Discovery of a novel Torque teno sus virus species: genetic characterization, epidemiological assessment and disease association

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The study describes a novel Torque teno sus virus (TTSuV) species, provisionally named Torque teno sus virus k2b (TTSuVk2b), originally found in commercial pig sera by applying the rolling-circle amplification technique. Full-length sequences of TTSuVk2b were obtained, annotated and used in the phylogenetic analyses, which revealed that TTSuVk2b is a novel Anellovirus species within the genus Kappatorquevirus of the family Anelloviridae. Quantitative PCR techniques were developed to determine total TTSuV DNA quantities as well as the prevalence and viral DNA quantities of TTSuV1, TTSuVk2a and TTSuVk2b. The mean total TTSuV load in seven commercial sera was determined at 6.3 log10 DNA copies ml−1 of serum, with TTSuVk2b loads being the lowest at 4.5 log10 DNA copies ml−1 of serum. Subsequently, prevalence and loads of TTSuVs were determined in pig sera from 17 countries. TTSuVk2b prevalence ranged from 0 to 100% with viral loads from 3.3 to 4.6 log10 copies ml−1 of sera. TTSuVk2a, so far the only species in the genus Kappatorquevirus, has been linked to an economically important swine disease, namely post-weaning multisystemic wasting syndrome (PMWS). Considering the grouping of TTSuVk2b in the same genus as TTSuVk2a, TTSuVk2b prevalence and viral DNA load were determined in PMWS-affected animals and healthy counterparts. This revealed that TTSuVk2a and TTSuVk2b are not only genetically related, but also that their viral loads in serum are elevated in PMWS animals compared with those of healthy pen mates. In summary, the present work describes a novel TTSuV species including its genetic characterization, epidemiological assessment and potential disease association.

INTRODUCTION

Anelloviruses are a highly diverse group of circular ssDNA viruses infecting vertebrates. Torque teno sus virus 1 (TTSuV1, genus Iotatorquevirus) and k2 (TTSuVk2a, genus Kappatorquevirus) are ubiquitous pig-infecting anelloviruses (Kekarainen & Segalés, 2012). These viruses are genetically very distinct (>56% sequence diversity), but share similar genome organization and expression strategy (Martínez-Guinó et al., 2011). Indeed, Torque teno virus (TTV) strains are considered species and genera if ORF1 nucleotide sequences differ more than 35 and 56%, respectively (Biagini, 2009; Biagini et al., 2011). TTSuVs are widespread and transmitted by vertical and horizontal routes (Martínez-Guinó et al., 2009, 2010; Pozzuto et al., 2009). Lately, TTSuVk2a has been associated with post-weaning multisystemic wasting syndrome (PMWS) (Aramouni et al., 2011; Blomström et al., 2010; Kekarainen et al., 2006; Nieto et al., 2011), an economically important multifactorial disease in pig production (Segalés et al., 2005).

After the first anellovirus was described in 1997 in humans, novel anelloviruses have been detected by using primers specific for the short, highly conserved sequences in the non-coding region of the genome (Okamoto et al., 2001, 2002). Recently, the rolling-circle amplification (RCA)-based sequence-independent approach has been applied to
identify TTVs (Biagini et al. 2007; Macera et al., 2011; Niel et al., 2005).

In this study, a novel TTSuV species, tentatively named Torque teno sus virus k2b (TTSuVk2b), originally found in pig sera by applying the RCA technique is described. Molecular and epidemiological studies were performed to further characterize this novel pig virus. For such purpose, the full-length genomic sequences of three TTSuVk2b strains were obtained. Furthermore, quantitative (q) PCR techniques were developed to detect and quantify all TTSuVs species together and separately. For epidemiological studies, the geographical distribution and viral loads of TTSuVk2b were determined and its association to PMWS assessed.

**RESULTS**

A novel pig-infecting anellovirus identified using the RCA technique

Commercial sera are generally pools of serum from several pigs and therefore a useful source for detecting viruses. To determine which porcine anelloviruses were present in commercial sera, the RCA technique with random primers was applied on extracted serum DNA. Amplicons of approximately 2.9 kb corresponding to the size of the full-length TTSuV genome were cloned, resulting in 21 clones of which 11 contained a full-length TTSuV genome and 10 a partial TTSuV sequence of 2.6–2.7 kb in length.

