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BRIEF REPORT

Identification and genetic characterization of two porcine astroviruses from domestic piglets in China

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Abstract Porcine astrovirus are divided into five genotypes. In this study, we identified two porcine astroviruses (AstV-LL-1 and AstV-LL-2) by using sequence-independent single-primer amplification (SISPA) on faecal specimens of healthy domestic piglets younger than 15 days. The detection rate for both was 2.82 % (14/497). AstV-LLs were then sequenced and characterised. Phylogenetic analysis revealed that they have the characteristics of porcine astrovirus (PastV) 2 and 5 and have some unique genetic features. Our findings show that the two astroviruses are novel lineages of PAstV2 and 5. The findings may be helpful in evaluating the ecology and evolution of astroviruses in pigs.

Keywords Porcine astrovirus · Domestic pig · Faeces

Astroviruses are small, non-enveloped viruses with positive-sense single-stranded RNA that belong to the family *Astroviridae*, which is divided into two genera, *Mamastrovirus* and *Avastrovirus*, which infect mammals and birds, respectively [6]. Currently, five distinct genotypes of porcine astrovirus (PAstV) have been characterized. All five porcine astroviruses have been detected in faecal samples from healthy pigs [8, 9, 13]. Additionally, PAstV4 has been identified in both domestic pigs and in wild boars [12]. PAstV2 and PAstV4 have also been detected in blood from healthy pigs of different ages [2]. PAstV3 was detected in a faecal sample from a pig suffering from diarrhoea [15]. Several outbreaks of porcine-associated zoonotic diseases have occurred in recent years, including outbreaks of Nipah virus [5] and *Streptococcus suis* [17]. With the rapid development of stock breeding in China, the potential threat of zoonotic diseases spreading to humans is growing; therefore, further research is needed to evaluate astrovirus ecology and its evolution in pigs.

This report describes the identification and genetic characterization of two novel lineages of PAstV2 and PAstV5 from healthy domestic piglets in China.

In 2006, 497 porcine stool samples from young healthy piglets (<15 days of age) were collected from several farms in Lulong County, China. All samples were stored at -80 °C until use.

The protocol used to identify novel PAstVs with an E-value cutoff of 10^{-5} was adapted from our previous study [3]. Two pooled faecal samples from 10 different pigs were amplified to produce two sequence-independent single-primer amplification (SISPA) products, which yielded 85 clones from one and 150 clones from the other. All 235 clones were sequenced and aligned against the NCBI Nr database using the BLASTn and BLASTx algorithms. The 85 clones from the first sample matched with viruses, bacteria, phages, eukaryotic cells, and unknown sources with constituent ratios of 7 %, 46 %, 11 %, 29 %, and 7 %, respectively. Of the six viral sequences ($E < 1 \times 10^{-5}$ in BLAST analysis), five matched other viruses with a high similarity (2 enteroviruses, 2 rotaviruses, and 1 sapovirus), and one sequence, which we named AstV-LL-1, showed 82 % identity to PAstV

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(HM756260.1) in the capsid region. Likewise, the constituent ratios of 150 other sequences were 12 %, 18 %, 24 %, 31 %, and 15 %, respectively. Of the 18 viral sequences, 17 sequences showed a high degree of sequence similarity to other viruses (3 enteroviruses, 11 kobuviruses, 2 caliciviruses, and 1 rotavirus), and one 475-bp sequence, which we named AstV-LL-2, had 74 % similarity to PAstV5 (JF713711.1). All other viruses fragments were 250-585 bp long and had more than 92 % identity to sequences of members of their own species in the GenBank database.

Two pairs of primers based on the sequences obtained from SISPA were designed to screen for viruses in a total of 497 faecal specimens. Only 14 samples were positive for either AstV-LL-1 or AstV-LL-2, with a detection rate of 2.82 %. Specific-primer PCR, genome-walking (kit D316; TaKaRa), and rapid amplification of cDNA ends (SMART^{TM} RACE from Clontech) were used according to the manufacturer's instructions to obtain the complete genomes of the two PAstVs. Overlapping fragments were amplified with special primers to exclude incorrectly assembled AstV-LLs. All of the primers used in this study are listed in the supplemental material.

