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BRIEF REPORT

Molecular characterization of a porcine astrovirus strain in China

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Abstract Pigs are increasingly recognized to harbor a wide range of viruses that apparently establish long-term persistence in these animals. They serve as reservoirs for a number of human zoonotic diseases. In this study, a porcine astrovirus (PAstV) strain, designated as PAstV JWH-1, is identified from a diarrheal pig in China, and it is partially characterized genetically. Sequence analysis shows that the PAstV JWH-1 strain contains divergent nucleotide sequences in both the open reading frame (ORF)1b/ORF2 consensus and the 3′-UTR regions (s2m motif), which are usually highly conserved among members of the family *Astroviridae*. Phylogenetic analysis indicates that the JWH-1 strain clusters closely with newly identified strains PAstV 12-4 and 14-4 and forms a group of mamastroviruses with the proposed novel deer astrovirus. Further recombination analysis shows that two possible interspecies recombination events between porcine and deer astroviruses occurred in the genome of the JWH-1 strain. This study further confirms that multiple lineages are present among PAstVs, and each lineage likely represents an independent origin. Additionally, the possibility of interspecies transmission among PAstVs is also suggested.

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Astroviruses, members of the family *Astroviridae*, are small non-enveloped viruses with 6.4–7.3-kb single-stranded positive-sense genomic RNA [20]. The entire astrovirus genome includes three open reading frames (ORFs), which are designated as ORF1a, ORF1b, and ORF2. ORF1a and ORF1b are situated at the 5′-end of the genome, and they encode the viral nonstructural serine protease and RNA polymerase proteins, respectively. ORF2, situated at the 3′-end of the genome, encodes the viral structural capsid protein [16].

The family *Astroviridae* is divided into two genera, namely, *Mamastrovirus* and *Avastrovirus*. The genus *Mamastrovirus* has six known species, i.e., human, bovine, feline, mink, ovine, and porcine astroviruses (PAstV) [12]. Six proposed novel AstV species, including deer, rat, dog, sea lion, bottlenose dolphin, and bat astroviruses, have recently been identified [4, 5, 20, 22, 23]. The viruses belonging to the genus *Avastrovirus* only infect avian hosts, and this genus includes chicken, duck, and turkey astroviruses [18]. In humans, eight classical human astroviruses (HAstV) are known [6, 25], and novel genotypes have continuously emerged in recent years [7, 8, 12]. PAstV was identified and isolated in 1980 [1] and 1990 [21], respectively, but the first molecular characterization of PAstV was not reported until 2001 [11]. Then, a closely related strain was also isolated from a pig diarrheal sample and successfully propagated in vitro in 2006 [9]. Recently, a number of genetically distinct strains of PAstVs have been identified, characterized, and proposed to represent novel lineages, types, groups or even tentative novel species of PoAstV [14, 19, 24].

In the present study, a porcine astrovirus (PAstV) strain in China is identified and partially characterized genetically. The existence of diversity and genetic recombination in PAstVs and the active role of pigs in the evolution and ecology of the *Astroviridae* were further confirmed.

Fecal samples were collected from 15 two-month-old piglets with diarrhea at a farm in Shanghai, China, in December 2009. The aim of the study was to characterize picobirnaviruses (PBVs) in diarrheal porcine fecal specimens using reverse transcription-polymerase chain reaction (RT-PCR) [2]. However, an unexpected band was observed by agarose gel electrophoresis when the full-length sequence of the RNA-dependent RNA polymerase (RdRp) gene of one porcine PBV strain was determined using the genome-walking PCR method. The genome walking PCR method is a useful method to obtain unknown sequences on either side of the known nucleotide sequence. We used it to determine the unknown sequences on either side of the RdRp gene of one porcine PBV strain. The whole process was performed using the commercial genome walking kit (Takara, Japan) as per the manufacturer's instructions. Briefly, three specific forward or reverse primers were designed based on the sequence of the amplified fragment obtained from detection PCR, and a thermally asymmetric PCR reaction was done using the four degenerate primers of low annealing temperature provided in the kit. Generally, at least one of the degenerate primers can be matched with specific primers, and the flanking sequences of a known sequence can be obtained by three nested PCR reactions. If one genome-walking PCR did not achieve the required length, we were able to obtain the flanking sequence by a subsequent round of genome-walking PCR based on the sequence information from the previous round.

