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D. Nieto, M. Aramouni, L. Grau-Roma, J. Segalés, T. Kekkarainen

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D. Nieto, M. Aramouni, L. Grau-Roma, J. Segalés, T. Kekkarainen. 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. *Veterinary Microbiology*, 2011, 152 (3-4), pp.284. 10.1016/j.vetmic.2011.05.020 . hal-00724196

**HAL Id: hal-00724196**

**<https://hal.science/hal-00724196>**

Submitted on 20 Aug 2012

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## Accepted Manuscript

Title: 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

Authors: D. Nieto, M. Aramouni, L. Grau-Roma, J. Segalés, T. Kekarainen



PII: S0378-1135(11)00298-7  
DOI: doi:10.1016/j.vetmic.2011.05.020  
Reference: VETMIC 5317

To appear in: *VETMIC*

Received date: 22-12-2010  
Revised date: 6-5-2011  
Accepted date: 10-5-2011

Please cite this article as: Nieto, D., Aramouni, M., Grau-Roma, L., Segalés, J., Kekarainen, T., 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, *Veterinary Microbiology* (2010), doi:10.1016/j.vetmic.2011.05.020

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 **Dynamics of Torque teno sus virus 1 (TTSuV1) and 2 (TTSuV2) DNA**  
2 **loads in serum of healthy and postweaning multisystemic wasting**  
3 **syndrome (PMWS) affected pigs.**

4

5 **D. Nieto<sup>a,1</sup>, M. Aramouni<sup>a,1</sup>, L. Grau-Roma<sup>a,b</sup>, J. Segalés<sup>a,b</sup>, T.**  
6 **Kekarainen<sup>a\*</sup>.**

7

8 <sup>1</sup> *Both authors contributed equally to the work*

9 <sup>a</sup> *Centre de Recerca en Sanitat Animal (CRESA), UAB-IRTA, Campus de la Universitat*  
10 *Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.*

11 <sup>b</sup> *Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona,*  
12 *08193 Bellaterra, Barcelona, Spain*

13

14

15

16

17

18 **\* Corresponding author**

19 Centre de Recerca en Sanitat Animal (CRESA), UAB-IRTA

20 Campus de la Universitat Autònoma de Barcelona

21 08193 Bellaterra, Barcelona, Spain

22 Tel. +34935814620

23 Fax. +34935814490

24 E-mail: Tuija.kekarainen@cresa.uab.es

25

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

71 significantly higher in PMWS affected pigs than in healthy animals (Kekarainen et al.,  
72 2006). Furthermore, experimental infection of gnotobiotic pigs with TTSuV1 and PCV2  
73 has been shown to trigger PMWS (Ellis et al., 2008). Combined infection of TTSuV1  
74 and porcine reproductive and respiratory syndrome virus (PRRSV) has been linked to a  
75 porcine dermatitis and nephropathy syndrome (PDNS)-like condition (Krakowka et al.,  
76 2008). On the contrary, in a recent study with limited number of animals (n=22), no  
77 association was found between PMWS and TTSuVs (Lee et al., 2010).

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

85

## 86 **2. Materials and Methods**

### 87 **2.1. Animals and samples**

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

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

99 Included animals were from 6 different Spanish herds. Blood was taken at 1, 3,  
100 7, 11 and around 15 weeks of age (time when the PMWS outbreak took place). For  
101 healthy animals at weeks 1 and 3 of age, 7 and 15 serum samples (out of the 17 pigs)  
102 were available, respectively, while for PMWS affected animals at week 1 only 6  
103 samples out of 18 pigs were available. Blood samples were individually identified and  
104 transported in refrigeration to the laboratory where serum was collected and stored at  
105 -80°C until further processed.

106

## 107 **2.2. Quantitative PCR (qPCR)**

### 108 **2.2.1. DNA extraction**

109 DNA was extracted from 200 µl of serum using Nucleospin Blood and eluted in  
110 100 µl of elution buffer (5mM Tris/HCl, pH 8.5) according to manufacturer's  
111 instructions (Macherey-Nagel). All DNA extraction procedure included a negative  
112 control, containing only PBS as extraction substrate.