The sequence of the complete AstV-LL-1 genome contained 6364 bp. Unfortunately, only a 6402-bp sequence of AstV-LL-2, which is not quite the complete genome, was obtained. The AstV-LL-1 and AstV-LL-2 ORFs were analysed using NCBI ORF Finder (Fig. 1). The complete sequence of AstV-LL-1 was predicted to encode three ORFs – ORF1a (nt 46–2520), ORF1b (nt 2469–3983), and ORF2 (nt 3976–6258) – as well as a 5′UTR of 45 bp, a 3′UTR of 76 bp, and a 31-bp poly-A tail. The AstV-LL-2 near-complete genome encoded three ORFs – ORF1a (nt 53–2620), ORF1b (nt 2575–4092), and ORF2 (nt 4076–6283) – and contained a partial 5′UTR (nt 1–52), a 3′UTR (nt 6284–6378), and a 24-base poly-A tail. BLASTn analysis showed 71–73 % identity between the nucleotide sequences of AstV-LL-1 and the published sequence of PAstV2 and 80 % identity between AstV-LL-2 and PAstV5.

The N-terminal domain (amino acids 1-415) of ORF2, which is involved in viral assembly, is highly conserved among all the human serotypes and some viruses of animal origin, but the C-terminal domain (amino acid 416 to the end), which is involved in the varied cellular adhesion capabilities of different astrovirus serotypes, is highly divergent among human serotypes [4, 7, 10, 14]. The sequences of the HAstV1 and HAstV8 strains are extremely similar, with 83 % identity in the conserved domain and 60 % identity in the variable domain [4]. A BLASTn analysis revealed a 60 % consensus region located at the two ends of the PAstV2 ORF2 and showed that PAstV2 has no significant similarity to the middle portion of the AstV-LL-1 ORF2 (nt 5210–6040). The amino acid sequences of AstV-LL-1 ORF2 showed low identities of 60–65 % with other PAstV2 strains and included many deletions/insertions (data not shown). A BLASTp analysis showed 84 % identity in the conserved domain (N-terminal domain, 415 bp) and 59 % identity in the variable domain (C-terminal domain) between AstV-LL-1 and other PAstV2 isolate. These data suggest that this isolate may be of a different PAstV2 serotype.

AstV-LL-1 has the same pentamer, CCAAA, in the 5′ terminus of its genome that has been found in other PAstV2 isolates. The predicted start codons of ORF1a in PAstV4 (GCGATGG) and PAstV5 (ATTATGG) have a Kozak sequence of RNNAUGG (A/G at position -3 and G at position +4), which has been reported to have the strongest positive effects on translation [16]. For the AstV-LL-1 ORF1a, the GTGATGT motif near the start codon is

