with acute respiratory symptoms [23]. In the present study, PAstV has been detected in spleen,

240   lung and kidney tissues. However, due to the limited number of extra-enteric samples

241   available for this study the obtained results require confirmation in additional studies which

242   should include larger numbers of pigs and a defined standard set of samples from each pig.

243   Nevertheless, these data further confirm that mammalian AstV have a wide tissue tropism and

244   are not restricted to enteric tissues. A potential role of PAstV in extra-enteric diseases

245   including neurologic disorder has been suggested previously [2].

246   In conclusion, the present study demonstrates the presence of PAstV1, PAstV2,

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247 PAstV4 and PAstV5 in domestic pigs located in the Hunan province, China. To our

248 knowledge, this is the first description of PAstV4 in Chinese pigs. Furthermore, the PAstV

249 genotype distribution in China appears to be substantially different compared to other

250 geographic regions with a high prevalence of PAstV5 in domestic pig herds instead of

251 PAstV2 or PAstV4. Within the PAstV genotypes from the Hunan province a high genetic

252 diversity has also been revealed.

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260 **Conflict of interest**

261 The authors declare that they have no conflict of interest.

262

263 **Compliance with Ethical Standards**

264 The experiments were approved and carried out in accordance with animal ethics guidelines

265 and approved protocols of the Hunan Agricultural University.

266
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Table 1. Primer information for porcine astrovirus (PAstV) detecting single or duplex RT-PCR assays.

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Genotype	Primer	Sequence (5' →3')	Position	Reference sequence	Size
PAstV1	AV1-F	5`-TCCTGTGCTATCAGTTGCTCTC-3`	4032-4053	GQ914773	
	AV1-R	5`-GATTGCTGGTTTTGGACCTGTG-3`	4430-4451		420
PAstV2	AV2-F	5`-AGCAGCTGGATCGTCTTTGGA-3`	3933-3953	JX556690	
	AV2-R	5`-AGATTCAGCATCCCAGGTTGTT-3`	4738-4759		827
PAstV4	AV4-F	5`-TGGCTTCAGGCCTTTGAGTTTT-3`	3588-3609	JX556692	
	AV4-R	5`-CACCGTCGTAGTAGTCGTGAC-3`	4126-4146		559
PAstV5	AV5-F	5`-TGGTACGTRCACAATCTGTTGAA-3`	3562-3584	JX556693	
	AV5-R	5`-TCAGTGTCTTCCCAACCRTC-3`	3724-3743		182

383

384 **Table 2.** Primers used to sequence porcine astroviruses (PAstV).

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Genotype	Sequence (5' →3')	Location	Position	Reference sequence	Expected size
PAstV4	F:GTCTATGGRGACGACAGATTGAC	ORF 1b	3660-3682	JX556692	425 bp
	R:TTATGCTTTGGTCCGCCCCTC	ORF 1b	4064-4084		
PAstV5	F:ACCAACTTCCCTCCCGACCC	ORF 1b	3778-3797	JX556693	368 bp
	R:TACGACAAGATCCTATCTGAAAAG	ORF2	4122-4145		

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389 **Table 3.** Porcine astrovirus (PAstV) genotypes detected in different regions/cities of the

