Prevalence of swine *Torque teno virus* in post-weaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs in Spain

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The present study was designed to investigate the prevalence of swine *Torque teno virus* (TTV) in post-weaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected Spanish swine. Nested PCR (nPCR) assays to detect two distinct TTV genogroups were applied. A significantly higher prevalence of TTV infection was found in sera from PMWS-affected animals (97 %) than in sera from non-PMWS-affected animals (78 %). Whilst PMWS-affected pigs (91 %) were more likely to be infected with TTV from genogroup 2 than non-PMWS-affected swine (72 %), no such difference was observed with genogroup 1. Nucleotide sequences of nPCR products were 91–99 % identical between strains within a genogroup. In contrast, inter-genogroup sequence identities were 49–58 %. Phylogenetic analyses demonstrated that genogroups form different clusters without association with PMWS or porcine circovirus type 2 infection status of the animals. These results indicate a high prevalence of both swine TTV genogroups in Spain, being present more frequently in PMWS-affected animals than in non-PMWS-affected animals.

*Torque teno virus* (TTV) belongs to the floating genus *Anellovirus*, which includes small, non-enveloped viruses containing a single-stranded, negative-sense, circular DNA genome (Okamoto *et al.*, 2002). The first TTV isolate was detected in a human Japanese patient with post-transfusion hepatitis of unknown aetiology (Nishizawa *et al.*, 1997). In humans, the virus is ubiquitous and several genogroups have been identified so far, although, to date, TTV has not been linked to any specific disease (Jelcic *et al.*, 2004; Peng *et al.*, 2002). More recently, TTV was also identified in domestic animals, including swine, chicken, cow, sheep, cat and dog (Leary *et al.*, 1999; Okamoto *et al.*, 2002). The complete genomic sequences of species-specific TTVs infecting human, non-human primate, tupaia, domestic pig, cat and dog have been determined, and sequence analysis showed a preserved genome organization in TTVs from different species (Inami *et al.*, 2000; Niel *et al.*, 2005; Okamoto *et al.*, 2001, 2002).

The genome of swine TTV is approximately 2.8 kb. To date, three full-length genomic sequences of swine TTV have been published (Niel *et al.*, 2005; Okamoto *et al.*, 2002). Two of the strains, Sd-TTV31 and Sd-TTV1p, shared relatively high nucleotide sequence identity (69.6 %), whereas strain Sd-TTV2p showed about 44 % sequence identity to the other two strains (Niel *et al.*, 2005). Due to the wide genetic diversity of these isolates, it has been suggested recently that Sd-TTV2p would be the prototype of a novel genogroup 2, whereas Sd-TTV31 would be the prototype of genogroup 1 (Niel *et al.*, 2005; Okamoto *et al.*, 2002).

Swine TTV is widespread and virus detection by genogroup 1-specific PCR in serum samples collected from different geographical regions, including Canada, China, Korea, Spain, France, Thailand and USA, has revealed a prevalence ranging from 33 to 100 % (McKeown *et al.*, 2004). Sequence analysis of partial genomic sequences within a conserved region of non-coding area has shown sequence identities ranging from 71 to 100 % (Bigarre *et al.*, 2005; McKeown *et al.*, 2004). Swine TTV, like its human counterpart, shows a marked degree of genetic diversity and, furthermore, one individual can be infected with at least two distinct subtypes (Bigarre *et al.*, 2005; Niel *et al.*, 2005). Swine TTV has not yet been shown to be pathogenic; however, its role during co-infection with other pathogens remains unknown.

Post-weaning multisystemic wasting syndrome (PMWS), an economically important swine disease worldwide, has been...
linked aetiotologically with Porcine circovirus type 2 (PCV2), a member of the family Circoviridae, genus Circovirus. PCV2, similarly to swine TTV, is a ubiquitous virus of pigs, indicating that PMWS is a multifactorial disease in which PCV2 infection is necessary, but not sufficient, to trigger the clinical outcome. Therefore, besides PCV2, other factors are needed for the full expression of PMWS in most cases. Management, genetics, concurrent viral and bacterial infections, and experimental stimulation of the immune system are some of the potential PMWS-triggering factors (Segalés et al., 2005). Specifically, it has been demonstrated experimentally that co-infection of Porcine parvovirus or Porcine reproductive and respiratory syndrome virus with PCV2 is able to trigger PMWS (Allan et al., 1999; Harms et al., 2001; Rovira et al., 2002). Moreover, it would not be surprising if other viral agents can also be found to trigger PMWS in PCV2-infected pigs.

Therefore, the main objective of the present work was to determine the prevalence of swine TTV genogroups 1 and 2 in PMWS- and non-PMWS-affected pigs to elucidate whether swine TTV could be considered as a putative PMWS trigger. On the other hand, although swine TTV genogroup 1 has been detected in Spanish swine (McKeown et al., 2004), a second objective of this study was to assess the prevalence of both genogroups of swine TTV in a larger number of pigs from Spain.