All clones were sequenced and compared with the sequence data available in GenBank to annotate the genomes. Eleven clones had an insertion that was closely related to TTSuV1. In Table 1 these clones were further divided in four different subtypes (Cortey et al., 2011). Five clones were annotated as TTSuVk2a subtypes. Five clones contained similar inserts that were identified as TTSuV sequences, but were considerably different from either TTSuV1 or TTSuVk2a sequences, suggesting that a novel TTSuV species was identified (Table 1).

**Table 1.** Characterization of 21 TTSuV clones derived from Hyclone porcine serum

It is indicated whether full-length (complete) or partial genome sequences were obtained.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Total</th>
<th>1a</th>
<th>1b</th>
<th>1c</th>
<th>1d</th>
<th>k2a</th>
<th>k2b</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTSuV1 (complete genome)</td>
<td>3</td>
<td>–</td>
<td>–</td>
<td>1</td>
<td>2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TTSuV1 (partial genome)</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TTSuVk2a (complete genome)</td>
<td>5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>–</td>
</tr>
<tr>
<td>TTSuVk2b (complete genome)</td>
<td>3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>TTSuVk2b (partial genome)</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2</td>
</tr>
</tbody>
</table>

Novel TTSuV sequences represented a new anellovirus species

The International Committee on Taxonomy of Viruses (ICTV) has classified TTVs into the family Anelloviridae based on the DNA sequence of the largest open reading frame, ORF1 (Biagini et al., 2011). According to the ICTV the ORF1 nucleotide sequence divergence cut-off values to distinguish species is >35 and >56 % for genera. To further classify the novel sequences, phylogenetic and pairwise sequence comparison (PASC) analyses were performed. The mean group distance of the novel ORF1 sequences with TTSuV1 isolates was 63.1 (61.9–65.6 %) and 41.7 % (40.26–43.59 %) with TTSuVk2a, while the phylogenetic distance between TTSuV1 and TTSuVk2a species is 59.5 % (56.6–62.0 %) (Fig. 1). Phylogenetic tree construction using nucleotide sequences of full-length genomes resulted in similar tree topology as obtained with ORF1. Therefore, the unclassified viral sequences were considered to represent a novel pig anellovirus species within the genus Kappatorquevirus. The novel virus was provisionally designated TTSuVk2b. Logically, and following the naming of Iotatorqueviruses, TTSuVk2 should be renamed as TTSuVk2a.

Next, the three obtained full-length TTSuVk2b sequences, 38E05, 38E19 and 38E23, were annotated and the deduced genomic features are presented in Table 2. Genomes 38E19 and 38E23 were 99 % identical on the nucleotide sequence level, whereas genome 38E05 was only 95–96 % identical to them. The TTSuVk2b genomes contained the characteristic GC-rich region and conserved sequences in the non-coding region. In addition, ORF1 and its splice variant ORF1/1, ORF2 and ORF3 were identified. The deduced amino acid sequences of ORF1 contained a typical arginine-rich N terminus and nuclear localization signals. Rolling-circle replication motif III located at aa position 473 (YxxK) (Huang et al., 2010b; Martínez-Guinó et al., 2011; Müeller et al., 2008; Mushahwar et al., 1999), while motif II (HxQ) (Huang et al., 2010b; Mankertz et al., 2004) could not be identified. This domain has only been detected in TTSuVk2a, but not in TTSuV1 (Huang et al., 2010b; Martínez-Guinó et al., 2011). Motif I (FTL) described in human TTV studies (Mueller et al., 2008) was not detected in any TTSuV species. TTSuVk2b-ORF2 contained a protein-tyrosine phosphatase motif (aa position 21–41) typically found in anelloviruses (Martínez-Guinó et al., 2011; Müller et al., 2008). The TTSuVk2b ORF1, ORF1/1 and ORF3 proteins were predicted to be nuclear with more than 90 % reliability, all of them containing bipartite, pat4 and pat7 nuclear localization signals (NLS). No NLS were detected in ORF2, which was predicted to be cytoplasmic with the reliability of 94 %. The predicted promoter region (‘-CTTTTTAAGTATATAAGT-AAGTGCACTGGCGAATGGCTGAGTTTATGCCG-3’) was highly conserved between TTSuVk2b and TTSuVk2a. The splicing site for ORF1/1 and ORF3 was similar to the one described in TTSuVk2a ORF1 (Martínez-Guinó et al., 2011). However, other splice sites mapped for TTSuVk2a in transfection experiments (Martínez-Guinó et al., 2011) could not be annotated for TTSuVk2b.
TTSuV species from different origins are found in commercial porcine sera