390 Hunan province, China.

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Regions	PAstV1	PAstV2	PAstV4	PAstV5	Co-infection	Overall
Changde	1/6 (16.6%)	0/6 (0%)	0/6 (0%)	2/6 (33.3%)	1/6 (16.6%)	2/6 (33.3%)
Changsha	8/85 (9.4%)	12/85 (14.1%)	21/85 (24.7%)	23/85 (27.1%)	18/85(21.2%)	46/85 (54.1%)
Xiangtan	4/22 (18.2%)	0/22 (0%)	4/22 (18.2%)	7/22 (31.8%)	5/22(22.7%)	10/22 (45.5%)
Chenzhou	2/19 (10.5%)	1/19 (5.3%)	2/19 (10.5%)	5/19 (26.3%)	2/19 (10.5%)	8/19 (42.1%)
Hengyang	8/39 (20.5%)	3/39 (7.7%)	4/39 (10.3%)	6/39 (15.4%)	8/39 (20.5%)	13/39 (33.3%)
Loudi	0/4 (0%)	1/4 (25%)	0/4 (0%)	2/4 (50%)	0/4 (0%)	3/4(75%)
Zhuzhou	3/15 (13.3%)	3/15 (20%)	1/15 (6.7%)	2/15 (13.3%)	3/15 (13.3%)	6/16 (40%)
Yiyang	2/16 (12.5%)	2/16 (12.5%)	1/16 (6.3%)	3/16 (18.7%)	4/16 (25%)	4/16 (25%)
Yueyang	4/12 (33.3%)	0/12 (0%)	2/12 (16.7%)	4/12 (33.3%)	1/12(8.3%)	9/12 (75%)
Total	32/218 (14.7%)	22/218 (10.1%)	35/218 (16.1%)	54/218 (24.8%)	42/218(19.3%)	101/218（46.3%）

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395 **Table 4.** Porcine astrovirus (PAstV) infection rate by genotype in pigs with a history of

396 diarrhea or without diarrhea.

Disease Status	PAstV1	PAstV2	PAstV4	PAstV5	Overall
No diarrhea	15.8% (19/120)	11.7% (14/120)	14.2% (17/120)	20% (24/120)	37.5% (45/120)
Diarrhea	12.2% (12/98)	8.2% (8/98)	18.4% (18/98)	31.6% (31/98)	57.1% (56/98)

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398

**Table 5.** Infection rate of different porcine astroviruses (PAstV) genotypes in selected samples types.

| Sample type	PAstV1	PAstV2	PAstV4	PAstV5	Overall
Small intestine	12.7% (16/126)	9.5% (12/126)	15.9% (20/126)	29.4% (37/126)	52.4% (66/126)
Fecal swab	17.6% (9/51)	7.8% (4/51)	25.5% (13/51)	27.5% (14/51)	49% (25/51)
Spleen	5.3% (1/19)	10.5% (2/19)	5.3% (1/19)	15.8% (3/19)	15.8% (3/19)
Lung	20% (4/20)	20% (4/20)	0% (0/20)	5% (1/20)	25% (5/20)
Kidney	50% (1/2)	50% (1/2)	50% (1/2)	0%	100% (2/2)