In total, 121 swine serum samples, corresponding to case submissions to the Pathological Diagnostic Service at the Veterinary School of Barcelona (Autonomous University of Barcelona, Spain), were used in this study. The studied pigs came from 34 different farms from northeastern Spain and were submitted because of different clinicopathological conditions, including wasting, respiratory distress and diarrhoea. PMWS diagnosis was established based on the following three criteria (Segalés, 2002): (i) clinical picture, characterized by wasting as well as other signs (respiratory and enteric ones, mainly), (ii) moderate to severe lymphoid lesions consistent with lymphocyte depletion and granulomatous inflammation, and (iii) a moderate to high amount of PCV2 antigen or genome in these lymphoid lesions. Histopathology and PCV2 detection by in situ hybridization (ISH) (Rosell et al., 1999) allowed us to classify 32 of the selected pigs as being affected with PMWS, 34 animals with mild or no PMWS-like lymphoid lesions and a low amount of PCV2 by ISH and, finally, 55 pigs that did not have PMWS-like lymphoid lesions and were PCV2 ISH-negative. PCV2 infection of these latter pigs was further ruled out by means of a PCR on serum (Quintana et al., 2002). Therefore, the two latter groups of pigs (n=89) corresponded to non-PMWS-affected ones.

Presence or absence of TTV sequences of genogroups 1 and 2 in the serum was determined by specific nested PCRs (nPCRs). DNA was extracted from 200 μl serum sample by using a NucleoSpin Blood DNA extraction kit (Macherey-Nagel) and eluted in 100 μl elution buffer. To amplify the non-coding region of swine TTV genogroups 1 and 2, primers for PCR were designed based on published (McKeown et al., 2004) and GenBank sequences, respectively. For genogroup 1, the first-round 20 μl PCR contained 4 μl serum DNA, 20 pmol of the primer pair forward-1 (5'-TACACTTCGGGTTCAAGGGCT-3') and reverse-1 (5'-ACTCCAGCGCTCAGGAAT-C-3'), 2·5 mM dNTPs, 2 mM MgCl2 and 0·75 U Taq DNA polymerase (Ecogen). The amplification was done by using 35 cycles of 94°C for 30 s, 52°C for 20 s and 72°C for 30 s. From this reaction, 4 μl amplification product was used as a template for nPCR by using the primer pair forward-nested-1 (5'-CAATTGGCGCTGCGTCCGCGC-3') and reverse-nested-1 (5'-TACTTATATTGCCTTGTGGAAC-3') (50 pmol each primer), 2·5 mM dNTPs, 2 mM MgCl2 and 0·75 U Taq DNA polymerase. The nPCR product (260 bp) was run on 2% TAE/agarose gel. For genogroup 2, the amplification was carried out as described above, using the primer pair forward-2 (5'-AGTTACATAACCCAAGCCA-AACC-3') and reverse-2 (5'-ATTACCGCTCGCCGAT AGCC-3') for the first round of PCR and primer pair forward-nested-2 (5'-CCAAACACAGGGAAACTGTGC-3') and reverse-nested-2 (5'-CTTGACTCCGCTCTCAGGAGG-3') for the nPCR, resulting in a product of about 230 bp. Twenty-two randomly selected amplification products (12 from genogroup 1 and 10 from genogroup 2) from nPCR were excised from the agarose gel and purified by using a QAquick gel-extraction kit (Qiagen). Sequencing reactions from both strands were done by using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and run on an ABI Prism 3100 sequence analyser (Perkin Elmer).

Sequences were aligned with the CLUSTAL W program. Phylogenetic and molecular-evolutionary analyses were conducted by using MEGA version 3.1 (Kumar et al., 2004). Alignments containing about 130 bp overlapping genogroup sequences were used to calculate phylogenetic distances using the Jukes–Cantor model. Phylogenies were inferred from distance matrices by using the neighbour-joining method. Statistical significance of the branching was estimated by using SEQBOOT (1000 resamplings) and, from these, a consensus tree was built.

Fisher’s exact and χ² tests (Epi Info version 3.2.2; Centers for Disease Control) were carried out to assess the prevalence of the different TTV genogroups in the different PMWS/PCV2 infection statuses. The level of significance was set to P values of <0·05. Relative risk and its 95% confidence interval were also calculated.

