Journal of Animal and Veterinary Advances 11 (20): 3755-3760, 2012
ISSN: 1680-5593

Cloning and Sequence Analysis of the Complete Genome of Porcine Torque Teno Sus Virus Type 1

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Abstract: According to the published genomic sequence of TTSuV1 (GenBank Accession No. AB076001.1), the pairs of specific PCR/nested-PCR primers were designed to amplify the full length of TTSuV1 complete genome from blood serum samples in Sichuan province. The amplified fragments were cloned, sequenced and named as TTSuV1-SC1 and TTSuV1-SC2, respectively. The sequencing results showed that the complete genome of them were 2852 bp and 2917 bp in length. And then, the correct target sequences gained were deposited in GenBank and the GenBank accession number JF694116 and JF694117 were generated. Homology analysis of TTSuV1-SC1, TTSuV1-SC2 and reference strain sequences retrieved in GenBank indicated that the complete genome of TTSuV1-SC1 and TTSuV1-SC2 from Sichuan isolated strains shared 81-98 and 82-99% homology, respectively with those reference sequences. The phylogenetic tree analysis indicated that the closest genetic relationship of TTSuV1-SC1 and TTSuV1-SC2 isolated strains were TTV1Bj10 strain HM633251.1 and TTVSH0822/2008 strain (GU450331.1). The study would lay a biological foundation to understand the epidemiology, genetic variation of the virus.

Key words: Porcine torque teno Sus virus type 1, cloning, sequence analysis, virus, genetic variation

INTRODUCTION

Nishizawa *et al*. (1997) discovered the Torque Teno virus using molecular methods from an atypical hepatitis patient with abnormal alanine amino transferance levels following a blood posttransfusion. This new DNA virus was named as Transfusion Transmitted Virus (TTV) for the patient who was infected through blood transfusion. The ninth International Committee on Taxonomy of Viruses (ICTV) classified this virus into family Anelloviridae which contains nine genus. It primarily infects vertebrates such as primates, livestock and companion animals (Leary *et al*., 1999; Kekarainen and Segales, 2009; Sibila *et al*., 2009a, b; Zhu *et al*., 2011). Due to the low sequence identity shown, the Porcine Torque Teno virus (Torque Teno sus virus) was separated as two species: *Torque Teno sus virus 1* (*TTSuV1*) and 2 (*TTSuV2*), belongs to genus *Iotatorquevirus and Kappatorquevirus*, respectively (Huang *et al*., 2010a; Cortey *et al*., 2011). Those species who have a broad tissue tropism and can cause persistent infections are prone to genetic mutations.

Torque teno virus is a small icosahedral, non-enveloped virus which contained a single-stranded circular, negative sense DNA genome and its genome length varies from 2.0 kb (cat) to 3.9 kb (human) depending on the host species (Itoh *et al*., 2000; Cortey *et al*., 2011). The genome of swine TTSuVs is approximately 2.8 kb and the entire genome is classified into coding and non-coding regions (UTR). The specific non-coding region is more conservative than the coding region; it has four open reading frames, ORF1, ORF2, ORF1/1 and ORF2/2 (ORF3) (Huang *et al*., 2010b). The region has six open reading frames in human, ORF1-ORF6 (Yokoyama *et al*., 2002) and the Untranslated Region (UTR) contains high G+C content. However, most research has been done on the three already identified reading frames, ORF1, ORF2 and ORF3. It is thought that ORF1 may encode the virus capsid and replication-associated protein (Biagini, 2009); ORF2 may encode non-structural protein (Leary *et al*., 1999). The ORF3 is spliced although the encoded protein function is still unknown.

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By now, TTSuVs is ubiqutions. It is confirmed that this virus is transmitted by the fecal-oral and vertical transmission primarily (Kekarainen *et al*., 2007; Brassard *et al*., 2008; Martinez-Guino *et al*., 2009; Sibila *et al*., 2009a, b). In this study, TTSuV1 was detected by nested Poly-merase Chain Reaction (nPCR) in blood serum samples from 244 pigs in different ages which were collected from Sichuan province. Then, a pair of specifical nested primers was designed for the positive serum to amplify, clone and sequence the complete TTSuV1 genome in order to perform comparative analysis of homology and phylogenetic tree with known genomes. The research aimed at laying the foundation to studying epidemiology, genetic variation of this virus.