Figure legends
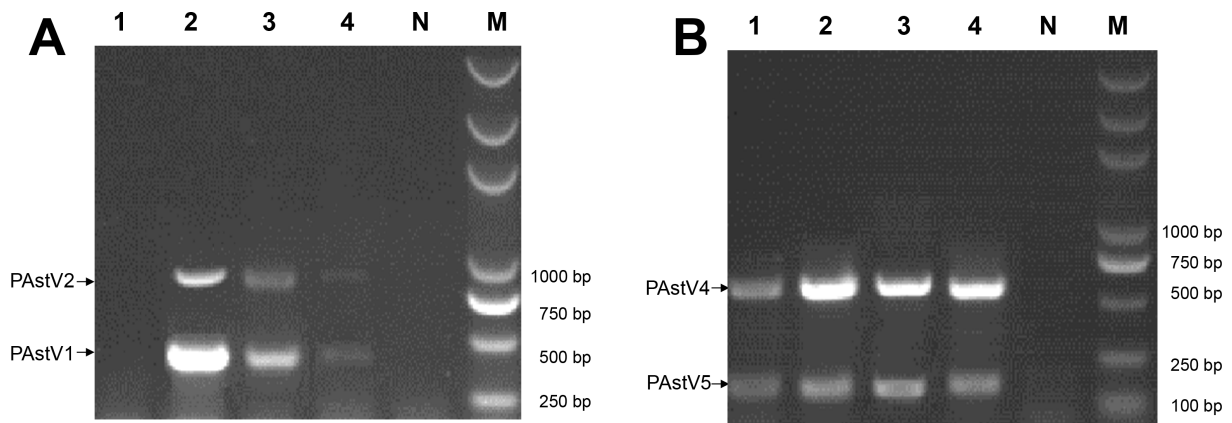
Fig.1 Gel-based electrophoresis of several selected samples to demonstrate the duplex differential RT-PCR outcome. The PCR products were separated on a 0.8 % agarose gel. (A) Duplex differential RT-PCR for PAstV1 (420bp) and PAstV2 (827bp). Lanes 1-4, selected clinic samples, lane 1 (negative), lanes 2-4 (positive for both PAstV1 and PAstV2); N, negative