Torque teno sus viruses: pathogenesis in co-infection with *Porcine circovirus type 2* and humoral immune responses during natural infection of pigs

Memòria presentada per **David Nieto Blanco**

per optar al grau de Doctor en Medicina i Sanitat Animals

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IRTA RECERCA I TECNOLOGIA AGROALIMENTÀRIES

CReSA Centre de Recerca en Sanitat Animal

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Doctoral thesis UAB - 2015

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La Dra. TUIJA KEKARAINEN, investigadora del Centre Recerca en Sanitat Animal (CReSA), i el Dr. JOAQUIM SEGALÉS COMA, professor titular del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona i investigador del CReSA,

Certifiquen:

Que la memòria de tesi doctoral titulada "*Torque teno sus viruses*: pathogenesis in co-infection with *Porcine circovirus type 2* and humoral immune responses during natural infection of pigs" presentada per David Nieto Blanco per l'obtenció del grau de Doctor en Medicina i Sanitat Animals, s'ha realitzat sota la nostra direcció al Centre de Recerca en Sanitat Animal (CReSA).

I per tal de que consti, als efectes oportuns, signem el present certificat a Bellaterra, a 16 de Setembre de 2015.

Els directors

Dra. Tuija Kekarainen

Dr. Joaquim Segalés i Coma

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CONTENTS

ABSTRACT

Torque teno sus viruses (TTSuVs) belong to the family *Anelloviridae* and they infect swine and wild boar. Currently, TTSuVs infecting swine are divided into two separated genera, *Iotatorquevirus* for TTSuV1, including species: TTSuV1a and TTSuV1b, and *Kappatorquevirus* for TTSuV2, including species TTSuVk2a and TTSuVk2b. Both genera are characterized by similar genomic organization and high genomic variability.

The impact of TTSuVs infection for the host is under discussion, since TTSuVs have been detected in high prevalence in healthy and diseased swine populations. Main described transmission routes are horizontal and vertical; nevertheless, the parenteral route is also possible due to the presence of TTSuVs contaminating veterinary vaccine products.

In the infected host, TTSuVs are able to infect a variety of tissues and organs. However, the pathogenic role of TTSuVs is not yet clear. It is assumed they can be associated with other well-known swine pathogens, potentially worsening the prognosis of the disease. One of the most studied and harmful viruses in pig production is *Porcine circovirus type 2* (PCV2), which is the essential cause of PCV2-systemic disease (PCV2-SD), a devastating disease for the swine industry. In fact, TTSuVs were linked to PCV2-SD triggering, based on the higher prevalence of TTSuVs observed in pigs suffering from PCV2-SD when compared with healthy counterparts. However, the high prevalence of TTSuVs in the swine population, the high genetic variability of TTSuVs, combined with high prevalence of other swine infecting viral pathogens constitute a hindrance in the understanding of the pathogenic role associated to TTSuVs infection. At the same time, the lack of effective diagnostic tools has been a barrier in the understanding of TTSuVs role or biology, as diagnosis of TTSuVs infection has been mainly based on polymerase chain reaction (PCR) detection.

The studies carried out in this Thesis aimed to contribute to the understanding of the role played by TTSuVs in the pig. To go further into the study of TTSuVs, it was necessary to develop effective tools to study the epidemiology of TTSuVs.

In the first study, the dynamics of TTSuV1 and TTSuV2 loads was studied. For this, serum TTSuV loads of pigs affected by PCV2-SD were compared with appropriate healthy control animals. Such study was carried out by means of a newly developed real-time quantitative PCR (qPCR) method. Results from this study showed that TTSuVs prevalence was high in all studied pigs. TTSuV2 viral load was significantly higher in PCV2-SD affected pigs. Such difference was not observed for TTSuV1. Importantly, it was observed that early TTSuV2 infected pigs were more prone to develop PCV2-SD; on the contrary, such correlation was not observed for TTSuV1. Altogether, obtained data reinforced the hypothesis of the association between TTSuVs infection and PCV2-SD development.

In the second study, TTSuV1 and TTSuV2 loads were investigated in tissues of 20 pigs (10 healthy and 10 PCV2-SD affected pigs). For each pig a total of 7 different tissues were analysed, including lung, kidney, liver, ileum, bone marrow, and mesenteric and mediastinal lymph nodes. TTSuV1 and TTSuV2 tissue loads were quantified by the previously developed qPCR. TTSuV2 load was significantly higher in tissues of PCV2-SD affected pigs when compared with healthy counterparts and with TTSuV1 load. For TTSuV2, the highest viral load was observed in bone marrow, mediastinal lymph node and liver, while it was bone marrow, lung and liver for TTSuV1. Regardless of the health status, bone marrow contained the highest viral load. It was observed that TTSuV2 loads in tissues were significantly higher than TTSuV1 tissue loads. Moreover, TTSuV2 tissue load in PCV2-SD affected pigs was significantly higher than TTSuV2 load in tissues of healthy pigs. Finally, TTSuVs had a wide range of tissue distribution, so, it is very likely that other tissues not included in this study would also be infected by TTSuVs.

In the third study, Enzyme-Linked ImmunoSorbent Assay (ELISA) assays to detect antibodies against TTSuVs were developed based on the open reading frame (ORF) 1-A recombinant protein of both viruses. Concomitantly, the viral loads in the same examined sera were quantified using the developed qPCR. The ELISA assay was used to study the development of the humoral immune response against TTSuV1 and TTSuV2 in longitudinally sampled clinically healthy pigs and their dams. Anti-ORF1-A IgGs were found in serum of pigs and sows for both TTSuVs. In case of TTSuV1, a high percentage of seropositive pigs were detected at 4 weeks of age; on the contrary, for TTSuV2, percentage of seropositive pigs at the same age was very low. It was concluded that, pigs

are able to mount a humoral immune response against TTSuVs. However, based on the high prevalence of viremic pigs in the presence of anti ORF1-A IgGs, it was suggested that these antibodies are not able to remove TTSuVs from blood circulation.

RESUMEN

Los *Torque teno sus viruses* pertenecen a la familia *Anelloviridae*, e infectan tanto a cerdos como a jabalíes. En la actualidad, los TTSuVs se dividen en dos géneros, *Iotatorquevirus* para los TTSuV1, que incluye las especies: TTSuV1a y TTSuV1b; y *Kappatorquevirus* para los TTSuV2, que incluye las especies: TTSuVk2a y TTSuVk2b. Ambos géneros se caracterizan por tener una organización similar del genoma y por una gran variabilidad genética.

Las consecuencias de la infección de los TTSuVs sobre su hospedador no son del todo claras. Ambos géneros se detectan con una prevalencia muy alta, tanto en poblaciones de cerdos sanos como enfermos. El virus se transmite principalmente tanto por vía horizontal como vertical; sin embargo, la ruta parenteral también sería posible, ya que se ha detectado la presencia de TTSuVs contaminando productos vacunales veterinarios.