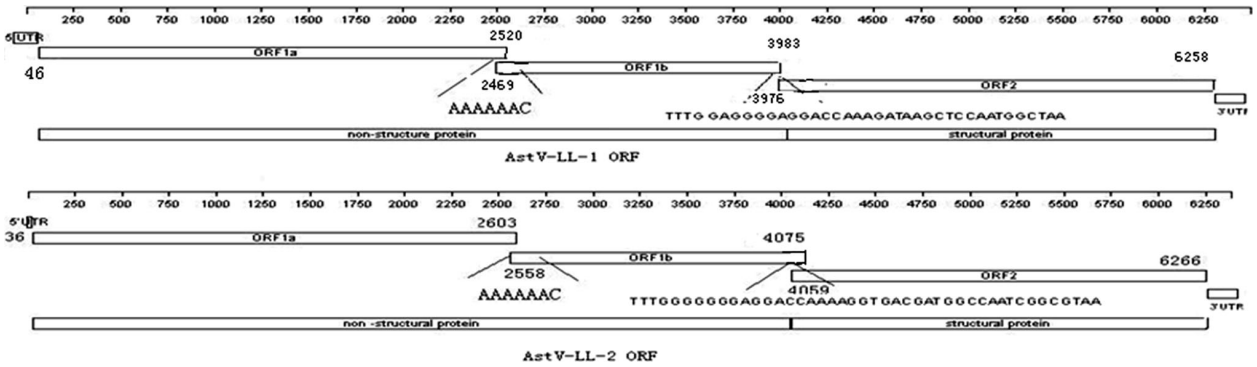


Fig. 1 The genome organization of AstV-LLs, AstV-LL-1 (top) and AstV-Ll-2 (bottom). The "slippery sequence" (AAAAAAC) required for inducing a ribosomal shift is identified at the junction between ORF1a and ORF1b. The conserved sequences (TTTGGAGGGGAGGACCAAAGATAAGCTCCAATGGC in AstV-LL-1; TTTGGGGGGGAGGACCAAAAGGTGACGATGGC in AstV-LL-2) are located at the junction between ORF1b and ORF2. A comparison of these sequences indicated that there was a conserved motif between these two PAstVs of UUUGG(A/G)GGGG(A/C)GGACCAAAN8/11AUGGC (N:A/T/C/G), with two differences, GGRG (R:A/G) instead of GGAG and N11/8 (AstV-LL-1/AstV-LL-2) instead of N4–8, as reported for PAstV2–5 [16]

