CORE
Metadata, citation and similar papers at core.ac.uk
Provided by Edinburgh Research Explorer

THE UNIVERSITY of EDINBURGH

Edinburgh Research Explorer

Prevalence of the Novel Torque Teno Sus Virus Species k2b from Pigs in the United States and Lack of Association with Post-Weaning Multisystemic Wasting Syndrome or Mulberry Heart Disease

Citation for published version:
Rogers, AJ, Huang, Y-W, Heffron, CL, Opriessnig, T, Patterson, AR & Meng, X-J 2017, 'Prevalence of the Novel Torque Teno Sus Virus Species k2b from Pigs in the United States and Lack of Association with Post-Weaning Multisystemic Wasting Syndrome or Mulberry Heart Disease' Transboundary and Emerging Diseases, vol. 64, no. 6, pp. 1877-1883. DOI: 10.1111/tbed.12586

Digital Object Identifier (DOI):
10.1111/tbed.12586

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Transboundary and Emerging Diseases

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 05. Apr. 2019

Prevalence of the novel *Torque teno sus virus* (TTSuV) species k2b from pigs in the United States and lack of association with postweaning multisystemic wasting syndrome or mulberry heart disease

Adam J. Rogers[1], Yao-Wei Huang[2], C. Lynn Heffron[1], Tanja Opriessnig[3], Abby R. Patterson[4], and Xiang-Jin Meng[1*]

[1]Department of Biomedical Sciences and Pathobiology, Virginia-Maryland College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA, U.S.A.

[2]College of Animal Sciences, Zhejiang University, Hangzhou, China;

[3]The Roslin Institute, University of Edinburgh, Midlothian, Edinburgh, U.K.;

[4]Boehringer Ingelheim Vetmedica Inc, Ames, Iowa, U.S.A.

Running title: Prevalence of TTSuVk2b in U.S. swine

***Corresponding author**

Dr. X.J. Meng, University Distinguished Professor

VA-MD College of Veterinary Medicine, Virginia Tech

1981 Kraft Drive, Blacksburg, VA 24061-0913

email: xjmeng@vt.edu

Word counts:

Abstract: 249 words; Text: 2559 words; Table: 3; Figures: 1

Abstract

The family *Anelloviridae* includes a number of viruses infecting humans (*Torque teno viruses*, TTV) and other animals including swine (*Torque teno sus viruses*, TTSuV). Two genetically-distinct TTSuV species have been identified from swine thus far (TTSuV1 and TTSuVk2), although their definitive association with disease remains debatable. In 2012, a novel TTSuV species was identified from commercial swine serum and classified in the genus *Kappatorquevirus* as TTSuVk2b. The other *Kappatorquevirus* species, TTSuVk2a, has been associated with postweaning multisystemic wasting syndrome (PMWS) when coinfected with *Porcine circovirus* type 2 (PCV2). Therefore, in this study we initially amplified a portion of TTSuVk2b ORF1 and, subsequently, assessed the molecular prevalence of the virus in pigs in the United States. A total of 127 serum and 115 tissue samples were obtained from pigs with PMWS or Mulberry Heart Disease (MHD) in 6 states, and tested by PCR for the presence of TTSuVk2b DNA. Approximately 27.6% of the serum and 21.7% of tissue samples tested positive for TTSuVk2b DNA and the positive products were confirmed by sequencing. However, we did not detect a correlation between TTSuVk2b infection and PMWS or MHD. The near full-length genomic sequence of U.S. TTSuVk2b was determined, and sequence analysis revealed that the U.S. TTSuVk2b isolates were 95% identical to the TTSuVk2b isolate from Spain, with most of the variations clustering in ORF1. We conclude that the novel TTSuVk2b species is present in pigs in the United States and its potential association with a disease warrants further investigation.

Keywords: *Torque Teno Sus Virus* k2b (*TTSuVk2b*); Postweaning Multisystemic Wasting Syndrome (PMWS); Mulberry Heart Disease (MHD).

