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Molecular Detection and Phylogenetic Analysis of TTSuV1 and TTSuV2 in Clinically Healthy Pigs of Different Ages Reared in Intensive Farms in Hunan province of China

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ABSTRACT

Torque teno sus virus (TTSuV) has been detected in pigs with and without diseases. As China's pig population is booming, it's important to understand the prevalence of TTSuV in healthy pigs. The aim of this study was to systematically investigate the TTSuV prevalence rate in healthy pigs of different age groups, reared in intensive farms in the Hunan province of China. We detected TTSuV in 401 sera samples of healthy pigs by using PCR with primers located in the untranslated region (UTR). The positive prevalence rates of TTSuVs, TTSuV1, TTSuV2, and co-infection with TTSuV1 and TTSuV2 were calculated. The nucleotide identities of TTSuV1 and TTSuV2 between the reference and obtained sequences were calculated using the P-distance method, and maximum likelihood phylogenetic trees were created using MEGA6.0. TTSuVs were detected with an average frequency of 32.17%, while the frequencies of TTSuV1, TTSuV2, and co-infection with both TTSuV genogroups were 7.99%, 28.92%, and 4.74%, respectively. TTSuV1 and TTSuV2 were detected in pigs mainly in the age range of 12 – 16 weeks (positive rate >10%) and 8 – 18 weeks (positive rate >40%), respectively. The nucleotide identities ranged from 92.9%~97.3% for TTSuV1 and 64.7%~99% for TTSuV2 among the obtained sequences. We also observed diversity in TTSuVs based on the phylogenetic analysis of the UTR of the gene. Thus, our study shows the importance of understanding the distribution of TTSuV, which are potentially pathogenic agents in healthy pigs.

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Abbreviations: TTSuV,Torque teno sus virus; ICTV, International Committee on Taxonomy of Viruses; UTR,Untranslated Region; PRV, Porcine Rabies Virus; CSFV, classic swine fever virus; PMWS, Postweaning Multisystemic Wasting Syndrome; PRDC, Porcine Respiratory Disease Complex; PPRSV, Porcine Reproductive and Respiratory Syndrome Virus

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Introduction

According to the International Committee on Taxonomy of Viruses (ICTV), Torque teno sus virus (TTSuV) includes TTSuV1 (genus Iotatorquevirus) and TTSuV2 (genus Kappatorquevirus), which are ubiquitous and distributed worldwide in swine samples[1]. TTSuV was first identified in 1997 in a Japanese patient with post-transfusion hepatitis[2], Thereafter, TTSuV infection was reported in pigs, cattle, dogs, and other animals[3]. TTSuV genomes encode several ORFs; of these, ORF1 and ORF2 are responsible for viral capsid protein and viral replication, respectively. TTSuV genomes share identities ranging from 35% to 56%, which is highly variable, while an untranslated region (UTR) is relatively conserved, which is frequently used for PCR detection and phylogenetic analysis[4, 5].

The role of TTSuV in disease is a debatable matter[6]. High detection rates have been reported by Sibila[7] in apparently healthy animals. However, a study by the same team showed that the number of stillborns was significantly higher in sows co-infected with both TTSuV genogroups compared with the non-co-infected ones[8]. Multiple clinical signs are associated with TTSuV1 infection. Moreover, co-infection of Porcine circovirus 2 (PCV2), with TTSuV1 or TTSuV2 resulted in signifi--cantly greater weight gain reduction than that with mono-infection. Studies have suggested that pigs with postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephro--pathy syndrome (PDNS) and porcine respiratory disease complex (PRDC) are TTSuV-positive[6], and TTSuV was detected in the T lymphocytes by multi--strained pooled probe-based in situ hybridization[9]. Furthermore, TTSuVs have been detected in pig livers with mild and moderate hepatitis lesions, which indicates that TTSuVs are potentially pathogenic to pigs[10].

