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

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

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

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.

D. Nieto[a,1], M. Aramouni[a,1], L. Grau-Roma[a,b], J. Segalés[a,b], T. Kekarainen[a*].

[1] *Both authors contributed equally to the work*

[a] *Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.*

[b] *Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*

*** Corresponding author**

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

Campus de la Universitat Autònoma de Barcelona

08193 Bellaterra, Barcelona, Spain

Tel. +34935814620

Fax. +34935814490

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

Summary

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

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

1. Introduction

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

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

Currently, the disease causing potential of anelloviruses is under debate. Human TTVs are apparently related to liver and respiratory diseases, haematological disorders and cancer (Okamoto, 2009b). In pigs, it has been suggested that TTSuVs infection could be a factor of aggravation in co-infection with other pathogens, mainly *Porcine circovirus type 2* (PCV2). PCV2 is the essential but not sufficient cause of postweaning multisystemic wasting syndrome (PMWS), the economically most important porcine 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 ™ (LUX ™) 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 -80ºC 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-

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

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

2.2.3. Standards

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

2.2.4. Quantitative PCR reaction

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

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

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

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

2.3. Statistical Analysis

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

3. Results

3.1 Quantitative PCR optimization

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

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

3.2 Reproducibility, specificity and sensitivity of the method

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

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

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

3.3 Prevalence of TTSuV1 and TTSuV2 in serum samples

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

3.4 TTSuV1 and TTSuV2 viral DNA load kinetics

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

Two different infection dynamics profiles were generated for each TTSuV species by the MeV software (figure 3). For TTSuV2, profile 1 included 16 pigs (11 healthy, 5 PMWS) on average with decreasing viral DNA loads throughout the study. At the final point (necropsy time), all the animals had mean viral loads below 5 $\log_{10}$. In the profile 2, 19 pigs (6 healthy, 13 PMWS) were included, which showed increasing viral loads with mean viral load at necropsy above 6 $\log_{10}$. The percentage of healthy and PMWS affected animals within each profile was statistically different ($p=0.03$). TTSuV1 profiles did not differ from a statistical point of view (data not shown).

4. Discussion

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

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

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

Few studies on TTV viral load have been published in humans. It has been shown that HIV-infected patients have higher TTV viremia and there is an association with decreased survivability when compared with healthy blood donors (Christensen et al., 2000). Another study suggested that TTV viremia is associated with the level of 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 no 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 myelosupressed leukaemia patients undergoing hematopoietic stem cell transplantation showed that during the immunesuppression 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 kynetics 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.

5. Acknowledgements

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

6. References

Allan, GM., Ellis, J.A., 2000. Porcine circoviruses: a review. J. Vet. Diagn. Invest. 12, 3-14.

Aramouni, M., Segales, J., Cortey, M., Kekarainen, T., 2010, Age-related tissue distribution of swine Torque teno sus virus 1 and 2. Vet Microbiol 146, 350-353.

Burra, P., Masier, A., Boldrin, C., Calistri, A., Andreoli, E., Senzolo, M., Zorzi, M., Sgarabotto, D., Guido, M., Cillo, U., Canova, D., Bendinelli, M., Pistello, M., Maggi, F., Palu, G., 2008, Torque Teno virus: any pathological role in liver transplanted patients? Transpl Int.

Cortey, M.M., L. Segalés, J. Kekarainen, T., 2010, Genetic variability and phylogeny of Torque teno sus virus 1 (TTSuV1) and 2 (TTSuV2) based on complete genomes. Vet. Microbiol. 2010 Aug 22. [Epub ahead of print].

Ellis, J.A., Allan, G., Krakowka, S., 2008, Effect of coinfection with genogroup 1 porcine torque teno virus on porcine circovirus type 2-associated postweaning multisystemic wasting syndrome in gnotobiotic pigs. Am. J. Vet. Res. 69, 1608-1614.

