

Molecular detection and prevalence of porcine caliciviruses in eastern China from 2008 to 2009

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Abstract Caliciviruses causing diarrhea have been reported in both industrial and developing countries, including China, in recent years. Porcine caliciviruses that are closely related to human sapoviruses (SaVs) and noroviruses (NoVs) have also been detected in swine, which has raised discussion about the animal reservoir and the potential risk for zoonotic transmission to humans. The objective of this work was to determine the frequency and age distribution of SaVs and NoVs in pigs and to characterize the strains prevalent in eastern China. A total of 904 stool samples from pigs of different ages were collected from eastern China from April 2008 to March 2009 and tested for both SaVs and NoVs using reverse transcription-polymerase chain reaction (RT-PCR). Our results indicate that 8 (0.9%) stool samples were positive for SaVs and 2 (0.2%) for NoVs. Phylogenetic analysis of partial sequences of the RNA-dependent RNA polymerase (RdRp) gene

indicated that all of the SaV strains belonged to the GIII SaVs, while the two NoV strains belonged to the GII NoV genogroup. The 8 SaV strains were further divided into two clusters, which clustered closely with the Netherlands isolate (AY615804) and the Chinese strain (EU599212), respectively. The two NoV strains shared about 67.3–67.6% nucleotide homology with a human norovirus strain (DQ369797), the only NoV strain from mainland China available in GenBank. Moreover, our results suggest that SaV infections are more frequent in 0–1 month-old pigs than in older ones. In conclusion, the present study provides evidence that PoSaVs and PoNoVs exist in swine in eastern China.

Introduction

Caliciviruses are small, non-enveloped viruses of 27–38 nm in diameter. They possess a single-stranded, plus-sense genomic RNA of 7.3–8.3 kb and a single 56–71-kDa capsid protein [8]. According to genetic analysis, they are divided into at least four genera: *Norovirus* (NoV), *Sapovirus* (SaV), *Vesivirus* and *Lagovirus*. They are phylogenetically related and have sequence similarities in the RNA polymerase and capsid regions [6]. SaVs are classified into five genogroups (GI–GV), of which GI, GII, GIV and GV cause human infection, while GIII infects pigs. The genomes of SaV GI, GIV, and GV are predicted to contain three major ORFs, whereas SaV GII and GIII have two. Based on phylogenetic tree topology and distance analysis of the capsid gene, the genus *Norovirus* is divided into five genogroups, GI–GV, which can be further subdivided into 29 genetic clusters (8 in G1, 17 in G2, 2 in G3, and 1 each in G4 and G5) [22, 29]. More and more studies have indicated that caliciviruses are among the

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major causes of gastroenteritis outbreaks in humans and several animal species worldwide [5, 7, 9, 20, 22–24]. Human caliciviruses, especially human noroviruses (HuNoVs), are the main cause of viral gastroenteritis of children in China (19%) [3]. A recent study reported the first Chinese SaV strain in piglets in Shanghai, China [28]. So far, few systematic investigations of the prevalence of porcine caliciviruses have been carried out in pigs in developing countries, including China. In the current study, in order to characterize the infection status of porcine caliciviruses in Chinese swine herds, fresh stool samples from pigs in three provinces in eastern China were collected and tested for SaVs and NoVs using RT–PCR assay, and molecular and phylogenetic analyses of the obtained sequences were performed. The prevalence of SaV infection at different ages was also evaluated.

Materials and methods

Sampling

A total of 904 stool samples from pigs of different ages were collected from 14 middle or large-scale pig farms (200–2,000 sows each) in eastern China from April 2008 to March 2009, of which 287 were collected from 4 farms located in Shanghai, 118 from 3 farms located in Jiangsu province, and 499 from 7 farms located in Anhui province (Table 1). Stool samples were freshly collected and immediately converted to 10% (w/v) suspensions in PBS (0.01 M phosphate, pH 7.2–7.4, 0.15 M NaCl, 0.1% DEPC) for RNA extraction.