Introduction

Torque teno virus (TTV) was first identified in a Japanese patient with post-transfusion liver disease in the absence of an infection with other known hepatic viruses (Nishizawa et al., 1997), and was first identified in pigs in Japan in 2002 (Okamoto et al., 2002). *Torque Teno sus virus* (TTSuV) is subdivided into two genera, *Iotatorqueviruses* and *Kappatorqueviruses* or TTSuv1 and TTSuVk2, with a relatively high intragenera nucleotide sequence identity but only approximately 50% intergenera identity (Kekarainen et al., 2006, Niel et al., 2005). Torque teno viruses are non-enveloped, single-stranded DNA viruses with genomes ranging from 2.1 to 3.8 kb in size (Okamoto et al., 1998b, Okamoto et al., 1998a, Mushahwar et al., 1999, Okamoto et al., 2002), and have a conserved genome organization (Inami et al., 2000, Okamoto et al., 2001, Okamoto et al., 2002, Niel et al., 2005). The untranslated region (UTR) consists of approximately one-third of the genome and is highly conserved, even within extremely divergent TTVs (Takahashi et al., 1998, Kamada et al., 2004, Suzuki et al., 2004). This particular genomic region, including a region with extremely high GC content proximal to the viral origin of replication, is predicted to form a number of secondary structures (Muhire et al., 2014). There exist at least five gene products, with ORF1 encoding the viral capsid protein, and ORF2 encoding a non-structural protein important for virus replication (Maggi and Bendinelli, 2009). TTSuV1 and TTSuVk2 are highly prevalent in pigs worldwide ranging from 24% to 100% (Bigarre et al., 2005, Kekarainen et al., 2006, Martelli et al., 2006, McKeown et al., 2004). The virus infects a relatively high proportion of otherwise healthy pigs (Sibila et al., 2009b). TTSuVs DNA can be detected in as early as 1-week-old piglets, but the likelihood of detecting TTSuV DNA or antibodies in a pig increases with age (Brassard et al., 2008, Sibila et al., 2009a, Aramouni et al., 2010a, Xiao et al., 2012). TTSuV's DNA can be detected in a number of tissues

including brain, lymph node, heart, liver, bone marrow, lung, spleen, and pulmonary epithelium indicating a broad tissue tropism (Reviewed in (Hino and Miyata, 2007, Aramouni et al., 2010a)). More recently in 2012, a novel *Kappatorquevirus* species was identified from commercial swine serum and classified as TTSuVk2b (Cornelissen-Keijsers et al., 2012). A subsequent study revealed that TTSuVk2b DNA was detected from 0 to 100% of pig serum samples from 17 different countries. However, whether the new TTSuVk2b species exists in pigs from the United States remains unknown.

Many studies have attempted to link TTSuVs to clinical symptoms, but thus far a definitive causal relationship between TTSuV infection and a particular disease in pigs is still lacking (Kekarainen and Segalés, 2012). TTSuV infection has been reportedly associated with respiratory diseases in experimentally-infected gnotobiotic pigs (Krakowka and Ellis, 2008) and contributed to the development of postweaning multisystemic wasting syndrome (PMWS) when coinfected with *porcine circovirus type 2* (PCV2) (Kekarainen et al., 2006, Aramouni et al., 2010b). PMWS and a collection of other symptoms collectively known as Porcine Circovirus Associated Disease (PCVAD) represent an economically significant disease for the pig industry (Reviewed in (Allan et al., 1998)). It has also been reported that TTSuVk2a, but not TTSuV1, has an association with PMWS in pigs (Aramouni et al., 2010b, Kekarainen et al., 2006, Nieto et al., 2011), and that there is an increased TTSuVk2a viral load in PMWS pigs (Aramouni et al., 2011). Additionally, TTSuVk2a copy numbers increased in pigs with experimental infection of classical swine fever virus while TTSuV1 did not, underscoring the differences in these two species (Nieto et al., 2013)

To determine whether the novel TTSuVk2b species is present in pigs in the United States (U.S.) and, if so, its prevalence, we tested a total of 127 serum and 115 tissue samples from pigs

that had been diagnosed for PMWS or Mulberry Heart Disease (MHD), a disease characterized by sudden death in piglets and characteristic discoloration of the cardiac muscle sometimes associated with Vitamin E deficiency (Pallarés et al., 2002), in 6 states by TTSuVk2b-specific PCR. The near complete genomic sequence of the U.S. strain of TTSuVk2b was also determined and analyzed.

Materials and Methods

Sample collection:

A total of 127 pig serum and 115 tissue samples were obtained for this study. Serum samples were obtained from pigs in Virginia and Iowa farms. At the time of sample collection, pigs were also scored for signs of PMWS. One pig herd from Iowa had been previously found to have a high incidence of MHD, prompting an investigation of potential disease correlation between TTSuVk2b infection and MHD. Additionally, heart or liver tissues from pigs with MHD were also obtained from 5 Midwestern states (Ohio, Illinois, Minnesota, Kansas, and Iowa).

PCRs to amplify TTsuVk2b DNA:

Nested PCR for initial amplification of TTSuVk2b DNA: To determine whether the novel TTSuVk2b species is present in pigs in the United States, viral DNA was extracted from serum samples using the ZR Viral DNA Kit (Zymo Research) as per manufacturer's protocols, and subsequently tested for TTSuVk2b DNA with a nested PCR using a combination of universal TTSuV primers and primers based on the sequence of the TTSuVk2b reference sequence (isolate 38e23) [NG343 + TTV2-mR first round, and NG 343+NG344 for second

round (Table 1)] using the Platinum HIFI Supermix Taq Polymerase (Invitrogen). Positive samples were subsequently used for Inverse PCR using the Herculase II Fusion Polymerase (Agilent) using primers k2b-IF and k2b-IR. The amplified product was sequenced at the Biocomplexity Institute of Virginia Tech (Blacksburg, VA).