Grau-Roma, L., Hjulsager, C.K., Sibila, M., Kristensen, C.S., López-Soria, S., Enoe, C., Casal, J., Botner, A., Nofrarias, M., Bille-Hansen, V., Fraile, L., Baekbo, P., Segalés, J., Larsen, L.E., 2009, Infection, excretion and seroconversion dynamics of porcine circovirus type 2 (PCV2) in pigs from post-weaning multisystemic wasting syndrome (PMWS) affected farms in Spain and Denmark. Vet. Microbiol. Accepted.

337 Huang, Y.W., Ni, Y.Y., Dryman, B.A., Meng, X.J., 2010, Multiple infection of porcine
338 Torque teno virus in a single pig and characterization of the full-length genomic
339 sequences of four U.S. prototype PTTV strains: Implication for genotyping of
340 PTTV. Virology 396, 289-297.
341 Kekarainen, T., López-Soria, S., Segalés, J., 2007, Detection of swine Torque teno virus
342 genogroups 1 and 2 in boar sera and semen. Theriogenology 68, 966-971.
343 Kekarainen, T., Segalés, J., 2009, Torque teno virus infection in the pig and its potential
344 role as a model of human infection. Vet. J. 180, 163-168.
345 Kekarainen, T., Sibila, M., Segalés, J., 2006, Prevalence of swine Torque teno virus in
346 post-weaning multisystemic wasting syndrome (PMWS)-affected and non-
347 PMWS-affected pigs in Spain. J. Gen. Virol. 87, 833-837.
348 Krakowka, S., Hartunian, C., Hamberg, A., Shoup, D., Rings, M., Zhang, Y., Allan, G.,
349 Ellis, J.A., 2008, Evaluation of induction of porcine dermatitis and nephropathy
350 syndrome in gnotobiotic pigs with negative results for porcine circovirus type 2.
351 Am. J. Vet. Res. 69, 1615-1622.
352 Lee, S.-s., Sunyoung, S., Jung, H., Shin, J., Lyoo, Y.S., 2010, Quantitative detection of
353 porcine Torque teno virus in Porcine circovirus-2-negative and Porcine
354 circovirus-associated disease-affected pigs. J Vet Diagn Invest 22, 261-264.
355 Martínez-Guinó, L., Kekarainen, T., Segalés, J., 2009, Evidence of Torque teno virus
356 (TTV) vertical transmission in swine. Theriogenology 71, 1390-1395.
357 Nishizawa, T., Okamoto, H., Konishi, K., Yoshizawa, H., Miyakawa, Y., Mayumi, M.,
358 1997, A Novel DNA Virus (TTV) Associated with Elevated Transaminase
359 Levels in Posttransfusion Hepatitis of Unknown Etiology. Biochemical and
360 Biophysical Research Communications 241, 92-97.
361 Okamoto, H., 2009a, History of discoveries and pathogenicity of TT viruses. Curr Top
362 Microbiol Immunol 331, 1-20.

363 Okamoto, H., 2009b, History of discoveries and pathogenicity of TT viruses. Curr Top
364 Microbiol Immunol. 2009;331:1-20.
365 Okamoto, H., Fukuda, M., Tawara, A., Nishizawa, T., Itoh, Y., Hayasaka, I., Tsuda, F.,
366 Tanaka, T., Miyakawa, Y., Mayumi, M., 2000, Species-Specific TT Viruses and
367 Cross-Species Infection in Nonhuman Primates. J. Virol. 74, 1132-1139.
368 Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa,
369 M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezantsev, A., Popov,
370 D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavich, A., Trush,
371 V., Quackenbush, J., 2003, TM4: a free, open-source system for microarray data
372 management and analysis. Biotechniques 34, 374-378.
373 Segalés, J., Allan, G.M., Domingo, M., 2005, Porcine circovirus diseases. Anim Health
374 Res Rev 6, 119-142.
375 Sibila, M., Martínez-Guinó, L., Huerta, E., Llorens, A., Mora, M., Grau-Roma, L.,
376 Kekarainen, T., Segalés, J., 2009a, Swine torque teno virus (TTV) infection and
377 excretion dynamics in conventional pig farms. Veterinary Microbiology 139,
378 213-218.
379 Sibila, M., Martínez-Guinó, L., Huerta, E., Mora, M., Grau-Roma, L., Kekarainen, T.,
380 Segalés, J., 2009b, Torque teno virus (TTV) infection in sows and suckling
381 piglets. Veterinary Microbiology 137, 354-358.
382 Taira, O., Ogawa, H., Nagao, A., Tuchiya, K., Nunoya, T., Ueda, S., 2009, Prevalence
383 of swine Torque teno virus genogroups 1 and 2 in Japanese swine with
384 suspected post-weaning multisystemic wasting syndrome and porcine
385 respiratory disease complex. Vet Microbiol 139, 347-350.
386 Thom, K., Petrik. J., 2007. Progression towards AIDS leads to increased Torque teno
387 virus and Torque teno minivirus titers in tissues of HIV infected individuals. J.
388 Med. Virol. 79, 1-7.