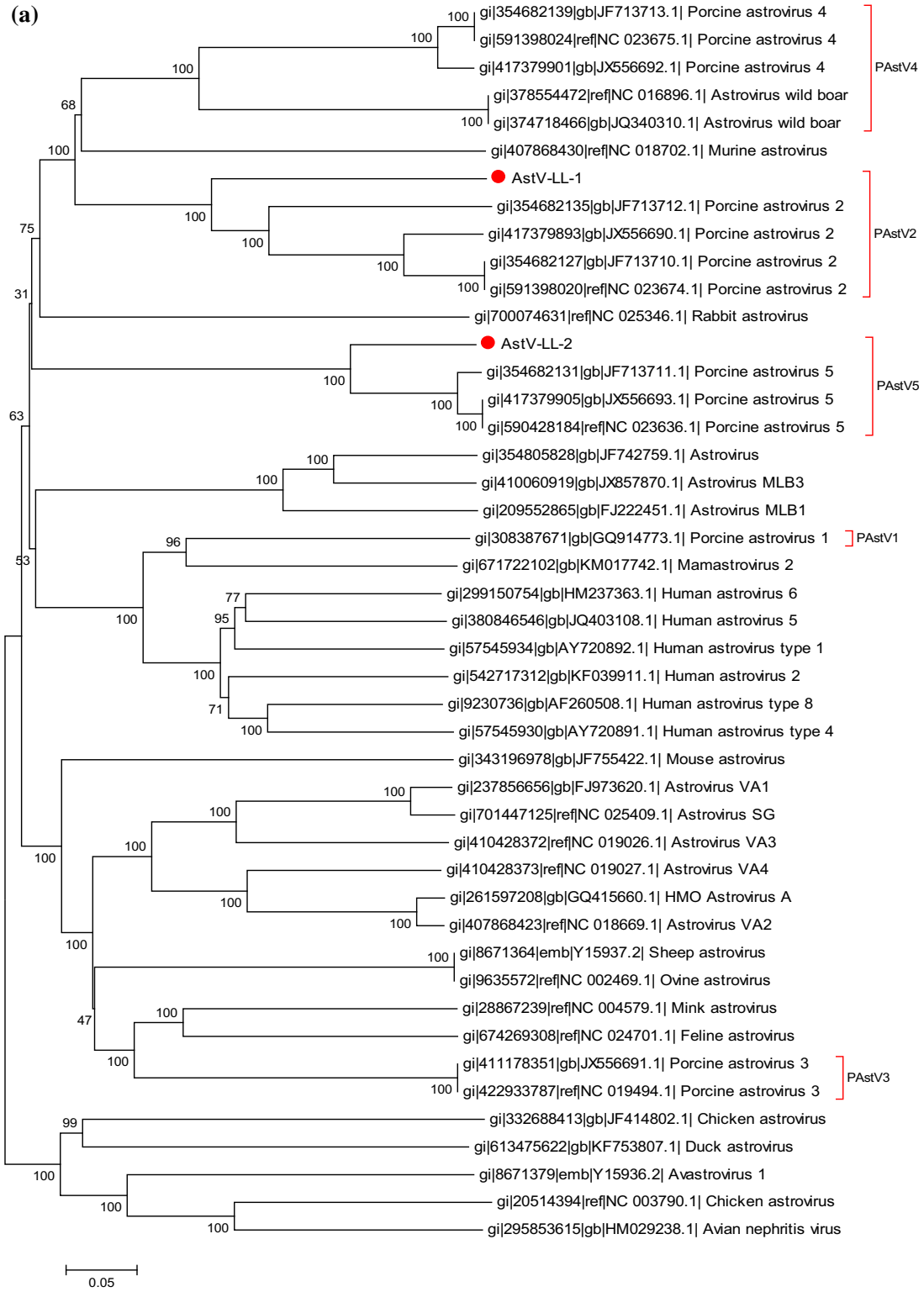
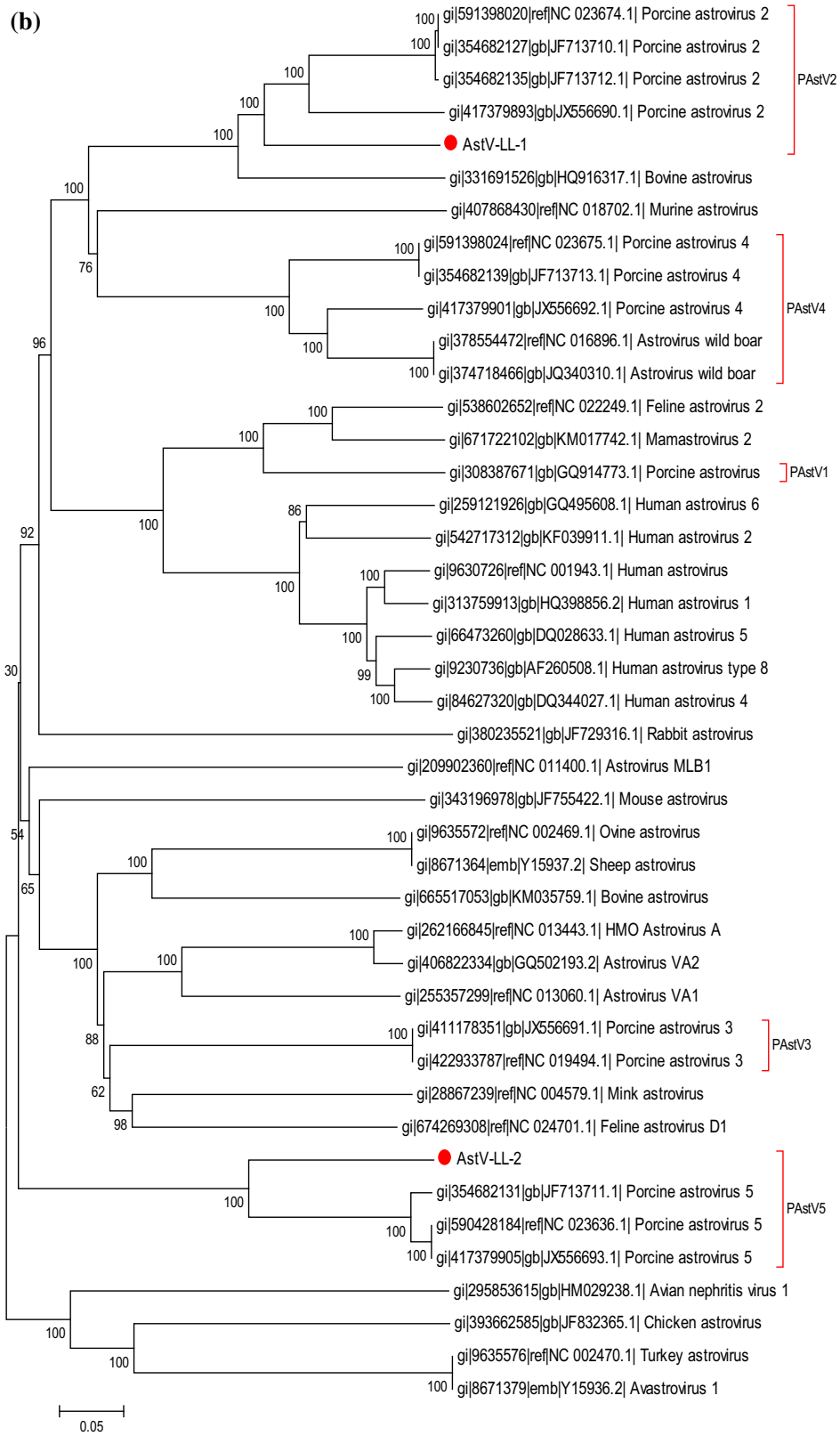


