Rescue of a porcine anellovirus (Torque teno sus virus 2) from cloned genomic DNA in pigs

Yao-Wei Huang1, Abby R. Patterson2, Tanja Opriessnig3, Barbara A. Dryman1, Andreas Gallei4, Kylie K. Harrall1, Eric M. Vaughn2, Michael B. Roof3, and Xiang-Jin Meng1*

Department of Biomedical Sciences and Pathobiology, College of Veterinary Medicine, Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, VA, USA1; Boehringer Ingelheim Vetmedica, Inc., Ames, IA, USA3; Department of Veterinary Diagnostic and Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA, USA3; BioScreen European Veterinary Disease Management Center GmbH, Mendelstrasse 11, D-48149 Münster, Germany4

Running title: Rescue of TTSuV2 in pigs

*Author for correspondence

X.J. Meng, M.D., Ph.D., Professor of Molecular Virology
Department of Biomedical Sciences and Pathobiology, College of Veterinary Medicine
Virginia Tech, CRC-Integrated Life Sciences Building
1981 Kraft Drive, Blacksburg, VA 24061-0913, USA

Phone: 540-231-6912; Fax: 540-231-3414; E-mail: xjmeng@vt.edu

Abstract: 247 words
Main text: 7,279 words
ABSTRACT

Anellovirus is a group of single-stranded circular DNA viruses infecting human and other animal species. Animal models combined with reverse genetics systems of anellovirus have not been developed. We report here the construction and initial characterization of full-length DNA clones of a porcine anellovirus, Torque teno sus virus 2 (TTSuV2), *in vitro* and *in vivo*. We first demonstrated that five cell lines including PK-15 cells are free of TTSuV1 or TTSuV2 contamination, as determined by a real-time PCR and an immunofluorescence assay (IFA) using anti-TTSuV antibody. Recombinant plasmids harboring monomeric or tandem-dimerized genomic DNA of TTSuV2 from the USA and Germany were constructed. Circular TTSuV2 genomic DNA with or without introduced genetic markers and tandem-dimerized TTSuV2 plasmids were transfected into PK-15 cells, respectively. Splicing of viral mRNAs was identified in transfected cells. Expression of TTSuV2-specific ORF1 in cell nuclei, especially in nucleoli, was detected by IFA. However, evidence of productive TTSuV2 infection was not observed in 12 different cell lines transfected with the TTSuV2 DNA clones. Transfection with circular DNA from a TTSuV2 deletion mutant did not produce ORF1 protein, suggesting that the observed ORF1 expression is driven by TTSuV2 DNA replication in cells. Pigs inoculated with either the tandem-dimerized clones or circular genomic DNA of the U.S. TTSuV2 developed viremia, and the introduced genetic markers were retained in viral DNA recovered from the sera of infected pigs. The availability of an infectious DNA clone of TTSuV2 will facilitate future study of porcine anellovirus pathogenesis and biology.

**Keywords**: Torque teno sus virus (TTSuV); TTSuV1; TTSuV2; porcine anellovirus; Torque teno virus (TTV); infectious clone
INTRODUCTION

Anelloviruses are small, single-stranded, circular DNA viruses that infect a wide range of animal species from humans to domestic animals including pigs (13, 35). Most recently, all human and other animal anelloviruses have been assigned into a newly established family Anelloviridae that includes nine genera (3). Human anelloviruses include Torque teno virus (TTV), Torque teno mini virus (TTMV) and Torque teno midi virus (TTMDV) that belong to three different genera. Human TTV, TTMV and TTMDV are non-enveloped spherical viruses with DNA genomes of 3.6-3.9, 2.8-2.9 and 3.2 kb in length, respectively (34). These three groups of human anelloviruses show a high degree of genetic diversity, and infections of TTV, TTMV and TTMDV at a high prevalence in human populations have been documented worldwide (33, 34). On the other hand, porcine anelloviruses or Torque teno sus viruses (TTSuV) is assigned into a new genus Iotatorquevirus comprising two species (TTSuV1 and TTSuV2), each also characterized by high genetic diversity with a genomic size of approximately 2.8 kb (17, 32). TTSuV1 and TTSuV2 are highly prevalent in pig populations in many countries (9, 21, 29).

Human and porcine anelloviruses share the same genomic structure, which consists of at least four presumed open reading frames (ORFs), ORF1, ORF2, ORF1/1 and ORF2/2, as well as a short stretch of high GC content in the untranslated region (UTR) (5, 17, 37, 39). The transcription pattern and related translational products of human TTV genogroup 1 have been experimentally determined by using two full-length TTV DNA clones (5, 31, 39). It was shown that the human TTV genome expresses three or more spliced mRNAs encoding at least six proteins, ORF1, ORF2, ORF1/1, ORF2/2, ORF1/2 and ORF2/3 (5, 31). The transcriptional analysis and protein expression profile using cloned full-length genomic DNA have not been
reported for TTSuV. The ORF1 of TTSuV is believed to encode a viral capsid and replication-associated protein (5, 17, 37). IgG antibodies against the ORF1 of TTV and TTSuV have been detected in human and pig sera, respectively (15, 18, 38).

The pathogenic potential of anellovirus is still controversial. Currently, human TTV is not considered to be directly associated with a particular disease, although recent studies suggested TTV may serve as an immunological trigger of multiple sclerosis (27). Similarly, whether TTSuV is associated with a swine disease is still debatable. TTSuV1 was shown to partially contribute to the experimental induction of porcine dermatitis and nephropathy syndrome (PDNS) and postweaning multisystemic wasting syndrome (PMWS or porcine circovirus associated disease, PCVAD) in a gnotobiotic pig model (6, 22). PMWS-affected pigs in Spain had a higher prevalence and viral loads of TTSuV2 than the PMWS-unaffected pigs (1, 21). Moreover, a significantly lower level of anti-TTSuV2 antibody was found in PCVAD-affected pigs than in PCVAD-unaffected pigs (15). However, results from other studies did not support a direct association of TTSuV1 or TTSuV2 with PCVAD or association of type 2 porcine circovirus (PCV2) and TTSuV with porcine reproductive failures (10, 16, 24, 40).

Due to the lack of a cell culture system to propagate anelloviruses, little is known regarding the molecular biology and pathogenesis of anelloviruses. In order to definitively characterize diseases associated with anellovirus infection, an appropriate animal model is needed. Since multiple infections of different genotypes or subtypes of human TTV or TTSuV are common events (9, 17, 33), a biologically pure and isolated form of a specific anellovirus generated from full-length infectious DNA clone is also required for a pathological study of a single phenotype. Although infectious DNA clones of human TTV in cultured cells have been reported (5, 19, 25), it is important to construct an infectious TTSuV DNA clone so that TTSuV can be used as a useful model to study the replication and transcription mechanisms and to dissect the structural
and functional relationships of anellovirus genes. More importantly, the availability of a TTSuV infectious DNA clone will afford us an opportunity to use the pig as a model system to study the replication and pathogenesis of TTSuV or even human TTV.