Quantitative broad spectrum and species-specific qPCR techniques were applied to screen seven commercial sera originating from either USA, New Zealand or Germany for the presence of TTSuVs (Table 3). All tested commercial sera, which are pools from a large number of pigs, contained each of the three viral species. The amount of total TTSuV varied between 5.93 and 6.65 log10 DNA copies ml⁻¹ serum. In each batch a similar pattern of prevalence of the three TTSuVs was found and the prevalence of TTSuV2b was much lower than that of the other two viruses. This can be confidently concluded...
since the three used qPCRs (TTSuV1, TTSuVk2a and k2b) had the same limit of detection and very similar amplification efficiencies (see Methods section). The relative percentage of TTSuVk2b detected was 0.7–2.1 %, while those of TTSuV1 and TTSuVk2a were 18.0–55.3 and 42.8–81.4 %, respectively (Table 3).

Global prevalence and viral DNA loads of TTSuV species in conventional pigs

Once the presence of TTSuVk2b in commercial pools of porcine sera was confirmed we aimed to determine its global distribution and its relation to the already well characterized TTSuV1 and TTSuVk2a (Cortey et al., 2012; McKeown et al., 2004). For this a total of 244 sera samples collected from farms located in 17 different countries were analysed by the different qPCRs.

In general, the total prevalence of TTSuV was 92.6 (226/244) and 74.6 % for TTSuV1 (182/244), 61.1 % for TTSuVk2a (149/244) and 41.0 % for TTSuVk2b (100/244) (Table 4). While TTSuV1 could be detected in sera from all countries analysed, TTSuVk2a was not found in the samples originating from Brazil and Vietnam, and TTSuVk2b was not present in sera from Brazil, Ukraine and Vietnam (Fig. 2 and Table 4). TTSuVk2a and TTSuVk2b global prevalences were correlated (P<0.05), while the prevalence of TTSuV1 did not correlate with that of the other two.

There was in general a very good correlation between the total viral loads determined by the broad spectrum qPCR and the sum of the viral loads determined by the TTSuV1-, TTSuVk2a- and TTSuVk2b-specific qPCRs: of 244 samples analysed, only 21 showed a greater than fivefold difference (data not shown). In three cases the broad spectrum qPCR detected TTSuV, while the specific qPCRs failed to detect TTSuV species. It was attempted by RCA to isolate TTSuV from the corresponding three sera, but this was unsuccessful, probably due to the low levels of TTSuV in these sera.

Viral DNA loads were in the range of 3.12–7.90 (mean 5.79) log_{10} DNA copies ml^{-1} serum for TTSuV1, 3.18–8.41 (mean 4.58) log_{10} DNA copies ml^{-1} serum for TTSuVk2a, and 3.13–6.87 (mean 4.14) log_{10} DNA copies ml^{-1} serum for TTSuVk2b in conventional pigs. There was no correlation of viral loads among the three TTSuV species. The mean TTSuV1 and TTSuVk2b loads in sera of individual pigs were similar to the loads observed in commercial sera (Table 3). However, the mean TTSuVk2a loads in individual sera were approximately 1.5 log_{10} lower than that in commercial sera.

### Table 2. Summary of the TTSuV genomic features

<table>
<thead>
<tr>
<th>Genomic length</th>
<th>ORF1 nt (aa)</th>
<th>ORF1/1 nt (aa)</th>
<th>ORF2 nt (aa)</th>
<th>ORF3 nt (aa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTSuVk2a</td>
<td>2735–2803</td>
<td>1875–1884 (625–628)</td>
<td>535 (176)</td>
<td>600–609 (200–203)</td>
</tr>
<tr>
<td>TTSuVk2b</td>
<td>2899–2901</td>
<td>1890–1893 (629–630)</td>
<td>577 (188)</td>
<td>673 (224)</td>
</tr>
</tbody>
</table>

### Table 3. TTSuV loads (log_{10} DNA copies ml^{-1} serum) in commercial batches of porcine serum determined by the broad spectrum and TTSuV-specific qPCRs

