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Chao-Ting Xiao[1,2*], Zhang Luo[2], Sun-Liang Lv[1], Tanja Opriessnig[3,4], Run-Cheng Li[2], Xing-Long Yu[2*]

[1]College of Biology, Hunan University, Changsha, China.

[2]College of Veterinary Medicine, Hunan Agricultural University, Changsha.

[3]The Roslin Institute and The Royal (Dick) School of Veterinary Studies, University of Edinburgh, Midlothian, Scotland, UK.

[4]Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, Iowa, USA.

[*]Corresponding authors: Chao-Ting Xiao, xiaocht@hnu.edu.cn;

Xing-Long Yu, xlyu999@126.com

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

recently, several PAstV2 and PAstV5 sequences were identified in domestic pigs or wild boars in the Sichuan province, China [8]. However, generally, the PAstV genetic diversity and prevalence of different PAstV genotypes in China is still not well understood.

In the present study, we report the discovery of PAstV4 in China and the prevalence of PAstV1, PAstV2, PAstV4 and PAstV5 in domestic pigs from Hunan province, China.

Materials and methods

Sample collection

Two-hundred-and-eighteen independent samples were randomly collected from January to August of 2014 and included 126 small intestines, 51 fecal swabs, 20 lung tissues, 19 spleens and two kidneys. The majority of the samples were from diagnostic case submissions to the College of Veterinary Medicine of Hunan Agricultural University. Samples were included based on availability at the time of sample collection resulting in limited numbers of lung, spleen and kidney samples compared to small intestines and fecal swabs. To avoid possible cross-contaminations, samples were collected with sterile instruments and whenever possible on different days. The samples came from 33 pig farms located in nine regions/cities of the Hunan province, China, including Changsha, Hengyang, Yiyang, Changde, Xiangtan, Chenzhou, Loudi, Zhuzhou and Yueyang. Among the samples, 98 were from pigs with a history of diarrhea, and the remaining 120 samples were from pigs without clinical signs of diarrhea. The age of the pigs sampled ranged from 7 to 60 days. The samples were shipped on ice and kept in a -80 ºC freezer until use.

Sample processing and viral RNA extraction

After re-suspending the fecal swabs in 1 ml sterile PBS, they were centrifuged at 4000 rpm for 10 min, and 0.2 ml of the suspension was transferred into a 2 ml Eppendorf tube and used for viral RNA extraction. For tissue samples, approximately 0.1 gram of each the sample was placed into a 2 ml Eppendorf tube containing four 1.5 mm steel balls and were grinding using the Mixer Mill MM 400 (Retsch, Germany) for 1 min. Then 1 ml TRIzol reagent (Invitrogen^{TM}) was added into the 2 ml tubes and RNA was extracted according to the manufacturer's instructions. The viral cDNA was synthesized by using the Transcriptor First Strand cDNA Synthesis Kit (Roche^{TM}) and random primers as described by the manufacturer. The obtained viral cDNA were stored at -80 °C freezer.

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

Based on the alignments of the available genome sequences of PAstV, four pairs of primers were designed within conserved regions aiming to amplify PAstV1 and PAstV2 and PAstV4 and PAstV5 (Table 1). As the prevalence of PAstV3 has been reported to be very low, only one genome of PAstV3 (JX556691) is available in GenBank, and as the genetic heterogeneity within PAstV genotypes is large [33], the prevalence of PAstV3 was not investigated in the present study. Initially, single-plex RT-PCR assays were designed and tested and once optimized two duplex differential RT-PCRs with the same primer pairs targeting PAstV1-2 and PAstV4-5 were developed. The PCR assays were carried out in a 30 µl total reaction volume containing15 µl PCR mix (TIANGEN, Beijing, China), 0.5 µl of 10 mM of each of the primers, 2 µl cDNA, and nuclease-free H_2O. The amplification reactions were performed under the following conditions: 2 min at 94^{o}C, 35 cycles of 30 s at 94^{o}C, 20 s

at 57°C and 30 s at 72°C, followed by 72 °C for 7 min. The PCR products were then checked on a 0.8 % agarose gel under UV light, and the samples with positive results were recorded.