In the present study, we describe the construction and initial characterization of full-length DNA clones of TTSuV2 in vitro and in vivo. We provide, for the first time, definite evidence of splicing of TTSuV2 mRNA and expression of the putative ORF1 capsid protein by transfection of the TTSuV2 full-length DNA clones in cultured cells. Furthermore, rescue of TTSuV2 containing the introduced genetic markers in pigs was confirmed by sequencing of viral DNA obtained from pigs experimentally inoculated with the circular TTSuV2 genomic DNA.

MATERIALS AND METHODS

Cell lines and cell cultures. A total of twelve continuous cell lines were used in this study. A type 1 porcine circovirus (PCV1)-free porcine kidney epithelial cell line PK-15 (8), a swine testis cell line ST (ATCC CRL-1746, passage 6), a baby hamster kidney fibroblast cell line BHK-21 (ATCC CCL-10, passage 62), and an African green monkey kidney epithelial Vero cell (ATCC CCL-81, passage 95) were each grown in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. A porcine monocytic cell line 3D4/31 (ATCC CRL-2844, passage 8), a porcine small intestinal epithelial cell line IPEC-J2 (a gift from Dr. Anthony Blikslager at North Carolina State University, Raleigh, NC) (41), and a hamster ovary cell line CHO-K1 (ATCC CCL-61, passage 12) were each cultured in Dulbecco’s modified Eagle’s medium (DMEM) and nutrient mixture F-12 (Ham) (1:1) with GlutaMAX™-I (Invitrogen, Carlsbad, CA) supplemented with 5% FBS and antibiotics. A monkey kidney cell line subclone MARC-145 (passage 42) derived from MA-104 (ATCC CRL-2378), a human
cervical cancer cell line HeLa (ATCC CCL-2, passage 10), two human hepatocellular carcinoma cell lines Huh-7 (subclone 10-3; a gift from Dr. Suzanne U. Emerson at NIAID, NIH) (7) and HepG2 (ATCC CRL-10741, passage 7) were each grown in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. A human 293 cell line, 293TT, engineered to stably express high levels of SV40 large T antigen (a gift from Dr. John T. Schiller, Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, MD) (4), was cultured in DMEM-10 medium (DMEM with 10% inactivated FBS, 1% non-essential amino acids and 1% GlutaMAX-I) supplemented with 400 µg/ml hygromycin B and antibiotics. All cells were grown at 37°C with 5% CO2.

Analysis of TTSuV1 or TTSuV2 contamination in cultured cells by real-time quantitative PCR (qPCR). To ensure that the porcine-derived cell lines used in the study were free of TTSuV contamination, five cell lines, PCV1-free PK-15, 3D4/31, IPEC/J2, BHK-21 and MARC-145, were tested for TTSuV1 or TTSuV2 DNA by using two singleplex SYBR green-based real-time qPCR assays (14). Briefly, total DNA was extracted from each cell line using the QIAamp DNA mini kit (Qiagen) and was subsequently subjected to TTSuV1 or TTSuV2 qPCR detection in a 25-µl PCR system using SensiMix SYBR & Fluorescein kit (Quantace Ltd) as described previously (14). A TTSuV1 or TTSuV2 standard template and a porcine serum sample from a commercial company used in cell culture, which is supposed to be OIE (The World Organization for Animal Health) diseases-free, were included as controls. We also tested if the porcine-derived trypsin and fetal bovine serum (FBS) products used for cell cultures were contaminated with TTSuVs. Two trypsin products from two different manufacturers and a fetal FBS product were tested including one trypsin product that was used for passaging of the cell
lines. The trypsin was heat-inactivated at 65°C for two hours before DNA extraction. All samples were run in duplicate on the same plate.

Generation of a rabbit anti-TTSuV2 ORF1 antiserum. We have previously expressed and purified a recombinant truncated ORF1 protein of TTSuV2 (PTTV2c-VA strain) (15). The purified protein products were used to immunize two New Zealand white rabbits as a custom antibody production service at Rockland Immunochemicals (Gilbertsville, PA). Serum samples from both rabbits were collected before immunization (pre-bleed) and at 45 days post-immunization. The specificity of the TTSuV2 ORF1 antiserum was demonstrated by a western blot (WB) analysis using the TTSuV2 ORF1 antigen and the bacterial control (cell lysis product from bacteria harboring the empty expression vector) as well as transfection experiments of PK-15 cells (see below) with the TTSuV2 ORF1 expression construct and two empty vectors, pTriEx1.1-Neo and pSC-B-amp/kan (Supplemental Fig. S1).

Construction of full-length genomic DNA clones of TTSuV2. Two PCR fragments (E and F) covering the full-length genome of the U.S. strain of TTSuV2 isolate PTTV2c-VA (GenBank accession no. GU456386) were re-amplified from the constructs reported previously (17), which were subsequently assembled into a full-length genomic DNA by overlapping PCR using the Herculase II Fusion DNA Polymerase (Stratagene) in the vector pSC-B-amp/kan (Stratagene). The monomeric TTSuV2 DNA fragment was flanked by a BamHI restriction site at both ends. The resulting construct was designated pSC-PTTV2c (Fig. 1A). The full-length PTTV2c genome was excised from the clone pSC-PTTV2c using BamHI digestion, purified and ligated head-to-tail to form concatemers. Two-copy concatemers were cloned into the BamHI-pre-digested pSC-B-amp/kan vector to produce a tandem-dimerized TTSuV2 DNA clone, pSC-2PTTV2c-RR (Fig.
Similarly, two plasmids harboring monomeric and tandem-dimerized TTSuV2 genomic DNA originated from German TTSuV2 isolate TTV-#471942 (GenBank accession no. GU188046) (9) were constructed with the EcoRV site on the same vector backbone, respectively. The monomeric TTSuV2 DNA fragment was flanked by an EcoRV restriction site at both ends (Fig. 1C and 1D). Since the TTV-#471942 strain was classified into the TTSuV2 subtype 2b together with the U.S. isolate PTTV-VA based upon phylogenetic analysis (data not shown), we designated these two clones pSC-TTV-#471942 (Fig. 1C) and pSC-2PTTV-RR (Fig. 1D), respectively.