Depending on the sample type and health status of pigs, TTSuV is detected in pigs with a varying prevalence rate of 24%-100%[1]. TTSuVs have been studied in different regions, and most of the cohorts have been associated with diseases[11-13]. Some studies have sampled serum from healthy pigs of different ages. Siliba et al[8]. detected TTSuVs in piglets (<3 weeks old), with a higher percentage of TTSuV1 (42.8%, 92/215) over TTSuV2 (19.1%, 41/215). A study on the serum of newborn pigs, weaned pigs, gilts, and sows showed that TTSuVs were prevalent in weaned pigs[14]. Moreover, Xiao[15] and Brassard[16] showed that pigs over 8 weeks of age have higher infection rate than weaning piglets. Age related distribution of TTSuV has also been reported in pigs of the age of 5 days and

5, 15 and 24 weeks[17]. A systematic investigation of TTSuVs in healthy pigs of different age groups is lacking, although some studies have focused on TTSuV prevalence in healthy pigs[18,19]. As China's pig population continues to increase, it is important to understand the distribution of TTSuV-potential pathogenic agents in healthy pigs of different ages. The aim of this study was to systematically investigate the TTSuV prevalence rate in clinical healthy pigs of different age groups from intensive farms in the Hunan province, China.

Material and method

1.Sample collection

From August to December 2016, we collected 401 sera samples from clinical healthy pigs in 4 intensive pig farms located in Shaoyang (SYJH, WX), Yueyang (ZH), and Zhuzhou (LH) in the Hunan province, China. The number of sows of WX, ZH, SYJH, and LH were 330, 1600, 2200, and 8000, respectively. We divided the cohort according to the age of the sampled pigs, which ranged from 2-20 weeks with an interval of 2 weeks, gilt group, and sow group. Blood was taken from the precaval vein (<16 weeks) or ear-vein (>16 weeks) using disposable syringes or evacuated blood collection tubes.

2.Serum separation and DNA extraction

The whole blood was shipped to the lab at 4°C. The whole blood was centrifuged at 3500g for 5 min. The serum was separated from the whole blood and the obtained sera were sub-packaged at 100μL/tube. These sera were stored in -80°C until virus DNA isolation. The virus DNA was isolated from 100μL of sera sample using DNAout Kit (TIANDZ, Beijing, China) according to the manufacturer's instruc--tion. The DNA was eluted from the column with 30μL of Tris-EDTA buffer solution. To minimize potential contamination, serum separation and DNA extraction were performed in different rooms. PBS was used as negative control during DNA extraction.

3.Development of PCR assays for TTSuV detection

Two primers were designed to amplify the 5′UTR of TTSuV1 (F: 5'TTTSMTGC CARGCGGACCT3', R: 5'GCGGCATA AACTCWGCCATTC3', 280bp) and TTSuV2 (F: 5'GCAGACGAATGGCT GAGTTTAT3', R: 5'GGAACCAGTGTC CGTAGCT3', 459bp) based on the align--ments of the sequences downloaded from Genbank.

PCR assays were carried out in a 20μl total reaction volume containing 10μl PCR mix and 0.2μL of each primer (0.5μM), 1μL DNA, and double distilled water. The amplification reactions were performed under the following conditions: 5 min at 94°C, 34 cycles of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C, followed by 72 °C for 7 min. PCR products were then

analyzed by 1% agarose gel.

The specificity of the primers was confirmed by amplification of DNA or cDNA of porcine circovirus 2(PCV-2), porcine parvovirus, porcine rabies virus (PRV), classic swine fever virus (CSFV), and porcine reproductive and respiratory syndrome virus(PPRSV). The sensitivity of the PCR assays was evaluated by amplification of the serially diluted plasmid.

4. Sequencing and phylogenetic analysis

Twelve PCR products of 5′UTR of TTSuV1 (280 bp) and 17 of TTSuV2 (459 bp) positive DNA were submitted for sequencing. The amplicons were purified using the Column DNAback Gel Extraction Kit (TIANDZ, Beijing, China) and the fragment was cloned into pEASY-T vector (TransGEN, Beijing), transformed into JM109 component cells, and cultured on Amp^+ LB plate. The transformants were identified by PCR with primers M13F and M13R, and the positive transformants were cultured in liquid LB for plasmid extraction.

