Identification and Genetic Characterization of Astrovirus in Wild Boar (Sus scrofa) in China

Fan-fan ZHANG [1,2,†]   Yu-xin TANG [1,2,†]   Yu YE [1,2]   Nannan GUO [1,2]   Min ZHANG [1,2]

Hao LI [1,2]   Dan LEI [1,2]   Qiong WU [1,2]   Dong-yan HUANG [1,2]   De-ping SONG [1, 2,a] ✎

[1] This work was carried out in Key Laboratory for Animal Health of Jiangxi Province in Jiangxi Agricultural University, Nanchang, Jiangxi, China
† These authors contributed equally to this work
1 Key Laboratory for Animal Health of Jiangxi Province, 330045, Nanchang, Jiangxi, CHINA
2 Department of Veterinary Preventive Medicine, College of Animal Science and Technology, Jiangxi Agricultural University, Nanchang, Jiangxi, CHINA
a ORCID: 0000-0001-5949-287X

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Abstract

Porcine astrovirus (PAstV) is a frequently detected virus in pigs suffering from diarrhea worldwide. Here, we report the first identification and complete genome sequence of astrovirus in wild boar (Sus scrofa) in Jiangxi, China. The complete genome sequence of a representative astrovirus, WBAstV/CH/2015, was amplified and determined. Sequence homology analysis showed that WBAstV/CH/2015 had 40.8% to 79.7% homology with PAstVs worlwide, and shared the highest homology (79.7%) with another wild boar astrovirus (WBAstV) strain WBAstV-1/2011/HUN from Hungary. Phylogenetic analysis showed that WBAstV/CH/2015 was closely related to WBAstV-1/2011/HUN and located in the cluster of PAstV 4.

Keywords: Porcine astrovirus (PAstV), Diarrhea, Wild boar (Sus scrofa), Genome, Phylogenetic analysis

Çin'de Yaban Domuzunda (Sus scrofa) Astrovirusların Tanımlanması ve Genetik Karakterizasyonu

Öz

Domuz astrovirusu (PAstV) dünya çapında ishalli domuzlarda en sıklıkla belirlenen bir virustur. Bu çalışmada, Çin'in Jiangxi eyaletinde yaban domuzunda *(Sus scrofa)* astrovirusun ilk tespiti ve tüm genom sekans analizi gerçekleştirilmiştir. Astrovirus WBAstV/CH/2015'un tüm genom sekansı amplifiye edildi ve belirlendi. Sekans homoloji analizi, WBAstV/CH/2015'in dünya çapında PAstVları ile %40.8 ile %79.7 arasında benzerliğe sahip olduğunu ve Macaristan'da tespit edilen diğer bir yaban domuzu astrovirus (WBAstV) suşu olan WBAstV-1/2011/HUN ile en yüksek benzerliğe (%79.7) sahip olduğunu gösterdi. Filogenetik analiz WBAstV/CH/2015'in WBAstV-1/2011/HUN ile yakın akraba olduğunu ve PAstV 4 topluluğuna yakın olarak yer aldığını göstermiştir..

Anahtar sözcükler: Domuz astrovirusu (PAstV), İshal, Yaban domuzu (Sus scrofa), Genom, Filogenetik analiz

INTRODUCTION

Astrovirus is a non-enveloped, single-stranded, positive-sense RNA virus that belongs to the family *Astroviridae*. Members of *Astroviridae* are associated with gastroenteritis, diarrhea, encephalitis and respiratory symptoms as those viruses could infect series of mammalian species (*e.g.* humans, bats, cattle, dolphins, deer, mice, mink, sheep, cats, pigs, and marine animals) and birds (*e.g.* duck, turkeys, and chickens) [1-4]. The entire genome of astrovirus is about 7 kb in length that includes 3 open reading frames (ORFs), ORF1a, ORF1b and ORF2. The ORF1a encodes the non-structural polyprotein 1a, while the longer ORF1b encodes polyprotein 1b, including the RNA-dependent RNA polymerase (RdRp), and ORF2 encodes the viral capsid structural polyprotein [5]. Porcine astrovirus (PAstV), a member of

✎ **İletişim (Correspondence)**

☏ +86 181 7080 7320 (C); Fax: 86 791 8382 8409 (O);

