Research Article

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Identification, Isolation And Genomic Characterization Of Porcine Astrovirus In Shandong Province, China, 2021-2023

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1.Abstract:

Astroviral infection has been described as one of important viruses causing diarrhea in humans and other animals. Porcine astrovirus (PAstV) is broadly distributed globally and exists at least five distinct genotypes. However, few studies have investigated in diarrheic piglets in Shandong area of China. Here, a total of 1025 samples of porcine diarrhea samples were collected from part areas of Shandong Province from January 2021 to October 2023 and tested by RT-PCR, followed by sequencing and phylogenetic analyses of the polymerase. The results showed that the total positive rate of PAstV was 34.6% (355/1025), of which the proportion of PAstV 1, PAstV 2, PAstV 4 and Past V 5 infection was 25.4% (90/355), 28.2% (100/355), 35.2% (125/355) and 22.5% (80/355), respectively, and also mixed infection existed. Meanwhile, 849 samples of healthy pigs were tested by RT-PCR, and the results showed that the total positive rate of Past V was 8.13% (69/849), of which the proportion of PAstV 1, PAstV 2 and Past V 4 infection was 27.5% (19/69), 37.7% (26/69) and 40.6% (28/69) and also mixed infection existed. Further sequencing and characterization of some the selected isolates revealed low sequence identities (56.2%) with known Past V strains, indicating novel types or genotypes of Past Vs. In addition, by optimizing the isolation conditions of porcine astrovirus, a pure PAstV-4 strain (named PAstV-4-GRF1) was obtained. Analysis revealed virus of typical astroviral morphology with 89.9 to 95.4% nucleotide identity with previously published PAstV-4 strains. Then, macrovirus transcriptome sequencing showed that 88.30% of the GRF1 samples were mammalian astroviruses, while by species classification, Past V 4 and Past V 2 accounted for 21.79% and 0.32%, respectively. Phylogenetic tree analysis showed that the c15050 fragment was the same as the GRF-1 sequencing fragment of the isolated strain, and had the highest homology with the Hunan Past V 4 sequence MK460231 in China. Altogether, this study investigated the prevalence of PAstV in Shandong Province and mainly analyzed the genetic evolution, which enriched the content of epidemiological investigation of porcine astrovirus. As the first isolated PAstV-4 strain, it provided critical material for the investigation of the biological and pathogenic properties of this virus as well as for future development of relevant biological and diagnostic reagents.

2. Keywords:
Porcine astrovirus, Epidemic situation, Genotype analysis, Isolation and identification.

3. Introduction:

Astrovirus (AstV) belongs to the Astroviridae family (Astroviridae), including Mamastrovirus and Avastrovirus, widely exists in humans, mammals and birds, causing diarrhea and neurological symptoms[1, 2]. The virions are about 28-30nm, non-enveloped, and contain a single-stranded, positive sense RNA molecule of 6.4 to 7.7 kb[3]. The genome is arranged in a 5' end that encodes non-structural proteins, three open reading frames (ORF1a, ORF1b, and ORF2) and 3′ end that encodes structural proteins[4]. As RNA viruses, they could facilitate new virus through gene reassortment or mutation that emerging cross-species transmission in the genotypes[5-7]. Porcine astrovirus (PAstV) was first discovered in 1980 by Bridger[8], and then spread to China[9], the United States[10], Japan[11], South Korea[12], India[13], Chile[14] and other countries. At present, five genotypes of PAstV (PAstV-1 to PAstV-5) with different prevalences have been identified worldwide[1, 15]. Except for PAstV-3, which is associated with neurological symptoms, all four types

are associated with diarrhea[16-18]. Although their pathogenicity is not well understood because of the difficulty in vitro isolation for experimental infections. To date, PAstV-1 was the first PAstV that has been isolated in cell culture[19]. Experimental infections with isolated strains of this virus have confirmed its pathogenicity, presenting as mild diarrhea in piglets with damage to the villi of the small intestinal mucosa and eventual growth retardation[20]. In 2020, a swine astrovirus type 5 from clinical swine fever virus (CSFV)-infected tissue samples was isolated possibly because CSFV counterinfection significantly enhanced the replication of PAstV5 [21]. However, it has been reported the most prevalent strain reported in China was PAstV-4, while PAstV-2 and PAstV-4 were the most common throughout Asia[22]. It is still that information available on the genetic characterization of PAstV in China is fairly limited. Therefore, it is necessary to identification, isolation and genetic diversity of PAstV currently in Shandong, China.

4. Methods:

4.1. Sample collection, cDNA synthesis and PAstV detection

To explore the prevalence of PAstV strains in Shandong Province of China, a total of 1025 diarrhea cases and 849 healthy pig samples from swine were collected from pig farms in 11 breeding regions, including Binzhou, Dezhou, Dongying, Heze, Jinan, Jining, Liaocheng, Tai'an, Weifang, Yantai and Zibo, were collected from January 2021 to October 2023. The RNA from these samples was purified from the supernatant of a previously homogenized 10% suspension in PBS with RNAiso Plus (Takara, Japan) according to the manufacturer's instructions. The cDNA was reverse-transcribed according to the supplier's instructions of the PrimeScript TM RT Reagent Kit (Accurate Biology, China) and subsequently used in PCR amplification using specific PAstV detection primers. Further, positive samples were analyzed and genotyped by PCR typing primers. All primer sequences from this study are listed in Table 1.

Table 1: Primers and PCR reaction conditions used in this study.

