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ORIGINAL PAPER

High genetic diversity and recombination events of porcine astrovirus strains identified from ill and asymptomatic pigs in 2017, Hunan Province, China

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Abstract

Astroviruses (AstV) are associated with enteric and systemic disease in mammals and birds. Astroviruses have received increased attention recently as they have been found to be associated with sporadic neurologic disease in mammals including humans. In pigs, porcine astrovirus (PoAstV) can be widely detected and has been grouped in five genotypes (PoAstV1 to PoAstV5). In the present study, we detected multiple PoAstVs in serum samples, nasal swabs, and fecal swabs collected from pigs suffering from respiratory disease or diarrhea but also from asymptomatic pigs, indicating a wide tissue tropism of the identified PoAstV genotypes. Coinfection of different genotypes in the same pig was commonly observed, and within an individual pig a high genetic diversity was observed for viruses belonging to the same PoAstV genotype. Two complete genomes of PoAstV2-WG-R2/2017 and PoAstV4-WG-R2/2017 were successfully obtained and characterized, with genome sizes of 6396 and 6643 nucleotides, respectively. The PoAstV2-WG-R2/2017 genome showed identities of 67.2–77.4% to other known PoAstV2 genomes, and the PoAstV4-WG-R2/2017 genome showed identities of 72.8–80.5% to other known PoAstV4 genomes. The predicted spike domain of open reading frame 2 (ORF2) of these strains showed the highest genetic heterogeneity, with amino acid identities of 13.7–70.9% for PoAstV2-WG-R2/2017 to other known PoAstV2 strains, and identities of 24.4–63.3% for the PoAstV4-WG-R2/2017 to other known PoAstV4 strains. Possible recombination events were identified in each of the two sequences. Two subclades of PoAstV2 and three subclades of PoAstV4 were defined in the present analyses. The obtained data provide further evidence for extraintestinal infectivity of PoAstVs, and confirmed the high genetic diversity of PoAstVs and the coinfection potential of different PoAstV types in a single pig.

Keywords Porcine astrovirus · RNA virus · Genetic diversity · Recombination

Introduction

Astroviruses are non-enveloped, single-stranded positive-sense RNA viruses of approximately 6.4–7.9 kb in length that belong to the family *Astroviridae*, which consists of two genera, *Avastrovirus* and *Mamastrovirus*, infecting birds and mammals, respectively [6]. Astrovirus infections are frequently associated with gastroenteritis in young mammals and birds, but have also been recognized in conjunction with extraintestinal manifestation in birds [6]. Recently, new types of mammalian AstV were associated with non-suppurative (aseptic) meningoencephalomyelitis in humans and other mammalian species [29], indicating extraintestinal pathogenicity of this virus in mammals.

The AstV genome contains three open reading frames (ORFs), ORF1a, ORF1b, and ORF2 [6]. ORF1a and ORF1b are located at the 5′ end of genome with a ribosomal frame

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shift signal of a "shifty" heptanucleotide (AAAAAAC) at the junction of ORF1a/1b [11, 12, 15, 22]. The entire ORF1 encodes for non-structural proteins including serine proteases and RNA-dependent RNA polymerases (RdRp), and ORF2 encodes for the capsid protein [11].

Porcine astrovirus (PoAstV) was initially identified by electron microscopy in the feces of piglets with diarrhea in 1980 [7] and was isolated in 1990 [31]. Up to now, PoAstV has been detected on a global basis and, besides the classical PoAstV1, four new genotypes (PoAstV2 to PoAstV5) have been defined sequentially [8, 12, 14, 19–21, 23, 28, 30, 32, 33]. Among the five PoAstV genotypes depending on geographic region, PoAstV4 or PoAstV2 have been identified as the predominant genotypes [18, 21, 32], and coinfection with other enteric viruses or other PoAstV genotypes can be observed frequently [9, 18, 32]. Moreover, PoAstV can be found in healthy pigs as well as in pigs suffering from enteric disease.

While PoAstV has a tropism for the enteric tract, PoAstV2 and PoAstV4 both have been detected in extraenteric locations including in serum samples from Croatian healthy pigs [8], and PoAstV2 and PoAstV5 were found in brain tissues from newborn Swedish piglets suffering from congenital tremor or healthy piglets from the same farm [4]. Moreover, PoAstV4 was found in nasal swabs from suckling pigs located in the US, exhibiting unexplained acute respiratory disease [27]. PoAstV1, PoAstV2, PoAstV4, and PoAstV5 were detected in spleen and lung samples in pigs from China [33]. Most recently, neurotropic PoAstV3 was identified to be associated with non-suppurative meningoencephalomyelitis [2, 5]. These data indicated that besides the neurotropic PoAstV3, other genotypes of PoAstVs may also play a role in the extraintestinal diseases in pigs; however, due to the absence of standardized in vitro and in vivo experimental models, the detailed pathogenesis and possible clinical significance of the newly identified PoAstV2 to PoAstV5 remain unclear. In the present study, we identified multiple PoAstV genotypes from sera, nasal, and fecal swabs collected from healthy and diseased pigs in China, and great genetic diversity was revealed based on genome analysis.