Among 121 serum specimens obtained from swine, 100 (83%) were TTV-positive by PCR assay (Table 1). Almost all PMWS pigs were infected with TTV (97% or 31/32), whilst 78% (69/89) of non-PMWS animals were TTV DNA-positive. Statistically, this difference was significant (P<0·05) and PMWS pigs had a 2·25 times higher risk of being infected with TTV than non-PMWS-affected pigs. Analysis of the prevalence of two TTV genogroups revealed that genogroup
2 was significantly \((P<0.05)\) more common in PMWS animals (91 %, 29/32) than clinically non-PMWS-affected ones (72 %, 64/89). Only few PMWS animals tested negative for genogroup 2 (9 %, 3/32), whilst almost one-third of non-affected pigs (28-1 %, 25/89) were negative for the same genogroup. No significant differences in swine TTV genogroup 1 prevalence in these groups were detected. Further division of animals into three categories according to histopathology and PCV2 detection by ISH indicated that PMWS animals were more frequently infected with TTV (97 %, 31/32) than animals that were diagnosed to be PCV2-free (76 %, 42/55, \(P<0.05\)). The correlation was not clearly linked to a specific genogroup. No statistical differences were observed in TTV genogroup prevalences between PMWS-affected pigs and pigs with mild PMWS lesions and a low amount of PCV2 nucleic acid in lymphoid tissues.

In a previous study, 90 % (18/20) of studied swine sera from Spain were shown to be TTV-positive by a PCR detecting only genogroup 1 variants (McKeown et al., 2004). The difference in the prevalence of genogroup 1 (60 %, 72/121) obtained in our study is probably due to the differences in the number of animals analysed; whilst 121 pig sera were analysed in the present study, only 20 pigs with unknown health conditions were analysed in the earlier study. It must be mentioned that, as there is no common method to detect swine TTV, the PCR method itself might be the source of differences between laboratories. Additionally, the large amount of intraspecies sequence variation evident within TTV complicates the detection of all existing genogroups in a given individual (Bigarré et al., 2005; Hu et al., 2005; Niel et al., 2005). Characteristically, anelloviruses contain considerable genetic diversity, showing, however, short highly conserved sequences within the untranslated region (Leary et al., 1999; Okamoto et al., 2002). When analysing the highly conserved sequences, low variation might be observed and clear distinction of different genogroups is not possible (Bigarré et al., 2005). For these reasons, in the present study, a variable region of non-translated area, flanked with conserved motifs, was amplified, allowing differentiation of viral isolates of the two genogroups.

Phylogenetic analysis of Spanish swine TTV strains demonstrated that strains of genogroups 1 and 2 form clearly distinct clusters (Fig. 1). Swine strains from this study were genetically related more closely to those of primates of lower order (tamarin) and dog isolates than to other animal species infected by anelloviruses. However, the analysed region is highly diverse between viruses from different species, thus complicating the sequence alignment and phylogenetic analysis.

To summarize, our results indicate that swine TTV isolates from both genogroups are widespread in Spain and that PMWS pigs are more likely to be infected with TTV, more specifically genogroup 2, than non-affected animals. The biological importance of this latter finding remains to be elucidated.

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**Table 1. Prevalence of TTV genogroups in Spanish swine**

Data are total no. positive animals/all animals tested. Percentage of infected animals is shown in parentheses.

<table>
<thead>
<tr>
<th>Category</th>
<th>Genogroup</th>
<th>1 or 2 TTV</th>
<th>1 TTV</th>
<th>2 TTV</th>
<th>1 and 2 TTV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total PMWS ((n=32))</td>
<td></td>
<td>31/32* (97)</td>
<td>21/32 (66)</td>
<td>29/32* (91)</td>
<td>19/32 (59)</td>
</tr>
<tr>
<td>Total no PMWS ((n=89))</td>
<td></td>
<td>69/89* (78)</td>
<td>51/89 (57)</td>
<td>64/89* (72)</td>
<td>47/89 (53)</td>
</tr>
<tr>
<td>Mild PMWS-like lesions; low PCV2 amount ((n=34))</td>
<td></td>
<td>27/34 (79)</td>
<td>21/34 (62)</td>
<td>26/34 (76)</td>
<td>20/34 (59)</td>
</tr>
<tr>
<td>No PMWS-like lesions; no PCV2 ((n=55))</td>
<td></td>
<td>42/55 (76)</td>
<td>30/55 (55)</td>
<td>38/55 (69)</td>
<td>27/55 (49)</td>
</tr>
<tr>
<td>All ((n=121))</td>
<td></td>
<td>100/121 (83)</td>
<td>72/121 (60)</td>
<td>93/121 (77)</td>
<td>66/121 (55)</td>
</tr>
</tbody>
</table>

*Statistically significant difference between animal groups.
References


Fig. 1. Phylogenetic tree based on nucleotide sequences of the untranslated region of anellovirus genomes. The reference number of each sequenced Spanish strain refers to the identification code of animals followed by year; ‘a’ (genogroup 1) and ‘b’ (genogroup 2) have been used to differentiate TTV sequences derived from the same pig. Filled and non-filled symbols indicate sequences of genogroups 1 and 2, respectively: ▲, ▲ are from PMWS-affected pigs; ○, ○ are from pigs with mild PMWS-like lesions and a low PCV2 amount; ♣, ♣ are from pigs with no PMWS-like lesions and no PCV2. Bootstrap values higher than 50% are shown at nodes. GenBank accession numbers for TTV originating from different species included in the phylogenetic analysis are indicated in parentheses.


