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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.

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25 **Summary**

26 Torque teno viruses (TTVs) are vertebrate infecting, small viruses with circular single
27 stranded DNA, classified in the *Anelloviridae* family. In pigs, two different TTV species
28 have been described so far, *Torque teno sus virus 1* (TTSuV1) and 2 (TTSuV2).
29 TTSuVs have lately been linked to postweaning multisystemic wasting syndrome
30 (PMWS). In the present study, TTSuV1 and TTSuV2 prevalence and DNA loads in
31 longitudinally collected serum samples of healthy and PMWS affected pigs from
32 Spanish conventional, multi-site farms were analyzed. Serum samples were taken at 1,
33 3, 7, 11 and around 15 weeks of age (age of PMWS outbreak) and viral DNA loads
34 determined by quantitative PCR. For both TTSuV species, percentage of viremic pigs
35 increased progressively over time, with the highest prevalence in animals of about 15
36 weeks of age. TTSuV1 and TTSuV2 viral DNA loads in healthy and TTSuV1 loads in
37 PMWS affected animals increased until 11 weeks of age declining afterwards. On the
38 contrary, TTSuV2 DNA loads in PMWS affected pigs increased throughout the
39 sampling period. It seems that TTSuV species differ in the *in vivo* infection dynamics in
40 PMWS affected animals.

41

42 Keywords: Anelloviridae, Torque teno sus virus 1 (TTSuV1), Torque teno sus virus 2
43 (TTSuV2), postweaning multisystemic wasting syndrome (PMWS), infection dynamics.

44

44 1. Introduction

45 Anelloviruses are vertebrate infecting, non-enveloped, icosahedral viruses with a
46 circular single-stranded DNA genome (Nishizawa et al., 1997). In swine, two
47 genetically distinct species have been identified so far, *Torque teno sus virus 1*
48 (TTSuV1) and 2 (TTSuV2), which are currently grouped into the genus *Iotatorquevirus*
49 (<http://www.ncbi.nlm.nih.gov/ICTVdb/>).

50 TTSuVs have been found in swine serum worldwide with prevalence rates
51 ranging from 24% to 100% (Bigarré et al 2005; Kekarainen et al 2006; Taira et al.,
52 2009; Gallei et al., 2010) and it is likely that both species are ubiquitous in domestic
53 pigs and wild boar (Kekarainen and Segalés, 2009). TTSuVs have been also found in
54 biological fluids such as semen, colostrum, nasal cavity and faeces (Kekarainen et al.,
55 2007; Martínez-Guinó et al., 2009; Sibila et al., 2009a), indicating the occurrence of
56 both vertical and horizontal transmission (Martínez-Guinó et al., 2009; Pozzuto et al.,
57 2009; Sibila et al 2009a; Sibila et al., 2009b; Aramouni et al., 2010). Viral prevalence
58 increases with age and most if not all animals get persistently infected (Sibila et al.,
59 2009a; Sibila et al., 2009b; Taira et al., 2009). Also, tissues have been found PCR
60 positive from the second third of gestation onwards (Aramouni et al., 2010). It was also
61 demonstrated by a semi-quantitative method that virus DNA loads in tissues increased
62 over age, until 15 weeks of age, and then maintained until slaughter age (Aramouni et
63 al., 2010).

64 Currently, the disease causing potential of anelloviruses is under debate. Human
65 TTVs are apparently related to liver and respiratory diseases, haematological disorders
66 and cancer (Okamoto, 2009b). In pigs, it has been suggested that TTSuVs infection
67 could be a factor of aggravation in co-infection with other pathogens, mainly *Porcine*
68 *circovirus type 2* (PCV2). PCV2 is the essential but not sufficient cause of postweaning
69 multisystemic wasting syndrome (PMWS), the economically most important porcine
70 circovirus disease (PCVD). TTSuV2, but not TTSuV1, prevalence has been found to be

significantly higher in PMWS affected pigs than in healthy animals (Kekarainen et al., 2006). Furthermore, experimental infection of gnotobiotic pigs with TTSuV1 and PCV2 has been shown to trigger PMWS (Ellis et al., 2008). Combined infection of TTSuV1 and porcine reproductive and respiratory syndrome virus (PRRSV) has been linked to a porcine dermatitis and nephropathy syndrome (PDNS)-like condition (Krakowka et al., 2008). On the contrary, in a recent study with limited number of animals (n=22), no association was found between PMWS and TTSuVs (Lee et al., 2010).