MATERIALS AND METHODS

Samples: A total of 244 blood serum samples were collected from 244 pigs in different ages from Sichuan province. These samples were collected and stored at -20°C until the testing was performed. Whereas the health status of the pigs is unclear.

Primers design and synthesis: According to TTSuV1 (AB076001.1) genome sequence, two pairs specific primers T1/T2 and T3/T4 for TTSuV1 complete genome were designed using primer premier 5.0 with some modifications. PCR primer pairs for each target gene and the amplicon sizes are summarized in Table 1.

Viral DNA extraction: About 1.5 mL of blood serum sample was injected into an EP tube. It was centrifuged at 3000 r min^{-1} for 10 min at room temperature and the supernatant was extracted to a clean EP tube. Viral DNA was extracted from 200 uL serum samples by using the genomic DNA extraction kit (Bomaide Biotechnology Co., Ltd.) following the instructions.

PCR amplification of TTSuV1 genome: First nPCR reactions were carried out for detection of TTSuV1 from 244 blood serum samples using a published nested PCR (nPCR) Method (Kekarainen *et al*., 2006) and then researchers choose two of positive serum randomly for the second nPCR amplification using primers T1/T2 and T3/T4. The second nPCR reactions were carried out as follows. For the first-step PCR amplification, the reaction consisted of a total volume of 25 μL, containing 2.5 μL of 10xLA PCR Buffer II, 2.0 μL of dNTP (2.5 mM), 1 μL of $MgCl_2$ (25 mM), 1 μL of upstream primer (26 pm μL^{-1}), 1 μL of downstream primer (25 pm μL), 2.0 μL of template, 0.5 μL of LA Taq enzyme (5 U μL^{-1}), 15 μL of ddH_2O. The PCR amplification was programmed for 95°C for 5 min, 35 cycles at 95°C for 45 sec, 55°C for 50 sec and 72°C for 3 min then 72°C for 10 min after the cycles. The first-step PCR products were used as template for the second-step (nested) PCR amplification, the reaction consisted of a total volume of 50 μL, containing 5 μL of 10xLA PCR Buffer II, 5 μL of dNTP (2.5 mM), 2 μL of $MgCl_2$ (25 mM), 1 μL of upstream primer (25 pm μL), 1 μL of downstream primer (25 pm μL), 5.0 μL of template, 1 μL of LA Taq enzyme (5 U μL), 30 μL of ddH_2O. The PCR amplification was programmed for 95°C for 5 min, 12 cycles at 95°C for 45 sec, 65°C for 50 sec then decreased from 65°C by 0.5°C per cycle and 72°C for 3 min. Then for 30 cycles PCR was programed at 95°C for 45 sec, 55°C for 50 sec, 72°C for 3 min then 72°C for 10 min after the 30 cycles. The PCR products were separated on a 0.8% agarose gel by electrophoresis analyzed. Then, recovering and purifying DNA fragments from agarose gel.

Cloning and identification of PCR products: PCR purified products were performed to clone into pMD19-T simple vector and transformation and the recombinant plasmids were isolated through Plasmid Mini kit (OMEGA, Beijing, China) and sequenced by Invitrogen (Shanghai, China).

Sequence analysis: The genome of TTSuV1 isolates in this study were analyzed using the MegAlign Software (DNAStar Inc., Madison, WI, USA). Phylogenetic trees were constructed by the alignment of the full-length genome of TTSuV1 isolated in this study and the referenced strains (GenBank number and source of regions are shown in the study.