Hi-Fidelity PCR screen for molecular prevalence of TTSuVk2b DNA: Based on the initial U.S. TTSuVk2b sequence generated by the Inverse PCR, we designed U.S. TTSuVk2b-specific primers 1569F and 2004R to determine by PCR the prevalence of TTSuVk2b in swine. To determine the prevalence of TTSuVk2b in U.S. swine herds, viral DNAs were extracted from serum and tissue samples using the ZR Viral DNA Extraction Kit as previously described, and then subjected to PCR amplification with TTSuVk2b-specific primers to detect TTSuVk2b DNA using the Platinum Hi-Fidelity Taq polymerase kit (Life Technologies™) with 1.5µL DMSO added. Primers were designed (Table 1) to amplify the 1569-2007 nt fragment within the ORF1 gene. The PCR parameters include 98°C for 2 min followed by 30 cycles of 98°C for 30s for denaturation, 57°C 30s for primer annealing, 68°C 30s for extension, and a final incubation at 68°C for 7 min. The PCR products were analyzed by gel electrophoresis, cloned into the PCR 2.1 sequencing vector (Life Technologies™), and sequenced using an M13F universal sequencing primer to confirm the authenticity of the amplified TTSuVk2b DNA products. Molecular prevalence was then calculated as Total Positive Samples divided by Total Samples for the entire data set of serum samples, tissue samples, total samples, and subdivided for calculating prevalence of TTSuVk2b infection in PMWS or MHD positive pigs. The results of the TTSuVk2b DNA molecular prevalence were then analyzed using Chi-square analysis to determine statistical significance.

Amplification of the genomic sequence of TTSuVk2b: Based on the results of the initial PCR screening of the pig samples, three positive samples were selected for further PCR amplification to determine the complete genome of TTSuVk2b. A panel of primers was designed for amplifying overlapping products of approximately 200-400 bp in length (Table 1) by PCR using the Platinum Hi-Fi Supermix kit (Invitrogen). To overcome the problem associated with the extremely high GC content in the TTSuVk2b genome, the PCR reaction was performed with the addition of DMSO. The amplified products were sequenced at the Biocomplexity Institute of Virginia Tech, and the sequences were assembled and analyzed using the DNAStar software suite.

Sequence analyses of TTSuVk2b:

The overlapping sequences of the U.S. TTSuVk2b amplified by multiple PCR reactions were assembled, and the resulting U.S. TTSuVk2b genomic sequence was compared with that of previously published TTSuVk2b isolates (38E23, 38E19, and 38E05) using the Clustal Omega software (Sievers et al., 2011, Goujon et al., 2010, McWilliam et al., 2013). To molecularly characterize the sites of sequence variation in the k2b genome, 100-bp segments of the US TTSuVk2b genomic sequence were compared to the corresponding regions of the k2b sequences deposited in the GenBank database. The ORF1 sequences from US TTSuVk2b were also compared to other TTSuV isolates using the Megalign Software from DNAStar via ClustalW alignment with 1000 bootstrap trials, and a phylogenetic tree was generated from the sequence alignment.

Results

TTSuVk2b is present in U.S. swine herds:

As a pilot screen for the presence of TTSuVk2b DNA in pigs in the United States, we initially tested 32 serum samples from pigs with or without signs of PMWS by nested PCR using primers designed from the well conserved UTR of TTSuVs (NG343 and NG344) as well as using primers specific for the TTSuVk2b sequences as described by Cornelissen-Keijsers et al (Table 1). PCR-positive samples were then subjected to Inverse PCR amplification using primers k2b-IF and k2b-IR in an attempt to amplify the complete TTSuVk2b genome. Initially we were able to amplify by Inverse PCR a 1.6 kb fragment of TTSuVk2b genome consisting primarily of ORF 1.

Prevalence of TTSuVk2b in pigs in the United States:

We tested 127 serum and 115 tissue samples, and found that the overall prevalence of TTSuVk2b was 24.7% (27.6% positivity in sera, 21.7% in tissues) (Table 2). Prevalence rate ranged between 10 and 54.5% among the investigated herds (Table 3).

Lack of correlation of TTSuVk2b infection with PMWS or MHD:

It has been previously reported that TTSuVk2a is associated with the development of PMWS in pigs when coinfected with PCV2 (Kekarainen et al., 2006, Ellis et al., 2008). In this study, we found that approximately 13.5% of the pigs with diagnosed PMWS were positive for TTSuVk2b DNA, however approximately 32.1% of the pigs without PMWS were also positive for TTSuVk2b (Table 2). Chi-square analysis confirmed that there was no positive correlation between TTSuVk2b infection and PMWS disease.