Taking into account the potential relationship between TTSuVs and PCVDs, a quantitative approach was considered in a longitudinal study of pigs developing PMWS. Therefore, the aim of the present study was to describe the kinetics of viral DNA loads of both TTSuV1 and TTSuV2 in serum of healthy and pigs developing PMWS from their first week of age until the disease outbreak. Such objective was accomplished by means of a newly developed real-time quantitative PCR (qPCR) based on The Light Upon Extension TM (LUX TM) technique.

2. Materials and Methods

2.1. Animals and samples

Clinically healthy animals (n=17) and PMWS animals (n=18) were chosen for this study. The pigs were originally included in an epidemiological study of PCV2 conducted in Spain (Grau-Roma et al., 2009). Pigs were followed from the 1st week of life until the development of PMWS-like clinical signs, time when diseased and age-matched healthy controls were euthanized and necropsied. Healthy pigs were chosen based on the good corporal condition, the absence of clinical signs and the lack of histopathological findings such as lymphocyte depletion and granulomatous inflammation in lymphoid tissues and lack, or very low amount, of PCV2 in lymphoid tissues measured by *in situ* hybridization (ISH) (Rosell et al., 1999). PMWS pigs were

chosen based on PMWS-like clinical signs confirmed subsequently by histopathological findings and amount of PCV2 measured by ISH (Segalés et al., 2005).

Included animals were from 6 different Spanish herds. Blood was taken at 1, 3, 7, 11 and around 15 weeks of age (time when the PMWS outbreak took place). For healthy animals at weeks 1 and 3 of age, 7 and 15 serum samples (out of the 17 pigs) were available, respectively, while for PMWS affected animals at week 1 only 6 samples out of 18 pigs were available. Blood samples were individually identified and transported in refrigeration to the laboratory where serum was collected and stored at -80°C until further processed.

2.2. Quantitative PCR (qPCR)

2.2.1. DNA extraction

DNA was extracted from 200 µl of serum using Nucleospin Blood and eluted in 100 µl of elution buffer (5mM Tris/HCl, pH 8.5) according to manufacturer's instructions (Macherey-Nagel). All DNA extraction procedure included a negative control, containing only PBS as extraction substrate.

2.2.2. Primer design

GenBank entries AB076001 and AY823990 for TTSuV1 and TTSuV2 genomes, respectively, were used for the design of the corresponding primers. The untranslated region (UTR) of the genome of both viruses was chosen for the primers design, since it is a highly conserved area of these viral genomes (Okamoto et al., 2000). TTSuV1 forward primer (TTSuV1F), TTSuV1 reverse primer (TTSuV1R), TTSuV2 forward primer (TTSuV2F) and TTSuV2 reverse primer (TTSuV2R) (Table 1) were designed using D-LUX™ Designer Desktop v.3.0 from Invitrogen and were predicted to work under universal conditions. TTSuV1F and TTSuV2F primers were labelled at the 3' with JOE™ (6-carboxy-dichloro-dimethoxy-fluorescein) and FAM™ (6-carboxy-

123 fluorescein), respectively. Amplicon sizes of TTSuV1 and TTSuV2 were 86 bp and 67
124 bp, respectively.

125 All primers were tested for cross-specificity to both TTSuV species, swine
126 genome, PK-15 cell line DNA, and the most common swine viruses like PRRSV, PPV,
127 porcine circovirus type 1 (PCV1), and PCV2 genotypes “a” (PCV2a) and “b” (PCV2b),
128 by using the BLAST software and in direct qPCR assays.