Table 1: Name and sequence of the primers used to amplified the full length genome of porcine TTSuV1

Primers name	The sequence of primers	Expected product (bp)
T1	5'-CTTCGCTCGCACCACGTTTGCTGCCAGGCGGACCT-3'	2878
T2	5'-AATTTTGAATCTAACGGTTTTCAGTCTTCAATCAG-3'	
T3	5'-GGCGGACCTGATTGAAGACTGAAAACCGTTAGATT-3'	
T4	5'-TGGCAGCAAACGTGGTGCGAGCGAAGCGAGCCAAA-3'	

RESULTS AND DISCUSSION

PCR amplification of TTSuV1 genome: According to the nPCR and electrophoretic analysis, 65 out of 244 pigs (25%) were infected with TTSuV1. Two extracted viral DNA from positive serum was used as template, designed primers were used to amplify the complete TTSuV1 genome. PCR products TTSuV1-1 and TTSuV1-2 both had lengths of about 2900 bp which was consistent with the expected fragment size (Fig. 1. TTSuV1-1 and TTSuV1-2 results following 0.8% agarose gel electrophoresis).

Cloning and sequencing of PCR products: In order to determine bacilli by PCR, the two amplified products were cloned into the pMD 19-T vector to construct recombinant T-TTSuV1-1 and T-TTSuV1-2. The results were in line with expectations (Fig. 2). The two TTSuV1 genomes amplified from two different samples were sequenced, evaluated accurately and labeled TTSuV1-SC1 and TTSuV1-SC2 eventually.

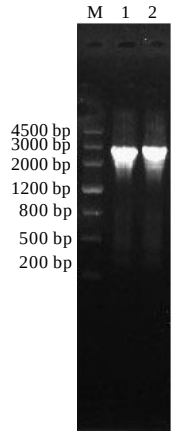

Fig. 1: PCR amplification of complete porcine TTSuV1 genome. M: DNA marker; 1: TTSuV1-1; 2: TTSuV1-2

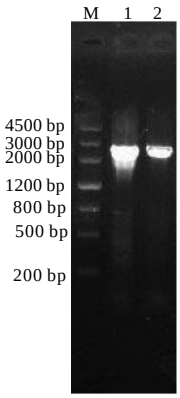

Fig. 2: Identification of recombinant plasmid T-TTSuV1-1, T-TTSuV1-2 by PCR; M: DNA marker; 1: TTSuV1-SC1; 2: TTSuV1-SC2

TTSuV1 genome sequence homology analysis: The result of sequencing showed the inserted gene for TTSuV1-SC1 had a length of 2852 bp and that for TTSuV1-SC2 had a length of 2917 bp. Blast was used on TTSuV1-SC1 and TTSuV1-SC2 via online comparison. Results indicated TTSuV1-SC1 and TTSuV1-SC2 share 81-98 and 82-99% similarity, respectively when they were compared with those reference sequences deposited in GenBank. The results showed that the TTSuV1-SC1 strain isolated in the study had a 98% similarity with HM633251.1 and a 97% similarity with GU570198.1. The TTSuV1-SC2 strand was most similar to GU450331.1 at 99%. TTSuV1-SC1 and TTSuV1-SC2 had a similarity of 71% with each other, the TTSuV1 genome in the same geographic area was less stable and its variability was greater.

Phylogenetic tree analysis of TTSuV1 isolates: From published TTSuV1 viral genome in NCBI, it seems that the variability of the virus, especially between TTSuV1 and TTSuV2 was large with <50% similarity. To further understand the hereditary characteristic of the TTSuV1 strain and related genetic relationship, a phylogenetic tree was constructed on the bases of the strains homology (Fig. 3). As can be seen from the figure, TTSuV1-SC1 and TTSuV1-SC2 belong to different branches that are distantly related. It is evident from the figure that TTSuV1-SC1 has a close genetic relationship with HM633251.1, GU570198.1, GU570199.1 and GU570200.1 as they were classified into the same group. TTSuV1-SC2 is closely related to GU450331.1, GU456383.1 and GQ120664.1 and they were grouped together. TTSuV1-SC1 and TTSuV1-SC2 are on two separate sub-branches, they're relatively distant in genetic relationship. Thus, different TTSuV1 isolates have a relatively distant genetic relationship.