Materials and methods

Sample collection

As part of a routine veterinary investigation in November 2017, serum samples, nasal swabs, and fecal swabs were collected from each of twenty pigs from a pig farm in central Hunan province, China. The sampled animals included two suckling pigs suffering from diarrhea, eight nursery pigs suffering from severe respiratory disease, and eight sows and two boars with no obvious clinical symptoms (Table S1). After being screened for causative pig pathogens (unrelated to this study), the samples were used to further investigate PoAstV. All samples were stored at − 80 °C until use.

Sample processing and viral RNA extraction

Right after collection, nasal and fecal swabs were placed in 3 ml 0.9% saline solution and were vortexed and centrifuged at 1500×*g* for 10 min. From each sample type, 200 µl of the supernatant were used for viral RNA isolation according to the protocol of the DNA/RNA extraction kit (Axygen).

RT-PCR and sequencing

For detecting PoAstV, degenerate semi-nested primers targeting the partial RdRp gene were used, which covered a fragment of about 422 bp [10]. Based on previous results obtained in our lab (data not shown), the degenerate primers do not cross-react with PoAstV1. Therefore, an additional pair of primers specific for PoAstV1 was designed, including the forward primer PoAstV1-DF: 5-GAATCACTCCATGGGAAACTCCTGT-3 and the reverse primer PoAstV1-DR: 5-CTGGTTTTGGACCTGTGACACCT-3, producing an amplicon of 434 bp size at the junction of ORF1b and ORF2 region. The PoAstV1 genome from positive samples was further amplified to obtain a longer fragment which overlapped with the region covered by the above degenerate primers for partial RdRp gene. The following primers were used: PAsV1-3055 F:5-GCTTGGTTCAGGGAGTTCTCCTAC-3 and PAsV1-4181 R:5-GAGTCACGAAG CTGCTTAGCAGTC-3. Moreover, to further investigate the genetic characterization of the present PoAstVs, the whole genomes of one PoAstV2 (CHN/WG-R2/2017) and one PoAstV4 (CHN/WG-R2/2017) from a fecal swabs from suckling pig 2 were further successfully sequenced as reported previously [32]. The PCR products of the expected size were cloned or directly sequenced with both primers using the Sanger dideoxy sequencing technology.

Sequencing analysis

The obtained sequences were analyzed with the software DNAMAN (Lynnon Corporation) and MEGA7.0 [17]. To identify possible recombination events within the PoAstV genomes, the Recombination Detection Program version 4 (RDP4) was used [24]. Currently, there is no clear subclade definition, and therefore in this study subclades were defined when located in distinct branches of the evolutionary tree with high bootstrap supports (over 90%) after 1000 replicates and with average p-distances between subclades above 0.4.

GenBank accession numbers

The PoAstV sequences obtained in this study are available in GenBank under the accession numbers MH414519-MH414525, MK802129-MK802138, MK460230, and MK460231.

Results and discussion

Among the 20 samples tested, 9 were positive for PoAstV (detection rate 45%), including 2 suckling pigs, 3 nursery pigs, and 4 sows (Table S1). Specifically, PoAstV2 was detected in 30% (6/20) of the pigs, followed by PoAstV4 with 20% (4/20), and PoAstV5 and PoAstV1 with 10% (2/20) each (Table S1). PoAstV3 was not found in the present study. Moreover, PoAstV2, PoAstV4, and PoAstV5 were found in serum samples and in nasal swabs, indicating that these viruses have a wide tissue tropism, while PoAstV1 was only found in fecal swabs. Coinfection of two or three PoAstVs was commonly observed in all age groups investigated, similar to the results indicated in a previous study which was only based on the fecal swabs [32]. Interestingly, the two partial RdRp sequences (MK802135 and MH414520) of PoAstV2 from nasal and fecal swabs from suckling pig 1 and the two sequences (MK802136 and MH414519) of PoAstV2 from sera and fecal swabs of suckling pig 2 showed a genetic distance of 0.098 and 0.131, respectively. Furthermore, these virus sequences clustered into different subclades (Fig. 1), which may suggest that a single pig could be infected with viruses from the same PoAstV type with rather big genetic divergences.

