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# 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. <https://doi.org/10.1111/tbed.12586>

### Digital Object Identifier (DOI):

[10.1111/tbed.12586](https://doi.org/10.1111/tbed.12586)

### Link:

[Link to publication record in Edinburgh Research Explorer](#)

### Document Version:

Peer reviewed version

### Published In:

Transboundary and Emerging Diseases

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

4  
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14  
15 **Running title:** Prevalence of TTSuVk2b in U.S. swine

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21  
22 **Word counts:**

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

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## 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 coinfecting 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).

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## 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

70 including brain, lymph node, heart, liver, bone marrow, lung, spleen, and pulmonary epithelium  
71 indicating a broad tissue tropism (Reviewed in (Hino and Miyata, 2007, Aramouni et al.,  
72 2010a)). More recently in 2012, a novel *Kappatorquevirus* species was identified from  
73 commercial swine serum and classified as TTSuVk2b (Cornelissen-Keijsers et al., 2012). A  
74 subsequent study revealed that TTSuVk2b DNA was detected from 0 to 100% of pig serum  
75 samples from 17 different countries. However, whether the new TTSuVk2b species exists in pigs  
76 from the United States remains unknown.

77 Many studies have attempted to link TTSuVs to clinical symptoms, but thus far a  
78 definitive causal relationship between TTSuV infection and a particular disease in pigs is still  
79 lacking (Kekarainen and Segalés, 2012). TTSuV infection has been reportedly associated with  
80 respiratory diseases in experimentally-infected gnotobiotic pigs (Krakowka and Ellis, 2008) and  
81 contributed to the development of postweaning multisystemic wasting syndrome (PMWS) when  
82 coinfecting with *porcine circovirus type 2* (PCV2) (Kekarainen et al., 2006, Aramouni et al.,  
83 2010b). PMWS and a collection of other symptoms collectively known as Porcine Circovirus  
84 Associated Disease (PCVAD) represent an economically significant disease for the pig industry  
85 (Reviewed in (Allan et al., 1998)). It has also been reported that TTSuVk2a, but not TTSuV1,  
86 has an association with PMWS in pigs (Aramouni et al., 2010b, Kekarainen et al., 2006, Nieto et  
87 al., 2011), and that there is an increased TTSuVk2a viral load in PMWS pigs (Aramouni et al.,  
88 2011). Additionally, TTSuVk2a copy numbers increased in pigs with experimental infection of  
89 classical swine fever virus while TTSuV1 did not, underscoring the differences in these two  
90 species (Nieto et al., 2013)

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

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

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99

## Materials and Methods

### 100 **Sample collection:**

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

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### 109 **PCRs to amplify TTSuVk2b DNA:**

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

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

120 ***Hi-Fidelity PCR screen for molecular prevalence of TTSuVk2b DNA:*** Based on the  
121 initial U.S. TTSuVk2b sequence generated by the Inverse PCR, we designed U.S. TTSuVk2b-  
122 specific primers 1569F and 2004R to determine by PCR the prevalence of TTSuVk2b in swine.  
123 To determine the prevalence of TTSuVk2b in U.S. swine herds, viral DNAs were extracted from  
124 serum and tissue samples using the ZR Viral DNA Extraction Kit as previously described, and  
125 then subjected to PCR amplification with TTSuVk2b-specific primers to detect TTSuVk2b DNA  
126 using the Platinum Hi-Fidelity Taq polymerase kit (Life Technologies™) with 1.5μL DMSO  
127 added. Primers were designed (Table 1) to amplify the 1569-2007 nt fragment within the ORF1  
128 gene. The PCR parameters include 98°C for 2 min followed by 30 cycles of 98°C for 30s for  
129 denaturation, 57°C 30s for primer annealing, 68°C 30s for extension, and a final incubation at  
130 68°C for 7 min. The PCR products were analyzed by gel electrophoresis, cloned into the PCR  
131 2.1 sequencing vector (Life Technologies™), and sequenced using an M13F universal  
132 sequencing primer to confirm the authenticity of the amplified TTSuVk2b DNA products.  
133 Molecular prevalence was then calculated as Total Positive Samples divided by Total Samples  
134 for the entire data set of serum samples, tissue samples, total samples, and subdivided for  
135 calculating prevalence of TTSuVk2b infection in PMWS or MHD positive pigs. The results of  
136 the TTSuVk2b DNA molecular prevalence were then analyzed using Chi-square analysis to  
137 determine statistical significance.

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

147

#### 148 **Sequence analyses of TTSuVk2b:**

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

159

160

## **Results**



161 **TTSuVk2b is present in U.S. swine herds:**

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

170

171 **Prevalence of TTSuVk2b in pigs in the United States:**

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

175

176 **Lack of correlation of TTSuVk2b infection with PMWS or MHD:**

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

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

191

#### 192 **Genetic variation of TTSuVk2b isolates:**

193           The 458 bp ORF1 sequence of U.S. TTSuVk2b initially amplified from the serum and  
194 tissue samples had approximately 83% nucleotide sequence identity with the corresponding  
195 region of the TTSuVk2b sequences available in GenBank (Cornelissen-Keijsers et al., 2012). By  
196 performing a phylogenetic analysis containing a consensus sequence of US TTSuVk2b and  
197 representative strains of all four other TTSuV species, the U.S. TTSuVk2b was found to cluster  
198 within the previously published TTSuVk2b sequences within the *Kappatorqueviruses* clade (**Fig.**  
199 **1**).