Tras la infección, los TTSuVs se distribuyen por la mayoría de los tejidos y órganos del cerdo. Aunque en la actualidad el papel patogénico de los TTSuVs aún no está claro, se cree que estos virus pueden agravar el curso de la enfermedad en asociación con otros patógenos comunes de los cerdos domésticos. Entre los virus concomitantes con los que se asocian destaca el *Circovirus porcino tipo 2* (PCV2), responsable de la circovirosis porcina (CP), una enfermedad con consecuencias devastadoras en la población porcina. La relación existente entre los TTSuV y el PCV2 se basa en la mayor prevalencia de los TTSuVs en cerdos afectados por CP comparada con la de cerdos sanos. Sin embargo, la alta prevalencia de los TTSuVs en la población porcina y su alta variabilidad genética, combinados con una alta prevalencia de otros virus que también infectan a los cerdos, constituyen varios obstáculos en la comprensión del papel patogénico asociado a la infección por los TTSuVs. Al mismo tiempo, la falta de herramientas de diagnóstico eficaces ha sido una barrera en el progreso del conocimiento de los TTSuVs. El diagnóstico de la infección de los TTSuV se ha basado principalmente en la detección mediante la técnica de la PCR.

El objetivo de los estudios realizados en esta Tesis fue el de contribuir a la comprensión del papel que desempeñan los TTSuVs en la infección del cerdo. Para poder llevarlo a cabo

fue necesario desarrollar técnicas de diagnóstico eficaces para estudiar la epidemiología de TTSuVs.

En el primer estudio se investigó la dinámica de la infección y la carga viral en suero de los TTSuV1 y TTSuV2. Se seleccionaron sueros de cerdos afectados de CP y, se compararon los valores de carga viral y prevalencia en sueros de cerdos sanos criados en condiciones equivalentes.

Para poder llevar a cabo el estudio, previamente se puso a punto una técnica de PCR cuantitativa a tiempo real (qPCR). Se observó que la prevalencia de los TTSuVs era alta en los dos grupos de cerdos estudiados y para los dos virus. En el caso del TTSuV2, la carga viral fue significativamente mayor en los cerdos afectados por la CP. Tal diferencia no se observó en el caso del TTSuV1. También cabe destacar que los cerdos infectados por el TTSuV2 a edades más tempranas (semanas 1 y 3 de vida) fueron más propensos a desarrollar la CP. Por el contrario, dicha correlación no se observó para TTSuV1. En conjunto, los resultados obtenidos refuerzan la hipótesis de la asociación entre la infección por los TTSuVs y el desarrollo de la CP.

En el segundo estudio se investigaron las cargas virales tanto del TTSuV1 como del TTSuV2 en tejidos de 20 cerdos (divididos en dos grupos: 10 sanos y 10 afectados por CP). De cada cerdo se investigaron un total de 7 tejidos, incluyendo pulmón, riñón, hígado, íleon, médula ósea, y los nódulos linfáticos mesentéricos y mediastínicos. La determinación de las cargas de TTSuV1 y TTSuV2 en tejidos se llevó a cabo usando la qPCR descrita anteriormente. Las cargas del TTSuV2 fueron significativamente mayores en los tejidos procedentes de cerdos afectados por la CP que en sus homólogos sanos y que las cargas virales del TTSuV1 para ambos grupos de cerdos. Las mayores cargas del TTSuV2 se observaron en la médula ósea, los nódulos linfáticos mediastínicos y en el hígado. Las mayores cargas del TTSuV1 se detectaron en la médula ósea, pulmón e hígado. Independientemente del estado de salud, la médula ósea fue el tejido donde se observaron las cargas virales más altas. En este estudio se concluyó que las cargas de TTSuV2 fueron significativamente mayores que las cargas de TTSuV1 en los tejidos analizados. Además, las cargas de TTSuV2 en tejidos de cerdos afectados por la CP fueron significativamente mayor que las cargas de TTSuV2 en los mismos tejidos procedentes de

cerdos sanos. Por último, los TTSuVs se hallaron en un gran número de tejidos diferentes, e incluso es muy probable que otros tejidos que no se incluyeron en este estudio también pudieran estar infectados por TTSuVs.

En el tercer estudio se desarrolló una técnica serológica de ELISA basada en la detección de la proteína recombinante ORF1-A de los TTSuVs. Al mismo tiempo, la carga de los TTSuVs en suero se cuantificó usando la qPCR desarrollada previamente. El ensayo ELISA se usó para estudiar el desarrollo de la respuesta inmune humoral frente a TTSuV1 y TTSuV2. Para ello, se realizó un estudio longitudinal en cerdos clínicamente sanos y en sus madres. Se detectaron IgGs anti ORF1-A en las muestras de suero, tanto frente al TTSuV1 como al TTSuV2. En el caso de TTSuV1, se observó una gran prevalencia de cerdos seropositivos en la semana 4 de vida; por el contrario, para el TTSuV2, el porcentaje de cerdos seropositivos en esa semana fue muy bajo. Se concluyó que los cerdos son capaces de desarrollar una respuesta inmune frente a la infección por los TTSuVs; sin embargo, la alta prevalencia de cerdos virémicos en presencia de IgGs anti ORF1-A sugiere que los anticuerpos no son capaces de eliminar los TTSuVs de la sangre.

LIST OF ABBREVIATIONS

α	alpha
BM	bone marrow
BP	bronchopneumonia
Can	chloramphenicol
CAV	*Chicken anaemia virus*
CV	coefficient of variation
E	amplification efficiency
ELISA	Enzyme-Linked ImmunoSorbent Assay
GC	guanine-cytosine
H	healthy group
HCV	*Hepatitis C virus*
HEV	*Hepatitis E virus*
HIS	histidine
HIV	*Human immunodeficiency virus*
H. pilori	*Helicobacter pilori*
HRP	horseradish peroxidase
HVR	hypervariable region
FAM™	6-carboxy-fluorescein
ICTV	International Committee for the Taxonomy of Viruses
IFN	interferon
IL	ileum
IPTG	β-D-1-thiogalactopyranoside
ISH	*in situ* hybridization
IPF	idiopathic pulmonary fibrosis
JOE™	6-carboxy-dichloro-dimethoxy-fluorescein
K	kidney
Kan	kanamycin
Kb	kilobases
KDa	kilodalton
LB	Luria-Bertani
LI	liver