**Introduction of genetic markers into the two TTSuV2 monomeric DNA clones and construction of a TTSuV2 deletion mutant.** An HpaI restriction enzyme site was engineered into the putative spliced region (intron) of TTSuV2 genome in the clone pSC-TTV-#471942 for introducing a genetic marker to discriminate between the cloned virus and the potential indigenous viruses in the subsequent animal study. To create the unique HpaI site (GTAAAC; mutations are underlined), three point mutations, C to T, C to A and T to A at nucleotide (nt) positions 1817, 1819 and 1820 corresponding to the TTV-#471942 genome were generated by a fusion PCR technique using two pairs of primers containing the desired mutations. The fusion PCR product replaced the corresponding region on the clone pSC-TTV-#471942 by using the cloning site KpnI at both ends. The mutations were located in the putative intron of the TTV-#471942 genome and did not change the putative ORF1 capsid amino acid sequence. The resulting full-length DNA clone was named pSC-TTV2-EU (Fig. 1E). Using the same strategy, two unique restriction sites, Psrl (CTGCAG) and Mfel (CATTG), were introduced into the putative intron of the PTTV2c-VA genome in the pSC-PTTV2c clone (Fig. 1F). The new clone, designed pSC-TTV2-US, contained three silent mutations at nt positions 1613 (A to T), 1784 (T
to C) and 1787 (C to T) corresponding to the PTTV2c-VA genome. A mutant clone pSC-TTV2-∆AA, with a 104-bp deletion (nt positions 332-437) from the putative TATA box to the ORF1/ORF2 start codon on the clone pSC-TTV2-US, was also generated by removing the short deletion fragment with double-digestion with the AccI and ApaI enzymes followed by formation of two blunt ends with a Klenow enzyme and self-ligation (Fig. 1G). All mutagenesis were confirmed by DNA sequencing.

In vitro transfection of TTSuV DNA clones. The PCV1-free PK-15 cells were seeded at 2×10^5 cells per well onto a 6-well plate and grown until 60%-70% confluency before transfection. Two micrograms of the tandem-dimerized clones pSC-2PTTV2b-RR, pSC-2PTTV2c-RR, and the vectors pTriEx1.1-Neo and pSC-B-amp/kan were directly transfected into the cells, respectively, using Lipofectamine LTX (Invitrogen) according to the manufacturer’s protocol. For monomeric clones pSC-PTTV2c, pSC-TTV2-#471942, pSC-TTV2-EU, pSC-TTV2-US and pSC-TTV2-∆AA, the respective genomic fragment was excised by BamHI or EcoRV enzyme, gel-purified, and re-ligated with the T4 DNA ligase overnight. The ligation mixtures (~2 μg) were used for transfection using Lipofectamine LTX, respectively. Cells were cultured for 3 to 5 days, and then subjected to an immunofluorescence assay (IFA) to detect the expression of ORF1. Alternatively, transfected cells were passaged into new 6-well plates and were cultured for 3 days before detection of ORF1 expression by IFA. Transfection of the other 11 cell lines and IFA detection were similar.

Immunofluorescence assay (IFA). Transfected or passaged cells on 6-well plates were washed 2 times with PBS and fixed with acetone. Five hundred microliters of the anti-TTSuV2 ORF1
antiserum at a 1:500 dilution in PBS, was added to the cells for each well and incubated for 1 hour at room temperature. Cells were washed 3 times with PBS and 500 μl Texas Red- or Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) at a 1:300 dilution was subsequently added. After incubation for 1 hour at room temperature, the cells were washed with PBS, stained with 500 μl DAPI (KPL, Inc.) at a 1:1000 dilution and visualized under a fluorescence microscope.

**RT-PCR.** Total RNA was extracted from PCV1-free PK-15 cells transfected with circular TTSuV2 DNA or the cloning vector pSC-B-amp/kan using the RNeasy mini kit (Qiagen) followed by an RNase-free DNase I treatment. The cDNA synthesis was performed using SuperScript II reverse transcriptase (Invitrogen) with oligo-dT as the reverse primer. PCR was performed in a 50-μL reaction with the Advantage 2 PCR kit (Clontech) using primers TTV2-448F (5'-GAAGAAAGATGGCTGACGGTAGCGTACT-3') and TTV2-2316R (5'-AGGTGCTTGAGGAGTCGTCGCTTG-3'). The PCR products were gel-purified, cloned into a pCR2.1 vector (Invitrogen) by TA cloning strategy and sequenced.

**In vivo transfection of colostrum deprived (CD) pigs with the tandem-dimerized TTSuV2 clones.** It has been previously demonstrated that the infectivity of infectious DNA clones for viruses with a circular genome can be tested by direct inoculation of dimerized full-length genomic DNA into animals (8). Therefore, in this study, a pilot animal study was initially conducted to determine the infectivity of the two tandem-dimerized TTSuV2 clones pSC-2TTV2c-RR and pSC-2TTV2b-RR. Briefly, six, 26-day-old, CD pigs that were seronegative and viral DNA-negative for TTSuV1 and TTSuV2 were assigned into three groups of two each. Each
group of pigs was housed separately and maintained under conditions that met all requirements of the Institutional Animal Care and Use Committee. The pigs in each group were injected by using a combination of intra-lymphoid (superficial inguinal lymph nodes) and intramuscular routes with the plasmid DNA of the full-length TTSuV2 clones. The two pigs (nos. 1 and 2) in group 1 were each given 1 ml of PBS buffer and used as the negative control. The two pigs (nos. 3 and 4) in group 2 were each injected with 200 μg of the pSC-2TTV2c-RR plasmid DNA whereas the remaining two pigs (nos. 5 and 6) in group 3 were each inoculated with 200 μg of the pSC-2TTV2b-RR clone.

Pigs were monitored daily for evidence of TTSuV2 infection for a total of 44 days. All pigs were necropsied at 44 days post-inoculation. Serum samples were collected from all pigs prior to inoculation and weekly thereafter until termination of the study. The samples were tested for the presence of TTSuV DNA and quantified for viral loads by a singleplex TTSuV2-specific real-time qPCR (14). Samples of tissues including brain, lung, lymph nodes, liver, kidney, thymus, spleen, small intestines, large intestines, heart, tonsil, bone marrow were collected during necropsies and processed for microscopic examination. The tissues were examined in fashion blinded to the treatment status of the pigs and given a subjective score for severity of tissue lesions ranged from 0 (normal) to 3 (severe) (8, 11).

**In vivo transfection of cesarean-derived, colostrum-deprived (CD/CD) pigs with the circularized TTSuV2 genomic DNA containing genetic markers.** To further verify the results from the initial pilot pig study, we introduced tractable genetic markers into the full-length DNA clones and conducted another CD/CD pig study. Approximately 600 μg of circular or concatamerized TTSuV2 genomic DNA derived from the clone pSC-TTV2-EU or pSC-TTV2-
US was generated by ligation of the linearized TTSuV2 genomic DNA. To determine the infectivity of the full-length DNA clones, we inoculated four, 40-day-old, CD/CD pigs (nos. 129, 135, 139 and 140 in group 1) each with 150 μg of concatamerized “TTV2-EU DNA” by a combination of both the intra-lymph node route and intramuscular route. Another four CD/CD pigs (nos. 133, 137, 138 and 141) in group 2, which were housed in a separate room, were each similarly inoculated with 150 μg of concatamerized “TTV2-US DNA”. The remaining four CD/CD pigs (nos. 127, 132, 136 and 142) in group 3 were each injected with 1.5 ml of PBS buffer and served as negative controls. All pigs were monitored for evidence of TTSuV2 infection for a total of 35 days, at which time they were necropsied. Total DNA was extracted without DNase I pre-treatment from all samples and with DNase I pre-treatment from selected samples. Viremia was tested by a TTSuV2 real-time qPCR (14). A TTSuV2 genomic region of 620 bp containing the engineered genetic markers in TTV2-EU or TTV2-US was amplified from the sera of inoculated pigs by PCR using primers TTV2-tagF (5’-TGACACAGGA/CGTAGGAAATGCAGT-3’) and TTV2-tagR (5’-TGAAGTATTTAGGGTCATTTGTAGCA-3’) from selected serum samples of pigs with viremia. The PCR products were gel-purified and cloned into a pCR2.1 vector by using the TA cloning strategy. The white bacterial clones on the X-gal-containing agar plates were picked up for subsequent DNA extraction and sequencing.