389 Touinssi, M., Gallian, P., Biagini, P., Attoui, H., Vialettes, B., Berland, Y., Tamalet, C.,
390 Dhiver, C., Ravaux, I., De Micco, P., De Lamballerie, X., 2001. TT virus
391 infection: Prevalence of elevated viraemia and arguments for the immune
392 control of viral load. J. Clin. Virol. 21, 135-141
393

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

Figure 2: TTSuV1 (A) and TTSuV2 (B) viral load dynamics in healthy (white bars) and PMWS (black bars) affected animals. Mean viral loads and standard deviation at different sampling times are represented in log_{10} scale. P-values for significant differences are shown. Different letters mean significant differences between ages within healthy (capital letters) or PMWS (case letters) groups.

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

Table 1: TTV1 and TTV2 LUX primer characteristics.

Primer	Tm (ºC)	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

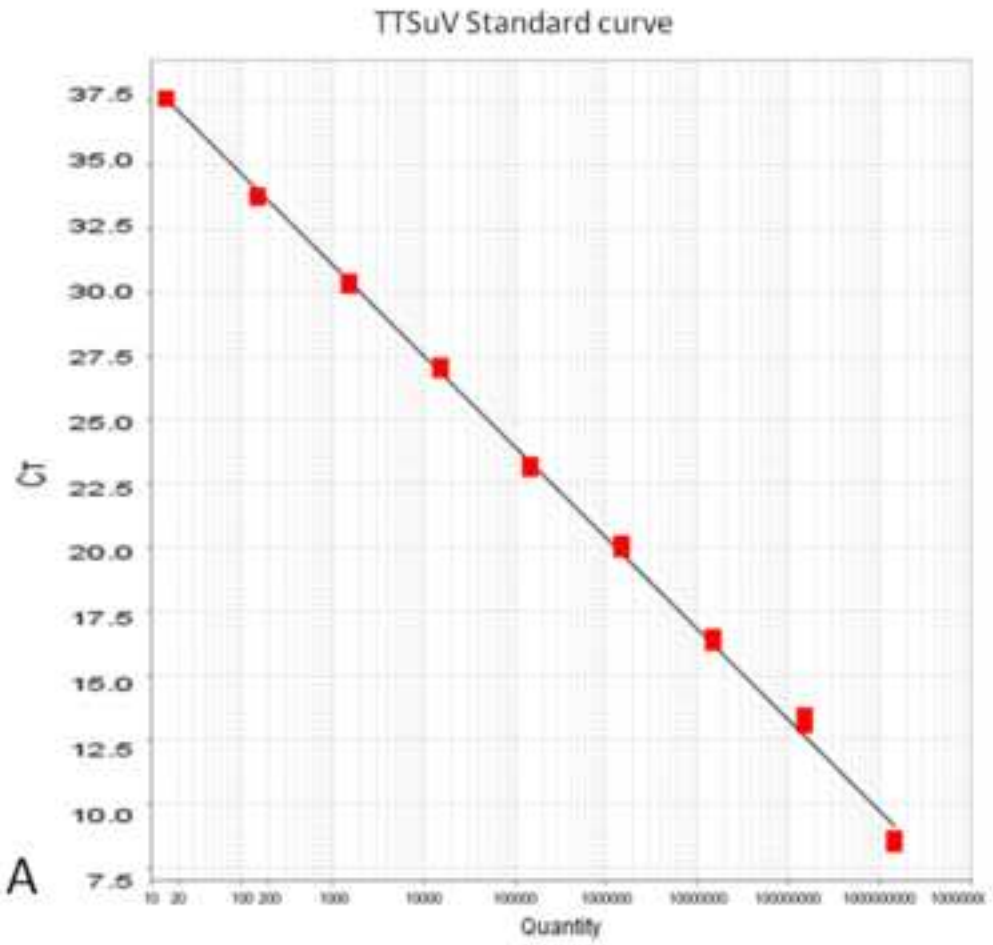
Table 2: Prevalence of TTSuVs at different weeks of age, expressed as qPCR positives/total serum samples studied and percentage of positives shown in parentheses.