The sensitivity of the two duplex differential RT-PCR was evaluated by serial dilution of the cDNA used in the single-plex PCR. The specificity of the primers was confirmed by BLAST analysis, and in addition, positive controls for other swine RNA viruses including porcine transmissible gastroenteritis virus (TGEV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine epidemic diarrhea virus (PEDV) and classical swine fever virus (CSFV) were tested with the two duplex differential RT-PCR assays.

DNA sequencing and sequence analysis

Four pairs of primers, located in open reading frame (ORF) 1b or ORF2, were designed to amplify the partial genome sequences of the four PAstV genotypes. The two pairs of primers used for PAstV1 and PAstV2 were the same as the single and duplex differential RT-PCR reactions (Table 2). The PCR products were checked under UV light, gel purified by QIAquick Gel Extraction Kit (QIAGEN Inc.), and the purified products were sequenced directly using the forward and reverse primers. The obtained sequences were analyzed by DNAMAN 7.0 (Lynnon Corporation) and the phylogenetic analysis was performed by the Neighbor-Joining method with the p-distance model through MEGA 6.0 [31].

Statistical analysis

Differences in prevalence were investigated by chi-square tests using the JMP® Pro12.0.1 software (SAS, Cary, NC, USA). A *P* value less than 0.05 was considered

136 significant.

137

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,

PAstV1-1 and PAstV1-2, and the sequences obtained in this study clustered all within PAstV1-2. PAstV1-1 includes old PAstV1 sequences identified before 2008 from Japan, Canada and China. Moreover, from the present analysis, PAstV1 showed a close relationship with AstVs recovered from cats, California sea lions, and the classic human AstV (Fig. 2).

For PAstV2, the present 12 sequences corresponded to four different regions and showed identities of 98.2%-100% between each other, with an average of 82.1% identity to other available PAstV2 sequences in GenBank. Phylogenetic analysis indicated that the present PAstV2 sequences from the Hunan province clustered into a monophyletic group (Fig. 3). Interestingly, AstV sequences recovered from porcupines (KJ571486) and roe deer (HM447046) clustered also with the PAstV2 group, possible indicating a recent cross-species transmission.

The 32 PAstV4 sequences were from seven regions of Hunan province with identities of 93.4% -100% between each other. In the phylogenetic analysis, the present sequences were clustered into two subgroups, designated as Hunan-1 and Hunan-2 groups (Fig. 4). The Hunan-1 group showed a close relationship with PAstV4 sequences from Spain, while the Hunan-2 group showed a closer relationship with some Italian PAstV4 strains (Fig. 4). PAstV4 sequences in GenBank have a global distribution and have been identified in Europe, Asia and North America (Fig. 4). Of note, except the PAstV4 sequences identified in the present study, no other PAstV4 sequence have been reported from China to date, although PAstV4 have high prevalence rates in other countries.

PAstV5 was detected in all of the nine investigated regions, with genetic identities of 92.7% to 100% between each other, and an average identity of 97.5% within the genotype.

Moreover, PAstV5 from the Hunan province formed a monophyletic branch in the phylogenetic analysis, and showed a closer relationship to PAstV5 from US pigs than to a PAstV5 sequence (KP747574) previously identified in China (Fig. 5).

Discussion

The present PAstV overall positive rate of 46.3% (101/218) in domestic pigs from the Chinese Hunan province is much higher than the 17.5 % (21/120) reported recently from the Chinese Sichuan province [8], 19.4% (25/129) from South Korea [14], 20.8% (25/120) from Germany [18], and comparable to 34.2%, (67/196) from the Czech Republic [11], while it is lower than the prevalence rate of 79.2% (76/96) from Canada [17], 89% (81/91) from Croatia [6], 67.4%(163/242) from Italy [19], 100% from both Spain (83/83) and Australia (136/136) [35], 85.7% (36/42) from Hungary [35], and 64% (326/509) from the USA [33]. Moreover, in previous studies, PAstV2 or PAstV4 were reported to be the predominant genotypes in domestic pigs [6, 14, 17, 19, 33, 35], while in the present investigation, PAstV5 was firstly revealed to be the predominant genotype (24.8%,54/218) followed by PAstV4 (16.1%, 35/218). This is also different compared to previous investigations in the Sichuan province, China, which indicated prevalence rates of 10% (12/120) for PAstV2 and 7.5% (9/120) for PAstV5 [8]. The different results may be due to differences in the efficacy of different primers used; moreover, PAstV has a large genetic divergence between genotypes but also within each genotype, which makes it is difficult to design universal primers (including degenerate primers) suitable for all know or unknown PAstV strains. On the other hand, the 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,