Primers	Primer sequences (5′→3′)	Annealing temperature (℃)	Product size (bp)
PAstV-H-F	GACATTTTGTGGATTTACAGTTGG	51	279
PAstV-H-R	TTGGTCCTCCCCTCCAAAG	56	
P1-F	TCCTGTGCTATCAGTTGCTCTC	61	419
P1-R	GATTGCTGGTTTTGGACCTGTG	61	
P2-F	AGCAGCTGGATCGTCTTTGGA	59	826
P2-R	AGATTCAGCATCCCAGGTTGTT	59	
P4-F	TGGCTTCAGGCCTTTGAGTTTT	59	558
P4-R	CACCGTCGTAGTAGTCGTGAC	61	
P5-F	TGGTACGTRCACAATCTGTTGAA	57	181
P5-R	TCAGTGTCTTCCCAACCRTC	57	
PEDV-F	TTGAACCTAACACACCTCCT	56	324
PEDV-R	TAAGCTTGTCAGGGTTTTCG	56	
RV-F	TGTACTAGCACCATTCGTCA	56	616
RV-R	TCTTATTGTGCATGTAGCGG	56	

4.2. Sequence alignment and genotype analysis

The PAstv-positive samples from the sick farms were further subjected to genome sequencing. To obtain the sequences, PCR was conducted using 2× Accurate Taq Master Mix (dye plus) (Accurate Biology, China) to amplify the overlapping fragments, using the primer pairs listed with the above. The reaction system was as follows: 94°C for 30 sec;35cycles of 98°C for 10 sec, 56°C for 30 sec and 72°C for 1 min; final extension at 72°C for 2 min. The amplified products were sent to Sangon Bioengineering (Shanghai) Co., Ltd. for sequencing. The obtained sequences were used to compare with the reference strains registered in GenBank (Table S1). Subsequently, phylogenetic trees were constructed by MEGA 6.0 using the neighbor-joining method and p-distance model were used to analyze the relationship between the PAstV gene and the reference strains.

Host	Times	Country	Genotype	GenBank login number
Cat	2012	Portugal	Feline astrovirus	KF374704
Cat	2012	China	Feline astrovirus	KF499111
Cat	2013	U.S.A	Feline astrovirus	KM017743
Cat	2017	China	Feline astrovirus	MK671306
Cat	2017	China	Feline astrovirus	MK671307
Cat	2017	China	Feline astrovirus	MK671308
Cat	2017	China	Feline astrovirus	MK671309
Cat	2018	China	Feline astrovirus	MK671310
Cat	2018	China	Feline astrovirus	MK671311
Cat	2018	China	Feline astrovirus	MK671312
Cat	2018	China	Feline astrovirus	MK671313
Cat	2018	Australia	Feline astrovirus	MW037841
Cat	2020	Canada	Feline astrovirus	MW164633
Tiger	2018	China	Tiger astrovirus	MN148428
Pig	2000	Japan	Porcine astrovirus 1	AB037272

Pig	2006	Canada	Porcine astrovirus 1	HM756258
Pig	2008	China	Porcine astrovirus 1	GQ914773
Pig	2013	China	Porcine astrovirus 1	KF787112
Pig	2010	U.S.A	Porcine astrovirus 2	JF713710
Pig	2010	U.S.A	Porcine astrovirus 2	JF713712
Pig	2011	U.S.A	Porcine astrovirus 2	JX556690
Pig	2012	U.S.A	Porcine astrovirus 2	KJ495986
Pig	2012	Uganda	Porcine astrovirus 2	KY940077
Pig	2014	Japan	Porcine astrovirus 2	LC201585
Pig	2015	Japan	Porcine astrovirus 2	LC201586
Pig	2015	Italy	Porcine astrovirus 2	MG930777
Pig	2017	Chile	Porcine astrovirus 2	MZ819164
Pig	2017	China	Porcine astrovirus 2	MK460230
Roe deer	2014	Slovenia	Roe deer astrovirus	MN150125
Bovine	2011	China	Bovine astrovirus	HQ916313
Bovine	2011	China	Bovine astrovirus	HQ916317
Bovine	2014	China	Bovine astrovirus	KJ620980
Bovine	2014	Japan	Bovine astrovirus	LC047796
Pig	2011	U.S.A	Porcine astrovirus 3	JX556691
Pig	2010	U.S.A	Porcine astrovirus 4	JF713713
Pig	2011	U.S.A	Porcine astrovirus 4	JX556692
Pig	2012	Kenya	Mamastrovirus 4	MT451918
Pig	2014	Japan	Porcine astrovirus 4	LC201603
Pig	2014	China	Porcine astrovirus 4	KX060808
Pig	2014	China	Porcine astrovirus 4	KX060809
Pig	2015	U.S.A	Porcine astrovirus 4	KU764484
Pig	2015	U.S.A	Porcine astrovirus 4	KU764486
Pig	2015	Japan	Porcine astrovirus 4	LC201607
Pig	2015	Japan	Porcine astrovirus 4	LC201613
Pig	2015	Japan	Porcine astrovirus 4	LC201614
Pig	2017	China	Porcine astrovirus 4	MK460231
Pig	2018	China	Porcine astrovirus 4	MK613068
Pig	2018	China	Porcine astrovirus 4	MT470220
Pig	2018	China	Porcine astrovirus 4	MH425243
Pig	2019	Belgium	Porcine astrovirus 4	MT642666
Pig	2020	China	Porcine astrovirus 4	MW962975
Wild boar	2011	Hungary	Mamastrovirus 3	JQ340310
Wild boar	2015	China	Mamastrovirus 3	KX033447
Pig	2010	U.S.A	Porcine astrovirus 5	JF713711
Pig	2011	U.S.A	Porcine astrovirus 5	JX556693
Pig	2014	China	Porcine astrovirus 5	MT642595
Pig	2015	China	Mamastrovirus 3	KP747574
Pig	2015	Japan	Porcine astrovirus 5	LC201619
Pig	2015	Japan	Porcine astrovirus 5	LC201620
Pig	2017	Chile	Porcine astrovirus 5	MZ819168
Pig	2017	Chile	Porcine astrovirus 5	MZ819171

4.3. Cells and Virus

PK-15 cell line (pig kidney epithelial cells, ATCCCCL-33), were ideal cells for isolation and culture for PAstV in vitro. They were maintained with 10% FBS DMEM medium at 37°C under 5%CO2. The supernatant of samples with positive PAstV was taken, filtered and sterilized through a 0.22 μm sterile filter, and then inoculated with PK-15 cells. Briefly, the prepared PK-15 cells (6×106 cells/mL) were treated with 500 μL of the sterilized sample supernatant and 500 μL of pre-configured TPCK trypsin solution (TPB 150 μL, trypsin 250μL, DMEM 50mL). After the incubation of 12h, the virus solution was discarded replaced with 2% FBS DMEM, and cultured in a 5% CO2 incubator at 37°C for 48 h. Total RNA was isolated from virus-infected cells which frozen-thawed for three times and detected the PAstV by RT-PCR. PAstV type 4 strain, named as PAstV4 GRF-1 was isolated and obtained from the diseased pigs in Jinan, Shandong Province of China. The virus was continuously passaged on PK-15 cells for more than 18 generations and virus titer was as high as 104.5 TCID50/0.1mL.