After purification and sequencing, the 865-bp unexpected fragment was found to share 70% and 67% nucleotide similarity with the RdRp motif of the HAstV type 1 and 4 (HAstV-1 and HAstV-4) strains, designated as HQ398856 and DQ070852, using the Blastn program. At that time, the recently identified sequences of PAstVs and the proposed novel deer astroviruses have not yet been submitted to the GenBank database. Two primers (424 bp), forward: 5′-AGTGCGGATGGACACCTTTTTA-3′ and reverse: 5′-GGAGTGGTTGTAAGCCTGTCATC-3′, were then designed from this sequence and synthesized to screen other porcine samples for the virus by PCR. Three of the 15 samples (20%) tested positive. These positive products were sequenced, and the resulting sequences were submitted to GenBank (accession numbers: HQ647381–HQ647383). Sequence analysis showed that the isolates shared 98.1–99.0% identity with each other, suggesting that they can be considered unique strains.

The entire 3544-nt, continuous sequences of ORF1b (1135 nt) and ORF2/3′-UTR (2386 nt) of the porcine strain JWH-1(HQ647383) were then determined using the genome-walking PCR method mentioned above and a 3′ RACE kit (TAKARA, Japan). Through two rounds of genome-walking PCR (primer sequences are shown in Table 1), a continuous sequence containing ORF1b (1135 nt) and most of ORF2 (2220 nt) was obtained. The remaining sequence of ORF2 up to the 3′ end was obtained using a commercial 3′ RACE kit (TAKARA, Japan) according the manufacturer's instructions. Briefly, the viral RNA was reverse-transcribed into cDNA using the 3′ RACE adaptor primer and M-MLV reverse transcriptase (RNase H^-) provided in the kit. Then, two specific forward nested primers were designed based on the ORF2 sequence obtained (primer sequences are shown in Table 1), and the 3′ end was amplified using nested PCR. The first PCR reaction was performed using the outer specific primer and the 3′ RACE outer primer (complementary to the outer part of 3′ RACE adaptor primer) provided in the kit. Based on the first-round product, the second nested PCR was performed using the inner specific primer and 3′ RACE inner primer (complementary to the inner part of 3′ RACE adaptor primer) provided in the kit.

The partial ORF1b sequence that encodes an RdRp was found to have 86%, 83%, and 80–81% nucleotide homology with the PAstV Hungary strain (GU562296), PAstV 14-4 strain (HM756260), and deer astrovirus (HM447045 and HM447046), respectively, using the Blastn program. A highly conserved sequence, UUUGGAGNGGNGGACCN AAN_{5-8}AUGNC, where the ORF2 AUG start codon is underlined and N stands for any of the four nucleotides, was located at the junction between ORF1b and ORF2. This sequence was proposed to be a regulatory element that serves as a promoter for subgenomic RNA transcription. The sequence has been found in members of different species of astrovirus [16]. Alignment of the corresponding region from all strains shown in Fig. 1 that were characterized in the present study revealed that this motif was conserved in all strains. Compared with other PAstV strains, PAstV JWH-1, PAstV 12-4, PAstV 14-4 and PoAstV Hungary contain a unique insertion of three nucleotides upstream of the ORF2 initiation codon (Fig. 1b). The function of this difference is unclear and needs further confirmation. The putative ORF2 contains 2313 nt that encode a 770-aa capsid protein, which is close to the average length of the mamastrovirus capsid proteins. The complete ORF 2 of JWH-1 shared the highest nucleotide identity (70.3%) and amino acid identity (70%) with that of the PAstV 12-4 strain. The 3′-UTR measured 73 nt, excluding the poly (A) tract. All human astrovirus strains except strain MLB contain a conserved stem-loop-II-like motif (s2m) in the 3′-end of the genomic RNA [10]. The exact role of this motif is unclear, but due to its presence in members of many viral families such as picornaviruses and coronaviruses, this motif has been suggested to have an important function in viral RNA replication [11]. However, the s2m motif was not found in the PAstV JWH-1 strain. Alignment of the corresponding region from the eight