<table>
<thead>
<tr>
<th>Porcine serum</th>
<th>Broad spectrum</th>
<th>TTSuV1</th>
<th>TTSuVk2a</th>
<th>TTSuVk2b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyclone</td>
<td>6.48</td>
<td>5.84 (20.8)</td>
<td>6.41 (77.2)</td>
<td>4.84 (2.1)</td>
</tr>
<tr>
<td>Sigma 026k8453</td>
<td>6.39</td>
<td>5.81 (24.6)</td>
<td>6.28 (73.6)</td>
<td>4.69 (1.9)</td>
</tr>
<tr>
<td>Sigma 064k8451</td>
<td>6.08</td>
<td>5.98 (55.3)</td>
<td>5.87 (42.8)</td>
<td>4.52 (1.9)</td>
</tr>
<tr>
<td>Kreaber 9050957-G50</td>
<td>6.65</td>
<td>5.92 (20.1)</td>
<td>6.52 (79.3)</td>
<td>4.45 (0.7)</td>
</tr>
<tr>
<td>Kreaber 9071172-G50</td>
<td>6.47</td>
<td>5.66 (18.0)</td>
<td>6.32 (81.4)</td>
<td>4.23 (1.7)</td>
</tr>
<tr>
<td>Kreaber SS02/06-1</td>
<td>6.09</td>
<td>5.63 (27.2)</td>
<td>6.04 (71.1)</td>
<td>4.41 (1.7)</td>
</tr>
<tr>
<td>Kreaber SS03/06</td>
<td>5.93</td>
<td>5.70 (42.6)</td>
<td>5.81 (55.2)</td>
<td>4.40 (2.1)</td>
</tr>
<tr>
<td>Mean</td>
<td>6.30</td>
<td>5.79 (26.3)</td>
<td>6.18 (72.2)</td>
<td>4.50 (1.4)</td>
</tr>
</tbody>
</table>
Table 4. TTSuV prevalence (positive samples/total number of samples) in pig sera from 17 different countries determined by qPCR
Percentage of the positive sera is shown in parentheses.

<table>
<thead>
<tr>
<th>Country</th>
<th>TTSuV1</th>
<th>TTSuVk2a</th>
<th>TTSuVk2b</th>
<th>Broad spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belarus</td>
<td>6/8 (75.0)</td>
<td>4/8 (50.0)</td>
<td>8/8 (100)</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>Brazil</td>
<td>3/8 (37.5)</td>
<td>0/8 (0.0)</td>
<td>0/8 (0.0)</td>
<td>3/8 (37.5)</td>
</tr>
<tr>
<td>Canada</td>
<td>25/30 (83.3)</td>
<td>30/30 (100)</td>
<td>25/30 (83.3)</td>
<td>30/30 (100)</td>
</tr>
<tr>
<td>Greece</td>
<td>38/40 (95.0)</td>
<td>28/40 (70.0)</td>
<td>23/40 (57.5)</td>
<td>40/40 (100)</td>
</tr>
<tr>
<td>China</td>
<td>7/8 (87.5)</td>
<td>7/8 (87.5)</td>
<td>6/8 (75.0)</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>Lithuania</td>
<td>8/8 (100)</td>
<td>5/8 (62.5)</td>
<td>3/8 (37.5)</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>Mexico</td>
<td>6/8 (75.0)</td>
<td>3/8 (37.5)</td>
<td>1/8 (12.5)</td>
<td>5/8 (62.5)</td>
</tr>
<tr>
<td>Mozambique</td>
<td>3/22 (13.6)</td>
<td>22/22 (100)</td>
<td>9/22 (40.9)</td>
<td>22/22 (100)</td>
</tr>
<tr>
<td>Netherlands</td>
<td>11/20 (55.0)</td>
<td>4/20 (20.0)</td>
<td>1/20 (5.0)</td>
<td>18/20 (90.0)</td>
</tr>
<tr>
<td>Philippines</td>
<td>7/8 (87.5)</td>
<td>1/8 (12.5)</td>
<td>2/8 (25.0)</td>
<td>7/8 (87.5)</td>
</tr>
<tr>
<td>Russia</td>
<td>6/8 (75.0)</td>
<td>1/8 (12.5)</td>
<td>1/8 (12.5)</td>
<td>5/8 (62.5)</td>
</tr>
<tr>
<td>South Africa</td>
<td>13/15 (86.6)</td>
<td>15/15 (100)</td>
<td>7/15 (46.6)</td>
<td>15/15 (100)</td>
</tr>
<tr>
<td>Spain</td>
<td>25/29 (86.2)</td>
<td>13/29 (44.8)</td>
<td>10/29 (34.4)</td>
<td>27/29 (93.1)</td>
</tr>
<tr>
<td>Thailand</td>
<td>3/8 (37.5)</td>
<td>7/8 (87.5)</td>
<td>2/8 (25.0)</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>Ukraine</td>
<td>8/8 (100)</td>
<td>2/8 (25.0)</td>
<td>0/8 (0.0)</td>
<td>7/8 (87.5)</td>
</tr>
<tr>
<td>Venezuela</td>
<td>6/8 (75.0)</td>
<td>5/8 (62.5)</td>
<td>2/8 (25.0)</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>Vietnam</td>
<td>7/8 (87.5)</td>
<td>2/8 (25.0)</td>
<td>0/8 (0.0)</td>
<td>7/8 (87.5)</td>
</tr>
<tr>
<td>Total</td>
<td>182/244 (74.6)</td>
<td>149/244 (61.1)</td>
<td>100/244 (41.0)</td>
<td>92.6% (226/244)</td>
</tr>
</tbody>
</table>