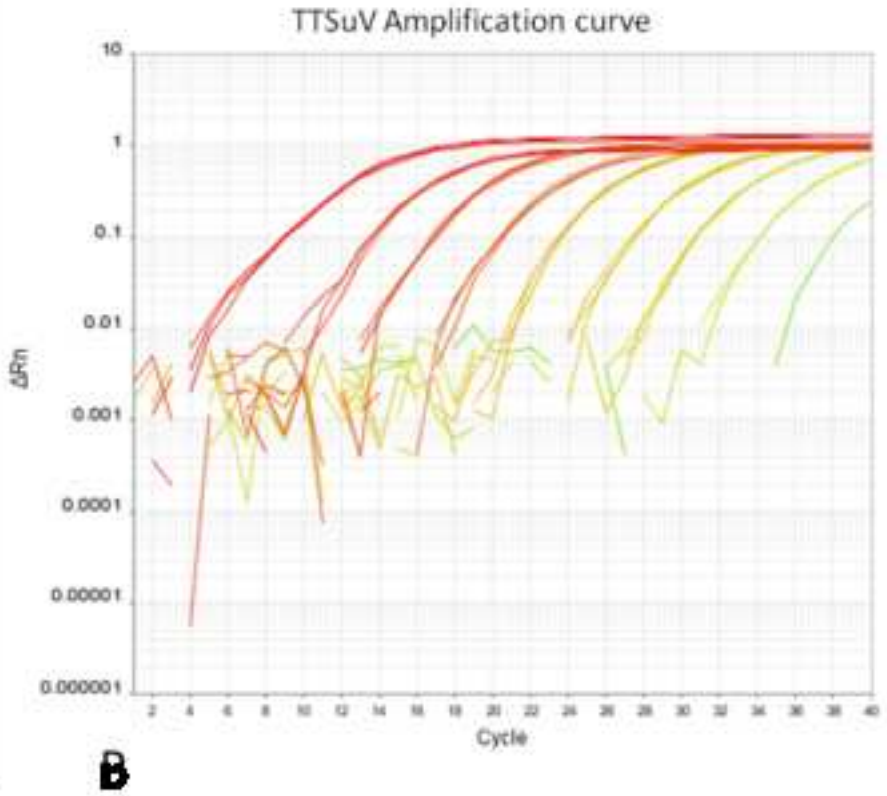
	Week 1		Week 3		Week 7		Week 11		Week N	
	TTSuV1	**TTSuV2**	**TTSuV1**	**TTSuV2**	**TTSuV1**	**TTSuV2**	**TTSuV1**	**TTSuV2**	**TTSuV1**	**TTSuV2**
Healthy	1/7 (14.3)	0/7 (0.0)	5/15 (33.3)	0/15 (0.0)*	10/17 (58.8)	8/17 (47.1)	13/17 (76.5)	12/17 (70.6)t	11/17 (64.7)	14/17 (82.4)t
PMWS	0/6 (0.0)	1/6 (16.7)	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)