PAstV4 and PAstV5 in domestic pigs located in the Hunan province, China. To our knowledge, this is the first description of PAstV4 in Chinese pigs. Furthermore, the PAstV genotype distribution in China appears to be substantially different compared to other geographic regions with a high prevalence of PAstV5 in domestic pig herds instead of PAstV2 or PAstV4. Within the PAstV genotypes from the Hunan province a high genetic diversity has also been revealed.

Acknowledgments

The author (Chao-Ting Xiao) received a funding from Natural Science Foundation of China (No. 31372459). Funding for T. Opriessnig was provided by the Biotechnology and Biological Sciences Research Council (BBSRC) Institute Strategic Programme Grant awarded to the Roslin Institute (BB/J004324/1; BBS/E/D/20241864).

Conflict of interest

The authors declare that they have no conflict of interest.

Compliance with Ethical Standards

The experiments were approved and carried out in accordance with animal ethics guidelines and approved protocols of the Hunan Agricultural University.

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380

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

382

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

383

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

385

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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388

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

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

400 types.

401

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)

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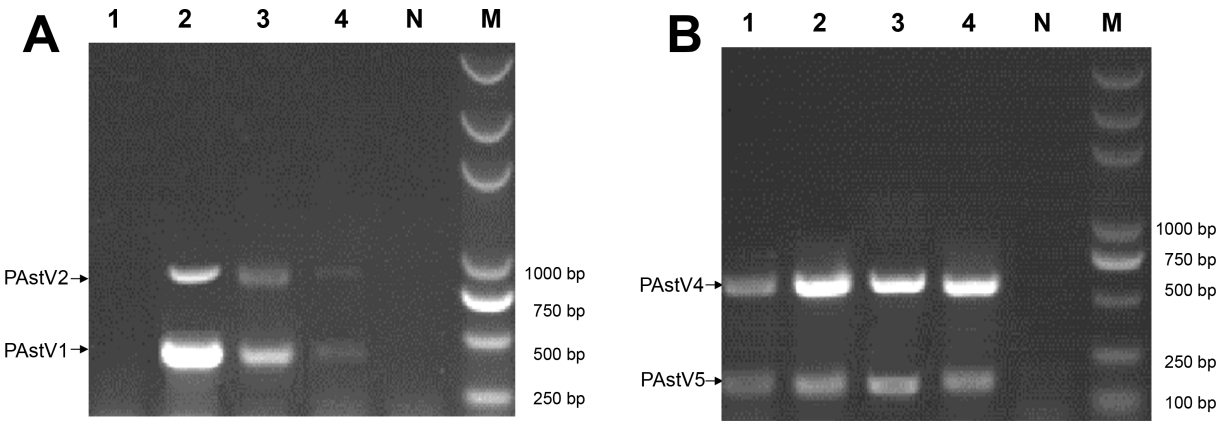
405 **Figure legends**



406

407 **Fig.1** Gel-based electrophoresis of several selected samples to demonstrate the duplex

408 differential RT-PCR outcome. The PCR products were separated on a 0.8 % agarose gel. (A)

409 Duplex differential RT-PCR for PAstV1 (420bp) and PAstV2 (827bp). Lanes 1-4, selected

410 clinic samples, lane 1 (negative), lanes 2-4 (positive for both PAstV1 and PAstV2); N,

411 negative control; M, DNA molecular marker. (B) Duplex differential RT-PCR for PAstV4

412 (559bp) and PAstV5 (182bp). Lanes 1-4, selected clinical samples, positive for both PAstV4

413 and PAstV5; N, negative control; M, DNA molecular marker.

414

415 **Fig. 2** Phylogenetic analysis of PAstV1 based on the 16 sequences identified in the present

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

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

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

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

replicates) are shown next to the branches (only >60% was shown). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated. There were a total of 304 positions in the final dataset. The PAstV1 strains identified presently were indicated with black diamonds. The evolutionary analyses were conducted in MEGA6.