Fig. 2. TTSuV loads and prevalences in pig sera collected from pig farms from several countries. TTSuV1, TTSuVk2a and TTSuVk2b log_{10} DNA copies ml^{-1} serum and error bars represent SD. Total number of analysed sera is shown after the name of the country and the number of positive samples in the corresponding bar.
High TTSuVk2b loads in serum of PMWS-affected pigs

TTSuVk2a has been associated in previous studies with porcine circovirus-associated diseases (Huang et al., 2011) and especially with PMWS occurrence (Aramouni et al., 2011; Nieto et al., 2011). Considering the molecular similarity of TTSuVk2a with TTSuVk2b, and the correlation of their prevalence in geographical samples, the possible association of TTSuVk2b with PMWS was explored. TTSuV1, TTSuVk2a and TTSuVk2b viral DNA loads were measured by the TTSuV-specific qPCRs in 34 serum samples of PMWS-affected pigs and 29 healthy control animals. The mean TTSuVk2a and TTSuVk2b loads were significantly higher \((P < 0.05)\) in PMWS-affected pigs than in healthy animals (Fig. 3). Such a difference was not observed for TTSuV1.

**DISCUSSION**

Three species were so far known to infect pigs before the present study, namely TTSuV1a and 1b within the genus *Iotatorquevirus* and TTSuVk2a within the genus *Kappatorquevirus*. Here, a novel anellovirus was identified in porcine sera, which, according to the degree of divergence with already known TTSuVs is sufficient to warrant classification of this virus as a new species in the genus *Kappatorquevirus*. Considering the current taxonomic organization of anelloviruses, this novel virus species was tentatively named TTSuVk2b.

Although there have been attempts to discover novel pig anelloviruses (Macera et al., 2011), TTSuVk2b was not detected before. Primers used in published TTSuV1- or TTSuVk2a-specific PCRs (Brassard et al., 2010; Gallei et al., 2010; Huang et al., 2010a; Nieto et al., 2011) are unlikely to amplify TTSuVk2b given the mismatch of these primers with TTSuVk2b sequences. Furthermore, sequencing of these amplicons is necessary to identify possible new species.

Different qPCR techniques were developed in this study. The wide spectrum qPCR was shown to be very useful in determining the overall TTSuV load (TTSuV1, TTSuVk2a and TTSuVk2b) in pig serum. This technique could be useful for diagnostic purposes and, when necessary, the species-specific qPCRs can be useful to determine the loads of each TTSuV species.

Bioinformatics analysis of full-length TTSuVk2b sequences showed that these viruses are rather distinct from, already known pig anelloviruses. However, a high similarity with TTSuVk2a was observed on genomic regions like splice sites and promoter elements (Huang et al., 2012b; Martínez-Guínó et al., 2011). These predictions may not reflect the reality, since a short intron of 91 bp could not be predicted from any genome of TTSuVk2 but has been shown to exist in genomes of TTSuVk2a by *in vitro* transfection experiments (Huang et al., 2012b; Martínez-Guínó et al., 2011), and therefore further experiments are necessary to characterize TTSuVk2b transcription and protein expression strategies. The relatedness to TTSuVk2a was supported by phylogenetic analysis that grouped TTSuVk2b closer to TTSuVk2a than TTSuV1. Considering the current criteria of the ICTV, TTSuVk2b would be the second species in the genus *Kappatorquevirus*.