4.4. Scanning Electron Microscopy

Briefly, the samples were fixed in 1% paraformaldehyde and 0.25% glutaraldehyde, dehydrated by ascending alcohol series and dried at the critical point with Balzers CPD 030 Critical Point Dryer (BAL-TEC, Schalksmühle, Germany). After coating samples with gold/carbon using a sputter coater SCD 050, scanning electron micrographs were taken with a LEO 1525 (Zeiss, Oberkochen, Germany).

4.5. Metaviral transcriptome sequencing analysis

Total RNA was extracted from the fecal supernatant by TRIzol, and ribosomal RNA was removed from the total RNA of the extracted sample to obtain mRNA. Then the obtained mRNA was randomly interrupted into 250-300bp short fragments with divalent cations in NEB Fragmentation Buffer. The fragmented RNA was used as the template and the random oligonucleotide was used as the primer to synthesize the first strand of cDNA, and then DNA Libraries were constructed applying the NEBNext® UltraTM DNA Library Prep Kit for Illumina (E7370L, New England Biolabs, Frankfurt am Main, Germany). The obtained raw data were subjected to sequencing quality analysis by removing adapters and low-quality reads, using FastQC for sequencing quality analysis. Kraken software was used to classify and annotate the quality-controlled raw data, and SPAdes software for de novo assembly to obtain the genome sequence. The spliced sequences were compared with the GenBank Virus RefSeq protein database using DIAMOND software to screen out viral sequences and align the sequence predicted by ORF with the virus sequence extracted from NR database of NCBI. The predicted PAstV sequence and the reference sequences of different genotypes of porcine astrovirus in GenBank were used to construct an evolutionary tree in MEGA7.0, and the Neibor-Joining method was used to construct the phylogenetic tree.

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5. Results

5.1. RT-PCR testing PAstV

RT-PCR detection of PAstV was performed on 1025 diarrhea pigs and 849 healthy pigs from 11 pig farms in Shandong Province of China. The overall prevalence of PAstV specific target genes in diarrhea pig samples was found to be 34.63% (355/1025) as the expected size of the target band of 279 bp (Figure 1A), the total incidence rate of healthy pigs is 8.13% (69/849), indicating that PAstV is widely distributed in Shandong Province and an important cause of piglet diarrhea. Also, the co-infection with other diarrhea viruses which most commonly detected was analyzed, such as PEDV and PRV. As a result, the rate of PEDV and PRV coinfection in PAstV-positive samples of diarrhea pigs was 46.5% (165/355) and 42.3% (150/355), respectively (Figure 1B), The co-infection rate of PEDV and PRV in PAstV-positive samples from normal pigs was 47.8% (33/69) and 43.5% (30/69), respectively (Figure 1C), indicating that they may have a potential role in PAstV-induced diarrhea in piglets.

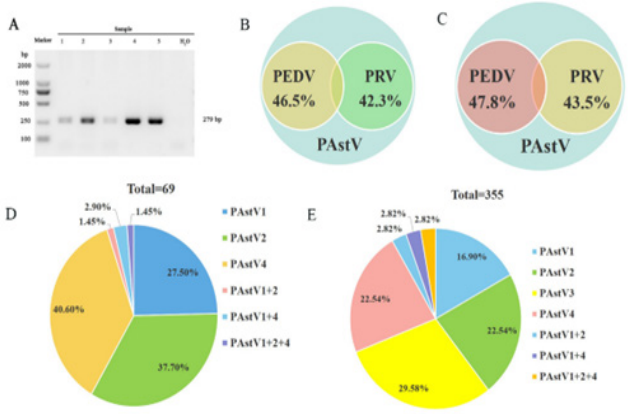

FIGURE 1: Prevalence of PAstVs in piglets. (A) Detection of astrovirus in diarrhea samples by RT-PCR. The intend band was 279bp. (B) The coinfection of PAstV with other diarrhea viruses in diarrheic piglets. (C) The coinfection of PAstV with other diarrhea viruses in heathy piglets. (D)The infection rate of the 3 porcine pathogens in the 69 PAstV-positive samples. (E) The infection rate of the 4 porcine pathogens in the 355 PAstV-positive samples.

In order to further genotype the positive samples detected above, RT-PCR with the specific primers was performed. As shown in Figure 1D, in heathy pig samples, the proportion of PAstV-1 was 27.5% (19/69), PAstV-2 was 37.7% (26/69) and PAstV-4 was 40.6% (28/69). PAstVs were divided into four groups in pig diarrhea samples, PAstV-1, PAstV-2, PAstV-4 and PAstV-5. Among them, the proportion of PAstV-1 was 25.4% (90/355), PAstV-2 was 28.2% (100/355), PAstV-4 was 35.2% (125/355) and PAstV-5 was 22.5% (80/355) (Figure 1E), of which PAstV-4 accounted for the highest ratio, indicating that PAstV-4 was predominant in diarrheic piglets in Shandong province.

5.2. Phylogenetic Analysis of PAstV

In the current study, a total of 29 strains were successfully sequenced from the 424 PAstV-positive samples. Here, a phylogenetic tree was constructed based on the ORF genes of the 29 identified PAstV strains and 75 reference AstV strains (Supplementary Table 1). For PAstV-1, eight sequenced fragments were compared for homology with the PAstV type 1 sequences in GenBank. The results showed that the homology between the eight strains in Shandong was 73.4%-95.2%, and the homology with the representative strains AB037272, HM756258, GQ914773 and KF787112 strains were 77.2%-94.4% (Supplementary Figure 1).