129 2.2.3. Standards

130 For the standard preparations, TTSuV1 and TTSuV2 full-length genomes were
131 amplified with proof reading activity polymerase (TaKaRa LA Taq™) and specific
132 pairs of primers (TTSuV1: sense: 5' TGA GTT TAT GCC GCC AGC GGT AGA 3';
133 antisense: 5' GCC ATT CGG AAC TGC ACT TAC T 3'; TTSuV2: sense: 5' GAA TTC
134 GCT AGA TTT TTA AAA GGA AAG 3'; antisense: 5' GAA TTC CAT TCC AAC
135 ATT ACT AGC T G 3') and then cloned into the pCR2.1 vector. Plasmid purifications
136 were made using the Qiaprep Spin Miniprep kit (Qiagen) according to the manufacturer
137 instructions. After a spectrophotometric quantification of the plasmids, standards were
138 prepared in 10-fold serial dilutions ranging from 10^9 to 10 molecules/ μ l and tested by
139 qPCR to ensure that standard curve parameters are in accepted values (figure 1). Two μ l
140 of the standards ranging between 10^5 and 10 molecules/ μ l were used subsequently for
141 the quantification of TTSuV1 and TTSuV2 in the studied samples.

142 2.2.4. Quantitative PCR reaction

143 Reactions were carried out in 96-well plates. Each sample and standards were
144 run in triplicate and a negative control was added between each three wells, using
145 autoclaved bi-distilled water instead of sample DNA. After optimization, each reaction
146 contained 2 μ l of sample or standard DNA, 200 nM of each primer, 10 μ l of Express
147 qPCR Supermix Universal™ (Invitrogen), 0.04 μ l of Rox in a total volume of 20 μ l.
148 Amplification and quantification were performed using ABI®7500 Fast Real Time PCR

149 System (Applied Biosystems™) under universal conditions: 10 min at 95°C, 2 min at
150 50°C and 40 cycles of 15 s at 95°C, 1 min at 60°C.

151 Quantitative PCR robustness and performance efficiency were assessed by three
152 parameters: the linear standard curve correlation coefficient (r) and coefficient of
153 determination (R^2), the amplification efficiency (E) and the inter-assay variability.
154 Results were validated in each qPCR reaction by the standard deviation (SD) of
155 threshold cycle of three replicates (intra-assay variability), the melting temperatures and
156 contamination of negative control.

157 To calculate the TTV genomic load per ml of sera, individual results from qPCR
158 were multiplied by 250 (100 μ l eluted from 200 μ l of serum x 2 μ l DNA input). Finally
159 the average $\log_{10}$ copies per ml of serum was used to compare data.

160

161 **2.3. Statistical Analysis**

162 The Chi-square test was used to compare the proportion of positive qPCR results
163 between the studied pigs. ANOVA was used to assess differences of viral loads between
164 healthy and PMWS groups. Student Newman-Keuls test was used to determine
165 differences of viral loads between weeks within animal groups. Statistical significance
166 level was set at $p = 0.05$, while tendency was set at $p = 0.1$. Multiple experiment viewer
167 software (MeV version 4.2, TM4 software suite, (Saeed et al., 2003) was used to group
168 animals according to their viral load dynamics. A K means algorithm was used with
169 Euclidean distance metric and 50 iterations, the different profiles were finally clustered
170 in two groups using Microsoft Excel software.

171

172 **3. Results**

173 **3.1 Quantitative PCR optimization**

174 Only qPCR reactions with a SD < 0.05 between triplicates (intra-assay
175 variability), standard curve with an accuracy of $R^2 > 0.97$, a slope measuring the

176 efficiency between - 3.2 and - 3.7 and a melting temperature of 77°C for TTSuV1 and
 177 82°C for TTSuV2 were accepted. Reactions not fulfilling those criteria or with
 178 contaminated negative controls were repeated.

179

180 ***3.2 Reproducibility, specificity and sensitivity of the method***

181 The reproducibility of the method was established with the inter-assay, measured
 182 as the coefficient of variation, (CV) of the threshold cycle of the standard curves
 183 generated in the different quantification assays. Inter-assay variations of detecting
 184 TTSuV standards range were calculated through all the experiments and the values were
 185 below 3.4% for TTSuV1 and below 3.8% for TTSuV2. The amplification efficiency (E)
 186 was 97.7% for TTSuV1 and 96.5% for TTSuV2.