Table 1 Primers designed for genome-walking PCR and the 3′ RACE method

Primer name	Primer and sequences (5′–3′)
GNW-1-S1[a]	TTCAGCGTTAAAATGTTGGTCG
GNW-1-S2	TCTTTGTCTGCCTTTGTGTCGA
GNW-1-S3	CATGGCAGTGATCCGTTTATCA
GNW-2-S1[b]	TCGACCAACATTTTAACGCTGA
GNW-2-S2	TGTATGGAACCCAGCATGACGT
GNW-2-S3	AACACGGTATGGGCTCTGGTTC
3′Race-GSP1[c]	GCTGTGAAATCACCACCCGATA
3′Race-GSP2	TCACTGGGTACCGTTTTATCCG

[a] Specific sense primers for the first round of genome walking

[b] Specific sense primers for the second round of genome walking

[c] Specific sense primers for the 3′ RACE method

representative PAstV strains characterized in the present study (Fig. 1c) revealed that the strains PAstV Japan, PAstV Tokushiman, PAstV 16-2, and PAstV 12-3 contain a conserved s2m motif, whereas the corresponding sequence has not been found in strains PAstV JWH-1, PAstV 12-4, PAstV 14-4, and PAstV Hungary.

Phylogenetic analysis was performed using either the RdRp region or the capsid protein region of the JWH-1 strain, prototypical PAstVs, and other referenced representative strains from different species. The GenBank numbers and sources are shown in Fig. 2. The results confirmed that JWH-1 clustered closely with the newly identified strain PAstV 12-4 and PAstV 14-4. They formed a subgroup that was divergent from the prototypical PAstVs and formed a novel group within the genus *Mamastrovirus*, together with the proposed new deer astrovirus. As shown in the phylogenetic tree, the existing PAstVs were distributed in several clades and were distributed in different species. Some strains were related to HAstVs, whereas some strains were only closely related to PAstVs, and the other strains were more closely related to strains infecting other animals, such as sea lions, mink and sheep. These results suggest that multiple lineages exist among porcine astroviruses, and each lineage is likely to represent an independent origin.

To identify sites of possible recombination events within the genome of the JWH-1 strain, the SimPlot program [13] and Recombination Detection Program (RDP 4) [15] were used to perform recombination analysis. Sequences were first aligned in the Clustal W program, and the SimPlot program was used to analyze similarities and to locate potential recombinant sequences. If the results were supportive of the presence of a recombination site, the RDP 4 program was employed to identify all possible recombination events. A similarity plot (Fig. 3) shows that the JWH-1 strain has a high degree of identity to the PoAstV Hungary strain at the beginning of the ORF 1b region, whereas it has a high degree of identity with PAstV 12-4,