✉ sdpjxau@hotmail.com

Astroviridae, was first observed in diarrheal feces of weaning piglets in 1980 by electron microscopy, and then was identified in 1990 [6,7]. To date, PAstV has been detected in diarrheal and/or healthy pigs in several countries, including the United States, Canada, China, South Korea, and many other countries. PAstV is a widely distributed virus that causes diarrhea, dehydration, and congenital tremor in pigs. The infection rates of PAstV are 17.5% to 89% in domestic pigs, including pigs with diarrhea and healthy pigs [2,8-10]. In China, there have been studies concerning PAstV, but these were limited only to molecular epidemiology and domestic pigs [10,11]. In this study, we investigated the infection rate of PAstV in diarrheal wild boars. To elucidate the genetic characterization and evolutionary relationships with PAstVs from other countries/areas, we identified and analyzed the full-length genome sequence of a representative astrovirus in wild boar (WBAstV).

MATERIAL and METHODS

Diarrhea Outbreak Information

In June, 2015, a sudden outbreak of diarrhea occurred at a wild boar farm (about 100 sows) in Jiangxi, China. The wild boars had no contact with domestic pigs and other animals, and were breed and raised in a closed community. Pigs in different ages showed watery diarrhea, including breeding sows, suckling piglets, and weaned pigs, with a morbidity of 40% in all of the boars and a mortality of 20% in suckling piglets. A total of 20 diarrheal feces were collected and submitted to Animal Disease Diagnostic Center, Key Laboratory for Animal Health of Jiangxi Province, China.

Pathogen Detection

To confirm the cause of the diarrhea, common diarrhea-associated pathogens, including porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), porcine rotavirus (PoRV), porcine deltacoronavirus (PDCoV),

porcine circovirus type 2 (PCV-2), classical swine fever virus (CSFV), porcine kobuvirus (PKV), porcine bocavirus (PBoV) and pathogenic *Escherichia coli* and *Salmonella* were investigated based on the previous methods [12,13]. Porcine astrovirus was further tested as previous studies unveiled its association with diarrhea in pigs [14].

Complete Genome Sequencing of A Representative WBAstV

To understand the genetic information of those astroviruses in diarrhea wild boars, five couples of overlapping primers targeting the complete genome of WBAstV were designed based on the conserved regions determined by a multiple alignment analysis of the reference PAstVs retrieved from GenBank *(Table 1)*. Total RNAs were extracted from the feces by RNAplus Reagent (TaKaRa, Japan) according to the manufacturer's instructions. The first-strand cDNA synthesis was performed at 42°C for 50 min and then 95°C for 5 min to inactivate the M-MLV reverse transcriptase (TaKaRa, Japan) and followed by 4°C for 5 min. The entire genome was amplified by five pairs of primer. Fragments were amplified using rTaq DNA polymerase (TaKaRa, Japan) on the conditions of a denaturation at 94°C for 4 min, 35 cycles (94°C x 45 sec, 53°C x 45 sec, 72°C x 1.5 min), and then with a final extension at 72°C for 10 min. The 5'- and 3'- rapid-amplification of cDNA ends (RACE) for the determination of the terminal sequences of WBAstV were performed by using 5'/3' SMARTer RACE kit (Clontech, Beijing, China) following the manufacturer's instructions. Positive PCR products were subjected to gel purification, and afterwards cloned into pMD 18-T vectors (TaKaRa, Japan). Three to five positive clones of each amplicon were submitted to a commercial sequencing company (Sangon Biotech, Shanghai, China) for sequencing at both directions by Sanger sequencing methodology.

Sequence Analysis

The raw sequence fragments of the representative WBstV,

Table 1. Primer information of complete genome of PAstV amplification				
Name	Sequence(5'-3')	Position	Product Size (bp)	Overlap (bp)
PAstv 1-F	CCAAGGTTGATTTAGCTGTC	44-63	1195	
PAstv 1-R	CCAGTTGGATCCCTTATCTC	1219-1238		
PAstV 2-F	CAGCAGCACTCATTTCCCTG	965-984	1282	273
PAstV 2-R	ATCGTCTTCAGGGTCACTCC	2228-2247		
PAstv 3-F	GAAGGGTAAGACCAAGCATGGC	2045-2066	1034	130
PAstv 3-R	ATTGCTGAAAAGGCAGACACATAGG	3055-3079		
PAstv 4-F	GTCATCCATATCTCAGCCACAGAG	2924-2947	1025	155
PAstv 4-R	ATCAGGAGTTGTAGGCTCAGGAG	3927-3949		
PAstv 5-F	AGGGATTATGACACCATCGTC	3657-3677	1755	292
PAstv 5-R	TGGCCAAGAGTATGGATTTCA	5349-5369		
5'RACE	GGTAACGTAGGGTCTCCTCAG	873-898	898	
3'RACE	GTGTGTGGGGTTTGGATTGGGGCCA	5300-5320	1345	