Fig. 2 Phylogenetic analysis of AstV-LLs. The tree was constructed using the maximum-likelihood method in MEGA5. GenBank accession numbers are shown on the tree, and AstV-LL sequences are indicated by red dots. (a) Maximum-likelihood phylogenetic analysis of ORF2 amino sequences of both AstV-LLs. (b) Maximum-likelihood phylogenetic analysis of complete/near-complete nucleotide sequences of both AstV-LLs

Fig. 2 continued



not a Kozak sequence, but the CCAATGG motif near the start codon for the AstV-LL-1 ORF2 is a Kozak sequence, and its characteristics are the same as those reported for PAstV2. In contrast, AstV-LL-2 has an optimal Kozak sequence for both the ORF1 and ORF2 start codon motifs (GCTATGG for ORF1a and ACGATGG for ORF2). These features are same as those reported for PAstV5 [16].

Pfam analysis (http://pfam.xfam.org/) revealed that AstV-LL-2 ORF1a has homology to a peptidase C4 domain, and AstV-LL-1 encodes peptidase S7, suggesting that the ORF1a of these two PAstVs encodes a non-structural polyprotein containing a serine-like protease motif. A conserved RdRp amino acid motif, YGDD, was also found in the RdRp region in both of these astroviruses. AstV-LL-1, but not AstV-LL-2, contains a nuclear localization signal (NLS) at amino acid 665 (RLAGAKKRK), similar to that of a DNA binding protein (R[PL]xGx[KR][KR]xK) (predicted with http://www.predictprotein.org/). The conserved VPg amino acid motifs (KGKNK and D/EEY) were found in ORF1a for both AstV-LL-1 and AstV-LL-2. Pfam analysis also showed that ORF1b of both PAstVs encodes an RdRp. The stem-loop-II-like motif (s2m) contained in the 3′UTR (AstV-LL-2 6267–6301: gCCGaGGCCACGCc GAGUAgGAUCGAGGGUACAGc, predicted with Rfam: http://rfam.xfam.org/) and the ORF2 stop codon located in front of the AstV-LL-2 s2m were both the same as for PAstV5 [11], but this motif is not included in AstV-LL-1 or PAstV2 [16].

Phylogenetic analysis was carried out on the near-complete nucleotide and amino acid sequences of ORF2. Five PAstV genotypes were clearly delineated, with all of the trees supported by high bootstrap values, similar to those described previously [8, 13, 16]. In the PAstV2 genotype, AstV-LL-1 was related much more distantly to the other PAstV2 lineages than to each other. In the PAstV5 genotype, AstV-LL-2 also appeared on a separate branch by itself (Fig. 2). Our results strongly support that AstV-LL-1 and AstV-LL-2 belong to the PAstV2 and PAstV5 genotype, respectively. Low sequence identities (<89.0 %) with known PAstV 2 and 5 isolates, some unique genetic features, and their phylogenetic positions revealed that they are novel lineages of PAstV2 and 5 [16].

There are five genotypes of porcine astroviruses, and these can co-circulate in the same area, even within one farm, but their sequences often vary widely from one another, even within the same genotype [16]. PAstVs have some mixed characteristics, such as containing or lacking an s2m, NLSs, or optimal Kozak sequences [16]. In the present study, AstV-LL-1 had a significant variable region in the middle of ORF2, which is related to the serotype classification of human strains, as described in previous reports [4, 7]. More work is needed to clarify whether AstV-LL-1 belongs to a different serotype with other PAstV2 strains.

PAstV1–PAstV5 have all been detected in healthy pigs, but PAstV3 can cause diarrhoea in pigs [15], and PAstV5 can cause congenital tremors [1]. In the present study, AstV-LL-1 and AstV-LL-2 were detected in healthy piglets, but the pathogenicity of these two viruses requires further analysis.

In conclusion, the present work shows that domesticated pigs harbour two different astrovirus genotypes with high genetic diversity and that these can co-circulate on one farm. More work is needed to understand the transcription, replication, ecology, and evolution of porcine astroviruses.

Accession number

The two full genome sequences have been submitted to GenBank under accession nos. KP747573 for AstV-LL-1 and KP747574 for AstV-LL-1, and the sequences from the positive samples have been deposited under accession nos. KP747575 to KP747601.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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