LMED	mediastinal lymph nodes
LMES	mesenteric lymph node
LU	lung
LUX	Light Upon eXtension
MDA	maternally derived antibodies
MeV	multiple experiment viewer software
miRNAs	micro RNAs
NF-κB	nuclear factor kappa beta
NMI	N-Myc interactors
nt	nucleotide
OD	optical density
o/n	overnight
ORF	open reading frame
P	PCV2-SD group
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PBoV	*Porcine bocavirus*
PCR	Polymerase chain reaction
PCVD	porcine circovirus disease
PCV1	porcine circovirus type 1
PCV2a	PCV2 genotype “a”
PCV2b	PCV2 genotype “b”
PCV2	*Porcine circovirus type 2*
PCV2-SD	PCV2-systemic disease
PDNS	porcine dermatitis and nephropathy syndrome
PEDV	*Porcine epidemic diarrhoea virus*
PK	porcine kidney
PNP	proliferative necrotizing pneumonia
PPV	*Porcine parvovirus*
PRDC	porcine respiratory disease complex
PRRSV	*Porcine respiratory and reproductive syndrome virus*
PToV	*Porcine torovirus*

R	correlation coefficient
R^2	coefficient of determination
RCA	rolling circle amplification
SIV	*Swine influenza virus*
SLE	systemic lupus erythematosus
S/P	sample to positive ratio
TBS	Tris buffered saline
TMB	tetramethylbenzine
TR	coding regions
T-TBS	Tris buffered saline with tween 20
TTDMV	*Torque teno midi virus*
TTMV	*Torque teno mini virus*
TTSuV	*Torque teno sus virus*
TTSuV1F	TTSuV1 forward primer
TTSuV1R	TTSuV1 reverse primer
TTSuV2F	TTSuV2 forward primer
TTSuV2R	TTSuV2 reverse primer
qPCR	real-time quantitative PCR
REP	replication associated protein
RCR	rolling circle replication
SD	standard deviation
ssDNA	single stranded DNA
UTR	untranslated region
WB	Western blot

CHAPTER I: INTRODUCTION

1. Introduction

1.1 Emerging pathogens

1.1.1 Importance of viruses in the process of emerging diseases

Emerging pathogens affecting humans, pigs or other vertebrate species are frequently reported. These emerging pathogens concern the public health sector; in most of the cases the impact on health is low, but the economic impact is usually high. Most of the novel pathogens described in the last decades are viruses. Between 1980 and 2001, from 87 discovered emerging pathogens infecting humans, a total of 58 (67%) were viruses (Woolhouse et al., 2001). In the same way, a study on pig pathogens described at least 77 novel emerging viral species since 1985 (Fournie et al., 2012). Emerging swine viruses described in the last decades include members of a variety of families like *Anelloviridae, Arteriviridae, Astroviridae, Caliciviridae, Circoviridae, Coronaviridae, Flaviviridae, Herpesviridae, Hepeviridae, Paramyxoviridae, Parvoviridae,* and *Picornaviridae* (Meng, 2012).

Some of these new emerging viruses are associated with disease in pigs, causing huge economic and health impacts in the swine population. Viruses included in this group are *Porcine respiratory and reproductive syndrome virus* (PRRSV), family *Arteriviridae*; *Porcine circovirus type 2* (PCV2), family *Circoviridae*; and *Porcine epidemic diarrhoea virus* (PEDV), family *Coronaviridae* (Baron et al., 1992; Segalés et al., 2005; Stevenson et al., 2013). Additionally, these viruses may produce re-emerging episodes due to highly virulent strains or due to new mutants with low identity with vaccine strains (Opriessnig et al., 2013; Song et al., 2014a; Zhou and Yang, 2010). Other pathogens have questionable clinical significance, like: *Torque teno sus virus* (TTSuV) (Leary et al., 1999), family *Anelloviridae*; *Porcine bocavirus* (PBoV), family *Parvoviridae* (Lau et al., 2008); and *Porcine torovirus* (PToV), family *Coronaviridae* (Kroneman et al., 1998). Apart from these viruses, there are other viruses with a zoonotic potential, including: *Hepatitis E virus* (HEV), family *Hepeviridae*; *Nipah virus,* family *Paramyxoviridae;* or *Norovirus*, family *Caliciviridae* (Meng, 2012; Song et al., 2014b; Terebuh et al., 2010).

1.1.2 Factors associated to the emergence of new viruses

The evolution of emerging infections towards overt disease is associated to two main factors: genetic factors, associated either to the virus or to the host, and ecological factors, unrelated to the host-pathogen interaction and considered as extrinsic factors. Altogether, it is known as the "host-agent-environment triangle" and is considered as the standard causal model by some researchers (Davies, 2012)

A) Genetic factors.

Linked to the virus. The host should prevent viral replication to avoid persistent infections or to prevent the viruses executing their pathogenicity. At the same time, viruses evolve to evade or overcome the host defences (Cann, 2005) by means of several mechanisms, including single point mutations of the genetic material, genome rearrangements, as well as recombination and translocation events (Witzany, 2006). Such evolution mechanisms affect the adaptability of the virus to the host. Genetic variability helps viruses to jump species boundaries, increase the likelihood to recognize a specific cell receptor of the host or a shift in receptor usage for virus entry, or render ineffective antiviral host response (Baranowski et al., 2001). In consequence, this new variants may have a higher capacity to establish productive infections in new host species or would be able to provide new variants with potentially significant epidemiological consequences in the already susceptible host (Holmes and Rambaut, 2004; Woolhouse et al., 2001).

Linked to the host. Unfortunately to the host, evolution occurs at a much slower pace than that of viruses. However, the resistance to infectious disease in pigs have a genetic component (Springbett et al., 2003; Zhao et al., 2012). The existing intensive pig industry selects pigs for meat production parameters, ignoring in most of the cases disease resistance traits, and reducing, at the same time, genetic variability of the pig population. Such homogeneity affects resistance to an invading pathogen (in favour or against) and may have inadvertently created selection indices that included undesirable effects, such as viral susceptibility (Edfors-Lilja et al., 1998; Zhao et al., 2012). Such pig viral susceptibility has been demonstrated for PRRSV, where studies have shown that the detrimental impact of PRRSV infection on growth varies between and within lines and breeds (Rowland et al., 2012). Another example would be PCV2, since susceptibility to the

development of PCV2-SD is genetic line-dependent (López-Soria et al., 2011). Therefore, genetic uniformity of the swine population might promote the emergence of swine-specific pathogens, causing putative catastrophic epidemics (Bishop and MacKenzie, 2003; Drew, 2011; Springbett et al., 2003).

B) Ecological factors.

The process of pathogen emergence is not solely based on the pathogen-host interaction, and other factors are involved in such mechanism. These other drivers are extrinsic ecological changes of host populations. Among them, demographic changes (especially those leading to increments in size and density of human and animal populations), international trade and intensification of farming are considered as the most significant ones (Conway and Roper, 2000; Holmes and Rambaut, 2004; Woolhouse et al., 2001). It is well known that the pig industry has experienced a rapid growth all over the world in the last decades, and nowadays, pig industry meets the ecological factors able to promote pathogen emergence.