The plasmids were sequenced using forward primer M13F and analyzed by the DNASTAR (Lasergene®). The substitution saturation was tested by DAMBE software using Xia's method[20], and the nucleotide substitution model was tested using the Jmodeltest software[21]. The distances were calculated by the method of p-distance. The maximum likelihood tree between sequences from Hunan and reference sequences downloaded from Genbank were drawn with a bootstrap test of 1000 replicates and the Hasegawa-Kishino-Yano model and Gamma Distributed model for TTSuV1 and for TTSuV2 with MEGA6 software[22]. The nucleotide sequences of Hunan TTSuV isolate described in this study were deposited in Genbank under the accession numbers MF058602-MF058613 (TTSuV1) and MF058614-MF058630 (TTSuV2).

5.Statistical analysis

Differences of TTSuV1 and TTSuV2 prevalence in different farms and different groups were investigated by one-way ANOVA tests using the IBM® SPSS Statistics 19 software (IBM, Armonk, NY, USA). Confidence interval was set as 95%, and a P value less than 0.05 was considered significant.

Results

1. Prevalence of TTSuV1 and TTSuV2

The amplification results of DNA or cDNA of PCV-2, PRV, PPV, CSFV, and PRRSV using the two primers were all negative (data not shown). The minimum amplification copies for TTSuV1 and TTSuV2 were 126 and 53 respectively.

The positive rate of TTSuVs detection in different farms was calculated (Table 1). The total positive detection rate of TTSuV, TTSuV1, and TTSuV2 was 32.17%, 7.99%, and 28.92%, respectively. Furthermore, the total coinfection rate detected was 4.74% (Table 1).

The total positive rate of TTSuV2

prevalence in the same farm was significantly higher than that of TTSuV1 (P=0.005).

The positive rates of TTSuV, TTSuV1, and TTSuV2 prevalence in pigs of different ages (2–20 weeks), gilts, and sows are shown in Table 2. The positive rate of TTSuV prevalence was 79.17% in the 16-week group, which was higher than TTSuV prevalence rate in other age groups (Table 2). TTSuV1 and TTSuV2 were dominant in pigs whose age ranged from 12 to 16 weeks (positive rate >10%) and 8-18 weeks (positive rate >40%), respectively. The positive prevalence rate differences of TTSuV1 and TTSuV2 in different groups were calculated. The positive prevalence rate of TTSuV1 in 16-week-old pigs was significantly higher than that of the other age groups ($p<0.05$) expect in 12 weeks ($p=0.10$). There was no significant difference in the positive rate of TTSuV2 detection among the 8–18-week-old pigs ($p>0.05$), but the positive rate of TTSuV2 detection in 8–18-week-old pigs was significantly higher than that of other age groups ($p<0.05$). Interestingly, TTSuV1 was detected in pigs less than 4 weeks of age, while TTSuV2 was absent.

Table1. Positive rate of TTSuVs detection in different pig farms

Farm	TTSuV1	TTSuV2	TTSuV1+TTSuV2	TTSuV
WX	1.25%(1/80)	45%(36/80)	12.5%(1/80)	45%(36/80)
ZH-4	1.12%(1/89)	24.7%(22/89)	0	25.8%(23/89)
SYJH-2	16.7%(18/108)	34.3%(37/108)	12%(13/108)	38.9%(42/108)
LH-4	9.7%(12/124)	16.9%(21/124)	4%(5/124)	22.6%(28/124)
Overall	7.99%(32/401)	28.92%(116/401)	4.74%(19/401)	32.17%(129/401)

Table 2. Positive detection rates of TTSuV, TTSuV1, and TTSuV2 in different age groups