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

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

207

## 208 **Discussion**

209 TTSuVs are a group of viruses that appear to be widespread in swine herds worldwide,  
210 although its definitive association with a particular disease remains debatable. Epidemiological  
211 evidence suggested that TTSuVk2, but not TTSuV1, may be associated with the development of  
212 PMWS (Cornelissen-Keijsers et al., 2012, Nieto et al., 2011, Huang et al., 2012), although direct  
213 experimental evidence is still lacking. More recently, a novel species of TTSuVs, designated  
214 TTSuVk2b, was first identified from commercial swine serum (Cornelissen-Keijsers et al.,  
215 2012), although its biological and pathogenic nature is unknown. Also, little is known about the  
216 prevalence of TTSuVk2b in pigs from countries outside of Europe. Therefore, the objective of  
217 this study was to determine the prevalence of TTSuVk2b from pigs in the United States and  
218 attempt to assess its potential association with diseases.

219 In this study, by using U.S. TTSuVk2b-specific primers, we tested 242 pig samples and  
220 found that approximately 24.7% were positive for TTSuVk2b DNA. This is the first report of the  
221 identification of TTSuVk2b from pigs in the United States. In the countries tested thus far,  
222 TTSuVk2b prevalence ranges from 0 to 100% with an average of 41% (Cornelissen-Keijsers et  
223 al., 2012). Variation in prevalence between herds examined in the study was relatively high,  
224 consistent with what has been previously observed. TTSuVk2b prevalence ranged from 10 to  
225 50% from U.S. herds where at least 5 pigs were sampled. All of the tested herds had at least one  
226 positive sample, giving an indication of how widespread TTSuVk2b infection in pigs is in the  
227 United States.

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

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

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

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

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

263

#### 264 **Acknowledgement**

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

268

## Figure Legends

269

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

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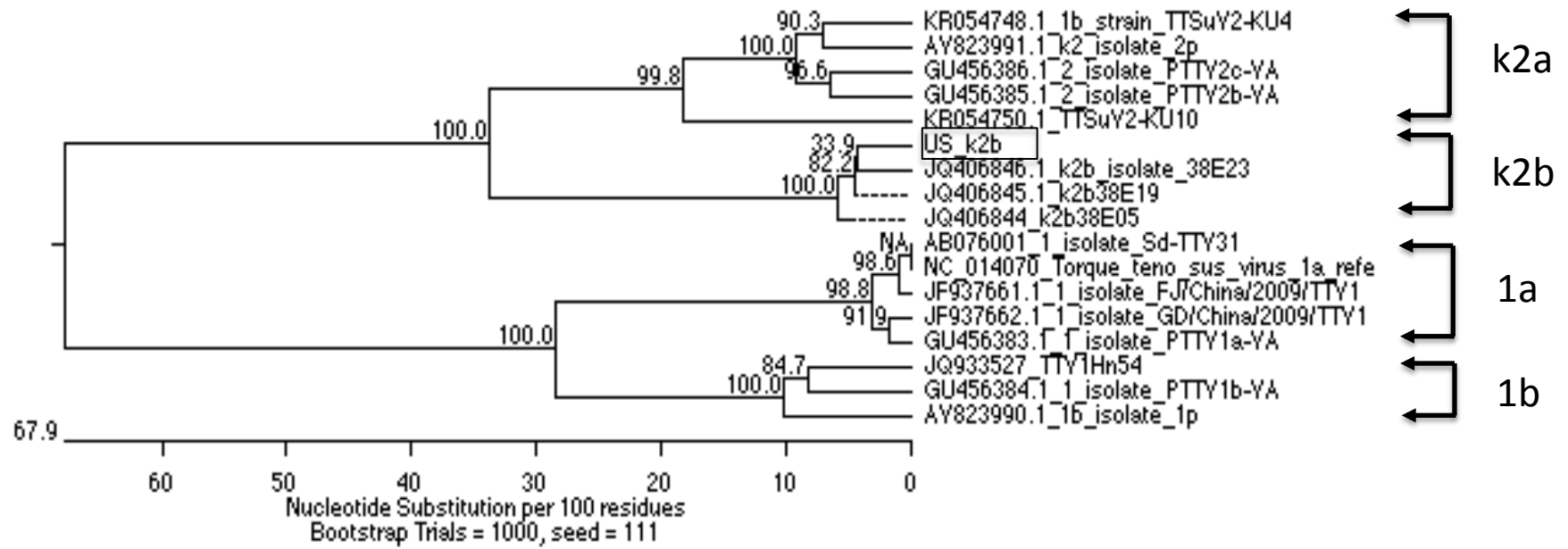
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**Table 1.** Oligonucleotide primers used for PCR and sequencing of the TTSuVk2b

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

<b>Sample Origin</b>	<b>Sample Type</b>	<b>No. Positive/No. Tested (%)</b>
Suffolk, VA	Serum	11/52 (21.2)
Ames, IA MHD	Serum	24/58 (41.4)
<u>Total</u>		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)
<u>Total</u>		25/115 (21.7)