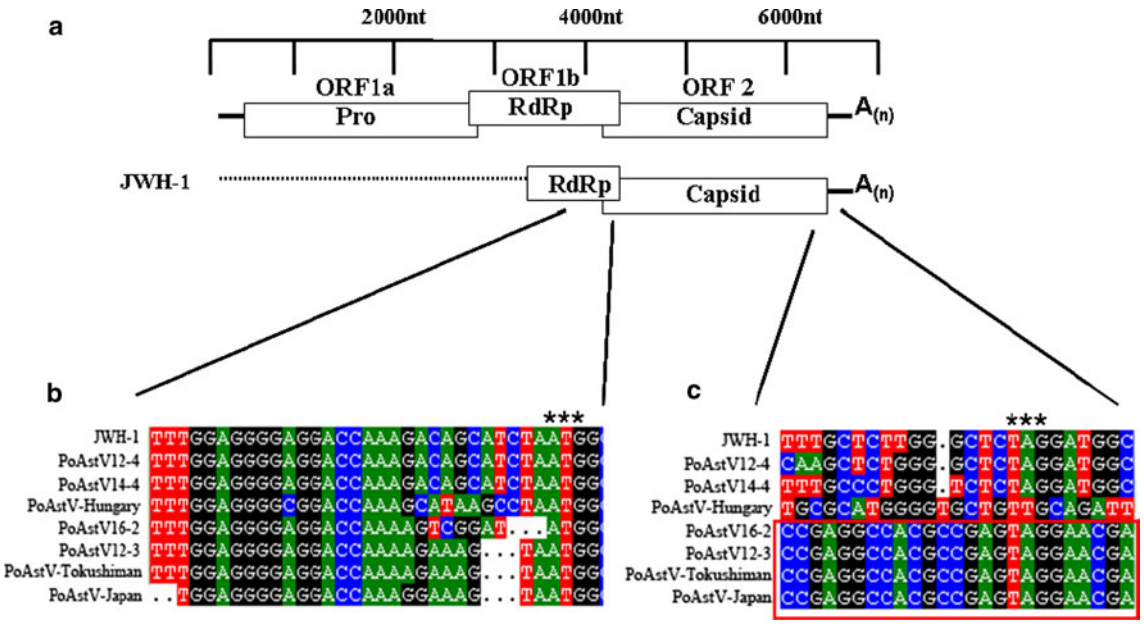

Fig. 1 Schematic representation showing the partial genome organization and nucleotide alignment of the newly identified PAstV strain JWH-1. **a** All ORFs, the 5′ UTR, the 3′ UTR and the polyA tail are shown in the diagram. Dotted lines represent uncharacterized regions. **b** Nucleotide sequence alignment of the conserved sequence at the ORF1b/ORF2 junction of the JWH-1 strain with those of prototypical PAstVs. The ATG initiation codon is indicated by asterisks. **c** Nucleotide sequence alignment of the conserved sequence motif at the 3′ ends of the JWH-1 strain with those of prototypical PAstVs. The ORF2 stop codon is indicated by asterisks. The stem-loop-II-like motif (s2m) is indicated by a red frame