During the initial TTSuVk2b screening, one herd with a high prevalence of TTSuVk2b in serum samples also had a higher than average incidence of MHD, thus prompting us to look for a potential correlation between MHD and TTSuVk2b infection as well. Heart and liver tissue samples from pigs that had been previously diagnosed for MHD by histopathology were subsequently tested for TTSuVk2b DNA. The results showed that approximately 13.5% of pigs with MHD were infected with TTSuVk2b, however 23.4% of pigs without MHD were also positive for TTSuVk2b DNA (Table 2). Statistical analysis confirmed that there was no positive correlation between TTSuVk2b infection and MHD.

Genetic variation of TTSuVk2b isolates:

The 458 bp ORF1 sequence of U.S. TTSuVk2b initially amplified from the serum and tissue samples had approximately 83% nucleotide sequence identity with the corresponding region of the TTSuVk2b sequences available in GenBank (Cornelissen-Keijsers et al., 2012). By performing a phylogenetic analysis containing a consensus sequence of US TTSuVk2b and representative strains of all four other TTSuV species, the U.S. TTSuVk2b was found to cluster within the previously published TTSuVk2b sequences within the *Kappatorqueviruses* clade (**Fig. 1**).

Pig samples which were strongly positive for TTsuVk2b DNA were subsequently used for PCR amplification of the complete viral genome. We were able to amplify a total of 2,263 bp TTSuVk2b genome from U.S. pigs. Sequence analysis revealed that this sequence corresponds to nt 411 to 2674 of the TTSuVk2b genome available in GenBank databse. The overall nucleotide sequence identity between the U.S. TTSuVk2b and the (Cornelissen-Keijsers et al., 2012)

sequences TTSuVk2b was 95%. Most of the variations were in the ORF1 regions, including one very diverse region between nucleotide 1200-1300.

Discussion

TTSuVs are a group of viruses that appear to be widespread in swine herds worldwide, although its definitive association with a particular disease remains debatable. Epidemiological evidence suggested that TTSuVk2, but not TTSuV1, may be associated with the development of PMWS (Cornelissen-Keijsers et al., 2012, Nieto et al., 2011, Huang et al., 2012), although direct experimental evidence is still lacking. More recently, a novel species of TTSuVs, designated TTSuVk2b, was first identified from commercial swine serum (Cornelissen-Keijsers et al., 2012), although its biological and pathogenic nature is unknown. Also, little is known about the prevalence of TTSuVk2b in pigs from countries outside of Europe. Therefore, the objective of this study was to determine the prevalence of TTSuVk2b from pigs in the United States and attempt to assess its potential association with diseases.

In this study, by using U.S. TTSuVk2b-specific primers, we tested 242 pig samples and found that approximately 24.7% were positive for TTSuVk2b DNA. This is the first report of the identification of TTSuVk2b from pigs in the United States. In the countries tested thus far, TTSuVk2b prevalence ranges from 0 to 100% with an average of 41% (Cornelissen-Keijsers et al., 2012). Variation in prevalence between herds examined in the study was relatively high, consistent with what has been previously observed. TTSuVk2b prevalence ranged from 10 to 50% from U.S. herds where at least 5 pigs were sampled. All of the tested herds had at least one positive sample, giving an indication of how widespread TTSuVk2b infection in pigs is in the United States.

There is some evidence that TTSuVk2a may be associated with the development of PMWS (Kekarainen et al., 2006, Aramouni et al., 2010b), therefore we decided to first assess the potential association of TTSuVk2b infection and PMWS using a set of samples from pigs with diagnosed PMWS. However, the results from this study showed a lack of statistically significant association of TTSuVk2b infection with PMWS. This finding contrasts with results published by Cornelissen-Keijsers et. al., who observed an increase in TTSuVk2b copy number within PMWS pigs. This discrepancy may be due to the subjective nature of PMWS diagnosis masking some correlation between PMWS and TTSuVk2b infection. Despite the contrasting results, our data suggests that no significant correlation between TTSuVk2b infection and PMWS exists in pigs in the United States.

Additionally, during our initial screening of TTSuVk2b infection in U.S. pigs, we observed a higher rate of TTSuVk2b from a limited number of serum samples obtained from a farm with a high rate of mulberry heart disease (MHD). Therefore, we further examined the potential role of TTSuVk2b in MHD using a larger set of MHD tissue samples. By using the liver and heart tissue samples from swine that had been diagnosed as having MHD, a statistically significant causal association of MHD with TTSuVk2b infection was not identified. Our results suggest that TTSuVk2b is not associated with PMWS/PCVAD or MHD in pigs in the United States. This data and the results regarding the lack of association with PMWS are similar to the lack of disease association observed with other Torque Teno viruses.