Currently, scientists are very interested in the research of TTSuVs (Kekarainen and Segales, 2009). There are two very different genotypes of TTSuV, TTSuV1 and TTSuV2 (Nie *et al*., 2005; Huang *et al*., 2010a). Several researchers have applied the PCR Method to detect TTSuVs UTR from pigs in the United States, Canada, Brazil, China, French, Italy, Spanish, German, Thailand, South Korean, Hungary, Australia, Cuba, etc. (McKeown *et al*., 2004; Bigarre *et al*., 2005; Nie *et al*., 2005; Kekarainen *et al*., 2006; Martelli *et al*., 2006; Takacs *et al*., 2008; Aramouni *et al*., 2010; Gallei *et al*., 2010; Lee *et al*., 2010; Liu *et al*., 2011; Perez *et al*., 2011). Among them, the difference of the serum positive rate of

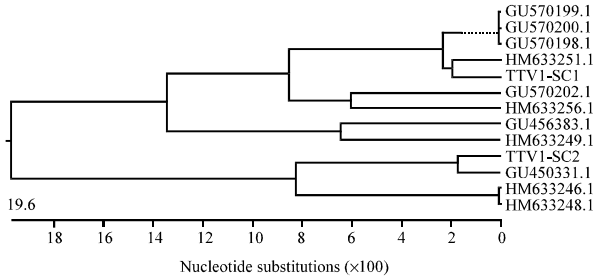

Fig. 3: Phylogenetic tree of TTSuV1 isolates

TTSuV1 is quite large for detecting virus nucleic acid between 24-100% (McKeown *et al*., 2004; Bigarre *et al*., 2005; Kekarainen *et al*., 2006; Martelli *et al*., 2006). Although, the torque teno virus has a higher positive rate in the various healthy pigs and human beings, whether it results in specific diseases and if it is related to the current known diseases is still being debated (Jelcic *et al*., 2004; Huang *et al*., 2011). On the other hand, the fact that the TTSuVs co-infects with other swine pathogens (PMWS, PCV2, PRRSV and PRV) (Rovira *et al*., 2002; Kekarainen *et al*., 2006; Krakowka *et al*., 2008; Lee *et al*., 2010; Martinez-Guino *et al*., 2010; Ritterbusch *et al*., 2011) is especially relevant to the Post-weaning Multisystemic Wasting Syndrome (PMWS) of piglets, it can increase the occurrence and the development of the diseases (Kekarainen *et al*., 2006; Ellis *et al*., 2008). Using a gnotobiotic pig as an animal model, it was found that the homogenate contains TTSuV1 can induce experimental Porcine Dermatitis and Nephropathy Syndrome (PNDS) and Post-weaning Multisystemic Wasting Syndrome (PMWS) (Ellis *et al*., 2008; Krakowka *et al*., 2008). Although, the Porcine Circovirus type 2 (PCV2) was considered as the primary factor that induced clinical PMWS or Porcine Circovirus 2 Associated Disease (PCV2AD), PMWS pigs not infected or slightly infected by PCV2 show a higher rate of TTSuV2 infection than pigs not displaying PMWS in France (Kekarainen *et al*., 2006). However, some researchers reported opposite results; they used qPCR to detect the viral load of TTSuVs in PCV2 pigs and PMWS pigs and found no significant differences in the two groups of pigs (Lee *et al*., 2010). Therefore, continuously monitoring the dynamic changes of TTSuVs infection in animals is useful to the understanding of TTSuVs and offers the effective technical support in the prevention and control of both TTSuVs and PMWS.

The pig serum samples used in this research were acquired from the different commercial pig farms in Sichuan province, China. The DNA virus was directly extracted from the pig serum, the published nested PCR (nPCR) Method (Kekarainen *et al*., 2006) were used to detect TTSuV1 and finally 64 positive samples were detected. In addition, another pairs of nested PCR primers T1/T2 and T3/T4 were designed by the sequence of Sd-TTSuV31 (AB076001.1) strain, found in Japan in order to amplify and clone the full length of TTSuV1 genome. Since, cell culture *in vitro* and animal model have not been developed for TTSuVs, the current TTSuVs detection methods such as nested PCR (nPCR) (Kekarainen *et al*., 2006) and quantitative PCR (qPCR) (Lee *et al*., 2010) were carried out based on its nucleic acids. It has been reported that a pig TTSuV2 ORF1 based ELISA Method was developed in the United States recently (Huang *et al*., 2011), however it hasn't been developed into reagent kits for clinical diagnosis.