Age	TTSuV1	TTSuV2	TTSuV	TTSuV1+TTSuV2
2W	8.82%(3/34)	0	8.82%(3/34)	0
4W	2.94%(1/34)	0	2.94%(1/34)	0
6W	2.94%(1/34)	14.71%(5/34)	17.65%(6/34)	0
8W	8.82%(3/34)	41.18%(14/34)	41.18%(14/34)	8.8%(3/34)
10W	5.88%(2/34)	47.06%(16/34)	52.94%(18/34)	0
12W	14.71%(5/34)	55.88%(19/34)	64.71%(22/34)	8.8%(3/34)
14W	12.12%(4/33)	57.58%(19/33)	57.58%(19/33)	12.1%(4/33)
16W	37.50%(9/24)	79.17%(19/24)	79.17%(19/24)	37.5%(9/24)
18W	8.33%(2/24)	41.67%(10/24)	50%(12/24)	0
20W	0	0	0	0
Gilt	4.76%(2/42)	21.43%(9/42)	26.19%(11/42)	0
Sow	0	8%(4/50)	8%(4/50)	0
Total	7.99%(32/401)	28.92%(116/401)	32.17%(129/401)	4.74%(19/401)

2. Phylogenetic analysis of TTSuV1 and TTSuV2

We obtained 12 TTSuV1 (1 from WX, 1 from ZH, 5 from LH and 5 from SYJH) and 17 TTSuV2 (4 from WX, 3 from ZH, 5 from LH and 5 from SYJH) sequences, respectively. The substitution saturation tested by DAMBE showed that selected sequences could be used for phylogenetic analysis and the p-distance value among the same genotypes was calculated.

For TTSuV1, the identities between obtained sequences and references ranged from 78.1% (MF058612-WX1 and KT 160265 from pigs in Nigeria infected with African swine fever viruses)-96.8% (MF058605-LH5 and GQ358967 from Zhejiang, China) with an average identity of 85%, while the identities in obtained sequences range from 89.3%-96.8% with an average of 93.7%. Three groups were established based on the phylogenetic analysis of TTSuV1 sequences including 12 obtained sequences in the present study and 10 reference sequences from Genbank. Group I included 7 sequences that showed a close relationship to 6 reference sequences from China between 2009-2013 and 1 from Canada in 2013(KF583457). One sequences (MF058607-LH2) with GU179315 from Germany in 2008 was clustered together for Group II. Group III contained 3 sequences which were close to JF694116 from Sichuan, China. Besides, the sequences MF058610-LH3 was out of all the 3 groups.

The identities of TTSuV2 between 17 obtained sequences and 14 reference sequences were 72.5% (MF058629-ZH4 and JF451586, from UK in 2010)-98.2% (MF058618-SYJH4 and JQ933529, from Henan in China in 2011), with an average of 89.6% among all sequences. The average identity among the 17 obtained sequences was 85.5%, ranging from 69.1%-98.5%. Phylogenetic analysis indicated that all 17 obtained sequences and reference sequences could be divided into 2 groups. Group I contained 14 sequences, and it could be further clustered into 2 clades. Clade I included 11 obtained sequences with 13 reference sequences, that is 1 from US, 1 from Spain, 1 from UK, and 6 from China. Clade II contained 3 obtained sequences (MF058514-S5, MF058519-L2 and MF058625-W3) and 2 reference sequences (JF451846_TTV2-KOR2 and HM632241_TTV2-lung). Sequences MF058628-ZH3, MF058621-L4 and MF058629-Z4, were clustered into Group II .