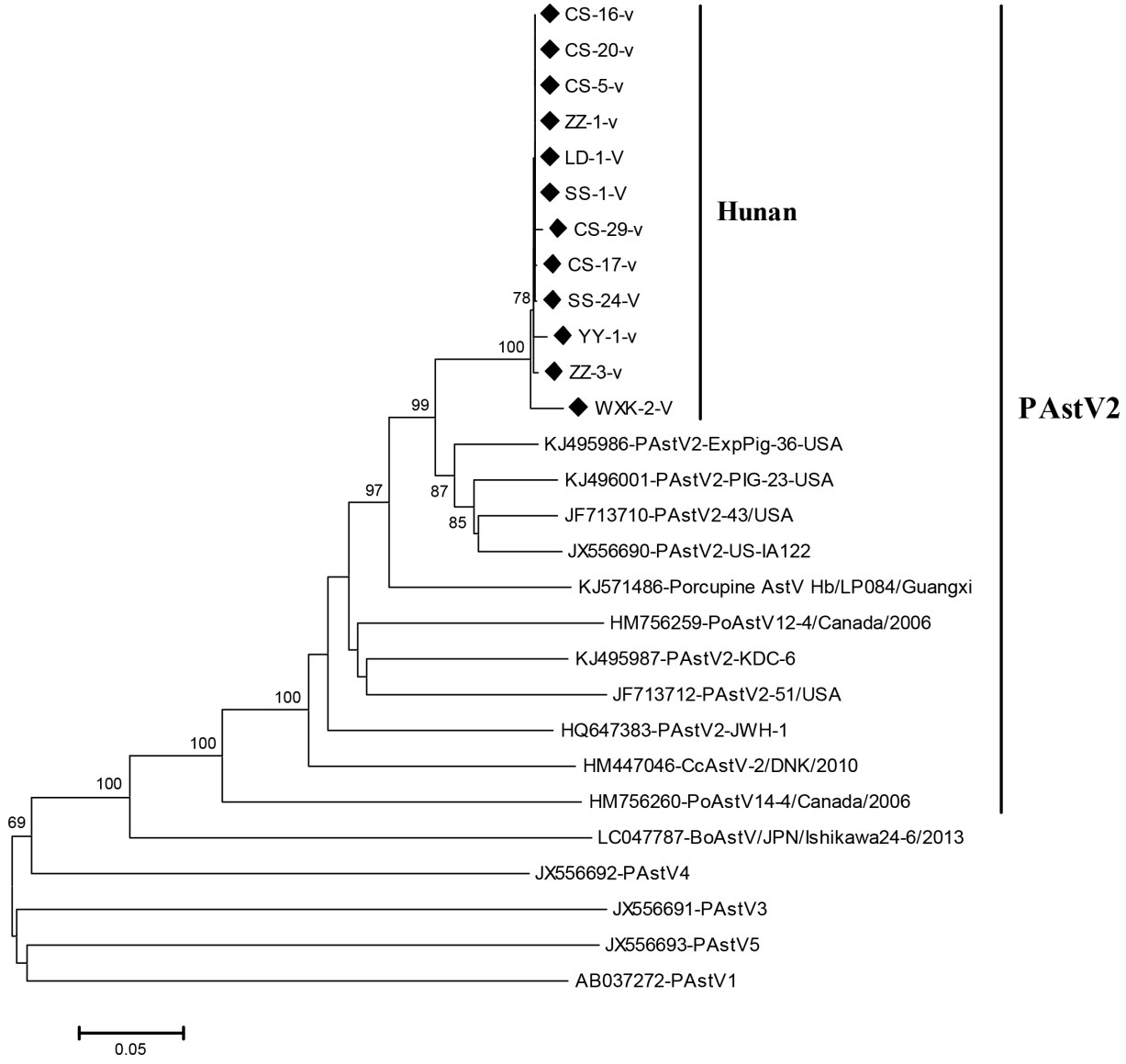