### 113 **2.2.2. Primer design**

114 GenBank entries AB076001 and AY823990 for TTSuV1 and TTSuV2 genomes,  
115 respectively, were used for the design of the corresponding primers. The untranslated  
116 region (UTR) of the genome of both viruses was chosen for the primers design, since it  
117 is a highly conserved area of these viral genomes (Okamoto et al., 2000). TTSuV1  
118 forward primer (TTSuV1F), TTSuV1 reverse primer (TTSuV1R), TTSuV2 forward  
119 primer (TTSuV2F) and TTSuV2 reverse primer (TTSuV2R) (Table 1) were designed  
120 using D-LUX™ Designer Desktop v.3.0 from Invitrogen and were predicted to work  
121 under universal conditions. TTSuV1F and TTSuV2F primers were labelled at the 3'  
122 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/ $\mu$ l and tested by  
139 qPCR to ensure that standard curve parameters are in accepted values (figure 1). Two  $\mu$ l  
140 of the standards ranging between  $10^5$  and 10 molecules/ $\mu$ 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  $\mu$ l of sample or standard DNA, 200 nM of each primer, 10  $\mu$ l of Express  
147 qPCR Supermix Universal™ (Invitrogen), 0.04  $\mu$ l of Rox in a total volume of 20  $\mu$ 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  $\mu$ l eluted from 200  $\mu$ l of serum x 2  $\mu$ 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  $5 \log_{10}$ . 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  $6 \log_{10}$ . 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

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

232 PMWS animals are known to be immunocompromised and when clinical signs  
233 appear, pigs suffer from leukopenia, have high viral DNA loads of PCV2 and low levels  
234 of PCV2 specific antibodies (Kekarainen et al., 2010). It seems that TTSuV2 viremia  
235 load was not counteracted by PMWS affected pigs, while healthy animals were capable  
236 of limiting the viremia load, most likely due to normal functioning immune system.  
237 Furthermore, TTSuV2 may benefit of the disease status by increased viral release or  
238 replication. In fact, it has been shown in humans that immunosuppression can induce an  
239 increase in TTV viral load (Burra et al., 2008). TTSuV1 was, however, not linked to  
240 PMWS occurrence. It has been proposed that some porcine and human anelloviruses  
241 might be more disease-linked than others (Kekarainen et al., 2006; Okamoto, 2009a),  
242 and co-infection with other viruses could affect the outcome or progression of some  
243 diseases (Fehér et al., 2009). Papillomaviruses are one of the best known examples of  
244 different virulence depending on the viral species (Knipe and Howley, 2007). A closer  
245 example in pigs in regards different virulent capabilities comes from pathogenic PCV2  
246 and non-pathogenic *Porcine circovirus type 1* (PCV1) (Allan and Ellis, 2000). A similar  
247 scenario could apply for TTSuVs, especially when considering the existing differences  
248 between the two species: the mean pair-wise nucleotide identities between the genomes  
249 of the studied TTSuV species is only 52% (Cortey, 2010; Huang et al., 2010) while 60-  
250 70% in papillomaviral species (de Villierds et al., 2004) and less than 80% in the case  
251 of porcine circoviruses (Meehan et al., 1998). Furthermore, different forces are shaping  
252 the evolution of the species; while the encoded proteins of TTSuV2 are mainly under  
253 neutral selection, positive selection is the main force in the case of TTSuV1 (Cortey et  
254 al., 2010). Unfortunately, with the currently existing techniques, it is not possible to  
255 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 1<sup>st</sup> and 3<sup>rd</sup> 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,

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

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

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

308

309 **5. Acknowledgements**

310 This work was funded by grants AGL2006-02778/GAN, TRT2006-00018 and  
311 CONSOLIDER-PORCIVIR CSD2006-00007 from the Spanish government. The  
312 authors want to thank Dr M. Cortey for his help in statistical analysis. T. Kekarainen  
313 was supported by the Spanish Government, Ramón y Cajal program.

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  $\log_{10}$  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.

<b>Primer</b>	<b>T<sub>m</sub> (°C)</b>	<b>GC%</b>	<b>bp</b>	<b>Sequence (5' to 3')</b>	<b>Location in genome</b>
<b>TTSuV1F</b>	71	50	26	CGA CCG GAG TCA AAT CTG ATT GGT [JOE] G	195-211
<b>TTSuV1R</b>	62	50	22	TAC TGG GAA CGC CCT AAT TCT G	259-281
<b>TTSuV2F</b>	69	50	28	CGG TTG AAC AGA GCT GAG TGT CTA AC[FAM] G	281-309
<b>TTSuV2R</b>	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.