Supplementary Figure 1

Percent Identity

Divergence	1	2	3	4	5	6	7	8	9	10	11	12		
1		79.0	79.5	78.8	79.7	75.8	76.7	73.4	77.2	80.4	78.5	80.9	1	625-7-1
2	24.6		88.5	90.7	95.2	86.8	82.7	83.4	90.2	90.7	89.4	91.7	2	719-5-1
3	24.0	12.5		92.4	89.5	85.9	81.9	79.7	85.9	86.4	84.3	86.7	3	719-6-1
4	25.0	10.0	8.0		90.4	85.1	81.5	78.1	86.4	86.9	84.8	87.4	4	719-26-1
5	23.7	4.9	11.3	10.3		87.7	81.9	82.6	92.6	93.4	91.8	94.4	5	811-3-1
6	29.3	14.7	15.6	16.8	13.5		79.3	79.9	86.3	88.4	86.9	86.1	6	1015-1-1
7	28.0	19.9	20.8	21.5	20.9	24.4		80.5	81.6	83.5	81.6	81.6	7	1015-12-1
8	33.0	18.8	23.8	26.1	19.8	23.5	22.6		82.1	83.4	82.1	82.1	8	1015-21-1
9	27.2	10.6	15.7	15.2	7.9	15.3	21.4	20.5		77.8	76.9	74.9	9	AB037272
10	22.8	10.0	15.0	14.5	7.0	12.7	18.8	18.8	26.7		75.4	81.8	10	HM756258
11	25.3	11.5	17.7	17.1	8.8	14.6	21.2	20.5	28.0	30.2		82.6	11	GQ914773
12	22.1	8.8	14.7	13.9	5.8	15.6	21.4	20.5	30.9	21.2	20.1		12	KF787112
	1	2	3	4	5	6	7	8	9	10	11	12		

One of the eight strains (811-3-1) in the branch of PAstV-1 showed higher genetic relationship with strain KF787112 from China 2013, sharing 94.4% of mean nt/aa identities. Phylogenetic trees were constructed based on these obtained nucleotide sequences compared with the selected AstV sequences from other species available in GenBank, respectively. With the expection of all other identified PAstV-1 strains (circles in red) together with the five reference strain sequences form the PAstV-1 clade, which is the closest relative to KF787112, with an average genetic distance of 0.137. Moreover, the lineage of PAstV-1 showed a close relationship to other astroviruses species recovered from cat and tiger (Figure 2A).

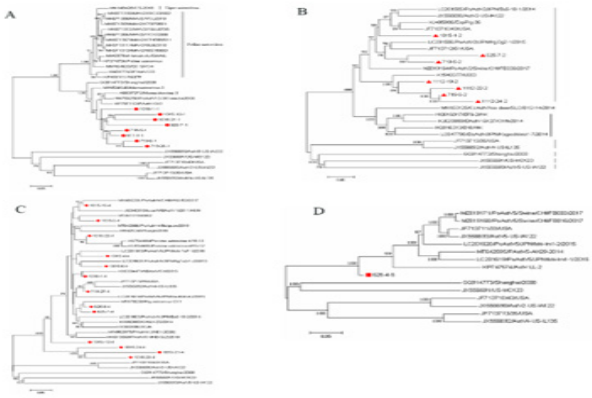

FIGURE 2: Phylogenetic Analysis of the newly identified PAstV strains. (A) Phylogenetic analysis based on the identified PAstV 1 with reference AstV strains. (B) Phylogenetic analysis based on the identified PAstV 2 with reference AstV strains. (C) Phylogenetic analysis based on the identified PAstV 4 with reference AstV strains. D) Phylogenetic analysis based on the identified PAstV 5 with reference AstV strains. Phylogenetic analysis

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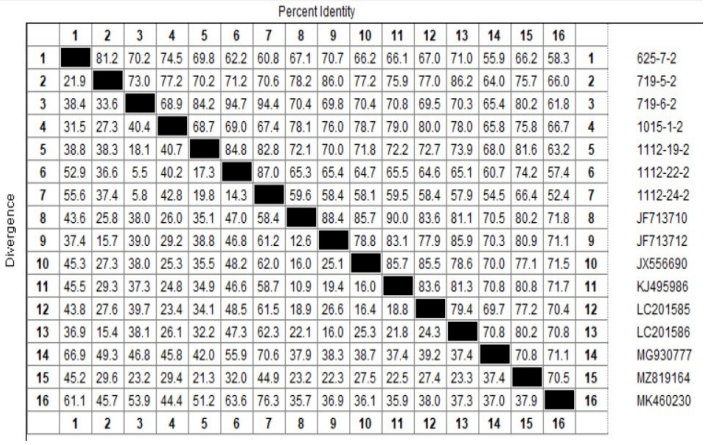
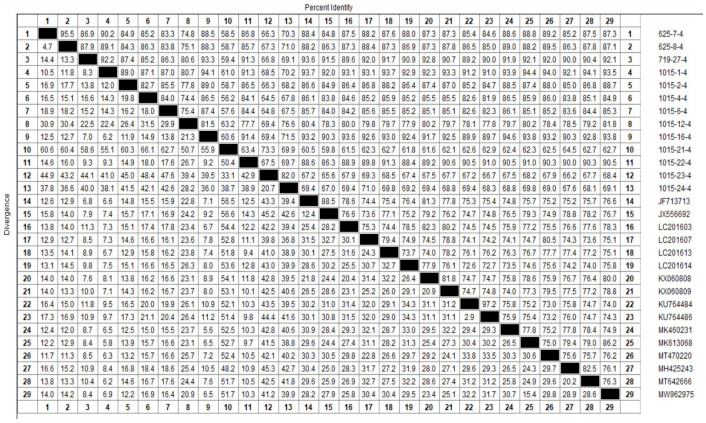
based on genome sequences of detected porcine astrovirus (PAstVs) and some other representative mammal astrovirus. The homology analysis and the trees were constructed by the neighbor-joining method with the p-distance model in MEGA 7.0 software. The PAstV isolate analyzed in the present study is indicated by circle in red. The GenBank accession number for each genome is shown.

For PAstV-2, the present 7 sequences showed identities of 60.8%~94.7% between each other, with 52.4%~86.2% identity to other available PAstV-2 sequences in GenBank (Supplementary Figure 2), meaning great variation of PAstV-2 in Shandong province. Phylogenetic analysis indicated that the present PAstV-2 sequences from Shandong province clustered into a monophyletic group. For example, the isolated strains 625-7-2 and 719-5-2 are closely related to the American strain JF713712 in 2010 and the Japanese strain LC201586 in 2015 while the strains 719-6-2, 1112-19-2, 1112-22-2 and 1112-24-2 clustered together with the Ugandan strain KY940077 in 2012. Interestingly, AstV sequences recovered from roe deer (MN150125) and cow (HQ916313) clustered also with the PAstV-2 group, possible indicating a recent cross-species transmission (Figure 2B).