RNA extraction

RNA was extracted from 200 μ l of 10% fecal suspension by using the TRIzol reagent (Invitrogen, USA) following

the manufacturer's instructions. RNA pellets were dissolved in 25 μ l RNase-free water, and reverse transcription was performed immediately.

RT–PCR

One set of primers was used for the detection of a partial RNA-dependent RNA polymerase gene of both human and porcine SaVs by adapting the universal primers mixture (Table 2) [4, 6]. Another set of primers, P289/P290, based on conserved sequences found in the polymerase gene of caliciviruses, was used to screen for NoVs (Table 2) [11]. Reverse transcription was performed in a 10- μ l reaction mixture containing 2 μ l 5 \times RT buffer, 0.5 μ l (200 units) of AMV reverse transcriptase (TaKaRa, Japan), 1 μ l (25 mM) of reverse primer and 6.5 μ l extracted RNA at 42°C for 1 h. Polymerase chain reaction (PCR) assays were carried out using 5 μ l of the synthesized cDNA with ExTaq DNA polymerase (TakaRa, Japan). The amplicons were analyzed by 2% agarose gel electrophoresis in TAE buffer, followed by staining with ethidium bromide (0.5 μ g/ml) and visualization under UV light.

Sequencing and sequence analysis

RT–PCR products were purified using a QIAquick gel extraction kit (Qiagen, Germany) following manufacturer's instructions. The purified PCR products were ligated into the PMD18-T vector (TakaRa, Japan) using T4 DNA ligase (TakaRa, Japan) at 16°C overnight. Competent *E. coli* DH5 α cells (TakaRa, Japan) were transformed with the recombinant plasmid. Four clones were sequenced for each sample. Similarity searches of the sequences were carried out using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>). After multiple alignment with CLUSTAL W (version 1.4), the phylogenetic relationships of the strains in the present study and the reference isolates were assessed employing

Table 1 Frequency of SaVs and NoVs detected by RT–PCR assay in stool samples from pigs of different ages in three provinces of eastern China

| Location (province) | Number of farms | Age (month) | Number of samples tested | Positive/tested samples for SaVs | Positive/tested samples for NoVs | Positive/tested farms for SaVs | Positive/tested farms for NoVs |
|---------------------|-----------------|-------------|--------------------------|----------------------------------|----------------------------------|--------------------------------|--------------------------------|
| Shanghai | 4 | >3 | 87 | 0 | 2/87 | | |
| | | 2–3 | 104 | 0 | 0/104 | 1/4 | 1/4 |
| | | <1 | 96 | 2 | 0/96 | | |
| Jiangsu | 3 | >3 | 25 | 0 | 0/25 | | |
| | | 2–3 | 33 | 1 | 0/33 | 2/3 | 0/3 |
| | | <1 | 60 | 2 | 0/60 | | |
| Anhui | 7 | >3 | 120 | 0 | 0/120 | | |
| | | 2–3 | 145 | 0 | 0/145 | 1/7 | 0/7 |
| | | <1 | 234 | 3 | 0/234 | | |

Table 2 List of the primers utilized in this study

| Primer | Sequence | Location | Polarity |
|--------|-------------------------------------|-----------|----------|
| SaV1 | 5'-GATTACTCCAGGTGGGAC(T)TCC(A)AC-3' | 4354–4376 | Positive |
| SaV2 | 5'-TGACGATTCATCATCA(C)CCA(G)TA-3' | 4663–4684 | Negative |
| SaVR1 | 5'-TGACAATGTAATCATCACCATA-3' | 4663–4684 | Negative |
| SaVR2 | 5'-TGACGATTCATCATCACCATA-3' | 4663–4684 | Negative |
| SaVR3 | 5'-TGACGATTCATCATCCCCGTA-3' | 4663–4684 | Negative |
| P289 | 5'-TGACAATGTAATCATCACCATA-3' | 4865–4886 | Negative |
| P290 | 5'-GATTACTCCAAGTGGGACTCCAC-3' | 4568–4590 | Positive |