We further characterized the U.S. TTSuVk2b isolate by attempting to determine its complete genomic sequence. We were able to successfully amplify and sequence the near full-length genome of the U.S. TTSuVk2b analogous to nucleotides 411 to 2674 from the previously described TTSuVk2b. The extreme high GC content in the viral genome prevented us from

obtaining the remaining 636 bp sequence in the UTR region despite our extensive efforts including the use of rolling cycle amplification, deep sequencing etc. The nucleotide sequence of U.S. TTSuVk2b was 95% identical to those from (Cornelissen-Keijsers et al., 2012). Most of the sequence variations were clustered in specific regions of the ORF1 capsid gene, which has been found in other TTSuV species to contain hypervariable regions, presumably as a potential means of immune evasion (Bendinelli et al., 2001).

Although TTSuVk2b has not been associated with any specific disease in this study or any other published literature, it is important to learn that the novel TTSuVk2b species is indeed present in pigs in the United States with a relatively high prevalence rate. Even though TTSuVs alone may not cause clinical disease in pigs, it is possible that TTSuVs may act as a co-factor or trigger in swine disease development. Therefore, further study of this virus regarding its biological and pathogenic nature is warranted.

Acknowledgement

This project is supported by a grant from Boehringer Ingelheim Vetmedica Inc, Ames, Iowa. We thank Dr. Dianjun Cao for his expert assistance in constructing the phylogenetic tree. Abby R. Patterson is an employee of Boehringer Ingelheim Vetmedica Inc, Ames, Iowa.

Figure Legends

Figure 1: Phylogenetic analysis of TTSuVk2b. The ORF1 sequences from representative strains of TTSuV were aligned with the US TTSuVk2b using the ClustalW software, and the resulting sequence alignment was used to generate a phylogenetic tree. Individual clades representing TTSuV species are indicated, and the US TTSuVk2b sequence obtained from this study is indicated with a box. Bootstrap values (1000 replicates) are indicated above each branch.

References

Allan, G. M., F. McNeilly, S. Kennedy, B. Daft, J. A. Ellis, D. M. Haines, B. M. Meehan and B. M. Adair, 1998: Isolation of Porcine Circovirus-like Viruses from Pigs with a Wasting Disease in the USA and Europe. *Journal of Veterinary Diagnostic Investigation,* 10, 3-10.

Aramouni, M., J. Segales, M. Cortey and T. Kekarainen, 2010a: Age-related tissue distribution of swine Torque teno sus virus 1 and 2. *Vet Microbiol,* 146, 350-353.

Aramouni, M., J. Segales, M. Sibila, G. E. Martin-Valls, D. Nieto and T. Kekarainen, 2011: Torque teno sus virus 1 and 2 viral loads in postweaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS) affected pigs. *Vet Microbiol,* 153, 377-381.

Aramouni, M., M. Sibila, G. Martin-Valls, N. D, C. Marti, J. Segales and T. Kekarainen, 2010b: Torque teno sus virus 1 and 2 viral loads in postweaning multisystemic wasting syndrome (PMWS) affected and non-affected pigs. *21st IPVS Congress*, p. 232. Viral Diseases, Vancouver, CA.

Bendinelli, M., M. Pistello, F. Maggi, C. Fornai, G. Freer and M. Vatteroni, 2001: Molecular Properties, Biology, and Clinical Implications of TT Virus, a Recently Identified Widespread Infectious Agent of Humans. *Clinical Microbiology Reviews*, 98-113.

Bigarre, L., V. Beven, C. de Boisseson, B. Grasland, N. Rose, P. Biagini and A. Jestin, 2005: Pig anelloviruses are highly prevalent in swine herds in France. *J Gen Virol,* 86, 631-635.

Brassard, J., M. J. Gagne, L. Lamoureux, G. D. Inglis, D. Leblanc and A. Houde, 2008: Molecular detection of bovine and porcine Torque teno virus in plasma and feces. *Vet Microbiol,* 126, 271-276.

Cornelissen-Keijsers, V., A. Jimenez-Melsio, D. Sonnemans, M. Cortey, J. Segales, E. van den Born and T. Kekarainen, 2012: Discovery of a novel Torque teno sus virus species: genetic characterization, epidemiological assessment and disease association. *J Gen Virol,* 93, 2682-2691.

Ellis, J. A., G. Allan and S. Krakowka, 2008: Effect of coinfection with genogroup 1 porcine torque teno virus on porcine circovirus type 2-associated postweaning multisystemic wasting syndrome in gnotobiotic pigs. *Am J Vet Res,* 69, 1608-1614.

Goujon, M., H. McWilliam, W. Li, F. Valentin, S. Squizzato, J. Paern and R. Lopez, 2010: A new bioinformatics analysis tools framework at EMBL–EBI. *Nucleic Acids Research,* 38, W695-W699.

Hino, S. and H. Miyata, 2007: Torque teno virus (TTV): current status. *Rev Med Virol,* 17, 45-57.