Supplementary Figure 2

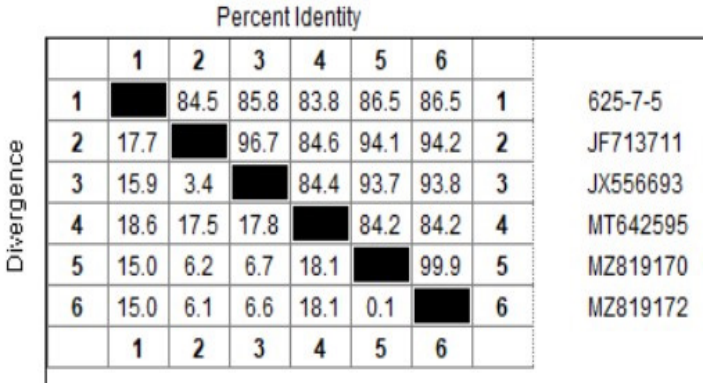


The 13 PAstV-4 sequences were identified of 56.2%~95.5% between each other. Among them, isolated strains 1015-21-4,1015-23-4 and 1015-24-4 had relatively large variation with the other 10 strains, and their homology with the reference strains was also very low (60.5%-71%) (Supplementary Figure 3).



In the phylogenetic analysis, the present sequences were quite different from different PAstV-4 reference strains distributed in all over the world. Of note, 1015-1-4 and 1015-4-4 were closely related to the GenBank wild boar astrovirus sequence (KX033447) from Jiangxi province in China, with genetic distances of 0.056 and 0.139, respectively (Figure 2C). Only one strain of PAstV-5 was obtained by sequencing, and the homology between this strain and the reference strain was 83.8%-86.5%, as shown in Supplementary Figure 4. The phylogenetic tree showed that the strain was closely related to the 2017 Chilean strains MZ819170, MZ819171, and MZ819172 (Figure 2D).

Supplementary Figure 4.



5.3. Isolation and identification of PAstV-4

To obtain the pure PAstV isolate from diarrhea viruses, a total of 29 PAstV-positive diarrhea samples were selected and treated as virus stock solution for virus isolation on PK-15 cells. However, neither RT-PCR nor cytopathic changes could be detected PAstV after passages 3, expect PAstV-4 (named GRF-1). After the GRF-1 sample infected the cells, the cells became larger and rounder, and gradually broke and fell off after 24 hours. Then after the cell liquid freezing and thawing repeated for 3 times, the next passage was continued to inoculate, and obvious cytopathic changes still appeared until the passages 11(Figure 3A).

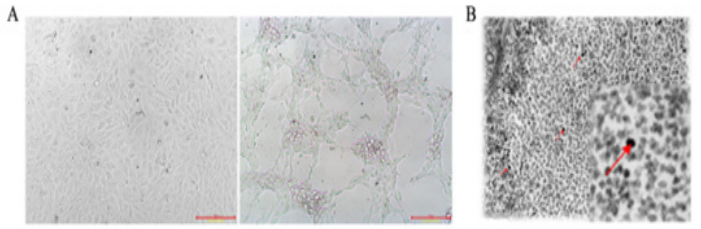


FIGURE 3: The pathogenicity of PAstV-4-GRF-1 to PK-15 cells. (A) Infected with 11th passage PAstV-4 GRF-1 induced the cells larger and rounder with gradually broke and fell off(right). The left picture was the control without virus. (Bar=10 μm) (B) Electron microscopy showed GRF-1 virions are nonenveloped and spherical, with a diameter of 30 nm (×60000).

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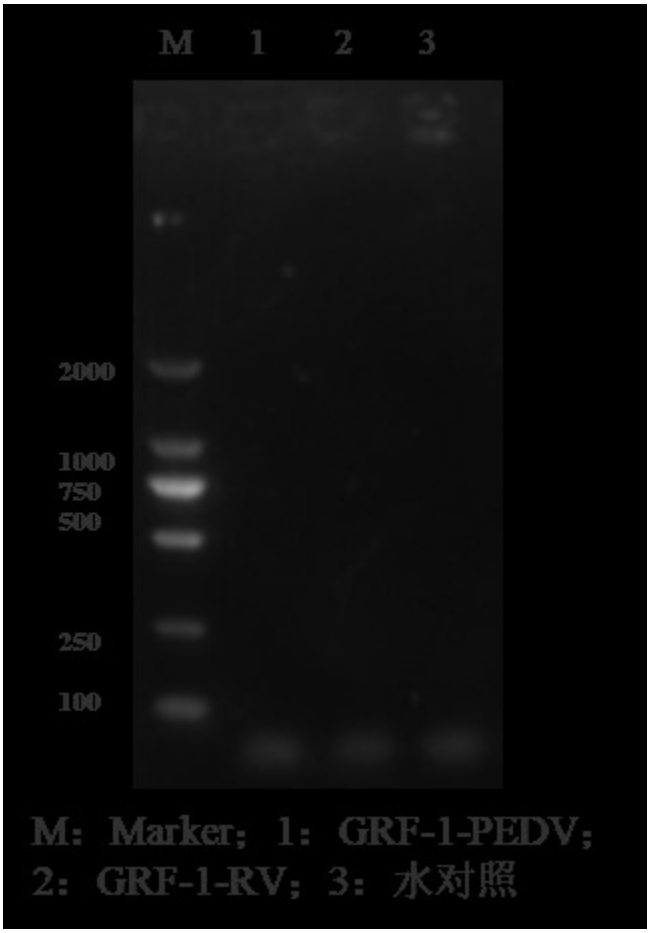
At present, the virus GRF-1 has been continuously passaged to the 18th generation and virus titer was as high as 104.5 TCID50/0.1mL. Furthermore, the isolated GRF-1 porcine astrovirus in the cell culture medium was detected and genotyped by RT-PCR according to the method above mentioned, the results showed that it could detect porcine astrovirus (279 bp) in the cell culture medium during continuous passage of GRF-1 to more than 18 generations and the genotype was astrovirus type 4. Also, the PEDV and RV were not detected by RT-PCR (Supplementary Figure 5). These results showed that the isolated PAstV-4-GRF-1 has been successfully purified, and its growth resulted in stable CPEs.