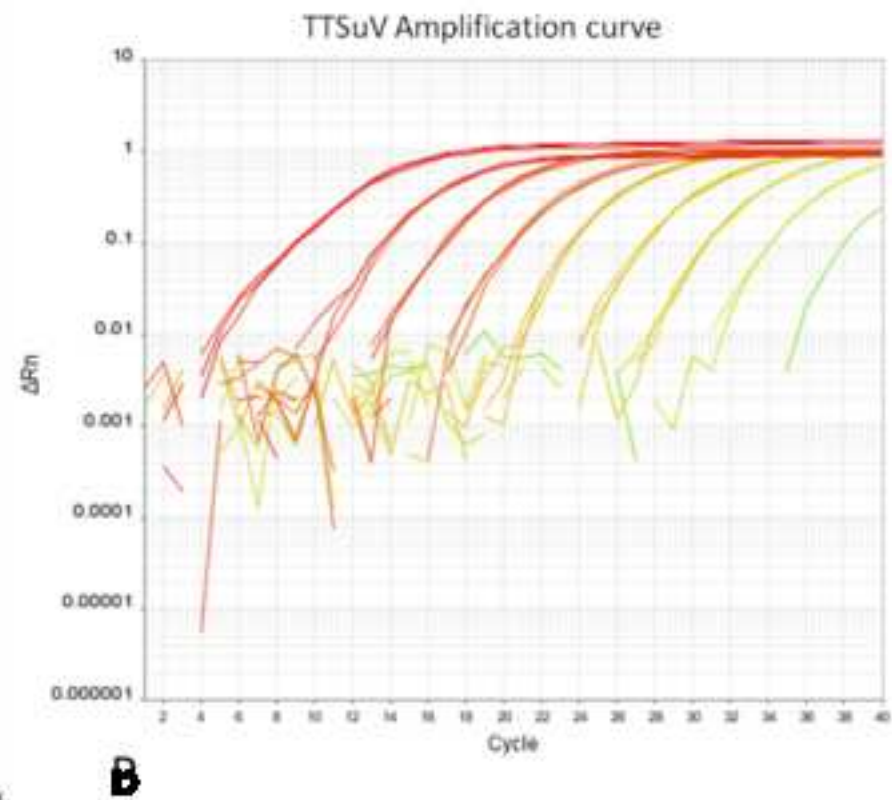
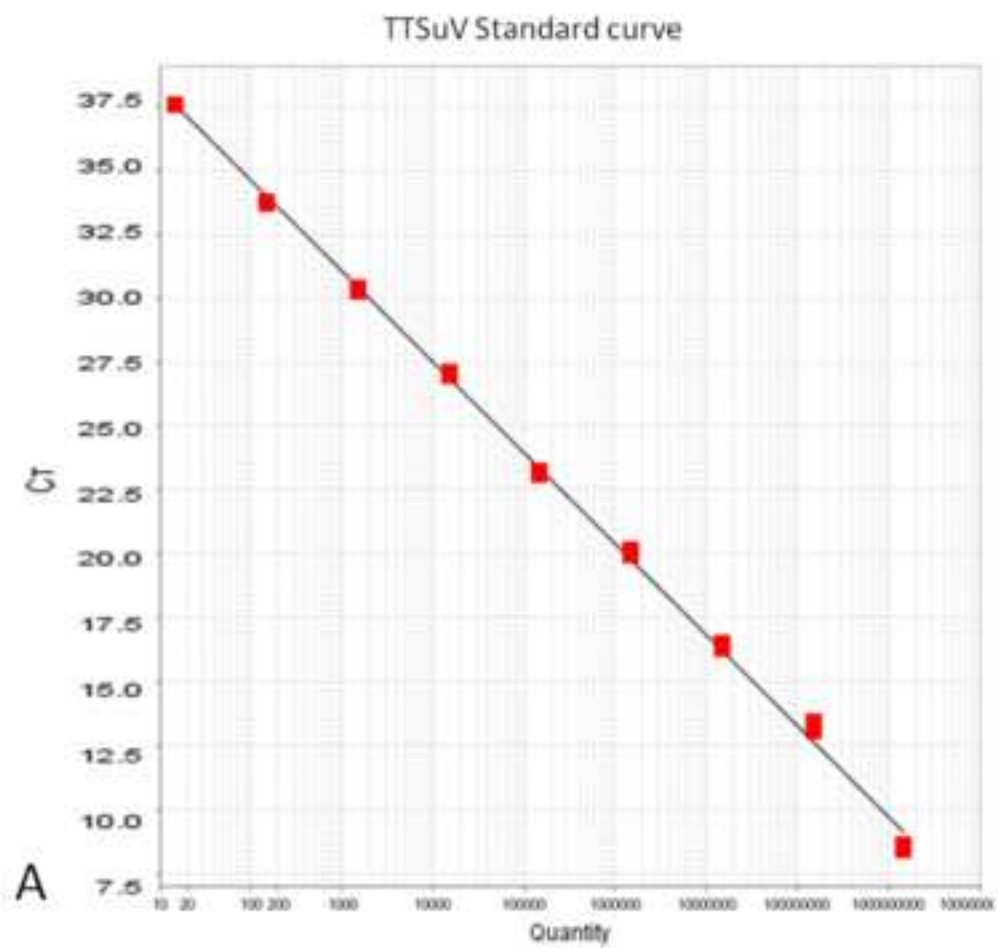
	Week 1		Week 3		Week 7		Week 11		Week N	
	TTSuV1	TTSuV2	TTSuV1	TTSuV2	TTSuV1	TTSuV2	TTSuV1	TTSuV2	TTSuV1	TTSuV2
<b>Healthy</b>	1/7 (14.3)	0/7 (0.0)	5/15 (33.3)	0/15 (0.0)*	10/17 (58.8)	8/17 (47.1)	13/17 (76.5)	12/17 (70.6) <sup>†</sup>	11/17 (64.7)	14/17 (82.4) <sup>†</sup>
<b>PMWS</b>	0/6 (0.0)	1/6 (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) <sup>†</sup>	13/18 (72.2)	18/18 (100) <sup>†</sup>
<b>Total</b>	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)