Huang, Y.-W., A. R. Patterson, T. Opriessnig, B. A. Dryman, A. Gallei, K. K. Harrall, E. M. Vaughn, M. B. Roof and X.-J. Meng, 2012: Rescue of a Porcine Anellovirus (Torque Teno Sus Virus 2) from Cloned Genomic DNA in Pigs. *Journal of Virology,* 86, 6042-6054.

Inami, T., T. Obara, M. Moriyama, Y. Arakawa and K. Abe, 2000: Full-length nucleotide sequence of a simian TT virus isolate obtained from a chimpanzee: evidence for a new TT virus-like species. *Virology,* 277, 330-335.

Kamada, K., T. Kamahora, P. Kabat and S. Hino, 2004: Transcriptional regulation of TT virus: promoter and enhancer regions in the 1.2-kb noncoding region. *Virology,* 321, 341-348.

Kekarainen, T. and J. Segalés, 2012: Torque Teno Sus Virus in Pigs: an Emerging Pathogen? *Transboundary and Emerging Diseases,* 59, 103-108.

Kekarainen, T., M. Sibila and J. Segales, 2006: Prevalence of swine Torque teno virus in post-weaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs in Spain. *J Gen Virol,* 87, 833-837.

Krakowka, S. and J. A. Ellis, 2008: Evaluation of the effects of porcine genogroup 1 torque teno virus in gnotobiotic swine. *Am J Vet Res,* 69, 1623-1629.

Maggi, F. and M. Bendinelli, 2009: Immunobiology of the Torque teno viruses and other anelloviruses. *Curr Top Microbiol Immunol,* 331, 65-90.

Martelli, F., A. Caprioli, I. Di Bartolo, V. Cibin, G. Pezzotti, F. M. Ruggeri and F. Ostanello, 2006: Detection of swine torque teno virus in Italian pig herds. *J Vet Med B Infect Dis Vet Public Health,* 53, 234-238.

McKeown, N. E., M. Fenaux, P. G. Halbur and X. J. Meng, 2004: Molecular characterization of porcine TT virus, an orphan virus, in pigs from six different countries. *Vet Microbiol,* 104, 113-117.

McWilliam, H., W. Li, M. Uludag, S. Squizzato, Y. M. Park, N. Buso, A. P. Cowley and R. Lopez, 2013: Analysis Tool Web Services from the EMBL-EBI. *Nucleic Acids Research,* 41, W597-W600.

Muhire, B. M., M. Golden, B. Murrell, P. Lefeuvre, J.-M. Lett, A. Gray, A. Y. F. Poon, N. K. Ngandu, Y. Semegni, E. P. Tanov, A. r. L. Monjane, G. W. Harkins, A. Varsani, D. N. Shepherd and D. P. Martin, 2014: Evidence of Pervasive Biologically Functional Secondary Structures within the Genomes of Eukaryotic Single-Stranded DNA Viruses. *Journal of Virology,* 88, 1972-1989.

Mushahwar, I., C. Erker, A. Muerhoff, T. Leary, J. Simmons, L. Birkenmeyer and M. Chalmers, 1999: Molecular and biophysical characterization of TT virus: evidence for a new virus family infecting humans. *PNAS,* 96, 3177-3182.

Niel, C., L. Diniz-Mendes and S. Devalle, 2005: Rolling-circle amplification of Torque teno virus (TTV) complete genomes from human and swine sera and identification of a novel swine TTV genogroup. *J Gen Virol,* 86, 1343-1347.

Nieto, D., M. Aramouni, L. Grau-Roma, J. Segales and T. Kekarainen, 2011: Dynamics of Torque teno sus virus 1 (TTSuV1) and 2 (TTSuV2) DNA loads in serum of healthy and postweaning multisystemic wasting syndrome (PMWS) affected pigs. *Vet Microbiol,* 152, 284-290.

Nieto, D., T. Kekarainen, M. Aramouni and J. Segales, 2013: Torque teno sus virus 1 and 2 distribution in tissues of porcine circovirus type 2-systemic disease affected and age-matched healthy pigs. *Vet Microbiol,* 163, 364-367.

Nishizawa, T., H. Okamoto, K. Konishi, H. Yoshizawa, Y. Miyakawa and M. Mayumi, 1997: A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. *Biochem. Biophys. Res. Commun.*, 92-97.

Okamoto, H., Y. Akahane, M. Ukita, M. Fukuda, F. Tsuda, Y. Miyakawa and M. Mayumi, 1998a: Fecal excretion of a nonenveloped DNA virus (TTV) associated with posttransfusion non-A-G hepatitis. *J Med Virol,* 56, 128-132.

Okamoto, H., T. Nishizawa, N. Kato, M. Ukita, H. Ikeda, H. Iizuka, Y. Miyakawa and M. Mayumi, 1998b: Molecular cloning and characterization of a novel DNA virus (TTV) associated with posttransfusion hepatitis of unknown etiology. *Hepatol. Res.,* 10, 1-16.