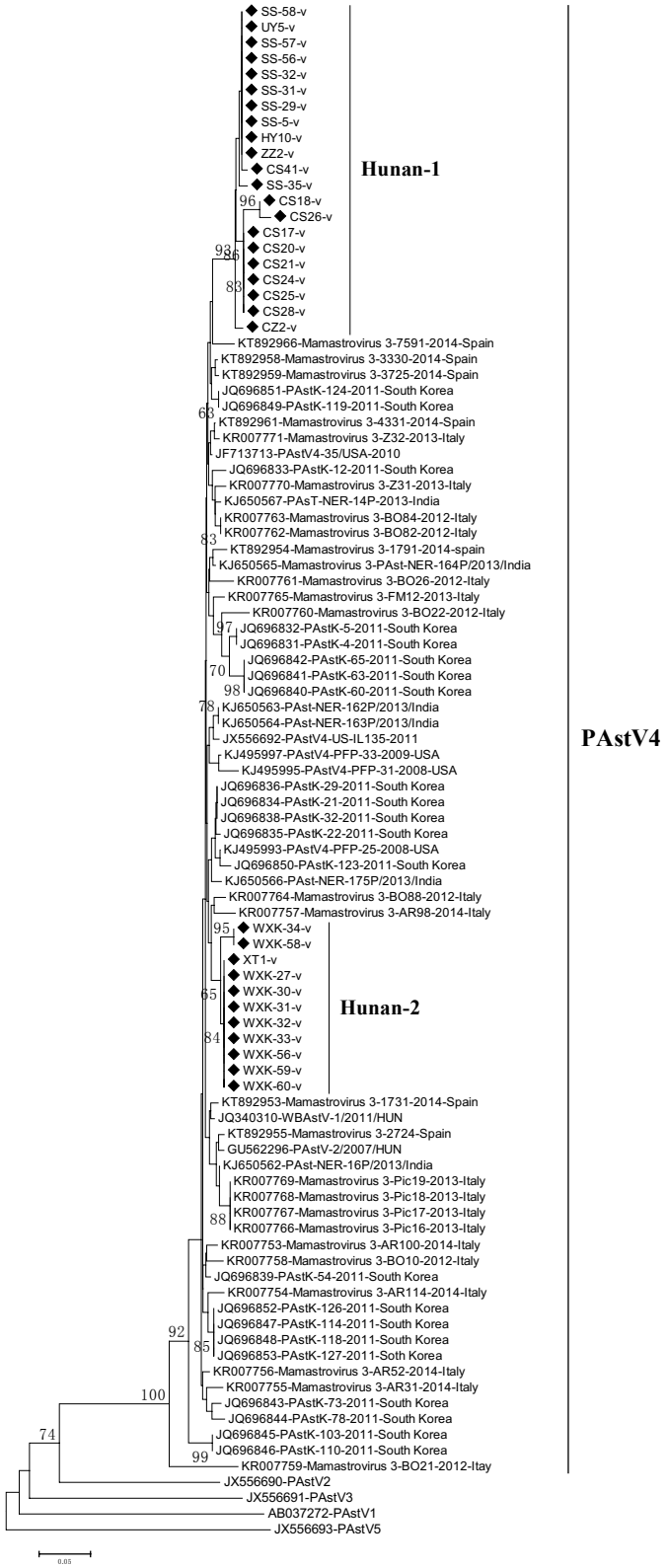