RESULTS

Neither the viral DNA nor the expression of the putative ORF1 capsid protein of TTSuV1 or TTSuV2 was endogenously present in five representative cell lines tested in this study. The present study first aimed to identify potential permissive cell lines supporting the TTSuV
propagation. We selected five commonly-used cell lines including three that are of pig origin: PCV1-free PK-15, 3D4/31 and IPEC-J2, and two other cell lines including BHK-21 and MARC-145. These cell lines are known to be permissive for a wide variety of animal virus infections. In order to rule out the possibility of endogenous contamination of TTSuV1 or TTSuV2 in cultured cell lines, both viral DNA and ORF1 protein expression were subjected to TTSuV1 or TTSuV2 real-time qPCR and IFA detections, respectively. An OIE diseases-free porcine serum, which had been shown to have a high level of anti-TTSuV2 ORF1 antibody, was also included as a control (15). The results obtained with the qPCR analysis showed that none of the five cell lines tested in the study were positive for TTSuV1 or TTSuV2 DNA, as determined by the analyses of fluorescence curves, melting curves and agarose gel electrophoresis, since their fluorescence curves were below the minimum detection limit, their melting curves did not overlap with that of the standards, and there were no specific bands corresponding to the expected PCR products (Fig. 2). The bands from the cell samples close to the standards in size were excised from the gel, sequenced and found to be porcine and mammalian genomic sequences. The porcine-derived trypsin and FBS products used for cell cultures were also negative (Supplemental Fig. S2). In contrast, as expected, the commercial porcine serum was positive for TTSuV1 and TTSuV2 DNA (Fig. 2).

To develop cell-based serological methods such as IFA or immunoperoxidase monolayer assay (IPMA) for TTSuV detection, we raised three specific antisera against the putative ORF1 capsid protein of TTSuV1a, TTSuV1b (16) or TTSuV2 in rabbits. When the five cell lines were stained with each of the three virus-specific antisera, respectively, no positive fluorescence signals were detected, indicating the absence of endogenous TTSuV1 or TTSuV2 ORF1 expression (Supplemental Fig. S3). The IFA results were consistent with the qPCR detection, which demonstrated that the five selected cell lines were not contaminated with TTSuV1 or
TTSuV2 and thus can be used for testing the susceptibility of TTSuV infection or replication by transfection with TTSuV2 DNA clones.

Construction and characterization of full-length TTSuV2 genomic DNA clones in porcine kidney PK-15 cells. We were particularly interested in characterizing the infectivity of TTSuV2 full-length DNA clone since TTSuV2 has been reported to be associated with PMWS or PCVAD at a high prevalence rate of viral DNA (21), a high viral load (1) and a low antibody level in disease-affected pigs with an unknown mechanism (15). We first generated two monomeric full-length TTSuV2 DNA clones, pSC-PTTV2c and pSC-PTTV2c#471942, derived from a prototype U.S. isolate PTTV2c-VA and a German isolate TTV2-#471942, respectively (Fig. 1A & 1C)(9, 17). Each of the full-length TTSuV2 genomic DNA was inserted into a cloning vector pSC-B-amp/kan that does not contain a eukaryotic promoter. The restriction site BamHI or EcoRV is the unique site on the PTTV2c-VA or TTV2-#471942 genome, which was engineered at both ends of genomic DNA to facilitate the generation of concatemers and thus to mimic the TTSuV circular DNA genome. BamHI or EcoRV single digestion of the plasmid DNA of each clone clearly resulted in two different fragments of 4.3-Kb and 2.8-Kb in size. The 4.3-Kb fragment represented the backbone vector whereas the 2.8-Kb fragment represented the inserted monomeric TTSuV2 genomic DNA (data not shown).

Subsequently, two copies of the full-length PTTV2c-VA genome from the clone pSC-PTTV2c were ligated in tandem into the pSC-B-amp/kan vector to generate the clone pSC-2PTTV2c-RR (Fig. 1B). Comparison of the AflII single digestion patterns between pSC-PTTV2c and pSC-2PTTV2c-RR showed that the latter clone had an additional 2.8-Kb fragment representing the intact single TTSuV2 genomic DNA (Fig. 3A, right panel). We utilized the same cloning strategy to produce a tandem-dimerized TTSuV2 DNA clone, pSC-2PTTV2b-RR,
derived from pSC-TTV2-#471942 (Fig. 1D). The construct pSC-TTV2-#4719421 has two
HindIII sites: one is located in the TTSuV2 genome, and another is located in the vector. The
close distance between them is 2.1 Kb. When digested with HindIII alone, the construct pSC-
TTV2-#471942 produced two fragments of 2.1-kb and 4.9-kb, whereas the construct pSC-
2PTTV2b-RR gave an additional 2.8-Kb fragment representing the intact single TTSuV2
genome (Fig. 3A, left panel), thus confirming the successful construction of the clone.

Circular TTSuV2 DNA was generated by tandem ligation of the purified linear TTSuV2
genomic DNA excised from the clone pSC-PTTV2c or pSC-TTV2-#471942. Typical monomer,
dimer and high-copy-molecules of concatemerized TTSuV2 DNA were observed in the ligation
products (Fig. 3B). The ligation mixture from PTTV2c-VA or TTV2-#471942 was transfected
into PCV1-free PK-15 cells. IFA conducted at five days post-transfection, using the rabbit
antisera against PTTV2c-VA ORF1, indicated that TTSuV2 ORF1 antigen was expressed in
the nuclei of the transfected cells with approximately 5% positive rate (Fig. 4A & 4C). No
fluorescent signal was observed in mock-transfected cells stained with the same anti-TTSuV2
serum (Fig. 4E) or in circular TTSuV2 DNA-transfected cells stained with the anti-TTSuV1a
ORF1, anti-TTSuV1b ORF1 (16) or pre-bleed rabbit serum (data not shown). Passaging of the
transfected cells for two times did not eliminate but reduced the fluorescent signal (data not
shown). When the transfected cells were continuously passaged for up to 20 passages, no
positive signal was detectable, suggesting that TTSuV2 infection did not occur (data not shown).