control; M, DNA molecular marker. (B) Duplex differential RT-PCR for PAstV4 (559bp) and PAstV5 (182bp). Lanes 1-4, selected clinical samples, positive for both PAstV4 and PAstV5; N, negative control; M, DNA molecular marker.
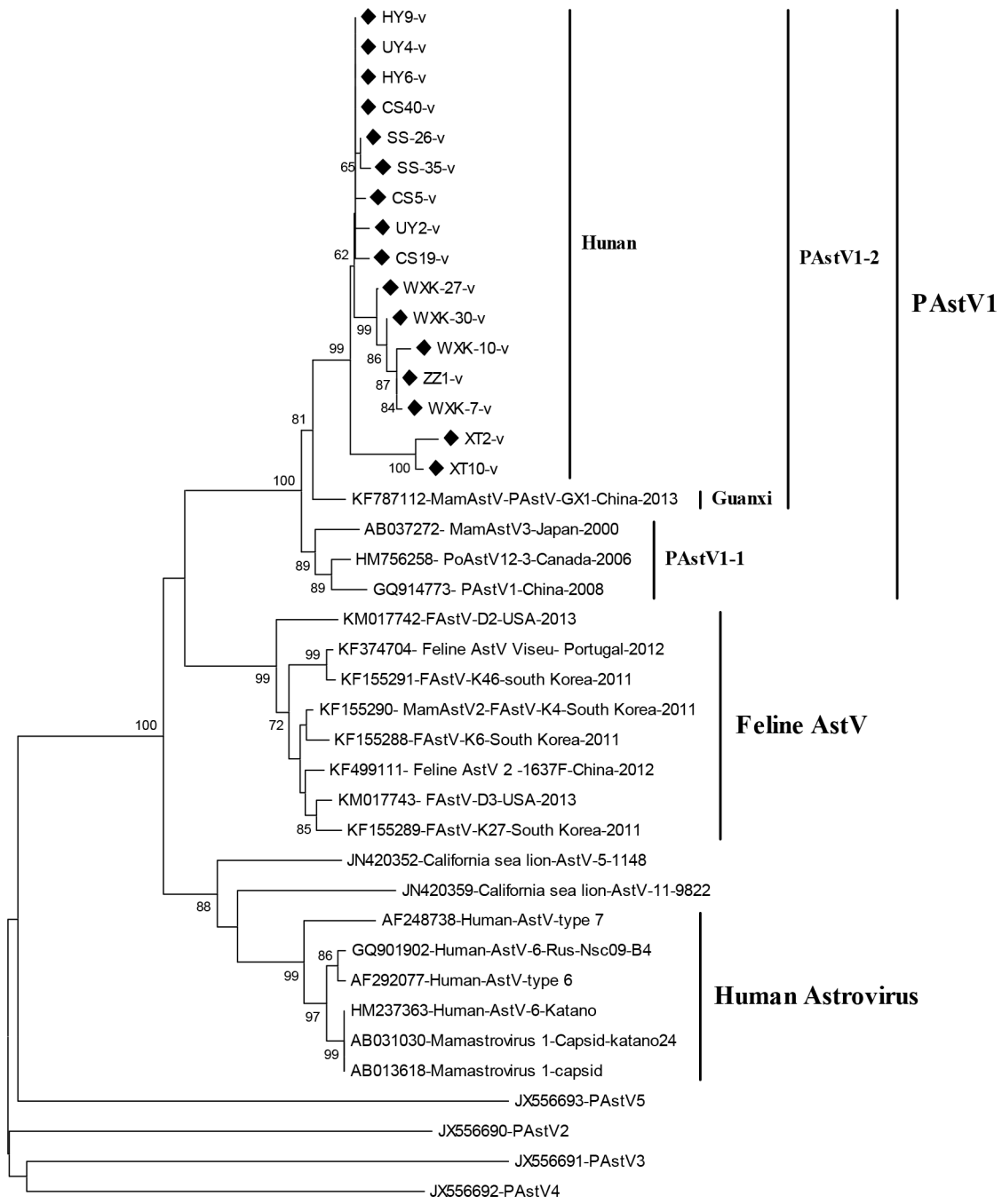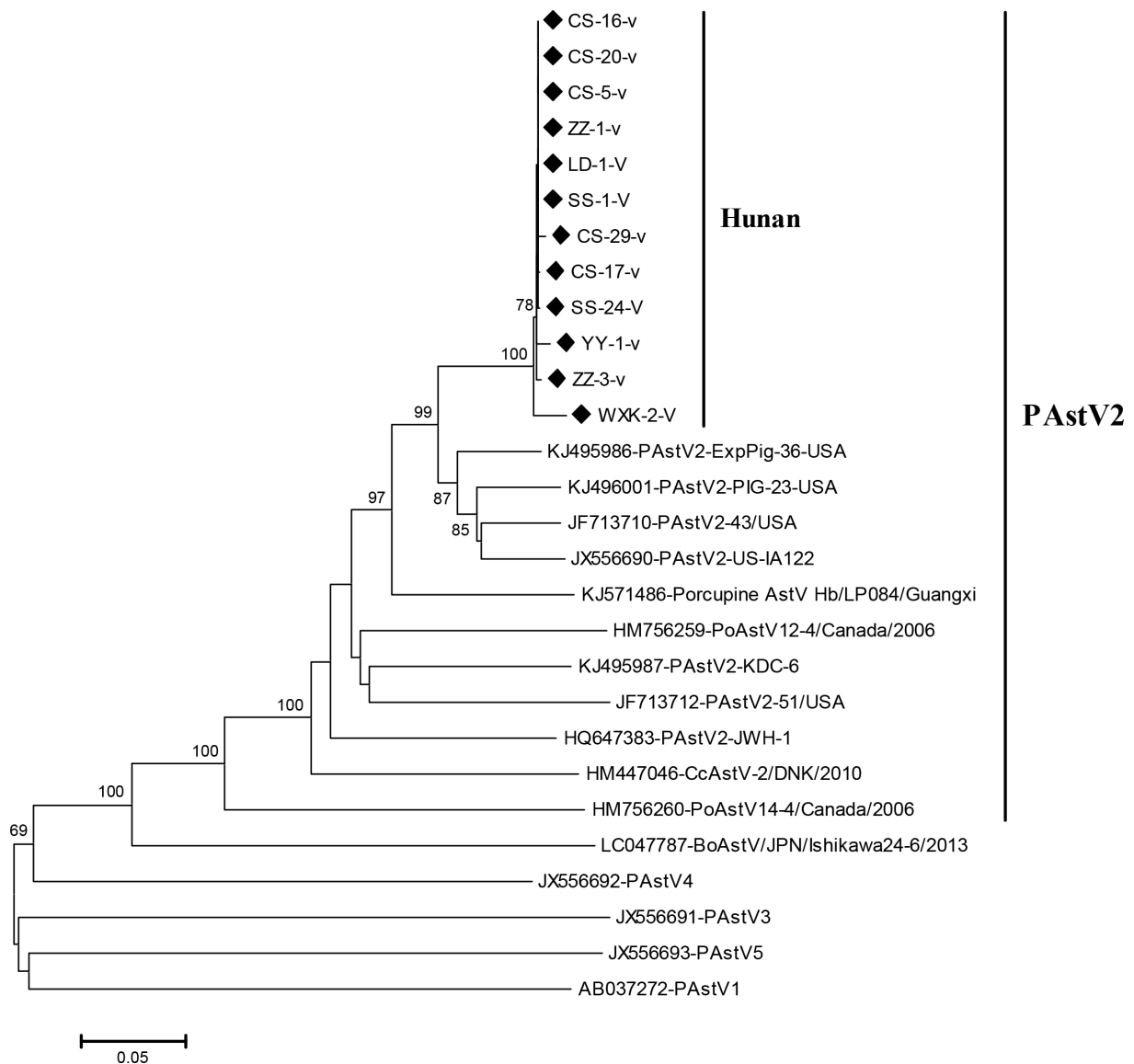
Fig. 2 Phylogenetic analysis of PAstV1 based on the 16 sequences identified in the present