Fig. 3 Phylogenetic analysis of PAstV2 based on the 12 sequences identified in the present study and nine additional PAstV2 sequences obtained through GenBank. The reference sequences of PAstV1, PAstV3, PAstV4, PAstV5 and AstV sequences obtained from porcupine,

roe deer, and cattle 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 replicates) are shown next to the branches (only >60% was shown). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated. There were a total of 748 positions in the final dataset. The PAstV2 strains identified presently are indicated by black diamonds. The evolutionary analyses were conducted in MEGA6.



437

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

present study and 61 PAstV4 sequences available through GenBank. The reference sequences of PAstV1, PAstV2, PAstV3 and 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 replicates) are shown next to the branches (only >60% was shown). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated. There were a total of 189 positions in the final dataset. The PAstV4 strains identified in the present study are indicated by black diamonds. The evolutionary analyses were conducted in MEGA6.

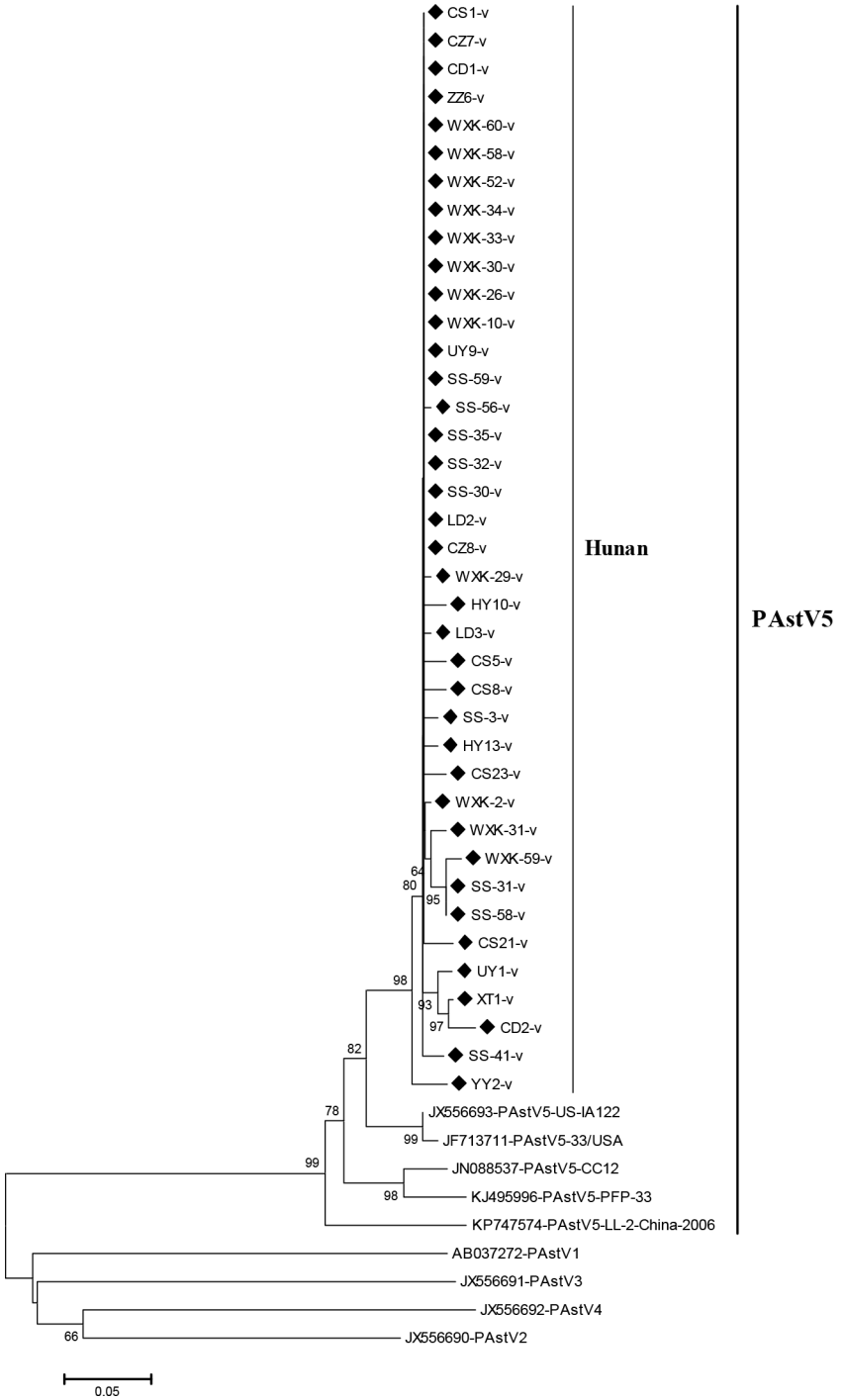


Fig. 5 Phylogenetic analysis of PAstV5 based on the 39 PAstV5 sequences identified in the present study and five PAstV5 sequences available through GenBank. The reference sequences of PAstV1, PAstV2, PAstV3 and PAstV4 were included for comparison. The evolutionary tree was inferred using the Neighbor-Joining 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.