Supplementary Figure 5



Electron microscopy showed that PAstV4-GRF-1 virions are nonenveloped and spherical, with a diameter of 30 nm. Star-like structures surrounding the icosahedral capsid were also observed, which is consistent with reports of human AstVs (Figure 3B).

5.4. Metaviral transcriptome sequencing

To better investigate the evolutionary history of the newly identified PAstV strain, the samples of GRF-1 were sequenced by macrovirus transcriptome sequencing. The RNA of the sample was sequenced by Illumina second generation sequencing platform Novaseq 6000 (Figure 4A). After the assembly was performed using the splicing software Trinity, the spliced sequences were first compared with the GenBank Virus RefSeq protein database using the DIAMOND software to screen out the viral sequences. The screening results showed a total of 37333610 Clean Reads were obtained, and 70787 transcripts were assembled by splicing software Trinity. 772 viral sequences were screened by comparing the spliced sequences with GenBank Virus RefSeq protein database by DIAMOND software.Statistical analysis showed that the proportion of Mamastrovirus in GRF samples was 88.30% which based on species annotation and PAstV 4 and PAstV 2 accounted for 21.79% and 0.32% by the species classification statistics (Figure 4B, C). Moreover, there was no other diarrhea-related viruses were found in sequencing results.

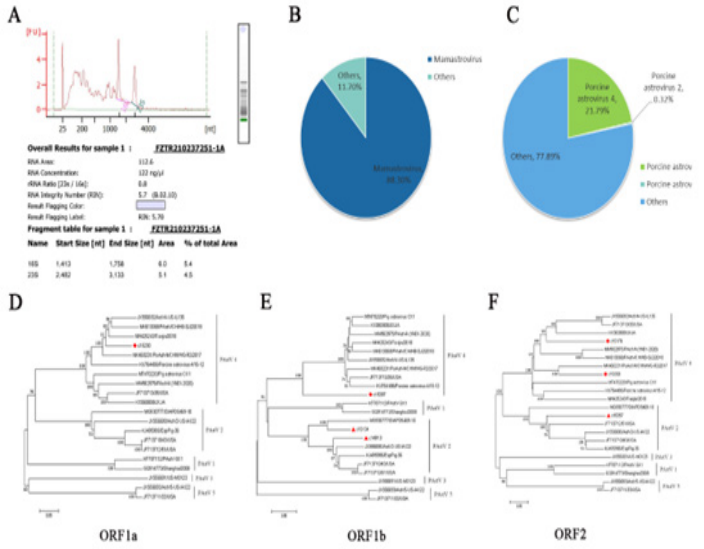


FIGURE 4: Metaviral transcriptome sequencing of isolated strain GRF-1. (A) RNA quality control results. (B)Genus classification statistics of GRF-1. (C) Species classification statistics of PAstV. (D) Phylogenetic tree based on the ORF 1a genes with the reference strains. (E) Phylogenetic tree based on the ORF 1b genes with the reference strains. (F) Phylogenetic tree based on the ORF2 genes with the reference strains. It is generated with the neighbor-joining method using the p-distance substitution model, with 1000 bootstrap replicates and a cut-off value of 70%, with the MEGA 7.0 software. The sequences of GRF-1 are marked with the red circle.

Finally, after splicing the obtained fragments, different ORF fragments were compared with the reference strains registered in GenBank and an evolutionary tree was constructed. The results showed that the fragment c15230 is located in the PAstV 4 ORF1a clade, and has the highest homology with the Tianjin strain MH425243 in 2018 (Figure 4D). The genetic evolution analysis of ORF1b found that c15134 and c14913 were located in the PAstV 2 clade, and c15397 was located in the PAstV 4 clade. Among them, c15134 has the highest homology with the Italian strain MG930777 in 2015 and c14913 has the highest homology with the American strain JF713710 in 2010.The c15397 fragment has the highest homology with the Chinese Jiangsu strain MT470220 in 2018 with the genetic distance was 0.259 (Figure 4E). For ORF2 fragments, c15357 was located in the PAstV 2 clade, and c15176 and c15050 were located in the PAstV 4 clade. C15357 has the shortest genetic distance from the

2010 American strains JF713712 and JX556690, with a genetic distance of 0.319 .c15176 has the shortest genetic distance from the 2014 Chinese Jiangxi strain KX060808, with a genetic distance of 0.322; the c15050 fragment was the same as the GRF-1 sequencing fragment of the isolated strain, and had the highest homology with the Hunan PAstV 4 sequence MK460231 in China. It was further verified that the strain GRF-1 isolated from PK-15 cells was PAstV 4 (Figure 4F).

6. Discussion:

Astroviruses are non-enveloped, positive single-stranded RNA viruses that could infect mammals such as human beings and avian species such as ducks. Astroviruses have a wide range of hosts and are prone to rapid genetic variations which increase their ability to spread across species and adapt to new hosts. It has been detected from 31 species of mammals and 6 species of poultry, and its host range is still expanding. Porcine astroviruses (PAstVs) belong to the genus of mammalian astrovirus, which has distributed throughout the world. In recent years, PAstV has been increasingly common in Chinese pig farms. However, there are few reports on the role of porcine astrovirus in Shandong Province, China. In this study, the prevalence of porcine astrovirus in porcine diarrhea cases and heathy pigs in Shandong province was investigated. RT-PCR results showed that the total positive rate of PAstV in porcine diarrhea cases collected from some areas of Shandong province from January 2021 to October 2023 was 34.6% (355/1025), while the total positive rate of PAstV in healthy pigs was 8.13% (69/849). Of the five known PAstV lineages, PAstV-3 mainly causes neurological symptoms while other types related to diarrhea. It has been reported PAstV2 and PAstV4 are the predominant genotypes reported in Canada, Hungary, China, USA, South Korea, Croatia, Italy, Kenya, Austria, Germany, Spain, and Sweden. PAstV5 has become increasingly prevalent in recent years. Consistently with our results, five PAstV lineages were detected in diarrhea cases, no PAstV3 strains were identified in this samples. Only PAstV-1, PAstV-2, PAstV-4 and PAstV-5 were found by the proportion of 25.4%, 28.2%, 35.2% and 22.5%, respectively. The proportions of PAstV-1, PAstV-2 and PAstV-4 in healthy pig samples were 27.5%, 37.7% and 40.6%, respectively. It seems that PAstV 4 had the highest infection rate and was the dominant strain in Shandong province. In addition, it was found that there were two or more different genotypes of mixed infection, indicating that gene recombination may occur between different genotypes of PAstV. As RNA viruses, are many reports of genetic variety and recombination events in human AstVs, few recombination was found within PAstV genotypes. Furthermore, PAstV was also reported to co-infect with other enteroviruses, resulting a decrease in the survival rate of piglets, which brings serious economic losses to the pig industry. We found that in PAstV-positive samples, the co-infection rates of PEDV and PRV in diarrhea-infected piglets were 46.5% and 42.3%, respectively, while the co-infection rates of PEDV and PRV in healthy piglets were 47.8% and 43.5%, respectively, suggesting that they may play a potential role in PAstV-induced diarrhea in piglets.