study and 20 additional PAstV1 sequences obtained through GenBank. The reference

sequences of PAstV2 to PAstV5 were included for comparison. The evolutionary tree was

inferred using the Neighbor-Joining method with the p-distance model. The percentage of

replicate trees in which the associated sequences clustered together in the bootstrap test (1000

420 replicates) are shown next to the branches (only >60% was shown). The tree is drawn to scale,

421 with branch lengths in the same units as those of the evolutionary distances used to infer the

422 phylogenetic tree. All positions containing gaps and missing data were eliminated. There were

423 a total of 304 positions in the final dataset. The PAstV1 strains identified presently were

424 indicated with black diamonds. The evolutionary analyses were conducted in MEGA6.

425

426 **Fig. 3** Phylogenetic analysis of PAstV2 based on the 12 sequences identified in the present

427 study and nine additional PAstV2 sequences obtained through GenBank. The reference

428 sequences of PAstV1, PAstV3, PAstV4, PAstV5 and AstV sequences obtained from porcupine,
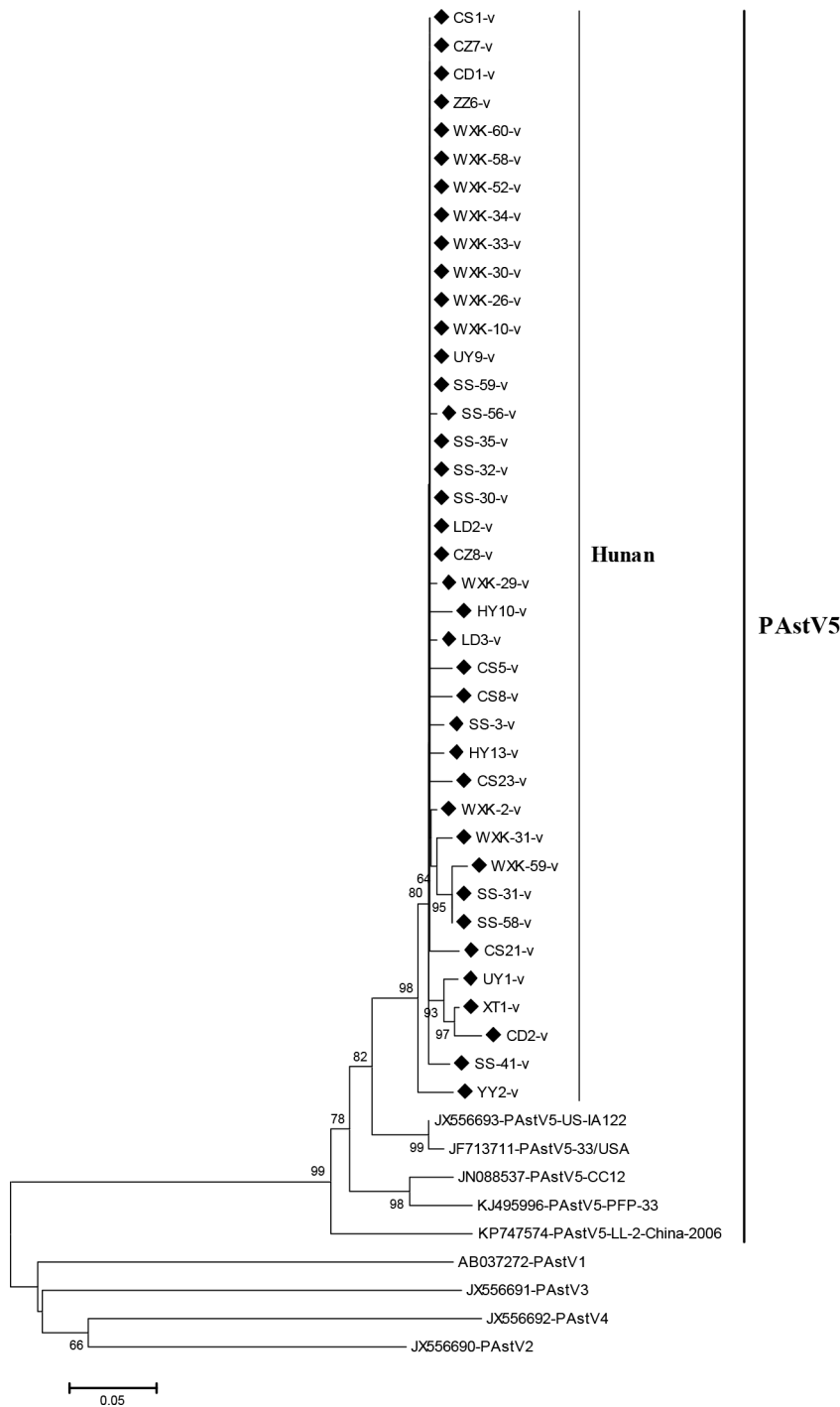
429   roe deer, and cattle were included for comparison. The evolutionary tree was inferred using