named as WBAstV/CH/2015, were assembled by SeqMan in DNAStar *Lasergene* V 7.10 (DNAStar, Inc., Madison, WI). Homology of nucleotide (nt) and deduced amino acid (aa) sequences of WBAstV/CH/2015 and reference PAstVs were comparatively analyzed. Phylogenetic trees based on the entire genomes, ORF1b and ORF2 of WBAstV/CH/2015 and reference astroviruses were constructed using the neighbor-joining method by the software of molecular evolutionary genetics analysis 6.0 (MEGA v. 6.0) (http://www.megasoftware.net/) with a bootstrap of 1.000 replicate datasets [5].

RESULTS

The diarrheal associated pathogens, PEDV, PDCoV, TGEV, PDCoV, PRoV, PCV-2, CSFV, PKV, PBoV, pathogenic *E. coli* and *Salmonella* were tested upon the 20 fecal samples from diarrheal wild boar, but all showed negative results. PAstV, a suspected diarrhea virus, was further tested. Unexpectedly, 60% (12/20) of these samples were found to be positive for PAstV. The weaning piglets showed the highest detection rate, 7 out of the 9 samples were positive; samples from the suckling piglets (2/5) and sows (3/6) were also found to be positive for PAstV, but with lower infection rates.

To investigate the genetic characterize and relationship of WBAstV with astroviruses from other countries/areas, a representative WBAstV, designated as WBAstV/CH/2015, was amplified and sequenced. Multiple sequences of WBAstV/CH/2015 were assembled and annotated using DNAStar *Lasergene* software. The entire genomic sequence of WBAstV/CH/2015 was 6, 644 nt in length, excluding the 3' poly(A) tail, and the sequence was deposited in GenBank under the accession number KX033447. The genome structure of WBAstV/CH/2015 was typical of astrovirus, and was arranged in the order of the 5' untranslated region (UTR) (nt 1 to 103), ORF1a (nt 104 to 2, 653), ORF1ab (nt 104 to 4, 099), and ORF2 (nt 4, 091 to 6, 572), and the 3'UTR (nt 6, 573 to 6, 644). The 5'UTR of WBAstV/CH/2015 was

103 nt; the replicase gene containing ORF1a and ORF1b was 3, 996 nt in length; ORF2, coding the capsid protein, was 2, 481 nt, and the 3' UTR was 107 nt. Similar to the astroviruses from domestic pigs and wild boars, WBAstV/CH/2015 contained a conserved start pentamer (CCAAA) at the beginning of the 5' terminus. We also found that the frameshift heptamer (AAAAAAC) followed by a stem-loop structure, present near the 3' end of ORF1a in the PAstV-4 genome, which is a potential signal for a ribosomal frameshift during translation to generate the replicase polyprotein ORF1ab *(Fig. 1)*.

Sequence homology analysis showed that WBAstV/CH/2015 had 40.8 to 79.7 % homology to PAstVs, and shared the highest homology (79.7%) with a wild boar astrovirus, WBAstV-1/2011/HUN *(Table 2)*. The ORF1ab gene of WBAstV/CH/2015 was 3, 395 nt long, encoding a protein of 1, 132-aa, and with a 31.9% to 92.4% homology with the reference strains, shared the highest homology with PAstV4-CH-2014, a PAstV determined in domestic pigs in Jiangxi province in 2016.

Phylogenetic analyses of WBAstV/CH/2015 and astroviruses from domestic pigs, wild boars and other host species were conducted based on the sequences of complete genome, ORF1b and ORF2. The phylogenetic trees showed that WBAstV/CH/2015 was located in the cluster of type 4 astroviruses with 3 other porcine astroviruses and evolutionarily closed to WBAstV-1/2011/HUN *(Fig. 2A)*. The phylogenetic results indicated that WBAstV/CH/2015 belongs to the lineage PAstV-4. Phylogenetic trees based on the sequences of ORF1ab and ORF2 also revealed the close relationship between strain WBAstV/CH/2015 and strain WBAstV-1/2011/HUN *(Fig. 2B,C)*.