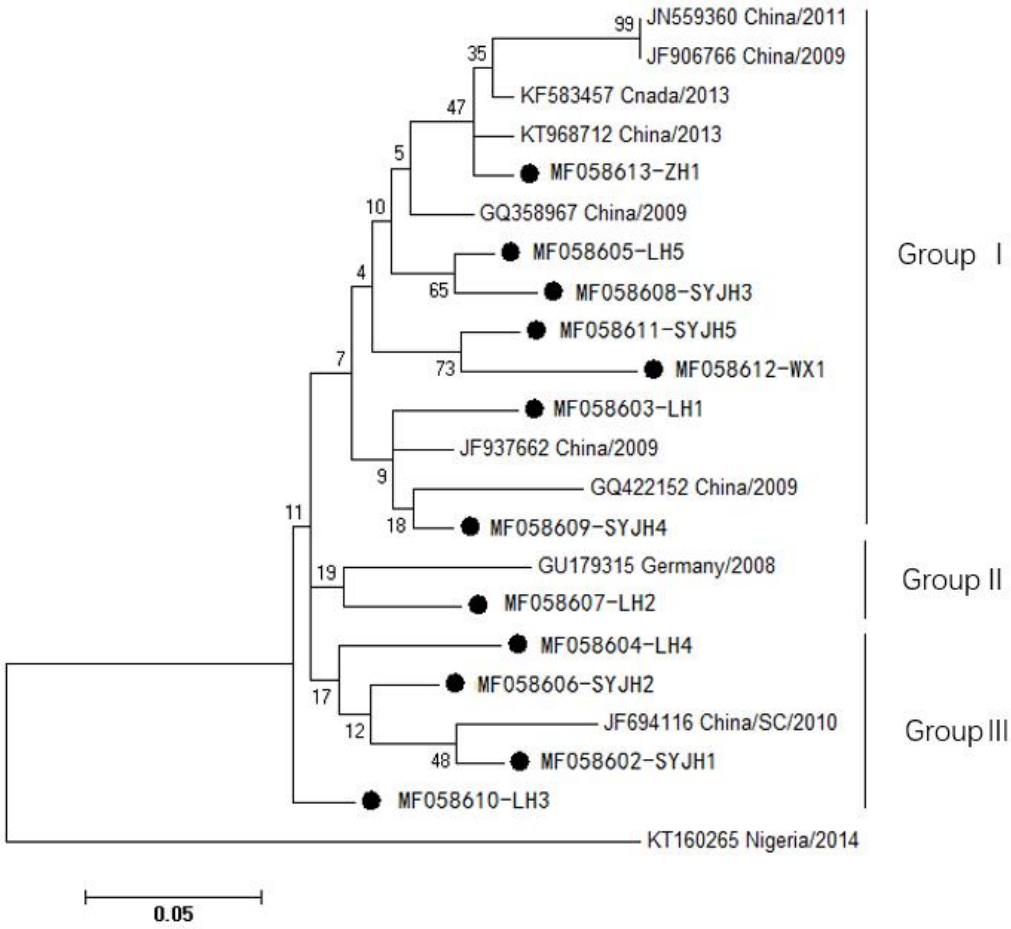

Figure 1: Evolutionary analysis of TTSuV1 based on 12 obtained sequences (marketed with circular symbol) in the present study and 20 reference sequences available through Genbank.

The evolutionary tree was inferred using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model and Gamma distributed model with rates of Gamma distributed. The tree with the highest log likelihood (-1031.6178) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3960)). The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. The analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were 247 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