In the epidemiological study, TTSuVk2b infection was confirmed in pigs from most of the countries. However, for some countries where TTSuVk2b appeared to be absent the number of serum samples analysed was low, so in order to conclude the presence of TTSuVk2b in these countries, more samples and farms should be analysed. There is also a possibility that the TTSuVk2b-specific primers do not detect all TTSuVk2b variants, since the primer binding sites were determined based only on the five TTSuVk2b sequences that were obtained in this study (see Table 1). Therefore, more viral genomic sequences should be obtained to confirm that the annealing sites of the used primers are highly conserved. Considering the global trading and its influence on the distribution of TTSuVs (Cortey et al., 2011).
2012), it is probable that each TTSuV species will be found in any given country.

Overall, it was seen for each TTSuV that the higher the prevalence the higher the viral load in serum. It is well documented that TTSuV1 and k2 viral loads and prevalences increase with the age of the animals (Aramouni et al., 2010; Nieto et al., 2011; Xiao et al., 2012). This is probably due to the efficient viral transmission by vertical and horizontal routes and the persistent nature of TTSuV infection (Martínez-Guiñó et al., 2009; Pozzuto et al., 2009) in pig and wild boar (Martínez et al., 2006). Therefore, the observed differences between countries in TTSuV prevalence and viral loads may reflect the age of the tested animals, which was not known in this study.

Due to the genetic similarity between TTSuVk2a and TTSuVk2b and the reported association of TTSuVk2a, but not TTSuV1, with PMWS (Aramouni et al., 2011; Kekarainen et al., 2006; Nieto et al., 2011), it was assessed whether TTSuVk2b was also associated with this disease. Indeed, both TTSuVk2a and TTSuVk2b DNA loads were significantly higher in PMWS-affected animals compared with healthy counterparts. No significant difference was seen in the case of TTSuV1, which confirmed previous findings. It has to be mentioned that the serum samples of PMWS and healthy animals tested in this study were the same as analysed before with different qPCR methods (Aramouni et al., 2011; Kekarainen et al., 2006; Nieto et al., 2011). However, in this study the samples were analysed with a SYBR Green-based qPCR technique, while in the earlier studies Light Upon Extension (LUX) qPCRs were used. With both assays association of TTSuVk2a loads with PMWS was confirmed, but the viral loads were quantified about 10-fold higher with the LUX assay. This is not surprising, considering that the techniques use different quantification methods and primer pairs.

With the description here of a novel TTSuV species, provisionally named TTSuVk2b, this group of pig-infecting viruses has become even more intriguing. These viruses are ubiquitous in pig and it has been demonstrated that the three species, TTSuV1, TTSuVk2a and TTSuVk2b, are often found with high loads in serum of the same animal. This may be due to a lack of cross-protection between species. Indeed, antigenic cross-reactivity has been demonstrated to exist between different genotypes (aa identity about 50\%) but are lacking between species (aa identity about 25\%) (Huang et al., 2012a). The aa identity of ORF1 between TTSuVk2a and TTSuVk2b is about 58.3\% and therefore cross-reactivity is likely to exist between these species. To get further insight into the possible cross protection, the infection dynamics of and antibody development against TTSuVk2a and TTSuVk2b should be investigated using longitudinal serum samples.

Since TTSuVs are known to cause persistent infections, important questions are raised about the role of these viruses in modulating the immune status of the animals. Although considered non-pathogenic by themselves, TTSuV is often present in pigs with diseases like PMWS, porcine respiratory disease complex (Rammohan et al., 2012) and porcine dermatitis and nephropathy syndrome (Aramouni et al., 2011; Krakowka et al., 2008). Studies are undertaken in our laboratory to further address the disease association of TTSuV.

**METHODS**

**Samples.** Commercial porcine sera were used to identify a novel TTSuV species applying the RCA technique. These sera were purchased from Hyclone (New Zealand origin, FSD29672), Kaebe (German origin, 9071172-G50, 9050957-G50, SS02/06-1 and SS03/06) and Sigma (USA origin, 026k8453 and 064K8451), which are pools from a large number of pigs.

For a preliminary epidemiological assessment, 224 pig serum samples were collected from commercial farms located in 17 different countries: Greece, the Netherlands, Brazil, Vietnam, China, Philippines, Lithuania, Mexico, Thailand, Russia, Belarus, Ukraine, Venezuela, South Africa, Mozambique, Canada and Spain. Furthermore, sera from 34 PMWS-affected pigs and 29 age-matched healthy pigs aged between 11 and 21 weeks from a previously published study (Aramouni et al., 2011; Nieto et al., 2011) were also tested. It has to be noted that in this study the 29 healthy pig sera were also used as the Spanish sera in the epidemiological study and are therefore the same.