To further investigate the genetic characteristics of the present PoAstVs, sequencing attempts to recover the whole genome were done on all positive samples, and the whole genomes of one PoAstV2 (CHN/WG-R2/2017) and one PoAstV4 (CHN/WG-R2/2017) were successfully obtained from the fecal swab of suckling pig 2. The entire genomic

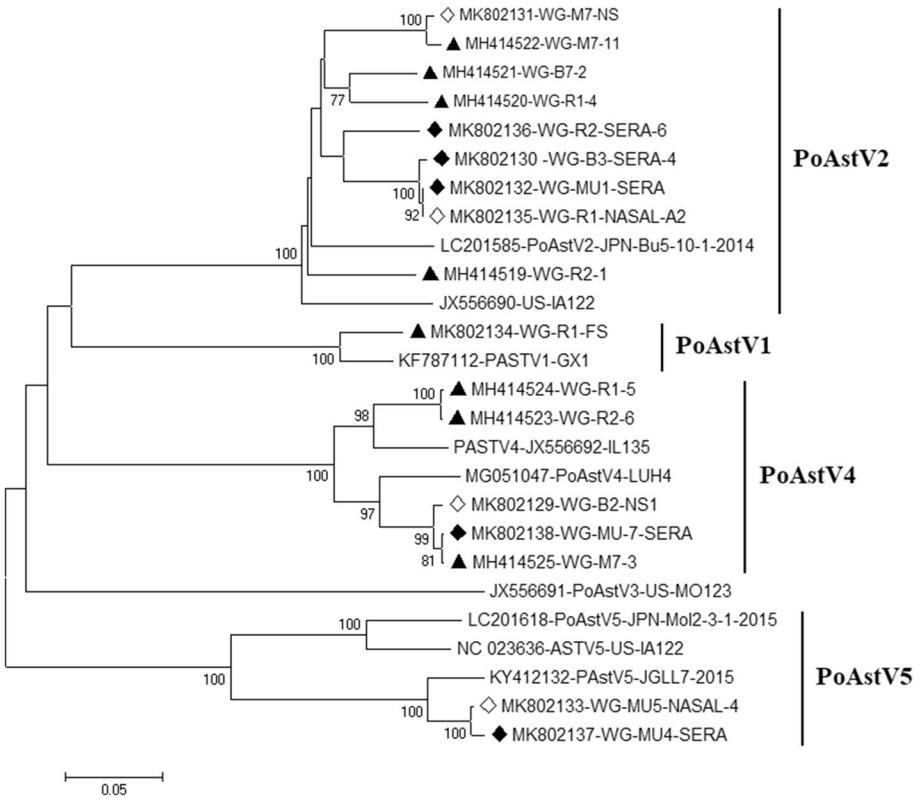


Fig. 1 The phylogenetic analyses, based on partial sequences of the RdRp gene of PoAstVs obtained in the present study and several reference strains from GenBank, were inferred using the Neighbor-Joining method with the evolutionary distances computed using the p-distance method. The percentage of replicates in which the associated virus clustered together in the bootstrap test (1000 replicates) is shown next to the branches (only values > 70% are shown) in each tree. 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. The GenBank accession numbers are shown on the tree. All positions containing gaps and missing data were eliminated. There were a total of 328 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. A solid triangle "filled triangle" indicates the PoAstV sequences obtained from fecal swabs; a solid diamond "filled diamond" indicates the PoAstV sequences obtained from serum samples; and an empty diamond "open diamond" the PoAstV sequences obtained from nasal swabs

sequences of PoAstV2/CHN/WG-R2/2017 and PoAstV4/CHN/WG-R2/2017 were 6396 and 6643 nucleotides (nt) in length, respectively, excluding poly(A) tails, with typical AstV genome organization. Specifically, the genomic structures for PoAstV2 WG-R2 and PoAstV4 WG-R2 were 5′UTR (nt 1-47)-ORF1a (nt 48-2522)-ORF1b (nt 2867-3985)-ORF2 (nt 3810-6323)—3′ UTR (nt 6324-6396) and 5′UTR (nt 1-21)-ORF1a (nt 22-2667)-ORF1b (nt 3015-4112)-ORF2 (nt 4105-6570)—3′ UTR (6571-6643), respectively. The present PoAstV2/CHN/WG-R2/2017 showed identities of 40.1–45.9% to the genomes of the other four PoAstV genotypes, and showed identities of 67.2–77.4% to other PoAstV2 genomes available from GenBank, with the highest identity of 77.4% to the Japanese PoAstV2 strain Iba-464-4-1/2015 (LC201594). The present PoAstV4/CHN/WG-R2/2017 showed 40.8–47.5% identities to the genomes of the other four PoAstV genotypes and low identities of 72.8–80.5% to other PoAstV4 genomes available from GenBank. Overall, this indicates a large genomic divergence of the two strains compared to other strains of the same PoAstV genotype.

The conserved heptameric slippery sequence "AAAAAAC," a signal for a ribosomal frameshift to translate ORF1ab, was found in both of the two genomes [22]. The presumed regulatory element, located at the junction of ORF1b and ORF2 just before the start codon AUG of ORF2, which serves as a promoter for subgenomic RNA transcription [26], was also identified in the present two strains with the conservative motif of UUUGGAGGGG(C/A)GGA CCAAAN$_{(11)}$AUGGC, which is identical with those of the PoAstV2 and PoAstV4 reported previously [32].The conserved sequences to form the canonical stem-loop-II-like motif (s2m) were not seen in the present 3′end of PoAstV2 and PoAstV4, while they were seen in PoAstV1, 2, and 3 [16, 23, 32] (Fig. 2a). However, as predicted by Mfold program [34], other stem-loop structure(s) could be formed by sequences near poly(A) of the genomes of PoAstV2 and PoAstV4, which may have similar function as s2m (Fig. 2b). The lack of the conserved canonical s2m sequences in PoAstV2 and PoAstV4 at the 3′ UTR may indicate a different origin of these two genotypes compared to the other three genotypes.