In the coming years, it can be anticipated, that new emerging diseases will appear in pigs; a different issue is to predict which virus will produce the next emerging disease. However, prediction of which factors from previous epidemics or current emerging infectious diseases have to be considered is feasible and such prediction might allow targeted modification of emerging pathogens (Conway and Roper, 2000).

1.1.3 Why studying swine anelloviruses as emerging viruses?

Porcine anelloviruses, known as TTSuVs, are considered emerging viruses (Meng, 2012). However, TTSuVs have been probably established in the swine population for a long period of time, since they were discovered around 15 years ago (Leary et al., 1999) and a retrospective study demonstrated evidence of TTSuVs infection in pigs at least since 1985 (Segalés et al., 2009). Anelloviruses fit very well with previous described characteristics as emerging viruses:

• They are worldwide distributed in human and swine populations (Abe et al., 1999; Bigarré et al., 2005; Cortey et al., 2012; Charlton et al., 1998; Maggi et al., 1999; McKeown et al., 2004; Viazov et al., 1998a; Wu et al., 2011) as species-specific viruses (Abe et al., 2000; Okamoto et al., 2002).

• Genetically, they are extremely variable and subjected to high mutation rates (Cortey et al., 2011; Huang et al., 2010b). In fact, mutation rate in anelloviruses is higher than expected for DNA viruses, as TTSuVs use host DNA polymerase for replication (Kakkola et al., 2007; Okamoto et al., 1998b; Suzuki et al., 2001). The nucleotide substitution/site/year is approximately 10^{-4} nucleotide substitution/site/year (Cortey et al., 2011; Umemura et al., 2002), similar to the variability observed in RNA viruses (between 10^{-2} to 10^{-5} nucleotide substitution/site/year) (Duffy et al., 2008; Shackelton and Holmes, 2006; Shackelton et al., 2005). Such high mutation rate enhances virus adaptability (Holmes and Rambaut, 2004). Genetic variation may occasionally, and fortuitously, produce new viral variants with an increased virulence or may produce new virulent variants (Abrantes et al., 2012; Chapman et al., 2008; Guo et al., 2012; Saha et al., 2012).

• Host immune response, according to current literature, is not able to neutralize anellovirus infections, resulting in a percentage of persistently infected individuals (Huang et al., 2011; Nishizawa et al., 1999; Okamoto and Mayumi, 2001).

So far, and despite the abovementioned properties, *Anelloviruses* have not yet been linked to any known disease. However, this scenario is not surprising since there are a number of diseases that epidemiologists consider of multifactorial aetiology (Buck et al., 1988). According to the "multiple causation" theory, each clinical outcome may have a number of causes, and a single cause may have multiple effects (Krieger, 1994). This multifactorial theory assumes that the patterns of health and disease in populations depends on a variety of risk and protective factors, which are interconnected between them (Krieger, 1994; Susser, 1985). Such scenario could be applied to *Anelloviruses*. Although the apparent lack of direct disease causality, anellovirus infections are considered as potential aggravating factors in several human and pig diseases (Aramouni et al., 2013b; Aramouni et al., 2011; Bando et al., 2001; Bando et al., 2008; Blomström et al., 2010; Figueiredo et al., 2007; Garbuglia et al., 2003; Hu et al., 2002; Krakowka et al., 2008; Miyamoto et al., 2000;

Moriyama et al., 2001; Pifferi et al., 2005; Szládek et al., 2005; Tokita et al., 2001, 2002; Zhong et al., 2001).

1.2 *Anelloviruses*

1.2.1 Discovering anelloviruses

Anelloviruses were the first discovered circular single stranded DNA (ssDNA) human infecting viruses. The first one, named as TTV (initially named following the initials of the patient but afterwards referred by the International Committee for the Taxonomy of Viruses (ICTV) as *Torque teno virus*) was discovered in 1997, in the blood of a Japanese patient. At the beginning, it was thought to be involved in the pathogenesis of a non A to G human hepatitis following blood transfusion (Nishizawa et al., 1997). The sequenced fragment was named as N22 (Nishizawa et al., 1997). Since their discovery, anelloviruses attracted significant interest, since a percentage of acute/chronic hepatitis could not be attributed to known hepatitis-causing viruses and, very likely, other agents should be involved in the genesis of these unknown hepatitis (Alter and Bradley, 1995). Based on Nishizawa and collaborators' works, TTV was postulated as a virus potentially responsible for hepatitis in humans (Nishizawa et al., 1997).

After the discovery of human anelloviruses, many studies tried to determine prevalence and pathogenic role of these viruses in the human population. Different populations were studied for the search of anelloviruses, including healthy people and patients with a variety of hepatic and extra-hepatic diseases. Overall, these epidemiological studies demonstrated that human anelloviruses were worldwide distributed (Biagini et al., 2000; Charlton et al., 1998; Figueiredo et al., 2007; Maggi et al., 1999; Naoumov et al., 1998; Tanaka et al., 1999; Viazov et al., 1998a; Yamamoto et al., 1998).

Soon became evident that infection with anelloviruses was not restricted to the human host, and positivity was confirmed in primates (Abe et al., 2000). Phylogenetic analysis demonstrated that primate anellovirus isolates were distinct from humans ones (Abe et al., 2000; Okamoto et al., 2000a). However, some of these studies also suggested that human

anelloviruses were able to infect chimpanzees (Mushahwar et al., 1999; Okamoto et al., 2000a; Okamoto et al., 2001b; Verschoor et al., 1999). Subsequently, other mammal species, including tupaias, pigs, dogs, cats, chicken, sheep and cattle, were investigated for the presence of anelloviruses, and evidence of infection confirmed (Kekarainen et al., 2006; Leary et al., 1999; Niel et al., 2005; Okamoto et al., 2001b; Okamoto et al., 2002).

Although genetic variability between anelloviruses infecting different mammalian species was high, phylogenetic studies demonstrated that livestock and human anelloviruses were genetically similar (Leary et al., 1999; Okamoto et al., 2002). Moreover, these studies allowed determining that genomic differences observed in viruses within the *Anelloviridae* family were mainly located in the coding regions (TR), sharing all of them a highly conserved untranslated region (UTR). To date, anelloviruses have also been identified in other vertebrate species, such as sea lions, camels, rodents, wild boars, pine martens and badger (Al-Moslih et al., 2007; Martínez et al., 2006; Ng et al., 2009; Nishiyama et al., 2014; van den Brand et al., 2012).

1.2.2 Taxonomy and classification

The family *Anelloviridae* includes TTV (Nishizawa et al., 1997), *Torque teno mini virus* (TTMV) (Takahashi et al., 2000) and *Torque teno midi virus* (TTMDV) (Jones et al., 2005). Current classification is based on the analysis of the complete ORF1 nucleotide (nt) sequence as the most convenient approach (King et al., 2011). *Anelloviridae* family comprises 11 genera according to the ICTV 2014 report (www.ictvonline.org/virus taxonomy.asp). Anelloviruses infecting pigs are grouped into two genera, namely *Iotatorquevirus* (including species TTSuV1a and TTSuV1b) and *Kappatorquevirus* (including species TTSuVk2a and TTSuVk2b) (Cornelissen-Keijsers et al., 2012; Cortey et al., 2011; Huang et al., 2010b). From now onwards, this Thesis will refer them as TTSuV1 (TTSuV1a and 1b) or TTSuV2 (TTSuVk2a and k2b).