Based on the phylogenetic analysis of 29 sequenced samples, it was found that there was a large genetic distance within the intra-genotypic of PAstV-1, PAstV-2 and PAstV-4. In particular, PAstV-4 roughly exists in 8 different branches of the evolutionary tree of this genotype, which indicates that there may be a large genetic variation of this strain in different regions of Shandong province. In parallel, it was found that there were large genetic differences between the PAstV-4 strains obtained in this study and the strains from different countries, and this situation was also found in PAstV-1 and PAstV-2, which may be related to the introduction of breeding pigs from abroad. Moreover, this study found that PAstV-1 belongs to the same branch as feline astrovirus (FeAstV) and tiger astrovirus (TigAstV) in the evolutionary tree, and PAstV-2 belongs to the same branch as Capreolus capreolus astrovirus (CcAstV) and bovine astrovirus (BoAstV), which indicates that PAstV may have occurred cross-species transmission.

Few research on the pathogenicity of PAstVs was reported because the virus is difficult to isolate in cells. So far, only few strains have been successfully isolated using cell lines, such as a porcine astrovirus strain on PK-15 cells was isolated by Xiaogui Shang in 2010, and the strain could cause cytopathic changes. PAstV-GX1 from pig feces in Guangxi was successfully isolated by Liu Huan in 2014. In 2020, a swine astrovirus type 5 from clinical swine fever virus (CSFV)-infected tissue samples was isolated possibly because CSFV counter infection significantly enhanced the replication of PAstV-5[21]. Up to now, PAstV-2 and PAstV-4 which the most common strains in China have not been isolated. In this experiment, 30 samples with higher viral load were selected from the 424 samples with positive porcine astrovirus detection for virus isolation. By explorating of trypsin concentration and optimizating of culture conditions on PK-15 cells, a porcine astrovirus type 4 strain named GRF-1 was successfully isolated though pathological changes, RT-PCR and Sequencing. After infection PK-15 cells for 24 hours, the cells became large, round and broken. Then cell culture medium was frozen and thawed for three times, and RNA was extracted. The target band of porcine astrovirus was detected by RT-PCR while the blank control cells of astrovirus could not be detected. At the same time, other diarrheal diseases (PEDV and RV) in the cell culture medium were detected and found to be negative, thus excluding the cytopathic changes caused by PEDV and RV. The phylogenetic tree showed it has the highest homology 95.4% with the Chinese Hunan strain MK460231 in 2017. Unfortunately, the lack of monoclonal antibodies limits our ability to further investigate the pathogenicity of PAstV-4-GRF1.And the clinical information will be carried out on the isolated strains. However, the isolation of this strain has provided a critical material for further studies into the role of PAstV-4 in diseases of pigs.

7. Author contributions:

Jiaqiang Wu and Shuqian Lin conceived and designed the work. Shifa Yang and Bin Yin coordinated technical support and funding. Yueyue Liu and Yu Zhang wrote the manuscript. The others performed the experiments and collected the samples. All authors have read and approved the published version of the manuscript.

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8. Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

9. Data availability:

The authors confirm that the data supporting the findings of this study are available within the article (and its supplementary materials).