Okamoto, H., T. Nishizawa, M. Takahashi, A. Tawara, Y. Peng, J. Kishimoto and Y. Wang, 2001: Genomic and evolutionary characterization of TT virus (TTV) in tupaias and comparison with species-specific TTVs in humans and non-human primates. *J Gen Virol,* 82, 2041-2050.

Okamoto, H., M. Takahashi, T. Nishizawa, A. Tawara, K. Fukai, U. Muramatsu, Y. Naito and A. Yoshikawa, 2002: Genomic characterization of TT viruses (TTVs) in pigs, cats and dogs and their relatedness with species-specific TTVs in primates and tupaias. *J Gen Virol,* 83, 1291-1297.

Pallarés, F. J., M. J. Yaeger, B. H. Janke, G. Fernandez and P. G. Halbur, 2002: Vitamin E and Selenium Concentrations in Livers of Pigs Diagnosed with Mulberry Heart Disease. *Journal of Veterinary Diagnostic Investigation,* 14, 412-414.

Sibila, M., L. Martinez-Guino, E. Huerta, A. Llorens, M. Mora, L. Grau-Roma, T. Kekarainen and J. Segales, 2009a: Swine torque teno virus (TTV) infection and excretion dynamics in conventional pig farms. *Vet Microbiol,* 139, 213-218.

Sibila, M., L. Martinez-Guino, E. Huerta, M. Mora, L. Grau-Roma, T. Kekarainen and J. Segales, 2009b: Torque teno virus (TTV) infection in sows and suckling piglets. *Vet Microbiol,* 137, 354-358.

366 Sievers, F., A. Wilm, D. Dineen, T. J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J.
367 Söding, J. D. Thompson and D. G. Higgins, 2011: Fast, scalable generation of high-quality protein
368 multiple sequence alignments using Clustal Omega. *Molecular Systems Biology,* 7.
369 Suzuki, T., R. Suzuki, J. Li, M. Hijikata, M. Matsuda, T. C. Li, Y. Matsuura, S. Mishiro and T. Miyamura,
370 2004: Identification of basal promoter and enhancer elements in an untranslated region of the
371 TT virus genome. *J Virol,* 78, 10820-10824.
372 Takahashi, K., H. Hoshino, Y. Ohta, N. Yoshida and S. Mishiro, 1998: Very high prevalence of TT virus
373 (TTV) infection in general population of Japan revealed by a new set of PCR primers. *Hepatology*
374 *Research,* 12, 233-239.
375 Xiao, C. T., L. Gimenez-Lirola, Y. W. Huang, X. J. Meng, P. G. Halbur and T. Opriessnig, 2012: The
376 prevalence of Torque teno sus virus (TTSuV) is common and increases with the age of growing
377 pigs in the United States. *Journal of virological methods,* 183, 40-44.

378

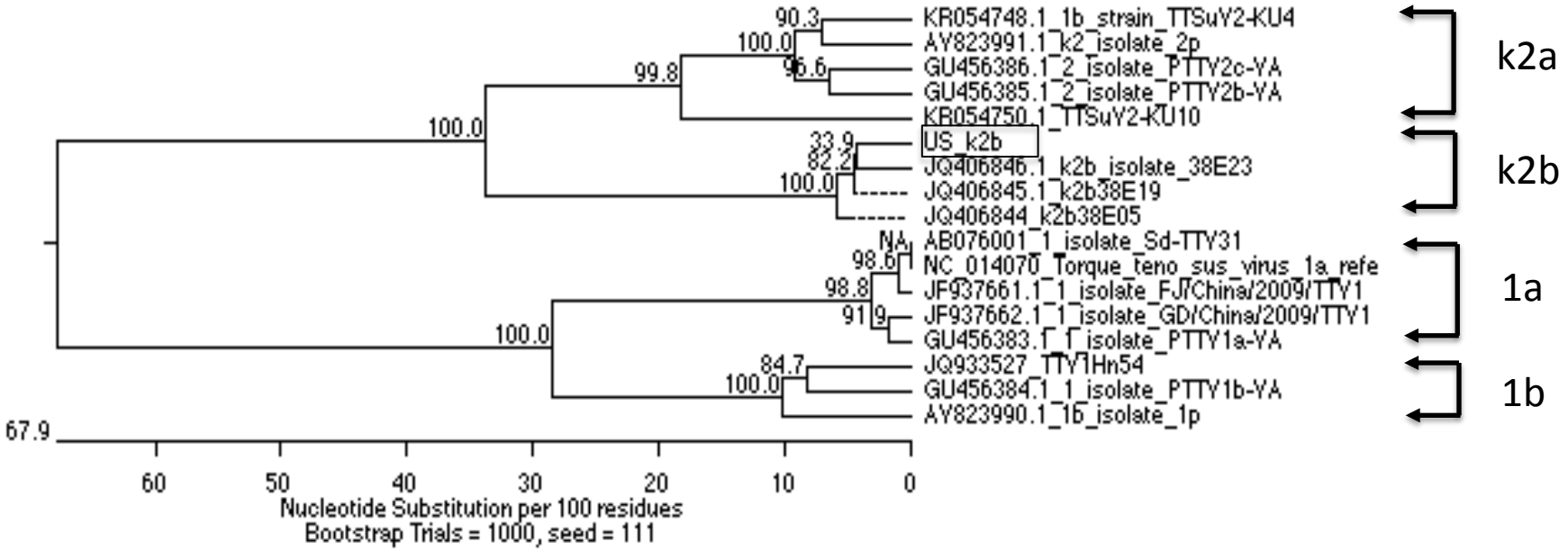
90.3
100.0
99.8
96.6
100.0
33.9
82.2
100.0
NA
98.6
98.8
91.9
100.0
84.7
100.0
67.9
KR054748.1_1b_strain_TTSuV2-KU4
AY823991.1_k2_isolate_2p
GU456386.1_2_isolate_PTTV2c-VA
GU456385.1_2_isolate_PTTV2b-VA
KR054750.1_TTSuV2-KU10
US_k2b
JQ406846.1_k2b_isolate_38E23
JQ406845.1_k2b38E19
JQ406844_k2b38E05
AB076001_1_isolate_Sd-TTV31
NC_014070_Torque_teno_sus_virus_1a_refe
JF937661.1_1_isolate_FJ/China/2009/TTV1
JF937662.1_1_isolate_GD/China/2009/TTV1
GU456383.1_1_isolate_PTTV1a-VA
JQ933527_TTV1Hn54
GU456384.1_1_isolate_PTTV1b-VA
AV823990.1_1b_isolate_1p
k2a
k2b
1a
1b
60
50
40
30
20
10
0
Nucleotide Substitution per 100 residues
Bootstrap Trials = 1000, seed = 111