1.2.3 Virion structure, genomic organization and predicted products

Anellovirus virions are icosahedral, non-enveloped, with a diameter of 30-32 nm and encapsidate the circular ssDNA genome of negative polarity (Mushahwar et al., 1999). The buoyant density in cesium chloride is 1.31–1.33 g/cm^3 for anelloviruses detected in serum, and 1.33–1.35 g/cm3 for anelloviruses from faeces (Okamoto et al., 1998a). Anelloviruses have a variable genome length depending of the host species, ranging from 2.1 kilobases (kb) in cats to 3.9 kb in humans (Okamoto et al., 2001b; Okamoto et al., 2002). TTSuV genomes are between 2.8 to 2.9 kb and 2.7 to 2.8 kb for TTSuV1 and TTSuV2, respectively (Cornelissen-Keijsers et al., 2012; Cortey et al., 2011; Martínez-Guinó et al., 2011; Niel et al., 2005; Wang et al., 2012b).

Anelloviruses have a wide range of sequence divergence. Considering this, and according to the ICTV Executive Committee, the proposed cut-off values for anelloviruses are: genus, which is defined when the entire ORF1 nucleotide sequence identity is between 36-55%; and species, which is defined when the entire ORF1 nucleotide identity ranges between 55-67%. The recognized viral taxonomy does not recognise additional taxa beyond the species level. However, some authors have proposed additional taxa for human and swine anelloviruses (Huang et al., 2010b; Jelcic et al., 2004). In case of pigs the proposed taxa are: 1) type, when nucleotide identity of full length genome ranges between 67-85%; 2) subtype, when TTSuVs share between 85-95% nucleotide sequence; and 3) variant, when nucleotide identity is higher than 95% (Huang et al., 2010b). Regarding TTSuVs, nucleotide identity with the corresponding human anelloviruses is less than 45% (Okamoto et al., 2002). Similarity between *Iotatorquevirus* and *Kappatorquevirus* ranges from 54-56% (Cortey et al., 2011; Huang et al., 2010b).

It is assumed that anelloviruses use the rolling circle replication (RCR) mechanism to replicate (Mushahwar et al., 1999), similarly to other ssDNA viruses infecting mammals and plants (Todd, 2000). After infection of permissive cells, the viral encoded replication associated protein (REP), binds to stem-loop structures presents in the UTR of anelloviruses, which is the origin of replication, and initiates the replication by cleaving the DNA (Mankertz et al., 1997; Niagro et al., 1998). Afterwards, the cellular DNA polymerase initiates viral DNA replication from the free 3' end, and the REP complex

remains linked to the 5' end. When the positive strand is replicated, the REP complex closes the DNA and releases a ssDNA which can be used for new round of replication or can be encapsidated (Faurez et al., 2009).

Despite the different genome size and the high genomic variability of the *Anelloviridae* family members, all anelloviruses share a similar genomic organization and transcription profile (Irshad et al., 2006; Martínez-Guinó et al., 2011; Mushahwar et al., 1999; Wang et al., 2012b). Their genomes are composed by an UTR and three overlapping ORFs (Cortey et al., 2011; Irshad et al., 2006; Kamada et al., 2004; Martínez-Guinó et al., 2011; Qiu et al., 2005; Wang et al., 2012b).

The UTR of anelloviruses represents one third of the genome (Cortey et al., 2011; Okamoto et al., 2002). Notwithstanding the high genetic variability of anelloviruses, the UTR is well conserved compared with the translated region; in fact, some regions of the UTR among family members share nucleotide identity as high as 90% (Erker et al., 1999). Important regulatory elements are located in this part of the genome (Kamada et al., 2004; Suzuki et al., 2004) and presumably a higher divergence is not tolerated in such region. In the middle of the UTR there is a guanine-cytosine (GC) rich stretch (of more than 150 nt in length), with successive repeated sequences forming GC-rich stem-loop structures; these latter structures are considered to play a pivotal role in viral transcription (Bendinelli et al., 2001; Okamoto et al., 1999a; Okamoto et al., 2002). The UTR also possess a polyadenylation site (Bendinelli et al., 2001; Hijikata et al., 1999). Both extremes of the genome are connected by a GC region, forming a closely circular molecule (Bendinelli et al., 2001; Miyata et al., 1999).

The TR is inserted between the motifs of TATA box and the polyadenylation signal. The TR is formed by 3 different ORFs. ORF1 gene translates into the largest predicted mRNA. ORF1 encodes a protein of 638-650 aa in length for TTSuV1 and 625-650 aa for TTSuV2 (Cortey et al., 2011; Martínez-Guinó et al., 2011; Okamoto et al., 2002). By analogy with *Chicken anaemia virus* (CAV) and with PCV members of the closely related family *Circoviridae,* ORF1 is believed to encode the putative coat protein (Bendinelli et al., 2001; Biagini and de Micco, 2008). Three hypervariable regions (HVR) have been described in the central part of ORF1, with sequence divergence of more than 73%; these regions are

considered to play a role in immune evasion (Mushahwar et al., 1999; Nishizawa et al., 1999; Wang et al., 2012b). ORF1 protein possesses two hydrophilic regions, one at the N-terminus and the other at the C-terminus (Huang et al., 2012a; Ott et al., 2000); hydrophilic regions are described as important for the antigenic properties of proteins (Hopp and Woods, 1981). Other functions attributed to the ORF1 protein are binding with viral DNA and transportation to the nucleus, which are supposed to be mediated by the Arg-rich region and the hydrophilic domain of the ORF1 protein (Davidson and Shulman, 2008; Okamoto and Mayumi, 2001). It has been also speculated that this protein might function as replicase based on the RCR motifs identified in the ORF1 nt sequence (Hino, 2002; Mankertz et al., 1997; Mueller et al., 2008; Mushahwar et al., 1999; Niagro et al., 1998). RCR domains have been identified in all swine TTSuV genomes (Cortey et al., 2011; Huang et al., 2010b; Okamoto et al., 2002).

The ORF2 gene generates the shortest predicted mRNA, and encodes a 73-74 aa protein in TTSuV1 and 69-81 aa protein in case of TTSuV2 (Cortey et al., 2011; Martínez-Guinó et al., 2011; Okamoto et al., 2002). Based in similarity with viral protein 2 of CAV (Hijikata et al., 1999), it is likely that this gene encodes a non-structural protein involved in viral replication and probably linked to gene transcription and regulation during infection (Bendinelli et al., 2001; Peters et al., 2002). Recent studies suggested that ORF2 protein may be involved in regulating the innate and adaptive immune responses of the host, specifically by suppressing nuclear factor kappa beta (NF-κB) activity (Zheng et al., 2007).