We next tested whether direct transfection of plasmid DNA of the tandem-dimerized clone
pSC-2PTTV2c-RR or pSC-2PTTV2b-RR into PK-15 cells resulted in the synthesis of TTSuV2
ORF1. The transfection efficiency of 50-60% in PK-15 cells was measured by using a GFP
expression construct with the Lipofectamine LTX reagent (data not shown). The tandem-
dimerized double-stranded DNA does not represent genomic anellovirus DNA but might
represent an infectious replicative intermediate. IFA at 5 days post-transfection using the same anti-TTSuV2 ORF1 antiserum confirmed that both DNA clones also expressed ORF1 in transfected PK-15 cells (Fig. 4B & 4D). Again, the ORF1 was expressed in cell nuclei. However, the fluorescent intensity and positive rate were lower than that in circular TTSuV2 DNA-transfected cells (Fig. 4B & 4D). We did not observe the localization of ORF1 antigen in the cytoplasm of the transfected cells.

**Experimental identification of two introns in the TTSuV2 genome.** Although the transcriptional profile using cloned TTSuV full-length genomic DNA has not been reported, we previously speculated that TTSuV likely expresses two essential viral mRNA transcripts, mRNA1 and mRNA2, to produce the four known ORF counterparts of human TTV (Fig. 5A) (17). The continuous mRNA1 encodes ORF1 and ORF2 whereas removal of the putative intron of 1341 nt (designated intron 1 here), corresponding to nt positions 648-1988 in PTTV2c-VA genome, generates the putative mRNA2 that encodes two discontinuous ORFs, ORF1/1 and ORF2/2 (17). We also speculated that more spliced mRNAs and their encoding proteins of TTSuV may exist, as shown in human TTV (31, 39).

To verify whether the splicing of the putative intron 1 in TTSuV2 occurred, total RNA was extracted in PK-15 cells transfected with circular PTTV2c-VA DNA followed by DNase I treatment and RT-PCR analysis. Two PCR product bands of approximately 500 bp and 600 bp in sizes were visualized by agarose gel electrophoresis. Sequencing of the cloned PCR fragments resulted in the identification of two sequences. As expected, the large cDNA fragment of 583 bp was exactly the intron 1-spliced product (Fig. 5B), whereas the small cDNA product of 492 bp contained two splicing regions including the intron 1 and an additional 91-nt intron, corresponding to nt positions 2103-2193 in PTTV2c-VA genome, which was designated intron 2.
in this study (Fig. 5C). The splicing sites are conserved among all published TTSuV2 sequences (data not shown). Therefore, in this study for the first time we experimentally demonstrated the existence of splicing of intron 1 and the viral mRNA2 transcripts. We also identified a novel viral mRNA transcript, termed mRNA3, which encodes two putative proteins, ORF1/1/2 and ORF2/2/3, and which switches reading frames from 1 to 2, and 2 to 3, respectively, due to splicing of intron 2 (Fig. 5A). The mRNA3 transcript contains at least three exons on the TTSuV2 genome. Since we failed to determine the 5'- and 3'-ends of the viral mRNA transcripts by rapid amplification of cDNA ends (RACE)-PCR, it is possible that there exists an additional TTSuV2 intron in the upstream of ORF2, as known in human TTV transcripts (31). However, human TTV genome does not contain a short intron corresponding to the TTSuV intron 2 in the downstream of the large intron (intron 1).

Nevertheless, transfection of PK-15 cells with circularized TTSuV2 genomic DNA resulted in the synthesis of viral mRNA transcripts and the expression of ORF1 protein, indicating that the TTSuV2 concatemers likely mimicked the transcription and protein expression from the natural circular genome of TTSuV2.

A tandem-dimerized TTSuV2 clone, pSC-2PTTV2c-RR, is infectious when inoculated into the CD pigs. To test the infectivity of TTSuV2 DNA clones in pigs, we first performed a pilot study with three groups of CD pigs with two pigs per group. The pigs were inoculated with PBS buffer (pig nos. 1 and 2) in group 1, the tandem-dimerized clone pSC-2TTV2c-RR (pig nos. 3 and 4) in group 2, and pSC-2TTV2b-RR (pig nos. 5 and 6) in group 3, respectively. Serum samples were collected from animals at 0, 7, 14, 21, 28, 35 and 42 days post-inoculation (DPI). Pig no. 2 died of septicemia due to an unidentified bacterial infection shortly after inoculation.
TTSuV2 DNA was detected in two pigs inoculated with pSC-2TTV2c-RR beginning at 28 DPI by real-time qPCR. The viral loads, although very low, increased weekly until 42 DPI before necropsy at 44 DPI in both pigs. The viral loads in serum of pig no. 3 increased from $1.93 \times 10^3$ at DPI 28 to $5.59 \times 10^3$ at DPI 35 and $4.36 \times 10^4$ at DPI 42 whereas the serum viral loads in pig no. 4 elevated from $5.07 \times 10^3$ at DPI 28 to $4.49 \times 10^4$ at DPI 35 and $8.87 \times 10^4$ at DPI 42.

Moderate microscopic lesions in brain (lymphoplasmacytic encephalitis mainly perivascular), liver (lymphohistiocytic hepatitis) and kidney (lymphoplasmacytic interstitial nephritis) were observed in pig no. 3 but not in no. 4. The remaining three pigs including pigs inoculated with the clone pSC-2TTV2b-RR did not develop viremia throughout the study. However, pig no. 5 had mild lymphohistiocytic multifocal hepatitis. The results from this pilot pig experiment indicated that the clone pSC-2PTTV2c-RR originated from a U.S. strain of TTSuV2 is infectious.

Characterization of two TTSuV2 full-length genomic DNA clones with engineered genetic markers and a derived mutant clone in vitro. To further rule out the possible contamination of other indigenous TTSuV2 infections in the pilot animal study, it is critical to introduce tractable genetic markers in the TTSuV2 genome so that the cloned virus and the potential indigenous contaminating virus in pigs can be discriminated in inoculated animals. We introduced a unique HpaI restriction site and two unique restriction sites, PstI and MfeI, into two TTSuV2 monomeric DNA clones pSC-TTV2-#471942 and pSC-PTTV2c to produce two new clones pSC-TTV2-EU and pSC-PTTV2-US, respectively (Fig. 1E and 1F). The positions of these sites, located in the intron 1, were expected to not change the putative ORF1 capsid amino acid sequence. PK-15 cells were transfected with ligation mixtures of the linear TTSuV2 genomic
DNA excised from these two marker clones, respectively. The ORF1 expression in nuclei of the transfected cells was detected by IFA at 3 days post-transfection, similar to the patterns of their parental clones (Fig. 6), indicating that the clones with introduced genetic markers are replication competent.