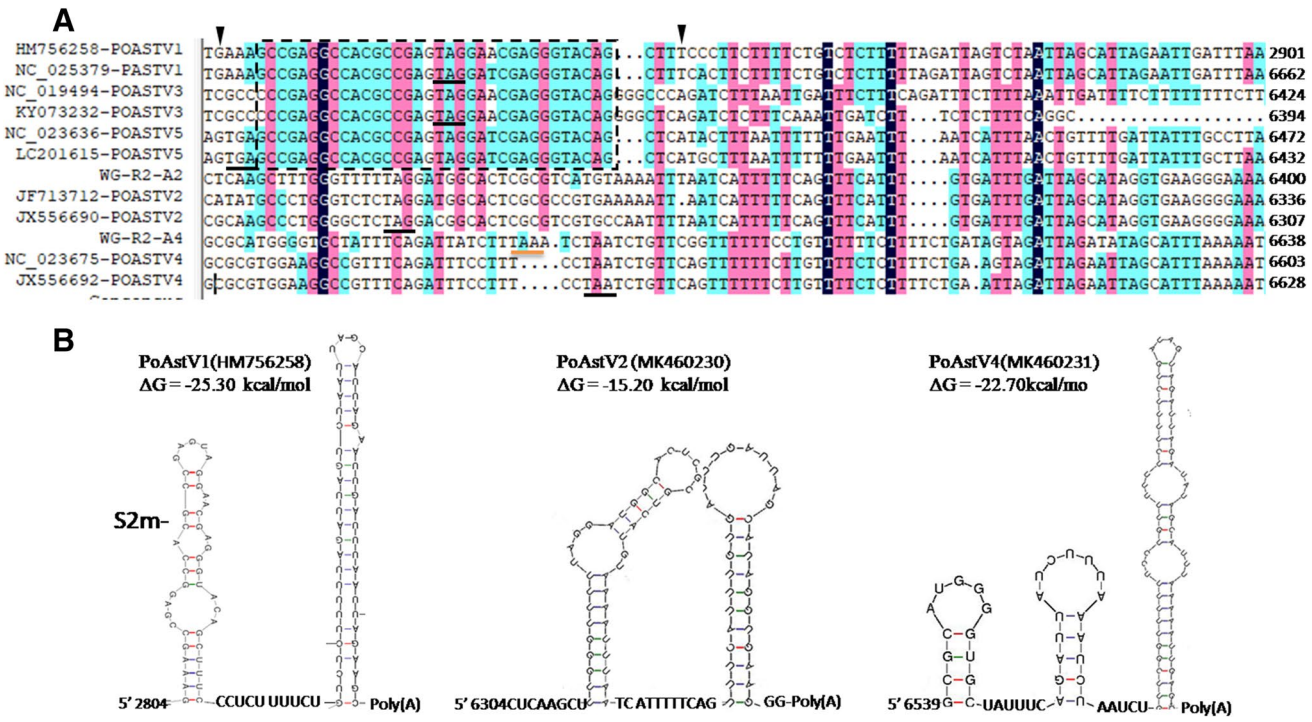


Fig. 2 **a** Alignment of the nucleotide sequences around the s2m motif near the 3′end of the genomes of the present PoAstV2 and PoAstV4 together with representative sequences of other genotypes. The proposed beginning and end of the canonical s2m motif [16] are indicated by arrowheads. Sequences in the dash-line box show the conserved regions in s2m. The ORF2 stop codons of each PoAstV type are underlined. Shading indicates that the sequences between different strains are different. **b** The predicted stem–loop structure is near the 3′end of the PoAstV genotype; PoAstV1, PoAstV2 and PoAstV5 have a conserved canonical sequence as shown in (**a**) which forms a typical s2m structure as indicated. PoAstV2 and PoAstV4 do not contain the conserved canonical sequences for s2m at the 3′end, while other stem-loop structures are formed as predicted by the program Mfold [34]. The nucleotide position numbers of each AstV sequence are indicated in **a** and **b**

There were many amino acid differences in the sequences of structural and non-structural proteins of the present PoAstV2 and PoAstV4, compared with other members from the same genotype. For PoAstV2/CHN/WG-R2/2017, the predicted ORF1ab is 1312 amino acids (aa) in length, showing identities of 80–95% to those of other PoAstV2 strains, with the highest identity of 95% to the recently identified Japanese strain PoAstV2 Iba-464-4-1/2015 (LC201594) and with the identities below 85% with other published PoAstV2 strains. The predicted ORF1ab of PoAstV4/CHN/WG-R2/2017 is 1363 aa in size, showing identities of 84–91% to those of other PoAstV4 published in GenBank, with the highest of 91% to a Japanese strain Ishi-Ya7-1/2015(LC201613) recently identified. The characteristic YGDD motif within the RdRp was identified in the ORF1b of the present PoAstV2/CHN/WG-R2/2017 and in PoAstV4/CHN/WG-R2/2017.