ORF3 protein of TTSuVs is generated after splicing, sharing its 5' half with the ORF2 gene. The ORF3 gene encodes a protein of 221-232 aa for TTSuV1 and 200-224 aa for TTSuV2 (Cortey et al., 2011; Martínez-Guinó et al., 2011). ORF3 protein function is still to be determined.

1.2.4 Epidemiology of anelloviruses

One of the best known aspects of anelloviruses is their epidemiology. Initially, anelloviruses were described in humans in 1997. However, the study of ancient samples showed presence of viral TTV-DNA in a 200-year-old dental pulp sample of a soldier

belonging to Napoleon's Great Army (Bédarida et al., 2010). In case of non-human primates, frozen samples collected in 1979 tested positive (Abe et al., 2000). In swine, the first TTSuV (TTSuV1) was reported in 1999 (Leary et al., 1999), although retrospective studies demonstrated the existence of TTSuVs already in 1985 (Segalés et al., 2009).

1.2.4.1 Prevalence

Infection by TTSuVs starts early in life, with a percentage of piglets born already infected (Martínez-Guinó et al., 2010; Pozzuto et al., 2009; Sibila et al., 2009b; Xiao et al., 2012). In general, the observed percentage of viremic pigs at first stage of life is higher for TTSuV1 than for TTSuV2 (Sibila et al., 2009a; Taira et al., 2009), and prevalence of both species increases over time (Martelli et al., 2006; Sibila et al., 2009a; Taira et al., 2009). Co-infection and persistence of the TTSuVs in the infected swine is commonly observed (Blois et al., 2014; Huang et al., 2011; Nieto et al., 2012; Sibila et al., 2009a; Sibila et al., 2009b; Xiao et al., 2012). According to published studies, health status, age of animals and swine breed are considered factors affecting TTSuVs prevalence (Aramouni et al., 2011; Blomström et al., 2010; Gallei et al., 2010; Kekarainen et al., 2006; Sibila et al., 2009a; Taira et al., 2009; Wu et al., 2011).

The reported prevalence in different countries greatly varies, ranging from 17% to 100% (Bigarré et al., 2005; Cortey et al., 2012; Gallei et al., 2010; Kekarainen et al., 2006; Lee et al., 2010; Martelli et al., 2006; McKeown et al., 2004; Takács et al., 2008; Wu et al., 2011). Moreover, it has been reported that international trade of live animals is closely related with propagation of TTSuVs worldwide (Cortey et al., 2012).

Prevalence in wild boars has also been studied, and is similar to farmed pigs, being of 58% and 66% in Spanish wild boars, and 20% and 49% in German wild boars, for TTSuV1 and TTSuV2, respectively (Gallei et al., 2010; Martínez et al., 2006).

1.2.4.2 Transmission

In pigs, many routes of viral transmission have been suggested, which would support the high prevalence and distribution observed. Vertical transmission is an important route of TTSuV transmission (Martínez-Guinó et al., 2009; Pozzuto et al., 2009; Sibila et al., 2009b). TTSuVs have been identified in samples collected from second third of gestation onwards, but also in aborted foetuses and stillborn piglets. Reported prevalence range from 10% to 17% in foetuses and 50% in stillborn piglets for TTSuV1; for TTSuV2, figures ranged from 30% to 40% in foetuses and just 7% in stillborn piglets (Aramouni et al., 2010; Martínez-Guinó et al., 2010; Martínez-Guinó et al., 2009; Pozzuto et al., 2009). These previous results are in contrast with a recent studied carried out in the USA, in which only TTSuV1 positive samples were detected in foetuses (Xiao et al., 2012).

In suckling pigs in Spain, prevalence of TTSuV1 was higher than for TTSuV2 (Sibila et al., 2009a). A similar conclusion were drawn from Japanese pigs farms, where no TTSuV2 infection was detected before 30 days of life (Taira et al., 2009). Rapid increase in prevalence was subsequently observed for both TTSuVs. The detection of PCR positive piglets born from PCR negative sows, and/or PCR negative piglets born from PCR positive sows (Pozzuto et al., 2009; Sibila et al., 2009b), suggests that horizontal transmission is another important route of spreading. Moreover, secretion and excretion of TTSuV1 and TTSuV2 trough respiratory and faecal routes have been documented (Sibila et al., 2009a), emphasizing the importance of horizontal transmission.

Additionally, boar semen and commercial vaccines against main pathogens affecting the pig industry (*Mycoplasma hyopneumoniae*, *Porcine parvovirus* (PPV) and PRRSV) have been shown to be PCR positive for TTSuVs (Huang et al., 2010b; Kekarainen et al., 2007; Kekarainen et al., 2009; Kulcsar et al., 2010). Recently, TTSuV2 genome has been detected by PCR in a commercial OIE (World Organization for Animal Health) disease-free porcine sera (Huang et al., 2011). Altogether, these data suggest that sexual and iatrogenic routes could be potential routes of transmission as well, probably of less importance compared to horizontal and vertical ones.

1.2.4.3 Distribution

Presence of anelloviruses in serum samples has been extensively documented, but not tissue distribution and cell type supporting replication in swine. TTSuV DNA has been found in tonsil, ileum, lymph nodes, brain, heart, lung, liver, spleen, kidney and bone marrow (Aramouni et al., 2010; Bigarré et al., 2005; Gallei et al., 2010; Novosel et al., 2012; Pozzuto et al., 2009; Takács et al., 2008). Among all studies carried out so far, just two of them investigated the presence of TTSuV in several organs/tissues of the same animal (Aramouni et al., 2010; Bigarré et al., 2005). Additionally, the presence of foetal PCR positive tissues has been documented from the second third of gestation onwards (Aramouni et al., 2010; Martínez-Guinó et al., 2010; Novosel et al., 2012).

Research on TTSuV infection in tissues indicates that prevalence of PCR positive tissues increases with age of animals (Aramouni et al., 2010). The same author found that viral load in different tissues was statistically higher in bone marrow and spleen at 5 weeks of age, when compared with the viral load in the rest of tissues (Aramouni et al., 2010). Such differences were not maintained in older pigs. Generally speaking, viral loads in tissues were low or very low in foetuses and newborn piglets, and incremented over age, in agreement with the increase in prevalence observed in serum (Sibila et al., 2009a). As a whole, studies detecting anelloviruses in swine tissues suggest that TTSuVs are able to spread all over the body, without any specific target tissue.

From the epidemiologic point of view, TTSuVs are common viruses in the swine population worldwide. Infection starts early in life and increase with age. Once pigs are infected, TTSuVs are able to establish chronic infections, although a percentage of them resolve spontaneously. However, a specific target tissue where TTSuVs can replicate has not been formally established.