430   the Neighbor-Joining method with the p-distance model. The percentage of replicate trees in

431   which the associated sequences clustered together in the bootstrap test (1000 replicates) are

432   shown next to the branches (only >60% was shown). The tree is drawn to scale, with branch

433   lengths in the same units as those of the evolutionary distances used to infer the phylogenetic

434   tree. All positions containing gaps and missing data were eliminated. There were a total of

435   748 positions in the final dataset. The PAstV2 strains identified presently are indicated by

436   black diamonds. The evolutionary analyses were conducted in MEGA6.

437

Fig. 4 Phylogenetic analysis of PAstV4 based on the 32 PAstV4 sequences obtained in the

439   present study and 61 PAstV4 sequences available through GenBank. The reference sequences

440   of PAstV1, PAstV2, PAstV3 and PAstV5 were included for comparison. The evolutionary tree

441   was inferred using the Neighbor-Joining method with the p-distance model. The percentage of

442   replicate trees in which the associated sequences clustered together in the bootstrap test (1000

443   replicates) are shown next to the branches (only >60% was shown). The tree is drawn to scale,

444   with branch lengths in the same units as those of the evolutionary distances used to infer the

445   phylogenetic tree. All positions containing gaps and missing data were eliminated. There were

446   a total of 189 positions in the final dataset. The PAstV4 strains identified in the present study

447   are indicated by black diamonds. The evolutionary analyses were conducted in MEGA6.

Fig. 5 Phylogenetic analysis of PAstV5 based on the 39 PAstV5 sequences identified in the

present study and five PAstV5 sequences available through GenBank. The reference

sequences of PAstV1, PAstV2, PAstV3 and PAstV4 were included for comparison. The

evolutionary tree was inferred using the Neighbor-Joining method with the p-distance model.

The percentage of replicate trees in which the associated sequences clustered together in the

454   bootstrap test (1000 replicates) are shown next to the branches (only >60% was shown). The

455   tree is drawn to scale, with branch lengths in the same units as those of the evolutionary

456   distances used to infer the phylogenetic tree. All positions containing gaps and missing data

457   were eliminated. There were a total of 229 positions in the final dataset. The PAstV5 strains

458   identified in the present study are indicated with black diamonds. The evolutionary analyses

459   were conducted in MEGA6.