A mutant clone pSC-TTV2-∆AA with a 104-bp deletion (nt positions 332-437) from the putative TATA box (nt positions 283-289; Fig. 5A) to the ORF1 (nt 528) and ORF2 (nt 445) start codons was generated based on the clone pSC-TTV2-US (Fig. 1G). When transfected into the PK-15 cells, the circularized DNA from this mutant clone did not express the ORF1 antigen (Fig. 6), suggesting that the deleted region likely contains a cis-acting element important for viral mRNA transcription or TTSuV2 ORF1 translation. The result of the deletion mutant clone also implied that the observed expression of ORF1 is likely driven by the replication-competent TTSuV2 DNA since the tandem-dimerized clone and concatemerized ligation products from the parental PTTV2c-VA genome were both infectious in pigs (see below).

Expression of the TTSuV2 ORF1 protein in various cell lines transfected with the circularized TTSuV2 DNA from the clone pSC-TTV2-US. From the in vitro transfection experiments described above, it appeared that, although the TTSuV2 putative ORF1 capsid protein is expressed, the PK-15 cells do not support the cell-to-cell spread of TTSuV2 recovered from the introduced TTSuV2 DNA clones. Alternatively, it is possible that the assembly of TTSuV2 virions in the transfected PK-15 cells may be deficient. To search for another cell line that may be permissive for TTSuV2 infection, we subsequently transfected eleven other different cell lines with the circularized TTSuV2 DNA from the clone pSC-TTV2-US, respectively. These cell lines included the four cell lines (3D4/31, IPEC-J2, BHK-21 and MARC-145) that were tested negative for TTSuV1 or TTSuV2 at both the DNA and protein levels. The plain cells of
the other seven cell lines (ST, Vero, 293TT, HeLa, Huh-7, HepG2 and CHO-K1) were also negative for TTSuV2 ORF1 as determined by IFA (Supplemental Fig. S3).

After transfection, all the eleven cell lines expressed the ORF1 protein at 3 days post-transfection (Fig. 7; the results of BHK-21 and CHO-K1 not shown). The percentages of transfected cells with positive IFA signals were subjectively categorized into three levels: IPEC-J2, ST, PCV1-free PK-15, Huh-7 and HepG2 with a high level of positive rates (>5%); 3D4/31, Vero, MARC-145 and 293TT with a middle level of positive rates (between 2-5%); Hela, BHK-21 and CHO-K1 with a low level of positive rates (<2%). In general, TTSuV2-specific antibody staining patterns of individual positive cells by IFA could be divided into three different types: (i) cells displaying dense nuclear staining; (ii) cells displaying large nuclear inclusion staining; and (iii) cells displaying punctate nuclear staining. The last two patterns indicated the localization of ORF1 antigen in cell nucleoli. No cytoplasmic staining was observed in the transfected cells.

To test if some of these IFA-positive cells were susceptible to TTSuV2 infection, supernatants collected from cell lysates of PK-15, ST and 293TT cells transfected with circularized TTSuV2 DNA were inoculated into all cell lines with high level positive rates and some with middle level positive rates including the 293TT cell line, respectively. The inoculated cells were cultured for 3 to 5 days and examined by IFA. No fluorescent signal was detected in these cells (data not shown), indicating that none of the tested cell lines are susceptible to TTSuV2 infection.

Rescue of TTSuV2 from concatamerized TTSuV2 genomic DNA of the clone pSC-TTV2-US in CD/CD pigs. With the introduced genetic markers in the full-length DNA clones that can be used to distinguish between infections caused by the cloned virus and potential indigenous
contaminating virus, we performed an additional study in CD/CD pigs to further verify the in
vivo infectivity of the TTSuV2 genomic DNA clones. Twelve CD/CD pigs were assigned into
three groups with four pigs each. Pigs in each group were inoculated with PBS buffer,
concatamerized “TTV2-EU DNA”, and “TTV2-US DNA”, respectively. Pre-inoculation serum
samples for all pigs (collected at 30 days prior to inoculation) were tested negative for TTSuV1
or TTSuV2 DNA by real-time qPCR. Serum samples were collected from all animals at 0, 7, 14,
21, 28 and 35 DPI.

TTSuV2 DNA was detected in all eight inoculated pigs, but unfortunately, it was also
detected in two negative control pigs (nos. 136 and 142), indicating contamination by other
indigenous strains of TTSuV2 in the research facility or the source pigs, which is not uncommon
(Supplemental Table S1). One pig (no. 133) inoculated with the concatamerized “TTSuV2-US
DNA” had a detectable viremia even at 0 DPI, whereas the other pigs developed viremia at 14 or
21 DPI (Supplemental Table S1). Except for pig no. 133, the seven TTSuV2 DNA-inoculated
pigs and the two TTSuV2-positive pigs in negative control group had an increased viral load
until necropsy, indicating active virus infection (Supplemental Table S1). We speculated that the
source of the TTSuV2 contamination was likely due to the 1-month waiting period between the
date of pre-inoculation serum sample testing (for which all animals were negative) and 0 DPI.
Since total DNA of these samples was extracted without DNase I pre-treatment, to rule out the
possibility of the presence of inoculated free DNA in the sera from pig no. 133, sera from this
pig, and from pigs no. 136 and 142 in the negative control group with positive PCR results, were
subjected to DNase I treatment or without treatment before DNA extraction followed by qPCR.
The result was consistent to supplemental Table S1 showing that there was no significant
difference in viral loads between DNase I treatment and non-treatment in the samples (Table S2).
However, thanks to the introduced genetic markers in the TTSuV2 DNA clones used in this study, we were still able to determine if the TTSuV2 DNA clones were infectious in pigs, which was the main objective of our study. Since we have previously demonstrated that a single pig can be infected by multiple strains of TTSuV2 and TTSuV1 (9, 17), then prior infection or concurrent infection of an indigenous TTSuV2 strain should not interfere with the infection of pigs by the TTSuV2 DNA clones we intended to test in this study. To determine if the genetic markers of TTSuV2-EU or TTSuV2-US were present in viruses recovered from the sera of infected pigs under the mixed TTSuV2 infection status, we amplified and sequenced a 620-bp region containing the engineered genetic markers from selected samples at 35 DPI from both inoculated and negative control pigs. The results showed that only the serum samples from pigs experimentally inoculated with the concatamerized “TTSuV2-US DNA” were found to have identical TTSuV2 sequences to the introduced genetic markers $PstI$ and $MfeI$, whereas serum samples from the negative control group and from pigs inoculated with concatamerized “TTV2-EU DNA” did not contain any introduced genetic markers (data not shown). Therefore, this pig study further confirmed the initial pilot pig study that the TTSuV2-US full-length DNA clone is infectious in pigs. The results also experimentally verified, for the first time, that pigs can be co-infected by different strains of TTSuV2.