DISCUSSION

Astroviruses have a wide range of host species. As emerging infectious diseases pose a continuous health
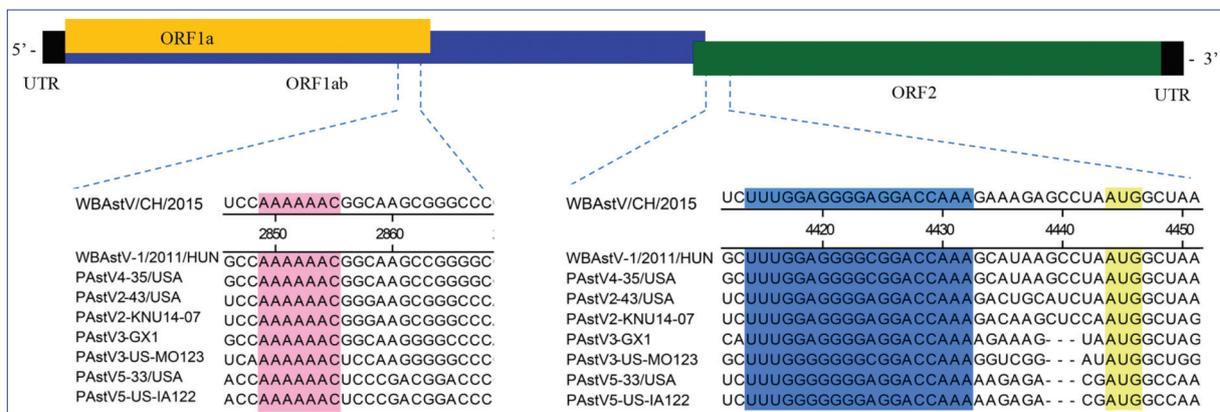
Fig 1. The schematic diagram of genomic structure, frameshift heptamer site and transcription initiation site of the subgenomic RNA of WBAstV/CH/2015. A frameshift heptamer AAAAAAC *(shaded in red)* and genotype conserved sequences *(shaded in green)* are present just upstream of the transcription initiation site *(shaded in yellow)* of the subgenomic RNA at the ORF1b and ORF2 junction

Table 2. *Nucleotide (nt) and amino acid (aa) sequence identities in percentage based on a comparison of the whole genome sequence*

Astrovirus Reference Strain	% Identity to WBAstV/CH/2015						
	Genome (nt)	ORF1a (nt)	ORF1a (aa)	ORF1ab (nt)	ORF1ab (aa)	ORF2 (nt)	ORF2 (aa)
PAstV2-43/USA	44.8	47	40.2	53.6	49.2	41.4	27.7
PAstV2-51/USA	44.5	47.1	40.2	53.6	49.1	40.1	25.9
PAstV2-US-IA122	44.2	46.8	39.8	53.4	49	39.6	26.8
PAstV2-KNU14-07	43.6	46.9	39.1	53.3	48.5	41.1	27.8
PAstV2-Bel-12R021	43.7	47.1	39.3	53.6	48.2	39.3	25.3
PAstV3-GX1	42.8	39.5	24.1	46	38.8	33.6	23.3
PAstV3-US-MO123	41.3	43.3	27.8	47.5	37.6	28	20
PAstV4-35/USA	77	81.6	84.8	85.9	90.4	65.3	58.4
PAstV4-US-IL135	76.7	80.6	81	84.9	87.7	66.2	58.5
PAstV4-CH-2014	79.7	85.4	88	88.4	92.4	67.9	62.4
WBAstV-1/2011/HUN	77.5	79.2	78.3	83.7	56.1	67.5	64.3
PAstV5-33/USA	40.8	41.4	21.5	45.2	32	37.1	22.4
PAstV5-US-IA122	40.9	41.8	21.5	45	31.9	31.8	20.9
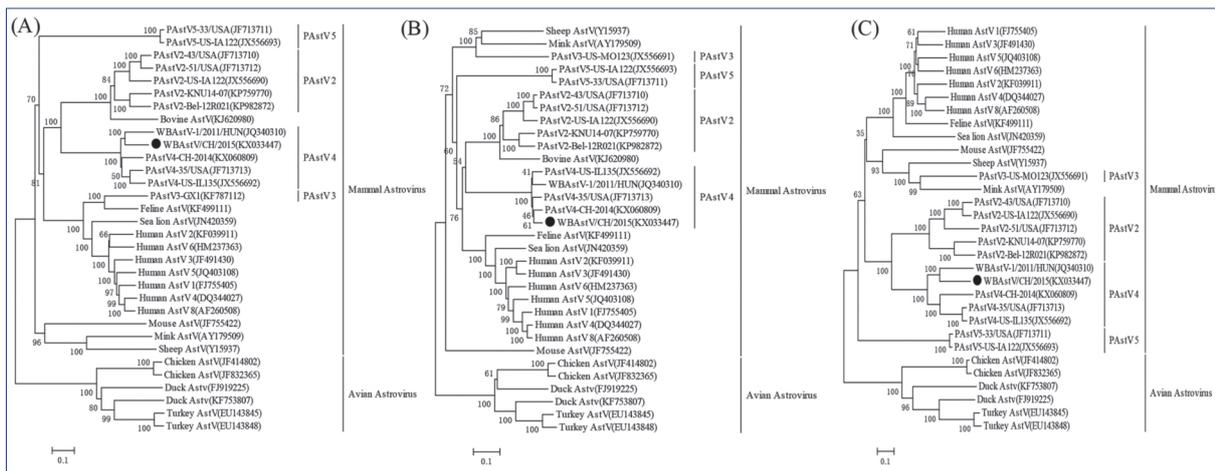
Fig 2. Phylogenetic trees of the entire genome (A), ORF1b (B) and ORF2 (C) sequence of Porcine Astroviruses. A bar of 0.1 indicates nucleotide or amino acid substitutions per site. "●" indicates the strain identified in this study