187 In regards to the specificity of the method, no cross-amplification was found
 188 with any of the tested pathogens by qPCR or by the BLAST analysis.

189 The quantification range of the method was between 10^9 and 20 TTSuV1 or
 190 TTSuV2 genome equivalents per reaction corresponding to $10^{9.60}$ and $10^{3.69}$ DNA
 191 copies/ml. At lower concentrations of virus, quantification was not always reproducible.

192

193 ***3.3 Prevalence of TTSuV1 and TTSuV2 in serum samples***

194 Prevalence of TTSuVs in healthy and PMWS affected pigs at different ages are
 195 shown in (table 2). Infections by TTSuV species increased with the age of animals,
 196 being highest at 11 and 15 week-old pigs for TTSuV1 and TTSuV2, respectively. No
 197 significant differences of TTSuV1 prevalence was observed between healthy and
 198 PMWS groups, while for TTSuV2 a tendency ($p<0.1$) was observed between healthy
 199 and PMWS affected pigs in the last two sampling points.

200

201 ***3.4 TTSuV1 and TTSuV2 viral DNA load kinetics***

202 TTSuV1 viral DNA loads increased in both studied animal groups from 1 or 3
 203 weeks of age until 11 weeks of age and declined by the last sampling point (figure 2). A
 204 similar pattern was observed for TTSuV2 in healthy animals. However, TTSuV2 loads
 205 in PMWS affected animals increased until last sampling point corresponding to the
 206 clinical manifestation of the disease. At that point, PMWS animals had significantly
 207 higher TTSuV2 viral DNA loads than healthy age-matched pigs ($p<0.05$). Such
 208 difference between studied groups was not evident in any other sampling point.

209 Two different infection dynamics profiles were generated for each TTSuV
 210 species by the MeV software (figure 3). For TTSuV2, profile 1 included 16 pigs (11
 211 healthy, 5 PMWS) on average with decreasing viral DNA loads throughout the study.
 212 At the final point (necropsy time), all the animals had mean viral loads below 5 log₁₀. In
 213 the profile 2, 19 pigs (6 healthy, 13 PMWS) were included, which showed increasing
 214 viral loads with mean viral load at necropsy above 6 log₁₀. The percentage of healthy
 215 and PMWS affected animals within each profile was statistically different ($p=0.03$).
 216 TTSuV1 profiles did not differ from a statistical point of view (data not shown).

217

218 4. Discussion

219 TTSuV infection in pigs is highly prevalent throughout the world. Currently,
 220 there is debate on its disease association, especially with PCVDs. Analysis of viral
 221 DNA loads can be helpful in understanding the *in vivo* dynamics of TTSuV infection in
 222 diseased and healthy animals. In the present study, new, handy, efficient, specific and
 223 sensitive qPCR methods to quantify TTSuV1 and TTSuV2 loads in serum have been
 224 developed. The utility of this new technique was assessed in this study by its application
 225 in an epidemiological study of TTSuVs in the context of PCVDs. Results from this
 226 study show that TTSuV2 viral loads continued increasing in pigs developing PMWS,
 227 while this was not the case in healthy animals, neither in the case of TTSuV1 in both
 228 groups of animals. The results displayed by the MeV software corroborated the different

behaviour of TTSuVs, since significant differences among generated profiles were observed only for TTSuV2, further suggesting a possible link between PMWS occurrence and TTSuV2.