Interestingly, the stop codon of the capsid gene of the present PoAstV4 is three nt ahead of most of the other PoAstV4 strains (Fig. 2a), which leads to a loss of serine at the carboxyl (C) terminus. This same change was also seen in several other strains, like PoAstV4/BEL/15V010 from Belgium (KY214437) and PoAstV-PFP-25 from the USA (KJ495993). Whether this mutation is related to virus function is unknown.

The predicted ORF2 of PoAstV2/CHN/WG-R2/2017 is 837 aa in length, and shows identities of 44–84% to other known PoAstV2 strains. The predicted ORF2 of PoAstV4/CHN/WG-R2/2017 is 821 aa in length, showing identities of 45.8–77.3% to the capsid of other known PoAstV4 strains, with several amino acid insertions and deletions (data not shown). Most differences were found located in the predicted outer core and in the spike domains of the capsid [1]. For the present PoAstV2/CHN/WG-R2/2017, its predicted spike domain (from T448 to D755 of capsid protein) showed only identities of 13.7–70.9% to other known PoAstV2 strains, with the highest identity of 70.9% to another Chinese PoAstV2 strain GXXZ5/2014 (KY412127) and 69.7% to a PoAstV2 strain BEL/15V010 from Belgium (KY214438) but it was below 60% compared to all other PoAstV strains. For the present PoAstV4/CHN/WG-R2/2017 predicted spike domain (from N403 to K708 of capsid protein), it also showed limited identities of 24.4–63.3% to other known PoAstV4 strains, with the highest identity of 63.3% to a PoAstV4 from a wild boar in Hungary (NC_016896) but below 50% compared to the remaining PoAstV4 strains. The lower aa identities of ORF1ab and ORF2 of the present two strains with other known strains, especially for the ORF2 sequences, demonstrates a large genetic diversity of these strains and also indicated the potential for considerable sequence differences within the same PoAstV genotype, which partially may result from the immune response pressure through the hosts during the virus adaption and evolution. However, as the spike domain of capsid is considered to be the dominant antigen and contains an essential receptor-binding site [1], whether these heterogeneities within the same PoAstV genotype are related to antigenic change or tissue tropism of the viruses needs further investigation.

Moreover, the phylogenetic analyses based on the complete/near complete genomes, the nucleotide sequences of ORF1ab and ORF2 of PoAstV2 and PoAstV4 were performed, respectively (Fig. 3. Figs. S1, S2). Interestingly, for PoAstV2/CHN/WG-R2/2017, in the trees constructed based on genomes, ORF1ab or ORF2, it was clustered together with different strains, that is, it clustered with a Japanese strain LC201594 in the trees of genome and *ORF1ab* gene, while it clustered with a Belgium strain KY214438 in the tree of *ORF2* gene, indicating a possible recombination event. To further investigate this, program RDP4 was used and the results indicated several possible recombination events. The one with the highest binomial probability (MC uncorrected = 5.2×10^{-36} and MC corrected = 8.0×10^{-33}) and with average bootstrap support of 96.2 was accepted and further confirmed by seven other methods within the RDP4 program. The predicted major and minor parent strains of the present PoAstV2 are KY214438 and LC201589, with the beginning and end breakpoints of 2498 and 4128, respectively, including the ORF of whole 1b (2867–3985) (Fig. 4a). Furthermore, a possible recombination event was also detected in PoAstV4/CHN/WG-R2/2017 with the predicted major parent of a PoAstV4 from wild boar (WBAstV-1/2011/HUN, JQ340310) and a minor parent KX060808, and with the beginning and end breakpoints of 1303 and 2846, respectively (Fig. 4b). Recombinant events in different regions of the genome have been observed in other AstVs, including in AstVs from humans and pigs [3, 13, 14, 25].

From the phylogenetic tree based on the nucleotide or amino acid sequences of ORF2 of PoAstV2, two distinct subclades within PoAstV2 (termed PoAstV2-1 and PoAstV2-2) could be identified (Fig. 3c, Fig. S2), which showed an average amino acid genetic distance (p-distance) of 0.418 between the two subclades, indicating a large genetic distance between the two subclades and within the genotype PoAstV2 (Table 1). For PoAstV4, three subclades (PoAstV4-1 to PoAstV4-3) were identified based on the nucleotide or amino acid sequences of ORF2, with the mean amino acid genetic distance (p-distance) of 0.433–0.453 between the three subclades, also indicating large genetic distance among the three subclades and within the genotype PoAstV4. Moreover, the mean amino acid genetic distance (p-distance) between the ORF2 of PoAstV2 and PoAstV4 is 0.674 ± 0.015 (Table 1), which is a little larger than the p-distance of 0.671 ± 0.016 between the two main genogroups of genus *Mamastrovirus* defined by the International