Table 1. Oligonucleotide primers used for PCR and sequencing of the TTSuVk2b

Primer Name	Sequence (5′ to 3′)	Purpose
NG343	5′-GCACTTCCGAATGGCTGAGTTT-3′	Nested UTR PCR
NG344	5′-TCCCGAGCCCGAATTGCCCCT-3′	Nested UTR PCR
K2b IF	5′-CTCCACTACAGGCCTCGGACTCCT-3′	Nested UTR PCR Inverse PCR
K2b IR	5′-GGTCTGGTTGTACATCCCCAT-3′	Nested UTR PCR Inverse PCR
1569-1592F	5′-GGTGGGCAGCAGAATGGTGGAATG-3′	PCR Screening
2004-2027R	5′-GGCAGGGAATGGTTTTGGGGTCTC-3′	PCR Screening
F1	5′-AGTCAAGGGGCTTATCGGGC-3′	Sequencing
F2	5′-CACTGGATGAGAAATGCTCTCCCT-3′	Sequencing
F3	5′-GCACAAATGCCACTTTTCAGACTC-3′	Sequencing
F4	5′-TGCATTATCTGGTGGGCAGC-3′	Sequencing
F5	5′-AAATTTCAGTGGGGAGGCCA-3′	Sequencing
F6	5′-CCGACTCCTCAACAAGAGACGA-3′	Sequencing
F7	5′-CGCGGCAGGGATACCAAAT-3′	Sequencing
R1	5′-TCCTACCAGGAGTCCGAGGC-3′	Sequencing
R2	5′-GGTTATAAAGAGTCCATACAGTCACA-3′	Sequencing
R3	5′-TGCTGTAAAAAGCATTGCCGC-3′	Sequencing
R4	5′-GGCCTCACATAATCATATTGCATGT-3′	Sequencing
R5	5′-GGTTAAAAATGTCTGGGCAGGG-3′	Sequencing
R6	5′-CCCTGTTTGGGGACCAGAGA-3′	Sequencing

Table 2. Detection of TTSuVk2b DNA in serum and tissue samples from pigs with or without post-weaning multisystemic wasting syndrome (PMWS) or mulberry heart disease (MHD)

Sample Type	No. positive/no. tested	%
Serum	35/127	27.6
PMWS+	7/52	13.5
PMWS-	24/75	32.1
Tissue	25/115	21.7
MHD+	7/51	13.5
MHD-	15/64	23.4

Table 3. Detection of TTSuVk2b from pig serum and tissues in different geographic locations

Sample Origin	Sample Type	No. Positive/No. Tested (%)
Suffolk, VA	Serum	11/52 (21.2)
Ames, IA MHD	Serum	24/58 (41.4)
Total		35/127 (27.6)
Ames, IA	Tissue	1/10 (10)
Sycamore, IL	Tissue	2/5 (40)
Parnell, IA	Tissue	5/15 (33.3)
Remsen, IA	Tissue	3/16 (18.8)
Abilene, KS	Tissue	6/11 (54.5)
Total		25/115 (21.7)