**DNA extraction.** DNA extraction from individual pig serum samples were performed on the MagNA Pure 96 Instrument using the MagNA Pure 96 DNA and Viral Nucleic Acid Small Volume kit (Roche Diagnostic). This procedure was performed automatically using the Viral NA Universal SV 2.0 protocol. Serum from PMWS-affected pigs, as well as pig serum from Spain and Mozambique were processed as described previously (Aramouni et al., 2011). The commercial porcine sera were processed manually by the QIAamp MinElute Virus Spin kit according to the manufacturer’s instructions (Qiagen).

**RCA.** The RCA-based sequence-independent approach was to identify a novel TTSuV species. For RCA, 10 µl of extracted viral DNA was denatured for 5 min at 95 °C. After cooling on ice, 10 µl of a mixture was added containing 10 U phi29 DNA Polymerase (New England Biolabs), 150 pmol exo-resistant random primers (Fermentas), 48 nmol dNTP (HT Biotechnology Ltd), 40 ng BSA and 2 µl of 10 × phi29 DNA Polymerase Reaction Buffer (New England Biolabs). Amplification was done for 18 h at 30 °C followed by 10 min at 65 °C to inactivate phi29 DNA polymerase. Subsequently, 5 µl of the amplification product was digested with 10 U BamHI or EcoRI. Restriction digests were separated by 0.8% agarose gel electrophoresis. Restriction fragments of approximately 2.9 kb were purified from the gel and inserted into the BamHI or EcoRI sites of vector pSC-A-amp/kan (Agilent). Inserts were sequenced using the Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems).

**Sequence and phylogenetic analyses.** The phylogenetic analyses of the obtained nucleotide sequences [GenBank accession numbers: JQ406844 (TTSuVk2b-38E05), JQ406845 (TTSuVk2b-38E19) and JQ406846 (TTSuVk2b-38E23)] were done using MEGA version 5 (Tamura et al., 2011), including TTSuV1 and TTSuVk2a sequences obtained from GenBank (Court et al., 2011; Huang et al., 2010b). The alignments were performed using a CLUSTAL W multiple alignment tool with a gap creation penalty of 10 and a gap extension penalty of 5. Phylogenies were inferred from p-distance matrices using the neighbour-joining method (Saitou & Nei, 1987). Statistical significance of the branching was estimated using bootstrap with 1000
replications and from this a consensus phylogenetic tree was built. Pairwise sequence comparison (PASC; http://www.ncbi.nlm.nih.gov/sutils/psicat/) was performed using available anellovirus sequences from GenBank (Bao, 2008).

The annotation of the TTSuVk2b genomes was based on the available animal TTV genomes already characterized (Okamoto, 2009; Okamoto et al., 2001). For gene predictions, the Open Reading Frame Finder program (http://www.ncbi.nlm.nih.gov/projects/orf/) was used. The NetGene2 program (http://www.cbs.dtu.dk/services/NetGene2/) (Brunak et al., 1991) was used to predict splice sites in TTSuVk2b genes and pSORT II (http://psort.hgc.jp) (Nakai & Horton, 1999) was used for protein localization predictions as described previously (Martínez-Guiño et al., 2011). The promoter region was predicted using the Neural Network Promoter Prediction program version 2.2 (http://www.fruitfly.org/seq_tools/) with a score cut-off of 0.80.

qPCR specific for TTSuV1, TTSuVk2a and TTSuVk2b. Species-specific qPCRs were developed to detect TTSuV1 (detecting both species a and b), TTSuVk2a and TTSuVk2b in porcine serum. The sequence of the species-specific primers and their location in the genome are listed in Table S1 available in JGV Online. All primers were tested to be species specific by BLAST analysis and challenging each primer set with using full-length TTSuV clones of each species as a template in qPCR (data not shown). The 25 μl PCR contained 12.5 μl of iQ SYBR Green Supermix (Bio-Rad), 10 pmol forward primer, 10 pmol reverse primer and 2 μl of DNA. The qPCR was performed on a CFX 96 Real-Time System (Bio-Rad) and the programme consisted of an initial denaturation step of 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 15 s at 56 °C (for TTSuV1 and TTSuVk2a) or 62 °C (for TTSuVk2b and broad spectrum TTSuV qPCR) and 30 s at 68 °C. The specificity of each PCR was determined by melting curve analysis: after an extra extension step of 7 min at 68 °C, the temperature was raised from 70 to 95 °C with 0.5 °C increments. Fluorescence was measured during each extension step and melting curve analysis. As a standard curve a dilution series of plasmids TTV001, TTV008 or 38E05 was used. The qPCR amplification efficiency was 94.6 for TTSuV1, 93.0 for TTSuVk2a and 1.8 % for TTSuVk2b; the limit of detection (LOD) was 10 copies reaction −1 for each qPCR, which corresponded to 1250 copies ml −1 serum.