1.2.4.4 Persistence in the infected host

The mechanisms by which anelloviruses are able to establish persistent infections and avoid clearance by the immune system remain elusive. Persistence of TTSuVs in infected

hosts suggest that immune responses might be ineffective, but also that no cross protection does exist among TTSuV species (Gallei et al., 2010; Huang et al., 2010b; Kekarainen et al., 2006; Martínez et al., 2006). According to the literature, *Anelloviridae* family members, and hence TTSuVs, have developed several effective mechanisms to subvert the host immune system and, therefore, ensure their persistence. Genetic variation has been suggested as a mechanism to escape the immune response; these viruses carrying mutations are able to actively replicate in the infected individuals as the immune system need some time to specifically respond against them, and when the immune response is generated against these escape mutants, new escape mutants may emerge (Bowen and Walker, 2005; Letvin, 2005). In human anelloviruses, but also in TTSuVs, the presence of HVRs in ORF1 have been documented (Huang et al., 2010b; Jelcic et al., 2004; Nishizawa et al., 1999; Wang et al., 2012b). In humans, HVR regions were observed in the virus population of individuals in which TTV was able to establish a persistent infection rather than individuals having an acute TTV infection; in such persistent infected individuals, a quasispecies distribution of TTV was observed (Nishizawa et al., 1999). In contrast, the quasispecies TTV nature in persistently infected individuals was not observed by Kakkola and collaborators (2008). In swine, the HVR of ORF1 is located in the central part of the ORF1 gene, but not in the C or N-ORF1 terminus, as these regions contain important key functions that would be compromised in case of high variability (Cortey et al., 2011). HVRs have been observed for other viruses establishing chronic infections, and are considered to contribute to immune evasion (Bowen and Walker, 2005; Chayama et al., 1999; Letvin, 2005). Also, HVRs are related with the pathogenic capacity of the virus, as demonstrated *in vitro* for CAV (Davidson and Shulman, 2008; Renshaw et al., 1996). Recombination, another mechanism that help to create variability, has also been demonstrated in anelloviruses, which would increase their genetic repertoire (Manni et al., 2002).

1.2.5 Interaction of anelloviruses with the immune system

During co-evolution with their host, viruses have developed different strategies to evade or overcome the immune system, making viral existence possible in an unfriendly environment. In case of anelloviruses, the following strategies have been suggested:

- Interferon (IFN) secretion. IFN is produced by infected cells and is used by the host immune response to destroy viruses; IFN also protects uninfected cells from virus infection, as it produces an antiviral state that help triggering an adaptive antiviral immune response (Bonjardim, 2005; Finlay and McFadden, 2006). Importantly, it seems that certain TTV genotypes are quite resistant to IFN alpha (α) therapy. Different studies have shown that IFN-α treatment had no effect on TTV load or such effect was TTV genotype specific, suggesting that TTV might possess mechanisms to evade the host defence by IFN-α (Chayama et al., 1999; Maggi et al., 1999).

- NF-κB response. Another mechanism used by TTV to escape immune responses is by interfering the NF-κB response. NF-κB contributes to the expression of over 100 target genes and it is involved (among other biological functions) in the host immune response (Ghosh et al., 1998; Hiscott et al., 2001; Silverman and Maniatis, 2001). A putative NF-κB binding site has been found in the ORF2 protein of TTV (Miyata et al., 1999). In vitro studies have proven that ORF2 protein can down-regulate or suppress activation of NF-κB in a dose-dependent manner (Zheng et al., 2007). This inhibitory effect might facilitate surviving of anelloviruses in the infected host and prevent TTV from being eliminated by the host.

- micro RNAs (miRNAs) release. miRNAs are small RNAs of approximately 22 nt that exert effects in the defence against viruses and other damaging genetic elements. In addition, several virus families are able to produce miRNAs that help viruses evading the immune response or playing a role in maintaining latent/persistent infections (Cullen, 2010; Grundhoff and Sullivan, 2011; Kincaid and Sullivan, 2012). In viruses, miRNAs have been proved to act as inhibitory of cell factors that play a role in the adaptative and innate antiviral immunity (Cullen, 2010; Nachmani et al., 2009). These viral miRNAs are speculated to play a role in prolonging the longevity of infected cells, evasion of the immune response, regulation the switch to lytic infection, and are invisible to the host immune response (Cullen, 2006). The abovementioned functions are particularly important during persistence of the viruses. The expression of miRNA has been demonstrated in vitro and in vivo for human TTVs (Kincaid et al., 2013); the identified target of the described TTV-miRNA is N-Myc interactor (NMI). In vitro, it has been proved that TTV-miRNA significantly inhibited the expression of the NMI (Kincaid et al., 2013). As NMI play a role in cytokine and interferon signalling but also in viral induced apoptosis (Wang et al.,

2012a; Zhu et al., 1999), it was inferred that TTV-miRNA targets NMI to inhibit interferon signalling and promoting immune evasion. In swine, the presence of miRNA has been recently studied in a ssDNA virus such as PCV2. No miRNA encoded by the viral genome was identified (Núñez-Hernández et al., 2015b), but the presence of PCV2 at tissue level affected the miRNA expression profile of the host, specifically in the mediastinal lymph node (Núñez-Hernández et al., 2015a). TTSuV-miRNAs presence has not been confirmed yet.

1.2.6 Anellovirus in humans

1.2.6.1 Pathogenic role

In humans, the pathogenic role of TTVs has been thoroughly studied. Initially, it was considered a potential hepatitis-causing virus (Kanda et al., 1999; Maggi et al., 2001; Okamoto et al., 1999a; Okamura et al., 2000; Savas et al., 2003; Viazov et al., 1998b; Yamamoto et al., 1998). Although several of those studies found a positive correlation, others did not find such relationship (Das et al., 2004; Matsumoto et al., 1999; Naoumov et al., 1998; Tanaka et al., 1999). Moreover, TTV has also been associated with cancer occurrence, as viral load was found significantly elevated in patients with different cancer types when compared with appropriate healthy control individuals (Zhong et al., 2002).

As one of the main routes of TTV infection is the oral route, it seems feasible that TTV can affect the digestive tract. In fact, a correlation was found between gastric disorders due to *Helicobacter. pilori* (*H. Pilori*) and TTV load. Moreover, such correlation was stronger when *H. pilori*-positive patients were carrying the cagA gene (Maggi et al., 2003a), which is considered a marker of pathogenicity for *H. pilori* and associated with gastric carcinoma

(Hatakeyama, 2004). However, other studies investigating such correlation failed (Toyoda et al., 2000).