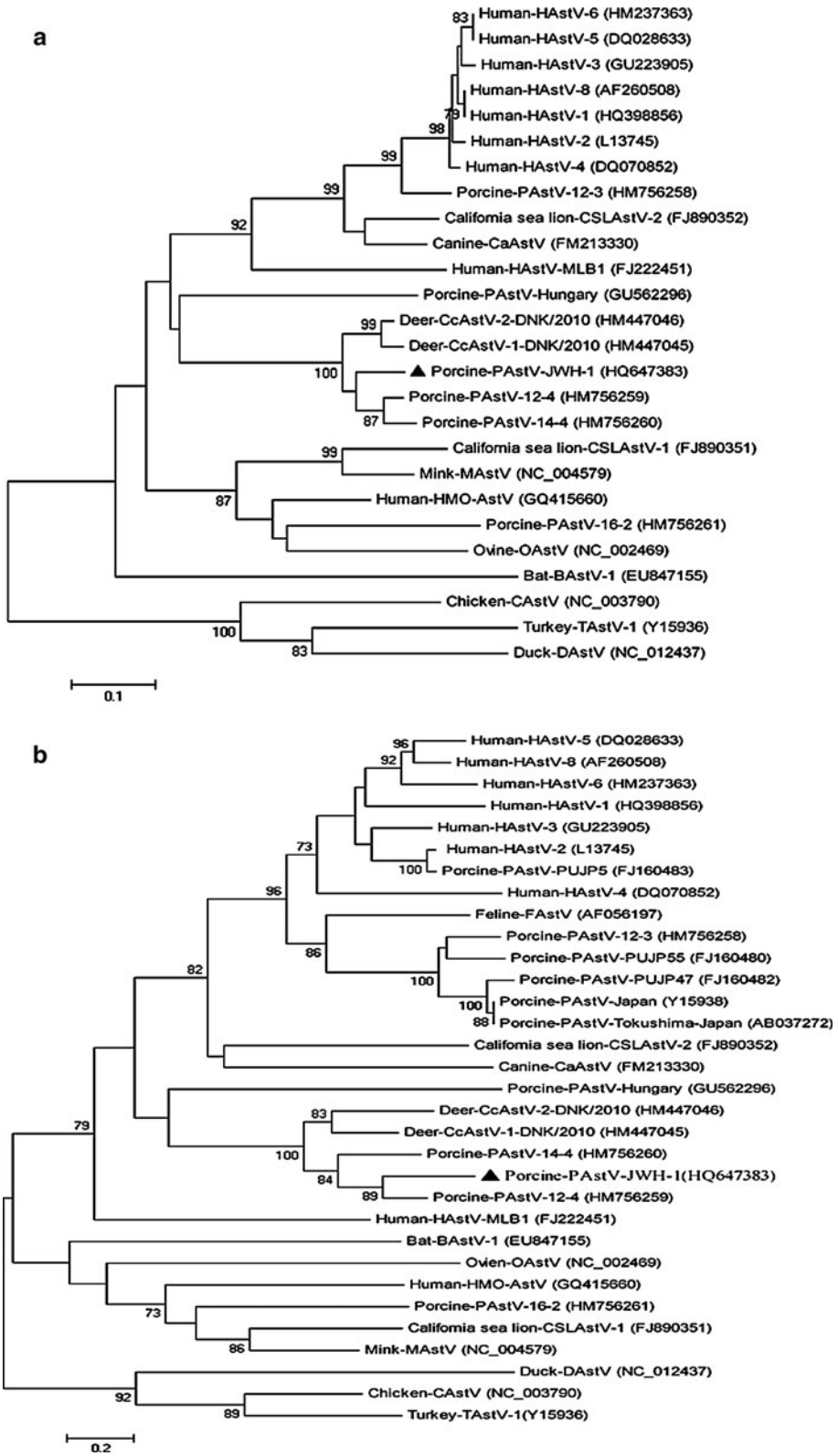

Fig. 2 Phylogenetic analysis of the newly identified PAstV strain JWH-1 and prototypical representatives of different astrovirus species. **a** A tree based on part of the RdRp region. **b** A tree based on the complete ORF2 coding region. Phylogenetic trees were generated by the neighbor-joining method using Mega 4 software. Bootstrap values of >70% are indicated for the corresponding nodes based on bootstrapping with 1000 replicates. The PAstV strain identified in this study is marked with a triangle. The GenBank accession number and species are indicated

PAstV 14-4 and deer astrovirus strains at the end of the ORF 1b region and in the ORF2 region. Further analysis of these highly similar sequences using the RDP4 program showed that there are two possible sites of recombination events in the JWH-1 strain, with a high degree of confidence ($p \leq 1.426 \times 10^{-4}$). Based on a bootscan plot (Fig. 4), one recombination event occurred between the PoAstV Hungary strain and the Deer–CcAstV-2 strain