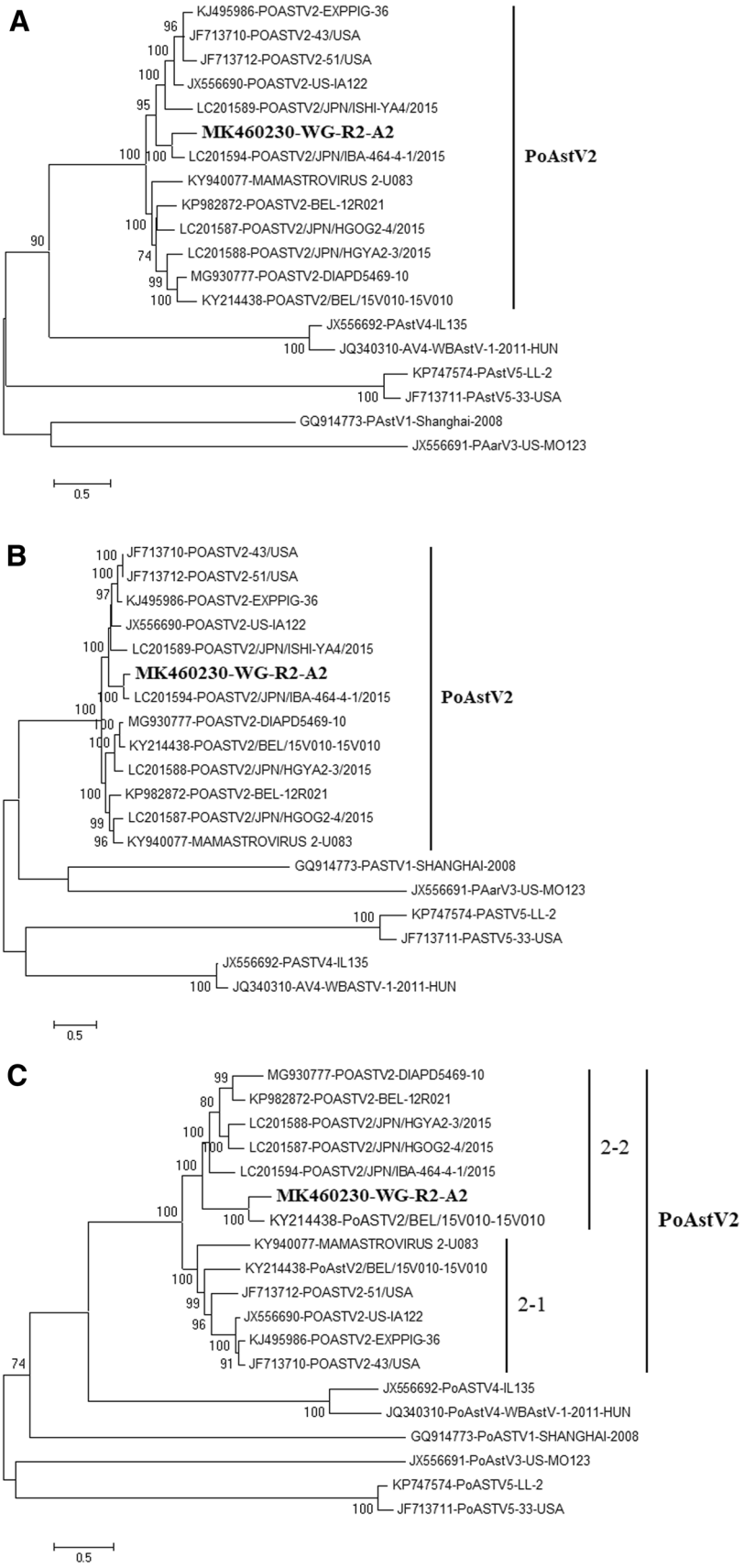
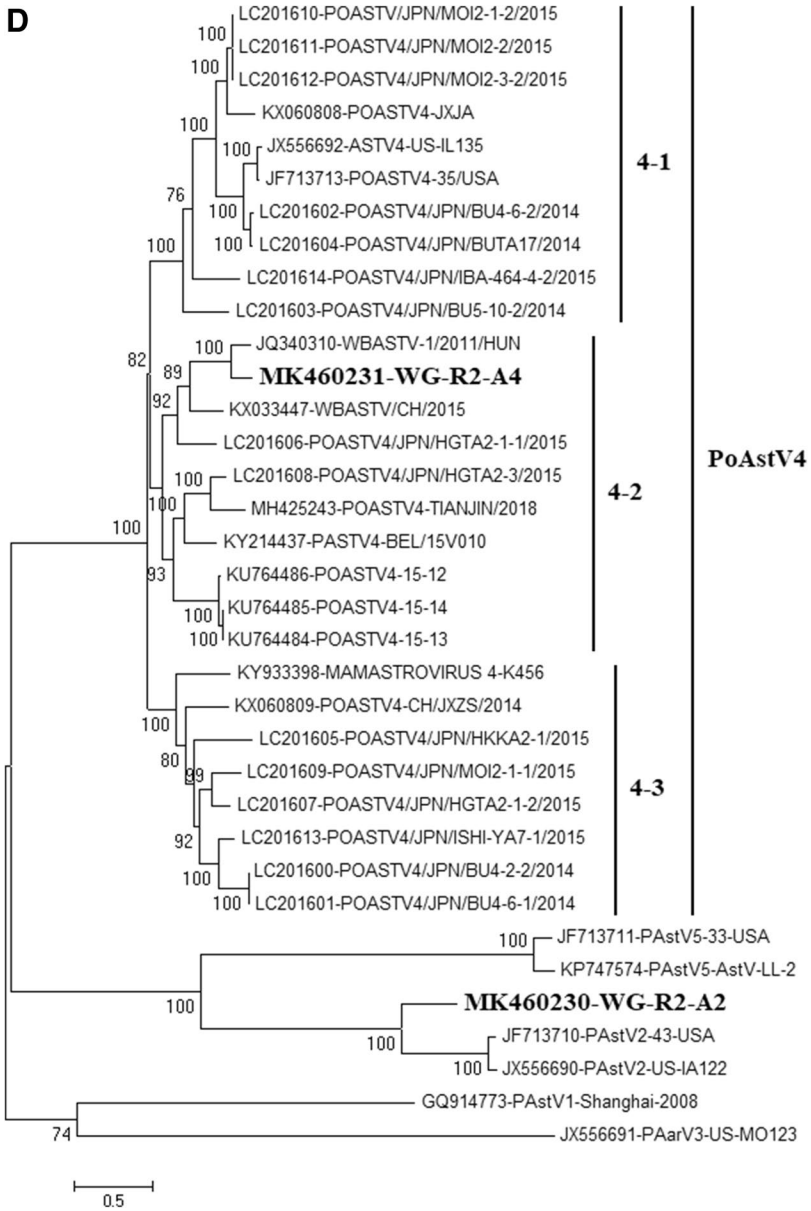