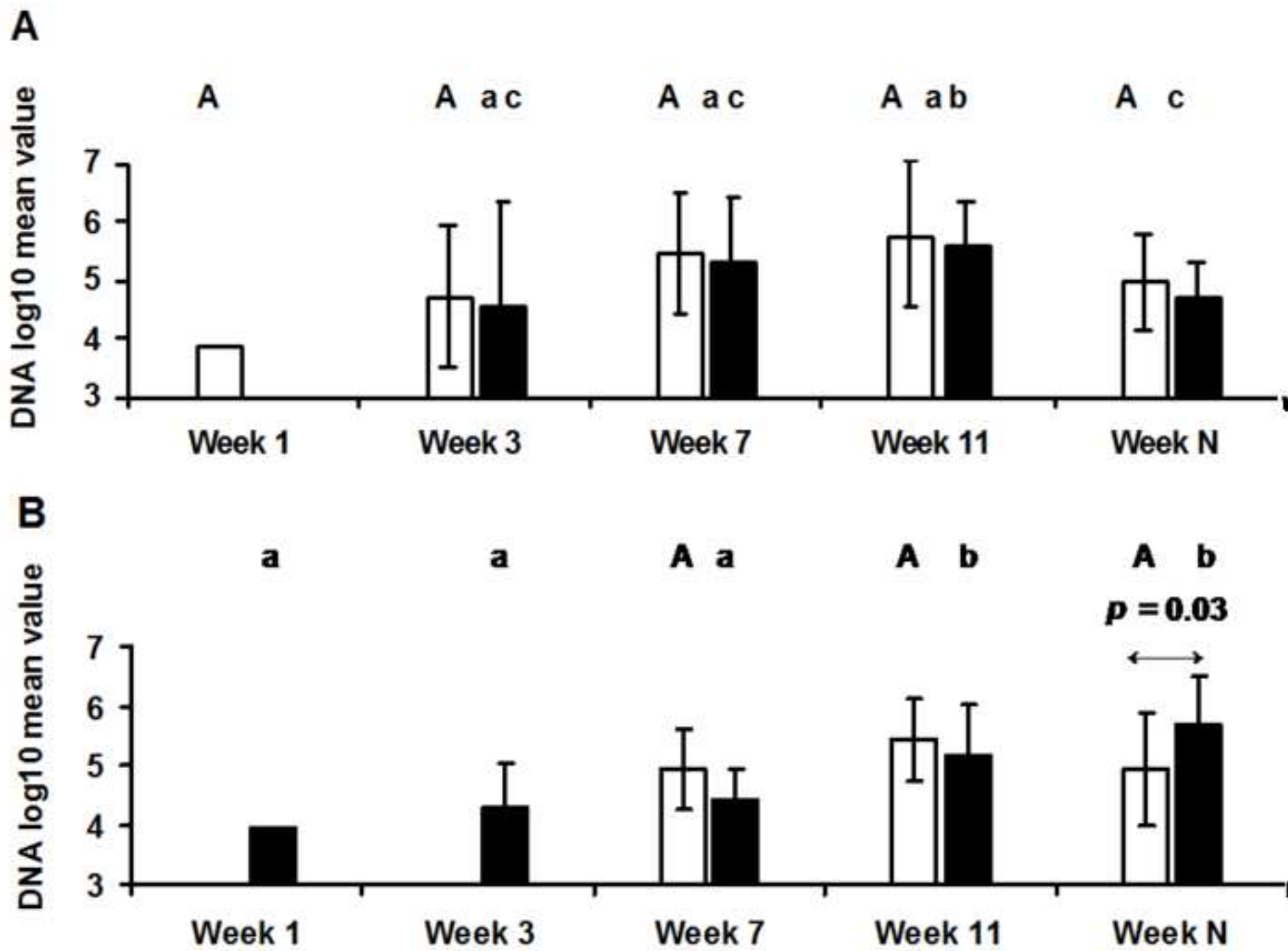
N: Date of necropsy.