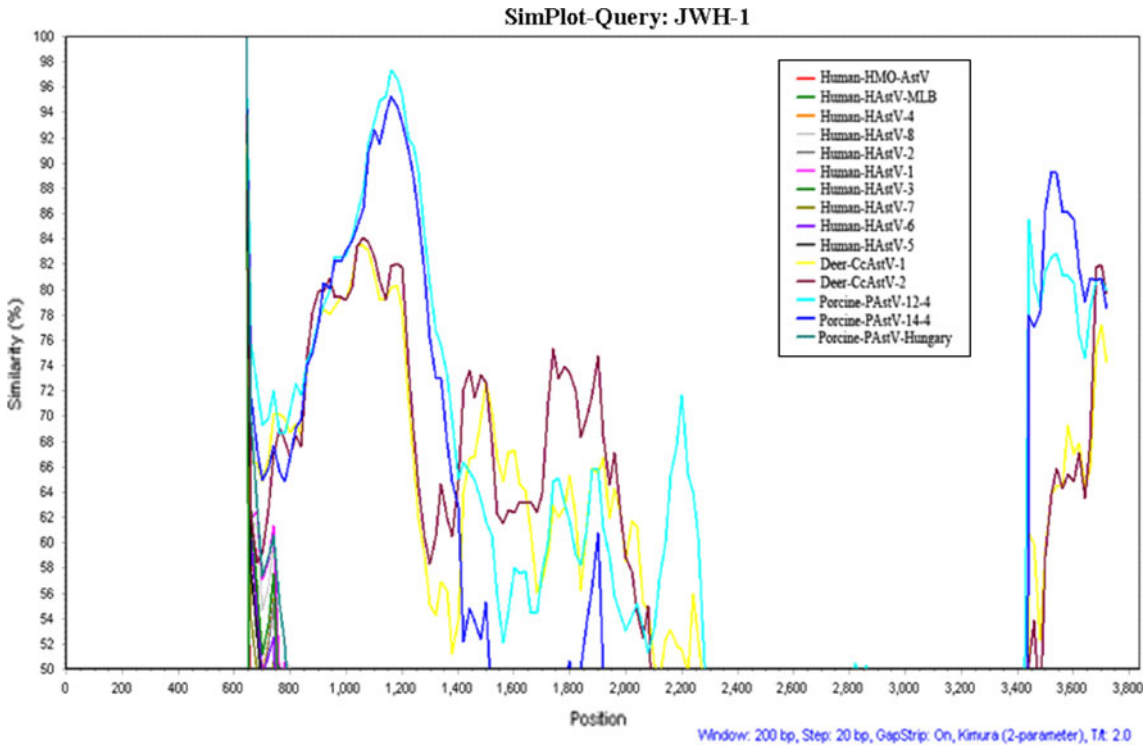

Fig. 3 Nucleotide similarity comparison of the newly identified PAstV strain JWH-1 with representative AstVs using the SimPlot program, showing a window size of 200 bp and a step size of 20 bp. The x-axis indicates nucleotide positions along the alignment, and the y-axis denotes similarity

(Fig. 4a), and another occurred between the PoAstV 14-4 strain and the Deer–CcAstV-2 strain (Fig. 4b), which led to the recombinant JWH-1 strain. To confirm the results, phylogenetic trees based on the recombinant regions and non-recombinant regions of the parental strains for each recombination event were constructed using MEGA4 (Fig. 4). The discrepancies between the phylogenetic trees provide direct evidence of recombination. The results described above indicate that recombination events have occurred within the genome of the JWH-1 strain.

In recent studies, novel astroviruses have been identified in species of many marine mammals, indicating that astroviruses have a wide range of host species [20]. Likewise, a number of genetically distinct strains of PAstV were recently identified and characterized. In the present study, we detected and characterized a PAstV strain in China. The PAstV strain was accidentally detected when the full-length RdRp gene of one porcine PBV strain was determined using the genome-walking method. The forward primer was a random primer from the commercial genome-walking kit, which was randomly combined with the sequence. The reverse primer was a specific primer that was designed for the RdRp region of PBV. Further comparison of the reverse primer and the PoAstV sequence in this study revealed that there is an 11-base-pair-long region of identity between the PoAstV sequence and the 3′ end of the primer sequence. We speculate that this identical motif, coupled with the randomness of the forward primer, led to the detection of the PAstV strain.

A highly conserved stem-loop-II-like motif (s2m) has been found in the 3′-end of the genomic RNA of all human astrovirus strains investigated except the human strain MLB-1. It has even been found in equine rhinoviruses, coronaviruses, and dog noroviruses [4, 12]. However, this motif was not found in the PAstV JWH-1 strain, and it is absent in the newly isolated strains PAstV 12-4 and PAstV 14-4. The biological role of the s2m motif requires clarification, because it is thought to be an indispensable element in the mamastrovirus life cycle [10]. We speculate that these strains without the s2m motif are able to form stable secondary structures, with a compensating element in a different part of the 3′-end of the genomic RNA that has a role similar to that of the s2m motif.

Phylogenetic analysis showed that the JWH-1 strain clustered closely with the newly identified strain PAstV 12-4 and 14-4, formed a novel group of mamastrovirus together with the proposed new deer astrovirus, and was different from the previously reported PAstVs. This result indicates that multiple lineages exist among PAstVs. Moreover, these lineages were distributed in different species. Likewise, recent reports about HAstVs have shown that they are genetically closer to animal AstVs strains and more distantly