**DISCUSSION**

Little is known about the biology and pathogenicity of anelloviruses due to the lack of a cell culture system to propagate human TTV or TTSuV and the lack of a suitable animal model combined with reverse genetics systems for anellovirus studies. Reports of TTSuV DNA sequences detected in commercial porcine vaccine products, porcine-derived human drugs and in
porcine-derived trypsin by nested PCR suggested a widespread contamination of TTSuV (20, 23). Cell cultures may be one of the major sources for TTSuV contamination in biological products of pig origin. Therefore, the present study was first aimed at examining whether five selected cell lines harbor endogenous DNA and protein antigen of TTSuV1 or TTSuV2, and to further identify TTSuV-negative cell lines that are potentially permissive for TTSuV propagation.

Surprisingly, none of the five cell lines tested in the study were found to be positive for TTSuV1 or TTSuV2 DNA or ORF1 antigen (Fig. 2). Furthermore, screening of seven additional commonly-used cell lines also yielded negative results as determined by IFA detection (Fig. S3), indicating that TTSuV contamination in cell cultures is probably not as common as we originally thought. Our result was distinct from a recent study by a Brazilian group that reported TTSuV DNA contamination in 15 out of 25 cell lines (42). In that study, the five cell lines that were also used here in our study, including PK-15, ST, BHK-21, Vero and MA-104 cells (from which the MARC-145 cell line is derived) had been shown to have detectable TTSuV1 and/or TTSuV2 sequences by using a one-round duplex PCR assay (42). It is unclear why there is such a major discrepancy between our results in this study and those by the Brazilian group. A reliable approach to prove the presence of a contaminating virus in cell cultures used in biological products is to determine its susceptibility to virus infection, which has been exemplified by PCV1 (2, 12, 26, 43). Theoretically, the possibility of TTSuV contamination in cell cultures is very low, since anellovirus has been shown to be extremely difficult to propagate in vitro. The present study utilized the (i) more sensitive qPCR assay (compared to the one-round PCR in the Teixeira et al study); (ii) the IFA; and (iii) transfection of circular TTSuV genomic DNA into the cells as the positive control (see below) to demonstrate the absence of TTSuV at both the DNA and amino acids levels in 12 representative cell lines including four of pig origin (PK-15, ST,
Therefore, based on the results from this study, we conclude that, contrary to what some may believe, there is very little, if any, endogenous TTSuV contamination in well-established continuous cell lineages. Instead, detection of contaminating TTSuV DNA sequences in biological products reported by other groups may come from the porcine-derived trypsin (20, 42) or serum products. The former was not detected (Fig. S2), but the latter was actually confirmed in the present study for the first time (Fig. 2).

Subsequently, we demonstrated that all of these TTSuV-free cell lines supported TTSuV ORF1 expression by transfection with the circular TTSuV2 genomic DNA or the tandem-dimerized TTSuV2 plasmids (Fig. 4, Fig. 6 and Fig. 7). The TTSuV2 ORF1 protein was expressed in cell nuclei, especially in nucleoli, which is consistent with the localization of human TTV ORF1 in Huh-7 cells transfected with the circular full-length TTV genomic DNA by immunoblotting with the ORF1-specific antibody (31). Most recently, it was also reported that TTSuV1 or TTSuV2 ORF1-GFP fusion protein expressed from the recombinant construct was accumulated in nucleoli of the PK-15 cells (28).

In addition, in this study TTSuV2-specific mRNA splicing events were detected in transfected PK-15 cells by RT-PCR, indicating the synthesis of viral mRNA transcripts in the transfected cells. While we experimentally demonstrated the existence of two viral mRNAs transcripts (mRNA2 and mRNA3) (Fig. 5), the putative mRNA1 encoding the full-length ORF1 of TTSuV2 was not detected (data not shown), which may suggest a lower quantity and integrity of mRNA1 than that of mRNA2 and mRNA3. In accordance with the result described by Martinez-Guino et al., splicing of the 91-nt intron 2 sequence in mRNA3 also occurred in the post-transcription of TTSuV2 ORF1-GFP fusion gene based on none-full-length viral clone (28).

The synthesis of viral mRNA transcripts and the subsequent expression of the ORF1 or ORF1-related viral proteins in transfected cells were driven by the endogenous TTSuV2
promoter. The processes were also regulated by the unidentified cis-acting elements, as we showed in this study that deletion of a 104-bp sequence downstream of the TATA box completely eliminated ORF1 expression (Fig. 6). To our knowledge, this is the first demonstration of porcine anellovirus viral mRNA and protein expression and mutagenesis analysis based on the viral DNA concatamers produced from circularized viral genomes or a tandem-dimerized full-length clone.

It appeared that both PTTV2c-VA and TTV2-#471942 DNA concatamers were replication-competent when transfected into cells since they mimicked the natural TTSuV2 circular genome. However, the rescue of PTTV2c-VA (“TTV2-US”), but not TTV2-#471942 (“TTV2-EU”), was only demonstrated in two in vivo animal experiments. The major sequence difference between these two TTSuV2 strains was in the GC-rich region. It has been proposed that the GC-rich region in anelloviruses forms unique stem-loop structures, which may play a significant role in viral replication (30, 36). Further in-depth mutagenesis analysis, which was not the scope of the present study, is required to explain this discrepancy between the two clones.

We also showed that, although the three cell lines (PK-15, ST and 293TT) tested in the study supported a limited level of TTSuV2 replication, the infection of these cells by TTSuV2, if any, was non-productive since the supernatants of the transfected cells did not induce a second-round infection. Most recently, the 293TT cell line was shown to be susceptible for human TTV propagation due to its expression of SV40 large T antigen at a high level (5). The authors proposed that the human TTV genome contains a conserved octanucleotide in the UTR forming a stem-loop as the putative origin of replication. Five 4-bp motifs (CGGG and GGGC) were found adjacent to the stem-loop, which may act as the recognition sites for the SV40 large T antigen to facilitate TTV replication (5). However, when we performed a sequence alignment analysis of the corresponding sequences among human TTV, TTSuV, Torque teno canis virus
(dog anellovirus) and Torque teno felis virus (cat anellovirus), neither the conserved octanucleotide nor the 4-bp motif was identified in the latter three anelloviruses (data not shown). Therefore, the SV40 large T protein expressed in 293TT cells likely does not provide the proposed helper effect on TTSuV replication. Further study is needed to screen whether additional cell lines are permissive to TTSuV infection.

Previous studies from our group and others have demonstrated that, even under strictly controlled experimental conditions in research facilities, TTSuV-negative pigs can easily acquire TTSuV infection due to the ubiquitous nature of this virus in pigs and environments (10, 15). Although our second in vivo experiment in the present study unfortunately “validated” these previous reports, our results did demonstrate the successful rescue of TTSuV2 in pigs inoculated with either the tandem-dimerized plasmids or circular TTSuV2 DNA with the introduced genetic markers. Unfortunately, due to the presence of indigenous TTSuV2 in the CD/CD pigs from the second animal study, we could not analyze or correlate any pathological lesions in the inoculated pigs to TTSuV infection. Therefore, a future study using the germ-free gnotobiotic pig and the infectious DNA clone is warranted to characterize the pathological lesions solely attributable to TTSuV2 infection. In addition, serum samples containing rescued TTSuV2 US strain of known viral loads can be used as virus stocks for experimental infection of TTSuV2-negative pigs in future studies. The availability of the pig model combined with the reverse genetics system of anellovirus described in this study will facilitate future studies of porcine and even human anellovirus biology and pathogenesis.