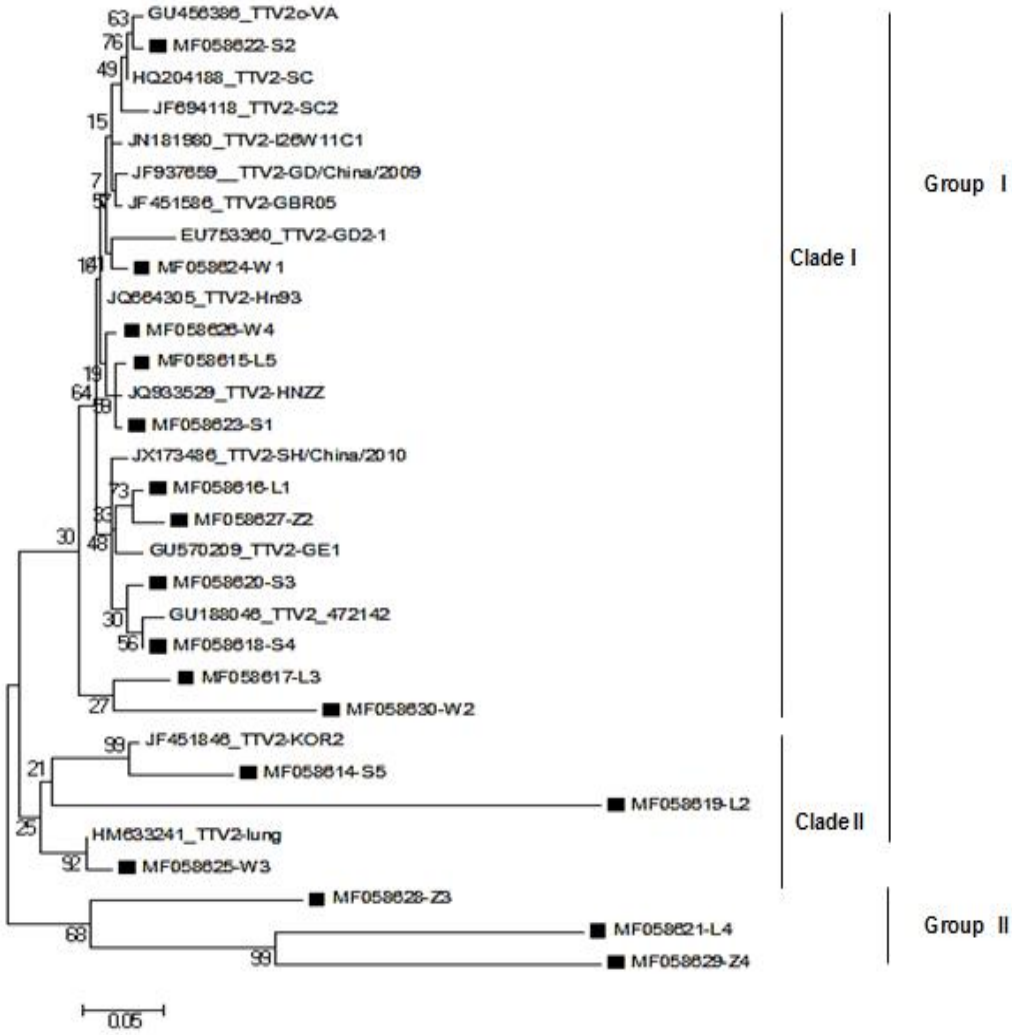

Figure 2: Phylogenetic analysis of TTSuV2 based on the 17 TTSuV2 sequences (marketed with square symbol) and 14 additional TTSuV2 sequences obtained from GenBank.

The evolutionary history was inferred using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model with rates of Gamma distributed. The tree with the highest log likelihood (-4797.7786) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution was used to model the evolutionary rate differences among sites (5 categories (+G, parameter = 5.7154)). The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. The analysis involved 24 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were 417 positions in the final dataset. Evolutionary analyses were conducted using MEGA6.

Discussion

The prevalence of TTSuVs in diseased pigs has been assessed in several tissue [9, 23, 24] and sera samples[25-27], and TTSuVs were thought to be pathogenic to pigs, especially in pigs co-infected with other pathogenic microbes[11, 13, 28, 29]. Therefore, since serum samples are widely used to investigate the prevalence of DNA virus, we investigated the prevalence of TTSuV in sera of clinically healthy pigs of the age range 2 to 20 weeks and the sera of gilts and sows.

TTSuVs have also been detected in lymphoid tissues, and they have been considered to be involved in the develop--ment of lymphoid lesions via alternating the host immune system[28]. Higher percentage of TTSuVs and viral loads have been detected in the bone marrow of healthy pigs and bone marrow, liver, mediastinal lymph nodes, and mesenteric lymph nodes of diseased pigs[17].

A prevalence rate of 52.33% of TTSuVs was calculated in lung tissues of 45 pigs with porcine respiratory disease complex. TTSuV1 detection rate (>75%) was significantly higher than that of TTSuV2 (26.67%) in these samples and TTSuV1 was found to be strongly associated with clinical PRDC[30]. TTSuVs have been detected in pigs with and without PCV-2 systemic disease[31]. The detection rates were higher in pigs with PMWS than in healthy pigs[25, 26]. An experiment of gnotobiotic pigs inoculated with tissue homogenate of TTSuV1 one week prior to PCV2 challenge showed PCVD[32]. PCV-2 infected models are unstable under experimental conditions, and therefore, the role of TTSuVs in PCV-2 infection is unclear.