10. Acknowledgments

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References

1. Lefkowitz EJ, Dempsey DM, Hendrickson RC, Orton RJ, Siddell SG, Smith DB. Virus taxonomy: the database of the International Committee on Taxonomy of Viruses (ICTV). Nucleic Acids Res. 2018 Jan 4;46(D1):D708-D717. doi: 10.1093/nar/gkx932. PMID: 29040670; PMCID: PMC5753373.
2. Cortez V, Meliopoulos VA, Karlsson EA, Hargest V, Johnson C, Schultz-Cherry S. Astrovirus Biology and Pathogenesis. Annu Rev Virol. 2017 Sep 29;4(1):327-348. doi: 10.1146/annurev-virology-101416-041742. Epub 2017 Jul 17. PMID: 28715976.
3. De Benedictis P, Schultz-Cherry S, Burnham A, Cattoli G. Astrovirus infections in humans and animals - molecular biology, genetic diversity, and interspecies transmissions. Infect Genet Evol. 2011 Oct;11(7):1529-44. doi: 10.1016/j.meegid. 2011.07.024. Epub 2011 Aug 5. PMID: 21843659; PMCID: PMC7185765.
4. Cubitt WD. Historical background and classification of caliciviruses and astroviruses. Arch Virol Suppl. 1996;12:225-35. doi: 10.1007/978-3-7091-6553-9_24. PMID: 9015119.
5. Nagai M, Omatsu T, Aoki H, Otomaru K, Uto T, Koizumi M, Minami-Fukuda F, Takai H, Murakami T, Masuda T, Yamasato H, Shiokawa M, Tsuchiaka S, Naoi Y, Sano K, Okazaki S, Katayama Y, Oba M, Furuya T, Shirai J, Mizutani T. Full genome analysis of bovine astrovirus from fecal samples of cattle in Japan: identification of possible interspecies transmission of bovine astrovirus. Arch Virol. 2015 Oct;160(10):2491-501. doi: 10.1007/s00705-015-2543-7. Epub 2015 Jul 28. PMID: 26212364.
6. Donato C, Vijaykrishna D. The Broad Host Range and Genetic Diversity of Mammalian and Avian Astroviruses. Viruses. 2017 May 10;9(5):102. doi: 10.3390/v9050102. PMID: 28489047; PMCID: PMC5454415.
7. Xiao CT, Giménez-Lirola LG, Gerber PF, Jiang YH, Halbur PG, Opriessnig T. Identification and characterization of novel porcine astroviruses (PAstVs) with high prevalence and frequent co-infection of individual pigs with multiple PAstV types. J Gen Virol. 2013 Mar;94(Pt 3):570-582. doi: 10.1099/vir.0.048744-0. Epub 2012 Dec 5. PMID: 23223616.
8. Bridger JC. Detection by electron microscopy of caliciviruses, astroviruses and rotavirus-like particles in the faeces of piglets with diarrhoea. Vet Rec. 1980 Dec 6;107(23):532-3. PMID: 6258286.
9. Lan Jianuan, Liu Lei, Guo Xuan, Zhang Minxiu, Lu Bingxia, Huang Fubiao, Qin Xionghui, Zhang Ning, Huang Weijian. Molecular epidemiological survey of pig astrovirus in Guangxi [J]. Chinese Journal of Preventive Veterinary Medicine, 2012,34 (08): 602-606.
10. Mor SK, Chander Y, Marthaler D, Patnayak DP, Goyal SM. Detection and molecular characterization of Porcine astrovirus strains associated with swine diarrhea. J Vet Diagn Invest. 2012 Nov;24(6):1064-7. doi: 10.1177/1 04063871 2458781. Epub 2012 Sep 5. PMID: 22956487.
11. Shimizu M, Shirai J, Narita M, Yamane T. Cytopathic astrovirus isolated from porcine acute gastroenteritis in an established cell line derived from porcine embryonic kidney. J Clin Microbiol. 1990 Feb;28(2):201-6. doi: 10.1128/jcm. 28.2. 201-206.1990. PMID: 2107200; PMCID: PMC269575.
12. Lee MH, Jeoung HY, Park HR, Lim JA, Song JY, An DJ. Phylogenetic analysis of porcine astrovirus in domestic pigs and wild boars in South Korea. Virus Genes. 2013 Feb;46(1):175-81. doi: 10.1007/s11262-012-0816-8. Epub 2012 Sep 11. PMID: 22965450; PMCID: PMC7089313.
13. Kattoor JJ, Malik YS, Saurabh S, Sircar S, Vinodhkumar OR, Bora DP, Dhama K, Ghosh S, Banyai K, Touil N, Abdel-Moneim AS, Vlasova AN, Kobayashi N, Singh RK. First report and genetic characterization of porcine astroviruses of lineage 4 and 2 in diarrhoeic pigs in India. Transbound Emerg Dis. 2019 Jan;66(1):47-53. doi: 10.1111/tbed.13058. Epub 2018 Dec 2. PMID: 30379411.
14. Flores C, Ariyama N, Bennett B, Mena J, Verdugo C, Mor S, Brito B, Ramírez-Toloza G, Neira V. Case Report: First Report and Phylogenetic Analysis of Porcine Astroviruses in Chile. Front Vet Sci. 2021 Nov 25;8:764837. doi: 10.3389/fvets.2021.764837. PMID: 34901251; PMCID: PMC8656452.
15. Laurin MA, Dastor M, L'homme Y. Detection and genetic characterization of a novel pig astrovirus: relationship to other astroviruses. Arch Virol. 2011 Nov;156(11):2095-9. doi: 10.1007/s00705-011-1088-7. Epub 2011 Sep 8. PMID: 21935627; PMCID: PMC7086720.
16. Shan T, Li L, Simmonds P, Wang C, Moeser A, Delwart E. The fecal virome of pigs on a high-density farm. J Virol. 2011 Nov;85(22):11697-708. doi: 10.1128/JVI.05217-11. Epub 2011 Sep 7. PMID: 21900163; PMCID: PMC3209269.
17. Boros Á, Albert M, Pankovics P, Bíró H, Pesavento PA, Phan TG, Delwart E, Reuter G. Outbreaks of Neuroinvasive Astrovirus Associated with Encephalomyelitis, Weakness, and Paralysis among Weaned Pigs, Hungary. Emerg Infect Dis. 2017 Dec;23(12):1982-1993. doi: 10.3201/eid2312.170804. PMID: 29148391; PMCID: PMC5708238.

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18. Matias Ferreyra FS, Bradner LK, Burrough ER, Cooper VL, Derscheid RJ, Gauger PC, Harmon KM, Madson D, Piñeyro PE, Schwartz KJ, Stevenson GW, Zeller MA, Arruda BL. Polioencephalomyelitis in Domestic Swine Associated With Porcine Astrovirus Type 3. Vet Pathol. 2020 Jan;57(1):82-89. doi: 10.1177/0300985819875741. Epub 2019 Sep 24. PMID: 31551018.
19. Liu Huan. Isolation and identification of porcine stellate virus and its whole genome sequence analysis and prokaryotic expression of capsid protein, 2014, Guangxi University.
20. Fang Q, Wang C, Liu H, Wu Q, Liang S, Cen M, Dong Q, Wei Y, Chen Y, Ouyang K, Wei Z, Huang W. Pathogenic Characteristics of a Porcine Astrovirus Strain Isolated in China. Viruses. 2019 Dec 13;11(12):1156. doi: 10.3390/v11121156. PMID: 31847270; PMCID: PMC6949928.
21. Mi S, Guo S, Xing C, Xiao C, He B, Wu B, Xia X, Tu C, Gong W. Isolation and Characterization of Porcine Astrovirus 5 from a Classical Swine Fever Virus-Infected Specimen. J Virol. 2020 Dec 22;95(2):e01513-20. doi: 10.1128/JVI.01513-20. PMID: 33115877; PMCID: PMC7944439.
22. Su M, Qi S, Yang D, Guo D, Yin B, Sun D. Coinfection and Genetic Characterization of Porcine Astrovirus in Diarrheic Piglets in China From 2015 to 2018. Front Vet Sci. 2020 Aug 14;7:462. doi: 10.3389/fvets.2020.00462. PMID: 32923463; PMCID: PMC7456941.