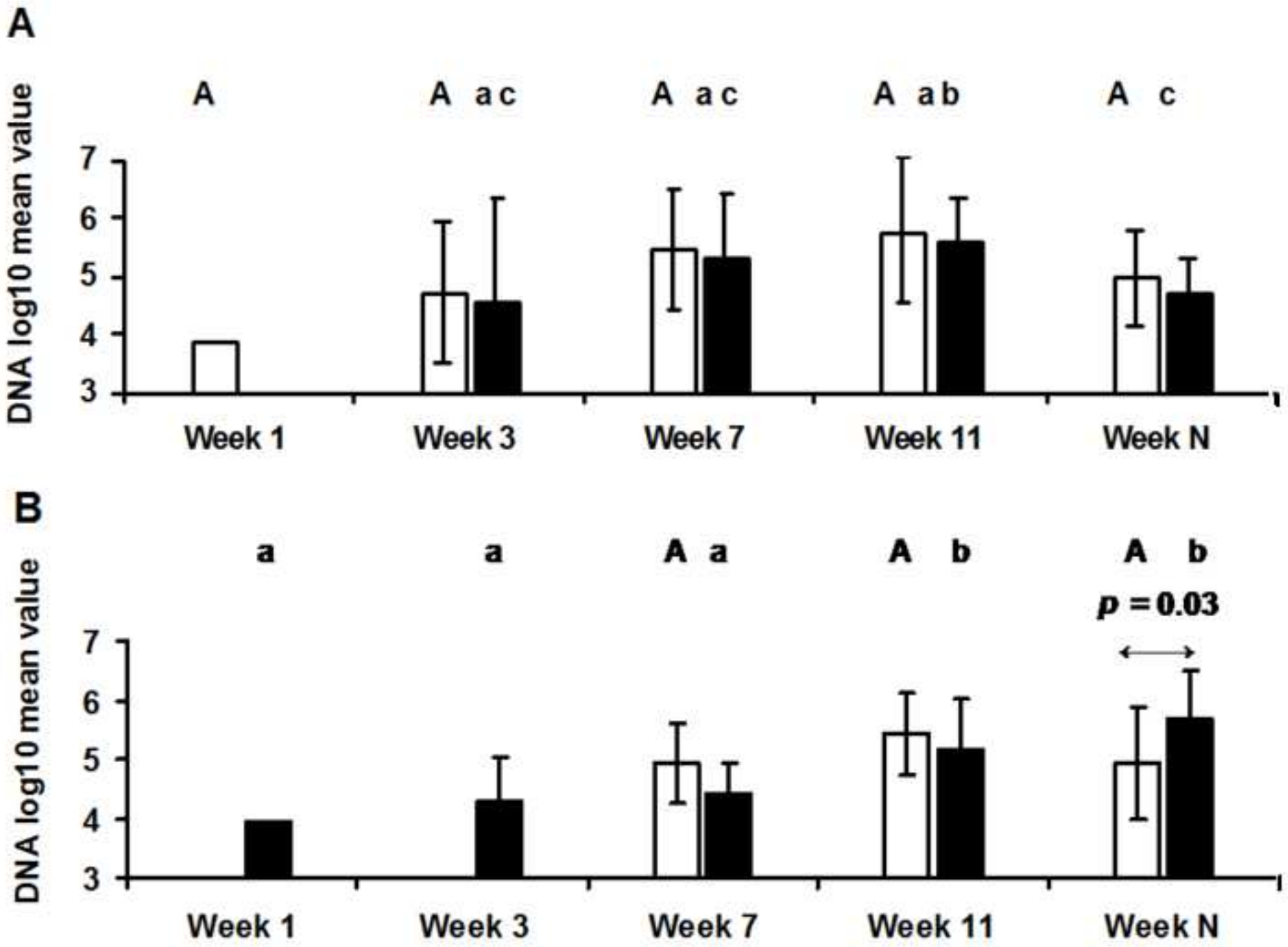
N: Date of necropsy.