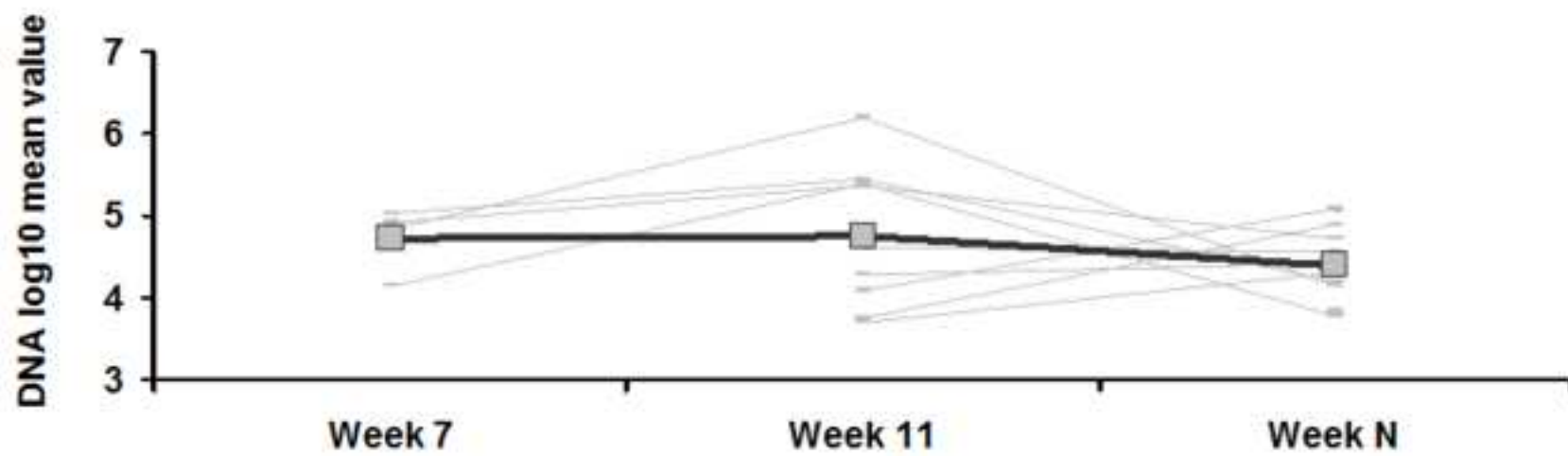
\* statistically significant differences between animal groups at a given age

t: tendency between animal groups at a given age

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**A****B**