PMWS animals are known to be immunocompromised and when clinical signs appear, pigs suffer from leukopenia, have high viral DNA loads of PCV2 and low levels of PCV2 specific antibodies (Kekarainen et al., 2010). It seems that TTSuV2 viremia load was not counteracted by PMWS affected pigs, while healthy animals were capable of limiting the viremia load, most likely due to normal functioning immune system. Furthermore, TTSuV2 may benefit of the disease status by increased viral release or replication. In fact, it has been shown in humans that immunosuppression can induce an increase in TTV viral load (Burra et al., 2008). TTSuV1 was, however, not linked to PMWS occurrence. It has been proposed that some porcine and human anelloviruses might be more disease-linked than others (Kekarainen et al., 2006; Okamoto, 2009a), and co-infection with other viruses could affect the outcome or progression of some diseases (Fehér et al., 2009). Papillomaviruses are one of the best known examples of different virulence depending on the viral species (Knipe and Howley, 2007). A closer example in pigs in regards different virulent capabilities comes from pathogenic PCV2 and non-pathogenic *Porcine circovirus type 1* (PCV1) (Allan and Ellis, 2000). A similar scenario could apply for TTSuVs, especially when considering the existing differences between the two species: the mean pair-wise nucleotide identities between the genomes of the studied TTSuV species is only 52% (Cortey, 2010; Huang et al., 2010) while 60-70% in papillomaviral species (de Villierds et al., 2004) and less than 80% in the case of porcine circoviruses (Meehan et al., 1998). Furthermore, different forces are shaping the evolution of the species; while the encoded proteins of TTSuV2 are mainly under neutral selection, positive selection is the main force in the case of TTSuV1 (Cortey et al., 2010). Unfortunately, with the currently existing techniques, it is not possible to study the biological differences between TTSuVs in more detail.

256 To date, the only longitudinal study investigating the dynamics of infection in
 257 pigs have used conventional PCR (Sibila et al., 2009a), just giving qualitative results.
 258 Similar prevalence rates and individual results were obtained with conventional PCR by
 259 Sibila et al. (2009a) and since some of the animals tested here were also included in
 260 such study, these two techniques can be considered consistent in prevalence studies.

261 This and the previous study (Sibila et al., 2009a) show that the TTSuV
 262 prevalence in serum increases with age, being lowest during the first weeks of life,
 263 which is in accordance with Martínez-Guinó et al. (2009) and also with Sibila et al.
 264 (2009b). Maximum prevalence was reached at 11 weeks for TTSuV1 and 15 weeks for
 265 TTSuV2, in accordance with Sibila et al. (2009a). It is expected to have viremic young
 266 animals since TTSuV is transmitted not only horizontally but also vertically (Martínez-
 267 Guinó et al., 2009; Pozzuto et al., 2009; Sibila et al., 2009a; Aramouni et al., 2010).
 268 Interestingly, in the present study, TTSuV2 viremia was not detected in healthy animals
 269 until 7 weeks of age, while 17% and 22% of animals that subsequently suffered from
 270 PMWS were infected already on their 1st and 3rd week of life, respectively. This may be
 271 only due to the low amount of animals studied since TTSuV2 has been detected in about
 272 10% of healthy piglets already during their first weeks of age (Sibila et al., 2009a). On
 273 the other hand, in Japanese pigs with PMWS-like clinical signs (the disease was not
 274 laboratorially confirmed) or porcine respiratory disease complex, TTSuV was
 275 undetectable in piglets below 30 days of age (Taira et al., 2009). Therefore, the
 276 difference on viral prevalence in young animals and its possible link to PMWS
 277 development should be further studied with larger populations.

278 Few studies on TTV viral load have been published in humans. It has been
 279 shown that HIV-infected patients have higher TTV viremia and there is an association
 280 with decreased survivability when compared with healthy blood donors (Christensen et
 281 al., 2000). Another study suggested that TTV viremia is associated with the level of
 282 immunocompetence of the populations studied (Touinssi et al., 2001). Moreover,

interferon (IFN) treatment for hepatitis C virus (HCV) results in decline, although sometimes short-lived, of TTV DNA viral loads (Maggi et al., 2001). However, the TTV load decrease was not correlated with the HCV decline, pointing to different factors involved in such viral load diminishment. Furthermore, the applied quantification technique determined total TTV viral DNA loads without knowledge on the specific viral species involved. Sequential sampling of myelosuppressed leukaemia patients undergoing hematopoietic stem cell transplantation showed that during the immunosuppression TTV loads were decreasing, while returned to high levels at the time of graft reconstitution (Maggi et al., 2010).