In our study, we found that the positive rate of detection of TTSuV1 was significantly lower than that of TTSuV2. Wu[33] obtained the same results of TTSuV1 and TTSuV2 in 2011, but higher detection rate was observed compared to our study. TTSuV1 with a lower positive detection rate than TTSuV2 has also been reported in China[19], Brazil[6], and Spain[23, 34], suggesting that TTSuV2 is more prevalent than TTSUV1. However, higher detection of TTSuV1 over TTSuV2 has been reported by Rammohan[30], Xiao[15] in a US pig farm, Zhai[5] in China, and Tshering[35] in Japan. We believe that the sample types, number of samples, health status of pig, and detection method may contribute to these differences.

In the present study, the pigs in farms LH and ZH were vaccinated against PCV-2 for over 4.5 years, pigs in SYJH for 2 years and 8 months, while pigs in WX were not vaccinated. Another study (unpublished) of PCV2 incidence in these four farms showed that PCV2 had the same incidence as TTSuV2: the highest was in farm WX, followed by SYJH, ZH, and LH. However, TTSuV1 detection was not consistent with this. PCV2 vaccination

seems to lead to a decrease in the infection by TTSuV2. Zhai[5] reported an overall TTSuV detection rate of 78.9% in samples collected in 2008-2009. Zhang[13] analyzed 1115 PRRSV-positive samples obtained during 2010-2013, and the TTSuV1 positive rate was 65.3%. Studies published in Chinese journals in 2014 showed that the detection rate of TTSuVs is lower than 60%[36,37]. The wide use of PCV2 vaccination in China started in 2011. The correlation of decreased detection rate of TTSuVs with PCV-2 vaccination is inconclusive, and it should be further investigated.

High detection rates of TTSuV1 and TTSuV2 were reported in nursery pigs in one study[6]. A study on the prevalence of TTSuVs in pigs of different ages by Xiao[15] indicated that finisher pigs (8-25 weeks of age) showed the highest prevalence rate. Zhai[5] reported that the total positive detection rate of TTSuVs in growing pigs (>60 days of age) was higher than that of TTSuVs in piglets (≤60 days of age). Similar results were reported by Brassard[16] and Sibila[7]. All of these results indicated that infection increased with age. In our study, the prevalence of TTSuV2 was more than that of TTSuV1. TTSuV2 prevalence increases with the age of pig from 2 weeks to 16 weeks and decreases from 18 weeks, and it was absent in 20-week-old pigs. The positive detection rate in 8 – 18 weeks of age was 53% (97/183). The detection rate at 18 weeks of age was 41.67%, which was obviously lower than that at 16 weeks, although it was not statistically significant (P=0.189). The positive detection rates in gilts and sows were quite low. The peak viremic state was observed at 16 weeks of age for both TTSuV1 (37.5%) and TTSuV2 (79.17%). TTSuV1 was found in suckling pigs (<4 weeks of age), indicating the presence of low level of maternal antibodies or even vertical transmission.

Although swine TTSuVs were discovered in 1999, they have existed in the pig herd since 1985, according to a retrospective study in Spain. Thus, TTSuVs shows a high genetic diversity [38]. In our study, 12 TTSuV1 and 17 TTSuV2 positive samples from 4 farms were sequenced and the average identity among the obtained sequences of TTSuV1 and TTSuV2 was 93.7% and 85.5%, respectively. Both TTSuV1 and TTSuV2 showed genetic diversity according to the phylogenetic analysis. This indicates that the global swine trade may have contributed to the high prevalence rate and diversity of TTSuVs, especially if TTSuVs are not the routinely inspected agent.

In summary, this is the first study to systemically investigate the TTSuV prevalence rate in healthy pigs from intensive farms in the Hunan province. We have shown that the TTSuV2 are

predominantly circulated in the pig herd, mainly in the age group of 8-18 weeks. We have also shown the diversity of TTSuVs based on the phylogenetic analysis of the UTR of the gene.

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