The forward detection primer for SaVs was SaV1, and the reverse primers mixture contained SaV2, SaVR1, SaVR2 and SaVR3

the software MEGA version 4.0. For analysis in MEGA, the Jukes–Cantor (JC) distance was utilized employing the neighbor-joining (NJ) algorithm [13]. The reliability of different phylogenetic groupings was evaluated by using the bootstrap test (1,000 bootstrap replications) available in MEGA.

Statistical analysis

Data analysis was performed by z test with confidence limits of 95%, $p < 0.01$, using the SPSS software package for Windows (version 13.0; SPSS, Chicago, IL, USA).

Results

SaVs were detected on 28.6% (4/14) of the farms in this study (Table 1). Eight of stool samples were found to be positive for SaVs, and all of the positive PCR bands were sequenced. For simplicity, SaVs strains with identical nucleotide sequences were treated as a unique strain in the analysis, leaving a total of 5 strains with distinct sequences (GenBank accession numbers: FJ374680 from Shanghai, FJ374681 from Jiangsu, FJ374682 from Anhui, FJ374683 from Anhui, FJ374684 from Jiangsu). Only two out of 904 (0.2%) stool samples were positive for NoVs RNA (Table 1). The positivity rates for SaVs in pigs of Shanghai, Anhui, and Jiangsu were 0.7% (2/287), 0.6% (3/499), and 2.5% (3/118), respectively (Table 1). The total positivity rate for SaVs was 0.9% (8/904), which was significantly lower than that in Brazil (30.1%), Venezuela (17.6%) and the USA (62%) [1, 15, 26]. The prevalence rates of SaV infection for different ages of pigs in eastern China were 0 (0/232; age \geq 3 months), 0.35% (1/282; age 2–3 months), and 1.79% (7/390; age \leq 1 month), suggesting that the prevalence rates for SaVs infection in pigs \leq 1 month of age was higher than that in those $>$ 1 month of age ($p < 0.05$).

Phylogenetic analysis indicated that all five SaVs strains belonged to GIII (Fig. 1a). They shared 85.1–99% nucleotide homology among themselves and 85.1–99% homology with another Chinese SaVs strain (EU599212) [28]. They were further divided into two different clusters in GIII. Four of the SaVs strains in the present study clustered with the Netherlands isolate (AY615804) and shared 89–89.3% nucleotide homology with it. The remaining one was closely related to another Chinese SaVs strain (EU599212), and they together clustered with the Korean isolate (DQ38962) and shared 94% nucleotide homology with it.