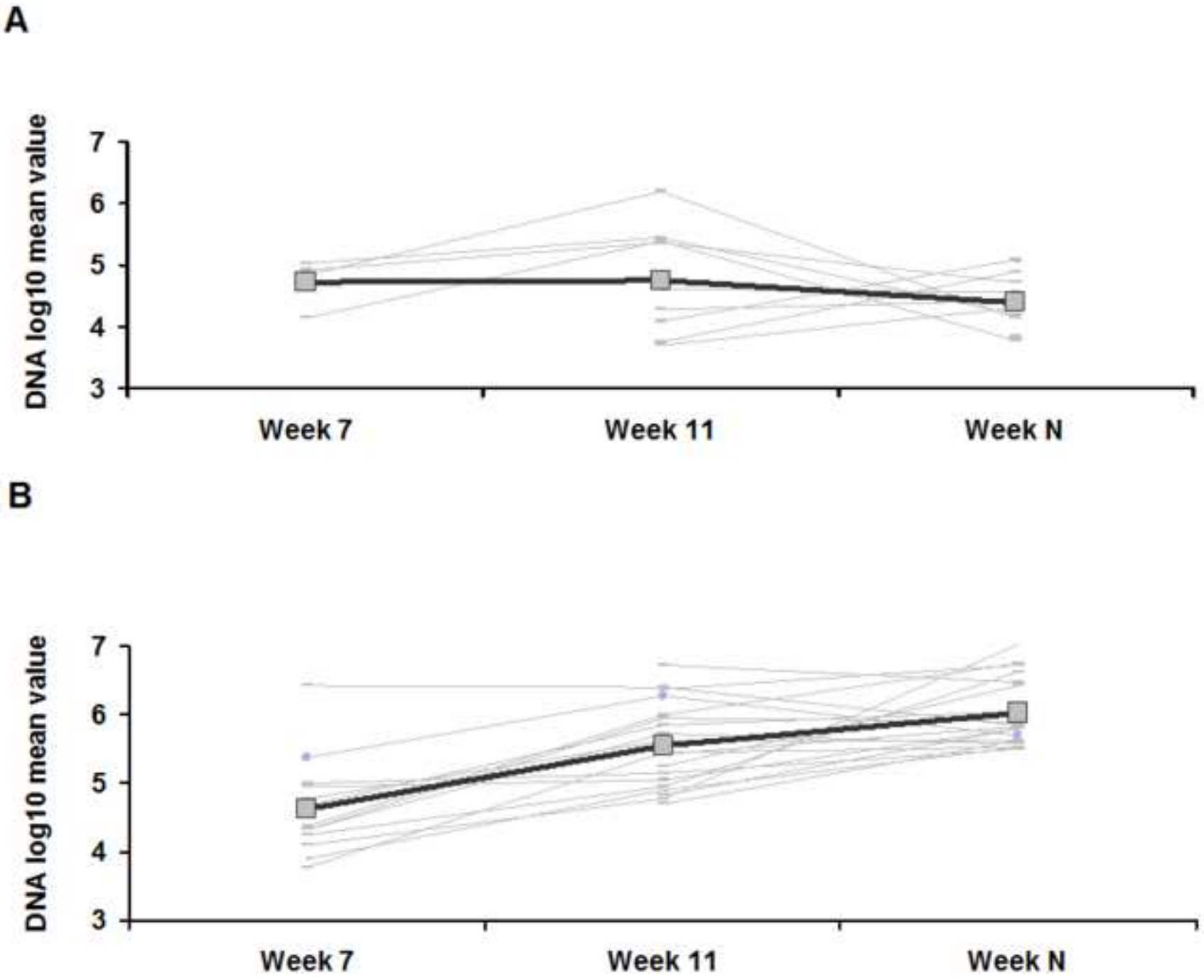
* statistically significant differences between animal groups at a given age

t: tendency between animal groups at a given age


TTSuV Standard curve
37.5
35.0
32.5
30.0
27.5
25.0
22.5
20.0
17.5
15.0
12.5
10.0
7.5
Ct
10 20
100 200
1000
10000
100000
1000000
10000000
100000000
1000000000
Quantity
A


TTSuV Amplification curve
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
ΔRn
Cycle
B


A
A
A ac
A ac
A ab
A c
DNA log10 mean value
7
6
5
4
3
Week 1
Week 3
Week 7
Week 11
Week N
B
a
a
A a
A b
A b
p = 0.03
DNA log10 mean value
7
6
5
4
3
Week 1
Week 3
Week 7
Week 11
Week N


A
DNA log10 mean value
7
6
5
4
3
Week 7
Week 11
Week N
B
DNA log10 mean value
7
6
5
4
3
Week 7
Week 11
Week N