threat to wild and domestic animals as well as to humans, continued characterization of astrovirus in different host species and areas will help our understanding of their origin and the possible mechanism of cross-species transmission. The study presented here is based on a field outbreak of diarrhea in wild boar herd. Our investigation showed the presence of astrovirus in wild boar in Jiangxi, one of the main pork producing province in China. Pathogen detection showed negative of eight porcine diarrheal-associated viruses (PEDV, PDCoV, TGEV, PDCoV, PRoV, PCV-2, CSFV, PKV, and PBoV), pathogenic *E. coli* and *Salmonella* in these diarrheal samples, and only WBAstV was found in 60% of these samples. The result was unlike the previous report in diarrheal domestic pigs and wild boars in another main pork produce province, Sichuan, in China. They found frequent coinfections of PEDV and PAstV in diarrheal pigs [15-17]. Shan et al.[18] reported the high

presence of astrovirus in healthy and diarrheal piglets in high density premises. Studies revealed astrovirus was associated with gastroenteritis and diarrhea in humans and animal species [19,20]. Our results revealed the astrovirus might be associated with diarrhea in wild boar. Further studies on virus isolation and pathogenesis are needed.

The entire genomic sequence of the representative astrovirus, WBAstV/CH/2015, was determined in this study. Sequence homology analysis showed WBAstV/CH/2015 was highly conserved with WBAstV-1/2011/HUN strain from wild boar in Hungry. The ORF2 showed high similarity to astroviruses in domestic pigs, but 20-aa shorter than the wild boar astrovirus from Hungry, WBAstV-1/2011/HUN [8]. The frameshift heptamer (AAAAAAC) at the ORF1a/1b junction was also found in WBAstV/CH/2015, and was conserved in PAstVs. The phylogenetic analysis

showed that WBAstV/CH/2015 was closely related to the wild boar astrovirus, WBAstV-1/2011/HUN from Hungary [8], and located in the cluster of type 4 astroviruses with 3 other porcine astroviruses. Recently, investigations have displayed up to five genotypes of PAstVs (PAstV-1~ PAstV-5) in domestic and wild pigs [17]. To our knowledge, this is the first report of type 4 astrovirus in wild boar in China. WBAstV/CH/2015 strain showed a 76.7%~79.7% nucleotide sequence identity to the recently discovered PAstV-4. Interestingly, we found the homology of ORF1ab of WBAstV/CH/2015 was highest with PAstV4-CH-2014, a astrovirus determined in domestic pigs in the same provine. Although the wild boars we investigated had not contacted with domestic pigs and other animals, we supposed humans, feeds or vechicles might carried this virus and transmitted to the wild boars. While further studies on the cross-species transmission of astrovirus are needed.

In conclusion, we firstly identified the presence of astrovirus in wild boars in Jiangxi, China. Then we determined and analyzed the genetic characterizations of the full-length genome sequence of a representative astrovirus in wild boar. Our results give further insight of into the presence of astroviruses in wild animals and provide information of the epidemiology and evolution of PAstV in China and other countries.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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