Although several recent studies have been reporting TTSuV viral loads (Lee et al., 2009; Brassard et al., 2009; Gallei et al., 2010), our study is the first one determining viral loads kinetics in healthy and diseased animals. Lee and co-workers concluded that TTSuV viral loads were not correlated with manifestation of postweaning multisystemic wasting syndrome (Lee et al., 2009). However, this study was based on one sampling point and only 6 TTSuV1 and 20 TTSuV2 positive animals and few animals were evaluated as diseased and non-diseased. In other studies, single samples of healthy animals were included (Brassard et al., 2009; Gallei et al., 2010) or a qPCR technique not able to differentiate the viral species (Brassard et al., 2009) were applied, therefore, not being usable to determine biological differences between TTSuVs.

In summary, the present study shows, for the first time, the *in vivo* load dynamics of any anellovirus in healthy and diseased subjects from their birth until disease occurrence. The amount of TTSuV2 viral DNA increased over time in diseased animals, which was not the case of healthy animals or for TTSuV1. The factors leading to these differences in viral load kinetics remain unknown.

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314

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393
 394 **Figure 1:** Generation of standard curve to assess reaction optimization by using a 10-
 395 fold dilution of a quantified TTSuV2 template and amplified by ABI®7500 Fast Real
 396 Time PCR System (Applied Biosystems™). Each dilution was assayed in triplicate. (A)
 397 Standard curve with the CT plotted against the log of the starting quantity of template
 398 for each dilution. (B) Amplification curves of the dilution series.

399
 400 **Figure 2:** TTSuV1 (A) and TTSuV2 (B) viral load dynamics in healthy (white bars)
 401 and PMWS (black bars) affected animals. Mean viral loads and standard deviation at
 402 different sampling times are represented in log₁₀ scale. P-values for significant
 403 differences are shown. Different letters mean significant differences between ages
 404 within healthy (capital letters) or PMWS (case letters) groups.

405
 406 **Figure 3:** Profiles generated by the MeV software (A and B) grouping TTSuV2
 407 infected animals according to individual viral load dynamics. Grey lines: viral load
 408 profile for each individual pig; black line: mean viral load of all pigs belonging to the
 409 same profile.

410

411

Table 1: TTV1 and TTV2 LUX primer characteristics.

Primer	T _m (°C)	GC%	bp	Sequence (5' to 3')	Location in genome
TTSuV1F	71	50	26	CGA CCG GAG TCA AAT CTG ATT GGT [JOE] G	195-211
TTSuV1R	62	50	22	TAC TGG GAA CGC CCT AAT TCT G	259-281
TTSuV2F	69	50	28	CGG TTG AAC AGA GCT GAG TGT CTA AC[FAM] G	281-309
TTSuV2R	65	65	20	CCC TTG ACT CCG CTC TCA GG	329-348

Table 2: Prevalence of TTSuVs at different weeks of age, expressed as qPCR positives/total serum samples studied and percentage of positives shown in parentheses.

	Week 1		Week 3		Week 7		Week 11		Week N	
	TTSuV1	TTSuV2	TTSuV1	TTSuV2	TTSuV1	TTSuV2	TTSuV1	TTSuV2	TTSuV1	TTSuV2
Healthy	1/7 (14.3)	0/7 (0.0)	5/15 (33.3)	0/15 (0.0)*	10/17 (58.8)	8/17 (47.1)	13/17 (76.5)	12/17 (70.6) ^t	11/17 (64.7)	14/17 (82.4) ^t
PMWS	0/6 (0.0)	1/6 (16.7)	4/18 (22.2)	4/18 (22.2)*	10/18 (55.6)	9/18 (50.0)	11/18 (61.1)	17/18 (94.4) ^t	13/18 (72.2)	18/18 (100) ^t
Total	1/13 (7.7)	1/13 (7.7)	9/33 (27.3)	4/33 (12.1)	20/35 (57.1)	17/35 (48.6)	24/35 (68.6)	29/35 (82.9)	24/35 (68.6)	32/35 (91.4)

N: Date of necropsy.

* statistically significant differences between animal groups at a given age

t: tendency between animal groups at a given age

A

B