TTSuV is one of members of family anelloviridae and has a small DNA genome, approximate 2.7-2.9 kb (Takahashi *et al*., 2002) thus, it is not difficult to amplify the entire genome. Researchers use nPCR or qPCR to detect nucleic acid of positively infected samples but there are also significant differences in positive/negative rates among different kinds of samples, especially in blood serums. This may possibly be due to the fact that some viral strains were not given priority to amplify as a result of interactions between viremia viral load and the detection primers used. Due to the limited quantity of sample, TTSuV1 has only been detected and analyzed in a few provinces in China (Liu *et al*., 2011), the geographic distribution of TTSuV1 infection cannot yet be assumed without more data from different geographical regions in the country.

In order to know more about the prevalence of TTSuVs in China, researchers should collect and analyze more samples from all cities and prefectures. In this study, the full genome of TTSuV1 were amplified and using homology analysis, it is known that TTSuV1-SC1and TTSuV1-SC2 have an 81-92 and 82-99% homology with the TTSuV1 genome reference sequence published on GenBank, respectively. Even though the strains in the same group have relatively conservative genome sequences, they still contain divergence. From the point of view of genome nucleotide length, almost no two strains have the same length in genome sequence, though all the sequences range between 2.7-2.9 kb. This showed that swine TTSuV1 mutate relatively quickly. Phylogenetic analysis and comparison with GenBank database showed that the two strains in this study, TTSuV1-SC1, TTSuV1-SC2 are closely related to HM633251.1 and GU450331.1, respectively. Both of the strains were isolated in China which meant the two strains in the study may be two typical strains prevalent in China. On the contrary some studies of TTSuV2 carried out in

the laboratory indicated that TTSuV2 is less closely related to the Chinese isolates published on GenBank. However, the TTSuV2 strain in the study has high genetic homology with PTTSUV2c-VA (GU456386.1 USA), 472142 (GU188046.1 Germany) and 2p (AY823991.1 Brazil). This phenomenon may affected by where these countries introduced the pigs, contamination in vaccines as well as the transportation of large number of pigs to and from different farms with varying conditions (McKeown *et al*., 2004). This also verified that there are genetic and geographical differences between TTSuVs in the world. The results of this research can offer some evidences to explain the possible origin of the strain and provide useful scientific basis for the study of Sichuan's TTSuV1 strains' epidemiology and disease prevention and control. As of now, it is not confirmed whether TTSuV1 can cause specific disease or what kind of disease it is related to, therefore it is not a quarantined object. Introduction of clinically or subclinically infected TTSuV1 positive pig could be introduced through inter-provincial trade and be rapidly spread all over the country.

CONCLUSION

Through TTSuV1 Blast online comparison, the genetic homology of ORF1 nucleotides sequences between Sichuan strains and other strains was higher than ORF2. This showed that the variation of TTSuV1 is mainly in ORF1 which means the *ORF1* gene codes for the major structural protein of the virus may have a larger mutation rate. Some scientists recommended using anellovirus with nearly full-length viral genome or the longest *ORF1* gene sequence to describe the mutation of the anellovirus. ORF1 sequence occupies at least 60% of the entire genome; they were thought to be the virus encoding Cap protein and Rep related proteins (Biagini, 2009). TTSuV1 contains two major ORFs, the larger ORF1 and the smaller ORF2 are both formed by transcription of forward DNA after it was spliced by the virus (Takahashi *et al*., 2002).

In this study, full-length genome of two TTSuV1 strains were sequenced and each of them was compared with the reference sequences published in GenBank for homology comparison and phylogenic analysis as well as the analysis of the homologous and evolutionary interactions between the TTSuV1 strains' ORF1 and ORF1 of current isolates in the world. This study set the foundation for further research for the mechanism of genetic variation and epidemiology of TTSuV1.

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