TTSuV broad-spectrum qPCR. The TTSuV broad spectrum qPCR was designed to detect and quantify total TTSuV load (including TTSuV1, TTSuVk2a and TTSuVk2b) in serum samples. The sequence of the primers and their location in the genome are listed in Table S1. The PCR mixture contained: 12.5 μl iQ SYBR Green Supermix (Bio-Rad), 10 pmol of the forward primer TTSuV-all-F1, 10 pmol of the reverse primer TTSuV-all-R4 and 2 μl of extracted DNA. The real-time qPCR was performed on the CFX 96 Real-Time System (Bio-Rad) and the programme started with an initial denaturation step for 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 15 s at 62 °C and 30 s extension at 68 °C. After an extra extension step of 7 min at 68 °C, a melting curve was made by raising the temperature from 70 to 95 °C with an increment 0.5 °C per s. Fluorescence data were acquired at each extension step and during the melt curve. As a standard curve a dilution series of plasmid TTV008 was used. The qPCR amplification efficiency was 102.8 %; the inter-assay variation of each primer set with using full-length TTSuV clones of each species as a template in qPCR (data not shown). The 25 μl PCR contained 12.5 μl of iQ SYBR Green Supermix (Bio-Rad), 10 pmol forward primer, 10 pmol reverse primer and 2 μl of DNA. The qPCR was performed on a CFX 96 Real-Time System (Bio-Rad) and the programme consisted of an initial denaturation step of 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 15 s at 56 °C (for TTSuV1 and TTSuVk2a) or 62 °C (for TTSuVk2b and broad spectrum TTSuV qPCR) and 30 s at 68 °C. The specificity of each PCR was determined by melting curve analysis: after an extra extension step of 7 min at 68 °C, the temperature was raised from 70 to 95 °C with 0.5 °C increments. Fluorescence was measured during each extension step and melting curve analysis. As a standard curve a dilution series of plasmids TTV001, TTV008 or 38E05 was used. The qPCR amplification efficiency was 94.6 for TTSuV1, 93.0 for TTSuVk2a and 1.8 % for TTSuVk2b; the limit of detection (LOD) was 10 copies reaction −1 for each qPCR, which corresponded to 1250 copies ml −1 serum.

Generation of PCR standard curves and viral DNA load calculation. The TTSuV1 PCR standard, TTV001, was generated by PCR amplification of a 390 bp sequence from DNA isolated from the Kreabær porcine serum (9071172-G50) using primers TTV1-1F and TTV1-390R (Table S1). For the generation of the TTSuVk2a and broad spectrum TTSuV qPCR standard, TTV008, a 320 bp PCR product was obtained from DNA isolated from Hyclone porcine serum (FSD29672) using primers TTV2-106F and TTV2-425R (Table S1). Both PCR products were cloned in vector pSC-A-amp/kan following the instruction manual of the StrataClone PCR cloning kit (Agilent). A 10-fold dilution series of plasmids TTV001 and TTV008 was used to generate PCR standard curves corresponding to 1 × 10−2 – 1 × 100 copies of DNA per reaction. To calculate the amount of TTSuVk2b DNA in sera, a 10-fold dilution series was used corresponding to 3.3 × 10−2 – 3.3 × 100 copies reaction −1 of plasmid 38E05 that contained the full-length sequence of clone 38E05 in vector pSC-A-amp/kan.

Finally, the mean log10 DNA copies ml −1 of serum was used to compare data.

Statistical analyses. The correlation test was used to compare the prevalence of viruses. Normality of data was assessed using the Shapiro–Wilk test. In order to assess differences of viral loads between healthy and PMWS groups for each TTSuV, Student’s t-test was used for normally distributed samples (TTSuV1 and TTSuVk2b) and Mann–Whitney U test was used for non-parametric data (TTSuVk2a). All statistical analyses were made using SPSS Statistics for Windows (SPSS Inc). Significance was set at P < 0.05 for all tests.

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