Fig. 3 The phylogenetic analysis of the identified PoAstV2/CHN/WG-R2/2017 (WG-R2-A2) with its complete genome (**a**), and the nucleotide sequences of ORF1ab (**b**) and ORF2 (**c**) including those of other PoAstV2 and representative sequences of genotypes of PoAstV1, PoAstV3 to PoAstV5. **d** Phylogenetic analysis of the ORF2 nucleotide sequences of the present PoAstV4/CHN/WG-R2/2017 (WG-R2-A4) with other PoAstV4 strains and representative strains of other AstV. The evolutionary trees were inferred using the maximum-likelihood method with the general time-reversible model for nucleotide sequences assuming gamma distribution and invariant sites (G + I) as implemented by MEGA7 [17]. The percentage of replicates in which the associated viruses clustered together in the bootstrap test (500 replicates) is shown next to the branches (only values > 70% are shown) in each tree. 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 GenBank accession numbers are shown on the tree. The complete genomes obtained in the present study are in bold font

Fig. 3 (continued)



Committee on Taxonomy of Viruses (ICTV) [6]. This confirmed that PoAstV2 and PoAstV4 are distinctly different AstV species, and their placement within *Mamastrovirus* 3 or 4 may not be appropriate; however, further analyses including of all of the five PoAstV genotypes and the references strains are needed to confirm this.

In summary, in this study we collected serum samples, nasal swabs, and fecal swabs from each of 20 pigs and investigated the PoAstV genotypes from these samples by conventional PCR. Using conventional PCR assays and not real-time PCR assays likely reduced the overall sensitivity but this has not been further investigated. The results confirmed that PoAstV2, PoAstV4, and PoAstV5 could be commonly detected in pigs outside the enteric system, and coinfections with these PoAstVs were also commonly observed. Moreover, from the genomic analysis, we also revealed that the present PoAstV2 and PoAstV4 showed a large divergence with other strains within the same genotype. Especially the heterogeneity of the spike domain of ORF2 was high between different strains within the same genotype, indicating possible pathogenicity difference. Possible recombination events were also detected from which these two strains emerged. Furthermore, based on the genetic distances and phylogenetic analysis, two subclades of PoAstV2 and three subclades of PoAstV4 could be defined under the study conditions. However, the significance of the large amino acid sequence differences including multiple indels within ORF1 and ORF2 of the present two strains compared to other strains in the same genotype remains unknown. The possible relationship of