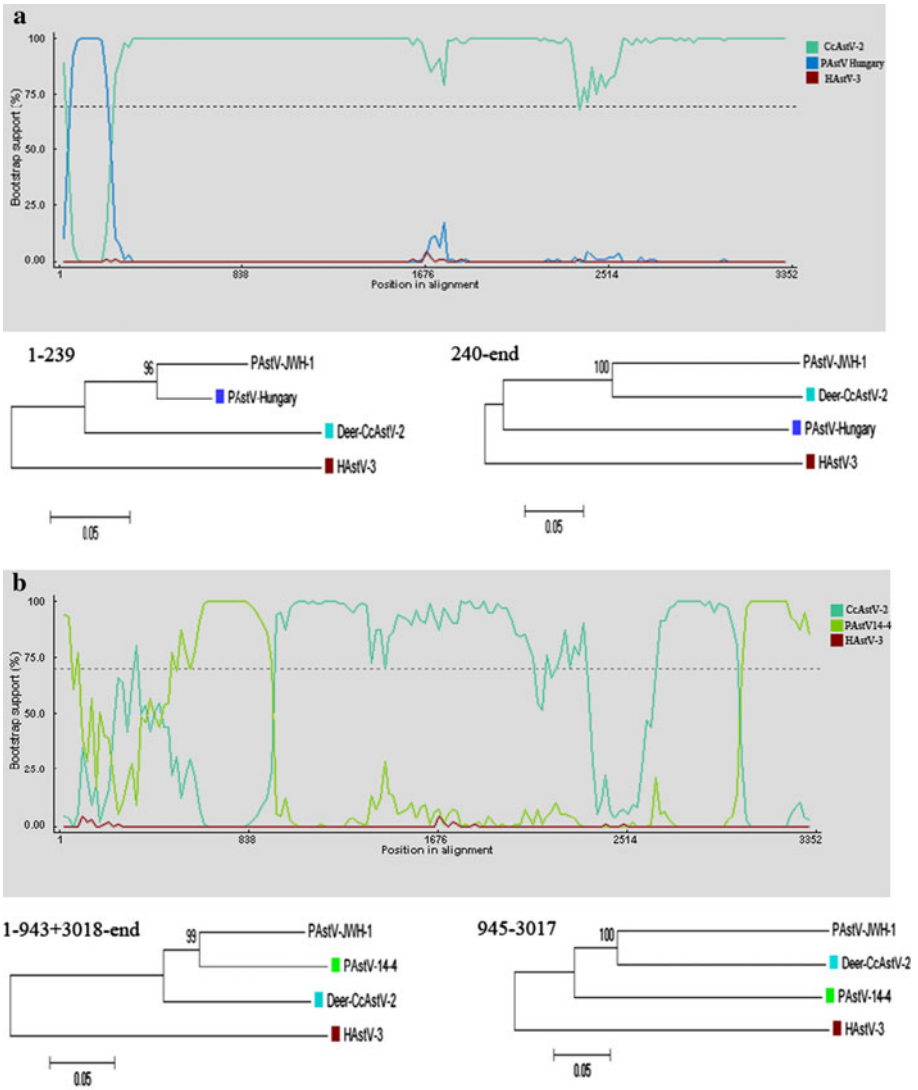

Fig. 4 Identification of the sites of two possible recombination events in the genome of the JWH-1 strain. **a** JWH-1 strain scan against strains PoAstV-Hungary and Deer–CcAstV-2, as well as HAstV-3 as the outgroup. **b** JWH-1 strain scan against strains PoAstV-14-4 and Deer–CcAstV-2, as well as HAstV-3 as the outgroup. The upper part of each panel shows BOOTSCAN evidence for recombination on the basis of pairwise distance, modeled with a window size of 200, a step size of 20, and 100 bootstrap replicates. The lower part of each panel shows phylogenetic trees constructed based on the recombination events

related to the type member HAstV [8, 12, 20]. This result suggests that PAstVs have distinct origins, and there is a possibility of interspecies transmission. Further recombination analysis indicated that the JWH-1 strain had possibly undergone two recombination events, and, interestingly, that these two possible recombination events involved interspecies recombination between porcine and deer astroviruses. Recombination is a relatively common phenomenon in positive-sense RNA viruses [3, 17], and understanding recombination can be helpful in unraveling the evolution of pathogens. Thus far, only one report has provided evidence of possible recombination events occurring between porcine and human astroviruses [24]. This result further confirms that interspecies recombination events occur in PAstVs.

In summary, we have identified and conducted partial genetic characterization of a PAstV strain in China. The results confirm that there are multiple lineages of porcine astroviruses and that these are distributed in different species. Moreover, recombination analysis showed that two interspecies recombination events between porcine and deer astroviruses had possibly occurred in the genome of the Chinese JWH-1 strain. These observations suggest that the pigs play an active role in the evolution and ecology of the family *Astroviridae*, and the possibility of potential interspecies transmission warrants further attention.

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