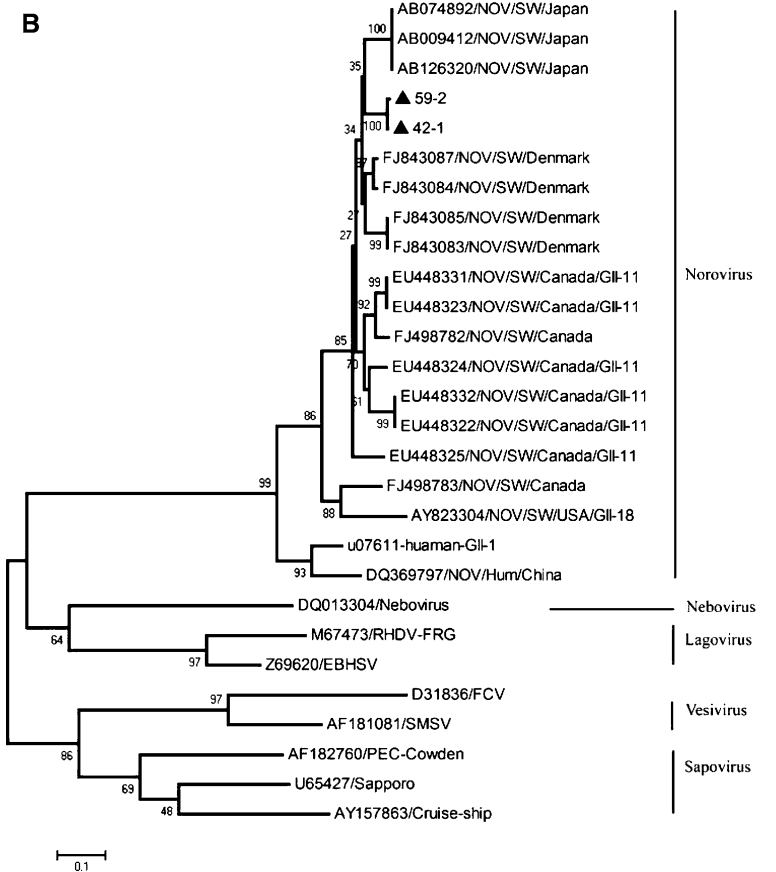
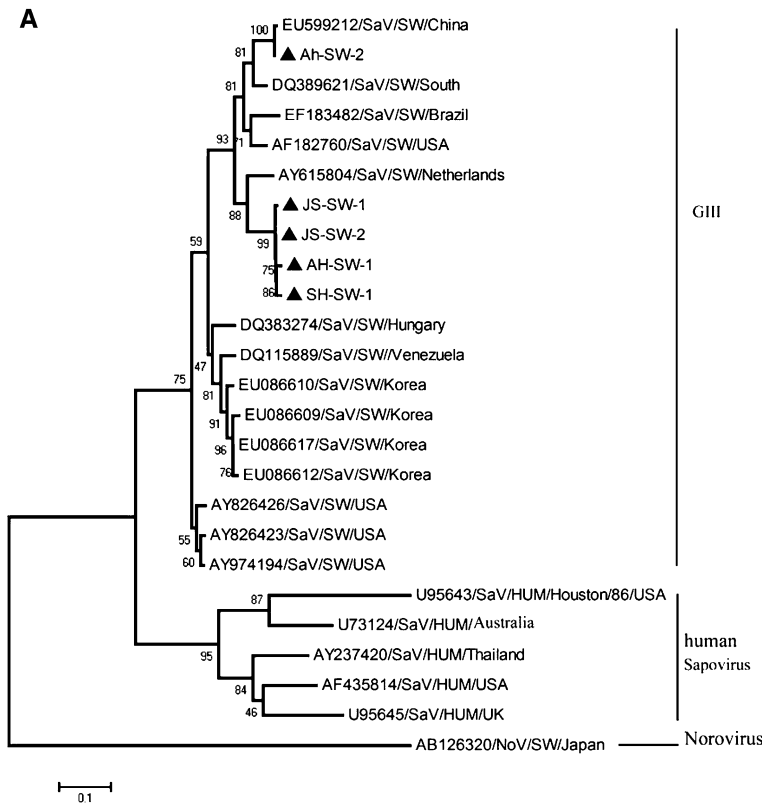
Two samples (0.2%) in the current study were positive for NoVs. The two NoVs strains shared 99 and 100% sequence homology in their nucleotide and predicted amino acid sequences, respectively (Fig. 1b). Phylogenetic analysis showed that they clustered closely with 19 PoNoV isolates referenced from different countries, sharing 89–99% nucleotide identities with them in the GII cluster, and they shared 67.3–67.6% nucleotide homology with a human norovirus strain (DQ369797), the only norovirus strain from mainland China available in GenBank (Fig. 1b).