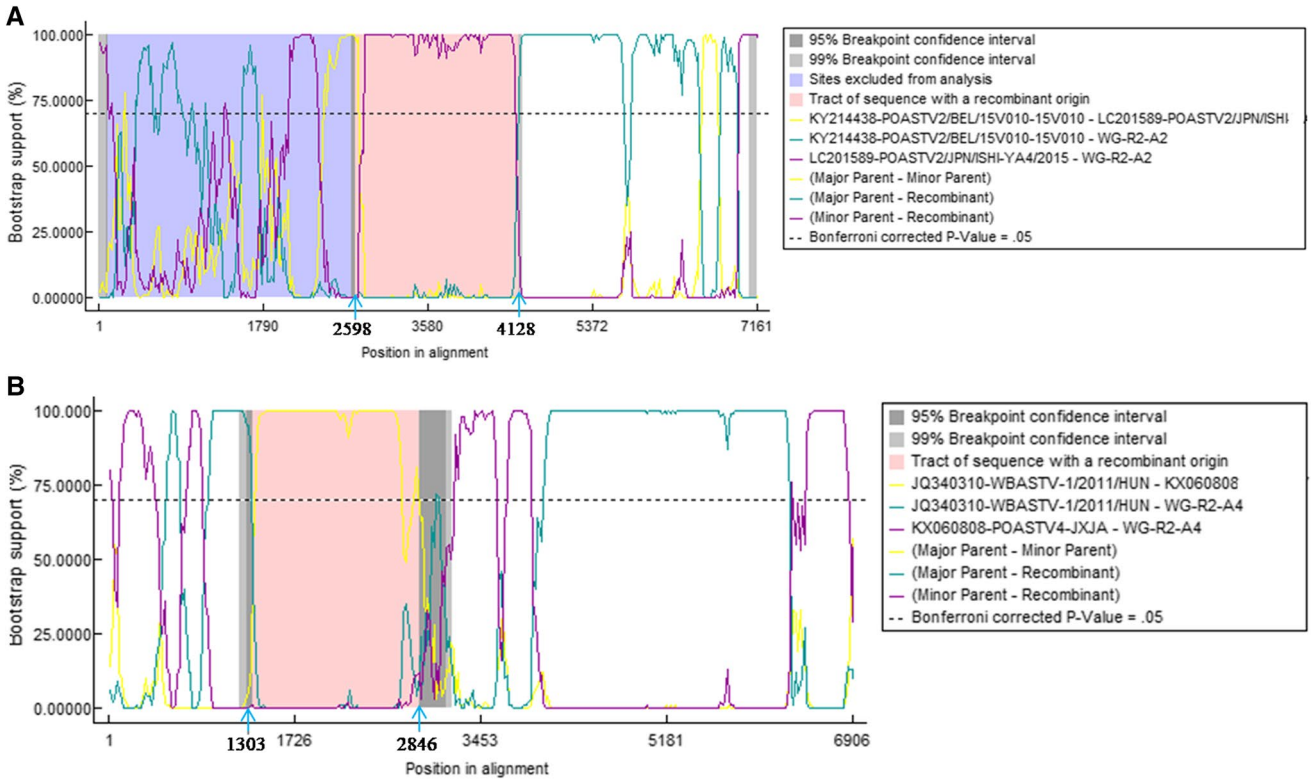


Fig. 4 Bootscan analysis (by RDP4 software package) of the present whole-genome sequences of PoAstV2 WG-R2(WG-R2-A2) and PoAstV4 WG-R2(WG-R2-A4).The X axis shows the nucleotide position number and Y axis shows the bootstrap support of the query sequence with reference datasets. The dashed line denotes a bootstrap cutoff of 70%; recombination breakpoints are numbered. **a** A recombination event starting at position 2598 and ending at position 4128 was predicted (indicated by arrows) in the present PoAstV2 with KY214438 being the major parent strain and LC201589 being the minor parent. **b** A recombination event was predicted in the present PoAstV4 with the major parent of a PoAstV4 from wild boar (WBAstV-1/2011/HUN,JQ340310) and the minor parent KX060808, and with the beginning and end breakpoints of 1303 and 2846, respectively (indicated by arrows).The analysis was done using a window size of 200 and a step size of 20

Table 1 The p-distance of the complete amino acid sequence of ORF2 between and within the subclades of PoAstV2 and PoAstV4 identified in the present study

p-Distance within subclade (mean ± SE)	Subclades	Average p-distance between subclades (mean ± SE)			
		PoAstV2-1	PoAstV2-2	PoAstV4-1	PoAstV4-2
0.266 ± 0.011	PoAstV2-1				
0.318 ± 0.012	PoAstV2-2	0.418 ± 0.013			
0.284 ± 0.01	PoAstV4-1	0.672 ± 0.015	0.680 ± 0.015		
0.361 ± 0.011	PoAstV4-2	0.672 ± 0.015	0.676 ± 0.015	0.433 ± 0.013	
0.29 ± 0.011	PoAstV4-3	0.667 ± 0.015	0.672 ± 0.015	0.453 ± 0.014	0.444 ± 0.013

The analyses were performed using MEGA7

AstV with clinic performance in pigs still needs further investigation.

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Author contributions CTX conceived and designed the study. SLL, HHZ, JYL, WQH, and YTS performed the experiments, SLL and CTX analyzed the data. SLL and CTX wrote the manuscript, and all authors read and approved the final manuscript.

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Compliance with ethical standards

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

Ethics approval Field samples used in this study were collected as part of routine health surveillance. This article does not contain any studies with human participants or animals performed by any of the authors.

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