ACKNOWLEDGEMENTS

This study is funded by a grant from Boehringer Ingelheim Vetmedica, Inc., Ames, Iowa.
REFERENCES


FIGURE LEGENDS

Figure 1. Schematic diagrams of TTSuV2 constructs containing full-length TTSuV2 genomic DNA. (A) pSC-PTTV2c (from the U.S. TTSuV2 isolate PTTV2c-VA; GenBank accession no. GU456386). (B) pSC-2PTTV2c-RR (tandem-dimerized PTTV2c-VA genomes). (C) pSC-PTTV2-#471942 (from the German TTSuV2 isolate TTV2-#471942; GenBank accession no. GU188046). (D) pSC-2PTTV2b-RR (tandem-dimerized TTV2-#471942 genomes). (E) pSC-TTV2-EU (derived from pSC-PTTV2-#471942). A HpaI site as the silent genetic marker was introduced in this clone. (F) pSC-TTV2-US (derived from pSC-PTTV2c). PstI and MfeI sites as the silent genetic markers were introduced in this clone. (G) pSC-TTV2-ΔAA. A 104-bp deletion mutation was introduced between the AccI and ApaI sites ranging from the putative TATA box to the ORF1 start codon on the clone pSC-TTV2-US. The restriction enzymes (BamHI or EcoRV) used for plasmids constructions are shown. The nucleotide positions of the restriction enzymes corresponding to the genomic sequences, the termini of the virus genome, ORF1 and ORF2 are labeled. The plasmid backbone used for cloning was the pSC-B-amp/kan vector (indicated by black). Grey arrows indicate the TTSuV2 genomic copies.

Figure 2. Detection of TTSuV1 or TTSuV2 contamination in five different cell lines (PCV1-free PK-15, 3D4/31, IPEC/J2, BHK-21 and MARC-145) and an OIE diseases-free porcine serum by real-time qPCR. Fluorescence curves (A and C) and melting curves (B and D) of TTSuV1 (A and B) or TTSuV2 (C and D) qPCR products are shown after 40 cycles of amplifications of the standard template with the minimum dilution limit (10⁻⁴ pg; indicated by red), five different cell lines (blue) and the porcine serum (green). For each sample, duplicate
determinations were made. (E) Detection of specific TTSuV1 or TTSuV2 qPCR products (marked by black arrowheads) by agarose gel electrophoresis.

**Figure 3. Identification and quality assessment of linear or circular TTSuV2 genomic DNA.**

(A) Comparisons of the *Hind*III single-digestion patterns between clones pSC-TTV2-#471942 and pSC-2PTTV2b-RR (left panel) and *Afl*III single-digestion patterns between clones pSC-PTTV2c and pSC-2PTTV2c-RR (right panel) by agarose gel electrophoresis. M: DNA markers. The results were consistent to the predicted patterns of the digested fragments (shown by black arrowheads). The 2.8-Kb fragments indicate the intact single TTSuV2 genomic DNA from the clone pSC-2PTTV2b-RR or pSC-2PTTV2c-RR. (B) Quality assessment of concatenated ligation products of the *Bam*HI-digested and purified PTTV2c genomic DNA. The samples were electrophoresed in a 1% agarose gel before (linear DNA) and after (ligation mixture) T4 DNA ligase treatment. Linear DNA (~2.8 Kb) and formations of the putative one-copy (monomer), two-copy (dimer) and high-copy-number circular DNA are indicated by arrowheads.

**Figure 4. Immunofluorescence assay (IFA) results on PCV1-free PK-15 cells transfected with the ligation mixtures of linear TTSuV2 genomic DNA derived from clones pSC-PTTV2c (A) or pSC-TTV2-#471942 (C), with plasmids pSC-2PTTV2c-RR (B) or pSC-2PTTV2b-RR (D), or with Lipofectamine LTX only (E).** Cells were stained with a rabbit anti-TTSuV2 ORF1 polyclonal antibody (Ab) and a Texas Red-conjugated goat anti-rabbit IgG (red) at 5 days post-transfection (the left panels). DAPI (blue) was used to stain the cell nucleus (the middle panels). The Ab and DAPI stainings are merged (right panels). Magnification = 200×.
Figure 5. The putative transcription profile and protein expression of TTSuV2 based on the PTTV2c-VA genome. (A) Schematic diagram of three putative viral mRNAs and six viral proteins. The TATA box, splicing sites (SD: splicing donor; SA: splicing acceptor) and the positions of primers TTV2-448F and TTV2-2316R are indicated at the top. The three open reading frames (ORFs) are depicted by colored boxes. The sizes of the six ORFs and two introns are also shown. (B) Sequencing of the RT-PCR products amplified by primers TTV2-448F and TTV2-2316R verified the splicing of the putative intron 1. The actual viral mRNA2 is illustrated and the nt positions of both ends of the mRNA2 and the splicing sites are marked. (C) Sequencing of the RT-PCR products amplified by primers TTV2-448F and TTV2-2316R identified an additional intron (intron 2). The actual viral mRNA3 is illustrated and the nt positions of both ends of the mRNA3 and the splicing sites are marked. Arrows and numbers indicate the joint site of the exons.

Figure 6. IFA results of PCV1-free PK-15 cells transfected with the ligation mixtures of linear TTSuV2 genomic DNA derived from clones pSC-TTV2-EU, pSC-TTV2-US or pSC-TTV2-ΔAA. Cells were stained with an anti-TTSuV2 ORF1 antibody (Ab) and an Alexa fluor 488-conjugated goat anti-rabbit IgG (green) at 3 days post-transfection. DAPI (blue) was used to stain the cell nucleus. Only merge of Ab and DAPI stainings are shown. Magnification = 200×.

Figure 7. Transfection of nine different cell lines with the ligation mixture of linear TTSuV2 genomic DNA derived from the clone pSC-TTV2-US. Alexa fluor 488-conjugated antibody (Ab) staining (green) merged with nuclear staining using DAPI (blue) are shown. Magnification = 200×.
Figure 1
Figure 2
Figure 3
Figure 4

<table>
<thead>
<tr>
<th>Anti-TTSuV2 ORF1</th>
<th>DAPI</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circular PTTV2c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSC-2PTTV2c-RR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Circular TTV2-#471942</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSC-2PTTV2b-RR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4
Figure 5
Figure 6

pSC-TTV2-EU  pSC-TTV2-US  pSC-TTV2-ΔAA
Figure 7