Discussion

Porcine calicivirus infections have been reported in both developed and developing countries. Our previous study indicated that SaV infection existed in piglets with diarrhea in China [24]; however, no NoVs infections in swine have been reported so far. Moreover, the prevalence rates of porcine enteric calicivirus infection in pigs of different ages were still not known. We therefore investigated the prevalence of both NoVs and SaVs in pigs of different age groups of in eastern China. The results indicated that both PoSaVs and PoNoVs were present in swine in eastern China. Phylogenetic analysis showed that these SaVs strains were divided into two different clusters in GIII. Phylogenetic

Fig. 1 a Neighbor-joining tree showing the phylogenetic relationship among SaVs strains based on a 309-nt nucleotide fragment of the RNA-dependent RNA polymerase gene. Bootstrap values, expressed as percentages of 1,000 replications, are given at the branch points. GenBank accession numbers for the reference strains are shown at each branch point. The five newly identified SaVs strains in the present study (GenBank accession numbers: FJ374680 for Sh-pig-1; FJ374681 for Js-pig-1; FJ374682 for Ah-pig-1; FJ374683 for Ah-pig-2; FJ374684 for Js-pig-2) are indicated by *triangles*.

b Neighbor-joining tree showing the phylogenetic relationship among NoVs strains based on a 274-nt nucleotide fragment of the RNA-dependent RNA polymerase gene. Bootstrap values, expressed as percentages of 1,000 replications, are given at the branch points. The two newly identified NoV strains in the present study (GenBank accession numbers: GQ149615 for 59-2, Q149616 for 42-1) are indicated by *filled triangles*. The NoV genogroups (G plus roman numerals) and genotypes (Arabic numbers following genogroup numbers) are indicated



analysis also suggested that the two clusters of SaVs strains in the present study may have common infection sources with Netherlands and Korean isolates, respectively.

Previous research showed that SaVs were detected more frequently in pigs between 22 and 28 days of age, and in equal frequencies in piglets with and without diarrhea in Brazil [1], which is consistent with the data of our study. Reports have indicated that the clinical signs of SaVs infection in swine were mainly concentrated in young animals, while PoNoVs have been detected mainly in adult pigs without clinical signs [24], which is consistent with our result that the two pigs that were positive for PoNoVs in the present study were adults (about 3 months old) and had no clinical signs. The infection rate in the present study for PoNoVs in swine in eastern China (0.2%, 2/904) is much lower than that in the USA (2.2%, 6/275) [22], Belgium (4.7%, 2/43) [19], Korea (23.1%) [27] and Hungary (5.9%, 1/17) [21], which suggests that although infections with porcine NoVs exist, they are not a major viral pathogen in this area.

SaVs and NoVs are commonly reported in animals and are sometimes genetically closely related to human strains [6, 17]. Specifically, PoNoVs are genetically closely related to some HuNoVs and HuSaVs [15, 16], and some potential recombinant NoVs and SaVs have been reported [10, 25]. Moreover, pigs have a closer relationship with humans than with other animals and are sometimes the hosts of co-infection with human and animal viruses [18]. The detection of SaVs and NoVs in pigs in many countries suggests a potential zoonotic risk, and the pig is considered an animal reservoir for such viruses [12, 14]. So far, enteric calicivirus sequences isolated from domestic pigs are limited. In this study, we report the molecular detection and phylogenetic analysis of PoSaVs in domestic pigs in eastern China. Detection and further analysis of porcine calicivirus strains from different geographic areas will be helpful for understanding the worldwide distribution and heterogeneity of calicivirus strains in swine and their potential zoonotic infection. This study confirms the prevalence of SaVs in swine in eastern China and provides additional information on their genetic diversity and infection status in pigs of different age groups. The low nucleotide sequence homology between the PoNoV strains in the present study and the previous HuNoV strain in China mainland suggest that PoNoVs are not the original infection source of human infection with NoVs in this area. So far, no Chinese HuSaVs sequences are available in GenBank; therefore, the relationship between HuSaVs and PoSaVs in China will have to be investigated.

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