Respiratory disease has been another field of research of TTV related disease. Idiopathic pulmonary fibrosis (IPF) has been associated with TTV infection (Bando et al., 2001), and such correlation was stronger in patients suffering IPF complicated with lung cancer when

compared with appropriate control groups (Bando et al., 2008). In children, TTV was found more frequently and with higher viral load in X-ray confirmed bronchopneumonia patients (BP) than in acute respiratory disease patients. Mean viral TTV load, as measured in nasal samples, was found statistically higher in X-ray BP patients of viral origin than BP patients of bacterial aetiology (Maggi et al., 2003b).

Besides the abovementioned diseases or lesions, some other disorders have been linked to TTV, like thrombocytopenia (Tokita et al., 2001, 2002), aplastic anaemia (Miyamoto et al., 2000), type II cryoglobulinemia (Cacoub et al., 2003) and autoimmunity, as is the case of systemic lupus erythematosus (SLE)(Gergely et al., 2005).

1.2.7 TTSuV and disease

1.2.7.1 TTSuV pathogenesis

One of the main constraints when studying the potential pathogenic role of TTSuVs is the long lasting viremia observed in a percentage of healthy pigs (Lee et al., 2010; Martelli et al., 2006; Sibila et al., 2009a). Pathogenesis of TTSuV infections has been studied in commercial pigs; however, due to the presence of other well-known pathogens co-infecting pigs with TTSuVs, it is very difficult to draw conclusions of the potential TTSuV pathogenic role.

To circumvent this situation, some researchers have studied TTSuVs pathogenicity in gnotobiotic pigs. These animals received a TTSuV1 PCR positive tissue homogenate, and some mild histological lesions were observed, including interstitial pneumonia, transient thymic atrophy, membranous glomerulonephropathy, and, modest lymphocytic to histiocytic infiltrates in the liver (Krakowka and Ellis, 2008). Regarding TTSuV2, gnotobiotic pigs inoculated with a TTSuV2 PCR positive (and TTSuV1 PCR negative) tissue homogenate showed subtle histopathological changes including interstitial pneumonia, membranous glomerular nephropathy, modest inflammatory cell infiltration in portal areas of the liver, foci of haemorrhages in some pancreas islets, infiltration of inflammatory cells (lymphocytes and eosinophils) in tonsil and hilar lymph nodes, and

necrosis or degeneration of lymphocytes in the paracortical zone in lymphoid tissues (Mei et al., 2011). In addition, caesarean-derived, colostrum-deprived pigs inoculated with an infectious TTSuV2 clone developed mild pathological lesions in brain, kidney and liver (Huang et al., 2012b). No clinical manifestations were displayed by any of the inoculated pigs.

1.2.7.2 TTSuV role in concomitant infections

Since their discovery in the swine population, many studies have been carried out to assess a potential association of TTSuVs with swine diseases. The studies, so far, have been inconclusive. The high TTSuV prevalence in studied pig populations, with about 60% prevalence in conventional pig farms (Bigarré et al., 2005; Kekarainen et al., 2006; McKeown et al., 2004; Wu et al., 2011; Xiao et al., 2012), jeopardizes the establishment of a potential disease causality. Also, the high genetic diversity described for each of the two TTSuV genera (Cortey et al., 2011; Huang et al., 2010b), which might possess different biological or pathogenic properties as pointed out by studies with human anelloviruses (Okamura et al., 2000; Sugiyama et al., 2000; Szládek et al., 2005), represents a serious hurdle in the elucidation of their clinical significance.

However, it is believed, based on existing human anelloviruses knowledge (Maggi et al., 2003a; Maggi et al., 2003b; Zhong et al., 2002), that TTSuVs may contribute by means of increasing the severity of disease induced by other agents in pigs. In this regards, the role of swine anelloviruses in co-infection with other pathogens, mostly with PCV2, has been extensively studied.

The first suggestion indicating that TTSuV2 might be involved in the genesis of disease came from Kekarainen et al. (2006), since TTSuV2 was more commonly found in pigs suffering from PCV2-SD than in non-affected pigs. Up to date, PCV2 is considered the necessary infectious agent for the PCV2-SD, although some other co-factors are needed for the full expression of the disease (Segalés et al., 2005). Subsequent studies found that TTSuV1 exacerbated the severity of the disease associated to PCV2 when gnotobiotic pigs were experimentally inoculated with both TTSuV1 PCR positive homogenate and PCV2

(Ellis et al., 2008). On the other hand, (Blomström et al., 2010) found that co-infection of PCV2, TTSuV1, TTSuV2, and *Porcine bocavirus* (PoBV) was the most frequent combination of viruses observed in pigs diagnosed as PCV2-SD (71% of PCV2-SD affected pigs), while such percentage was only 33% for the non PCV2-SD affected pigs.

In addition to PCV2, TTSuVs co-infection has also been studied in the background of other swine disease outcomes. In one study, piglets dually inoculated with a TTSuV1 PCR positive tissue homogenate and PRRSV developed a porcine dermatitis and nephropathy syndrome (PDNS)-like condition (Krakowka et al., 2008). However, a closer look to the pathology generated in these animals indicates that observed lesions were mild and not really compatible with classical cases of PDNS. Moreover, it has been found that TTSuV2 load in serum was higher in PDNS affected pigs than in healthy pigs, while for TTSuV1 such differences were not observed (Aramouni et al., 2011).

The role of TTSuVs in the occurrence of porcine respiratory disease complex (PRDC) has also been assessed. PRDC is a clinical entity of multifactorial aetiology, in which PCV2, PRRSV, *Swine influenza virus* (SIV) and/or *Mycoplasma hyopneumoniae* could be involved (Thacker, 2001). The lesion severity in PRDC seems to be associated with the number of different pathogens involved (Ramamoorthy and Meng, 2009). The TTSuV role was investigated in PRDC cases and it was concluded that TTSuV1, but not TTSuV2, was epidemiologically linked to PRDC (Rammohan et al., 2012).

Another factor to be considered is the study of TTSuVs at tissue levels, as different genotype might possess different tissue tropism and likely different pathogenic capacities. In humans, differential association of anellovirus genotype/subtypes in tissues with different disease or disorders are observed; importantly, when such correlation was investigated analyzing blood samples, instead of the tissue samples of such individuals, TTV association was not observed (Bando et al., 2001; Fehér et al., 2011; Kakkola et al., 2004; Maggi et al., 2003a; Okamoto et al., 2001a; Okamoto et al., 2000c; Pollicino et al., 2003; Szládek et al., 2005; Yamamoto et al., 1998). All together complicates, definitely, research on TTSuVs disease association.

The role of both TTSuVs in inflammatory lungs lesions of unknown aetiology has been also investigated. It was observed that TTSuV2 was more prevalent in inflammatory lungs

lesions than TTSuV1. Moreover, TTSuV2 mean load was statistically higher in lung lesions associated to a viral origin when compared to those of bacterial origin or normal lungs (Aramouni et al., 2013b). This study suggested a possible role of TTSuV2 in the pathogenic mechanism of inflammatory lung lesions. Similarly, human pneumonia, positive to common respiratory virus, has also been associated to anellovirus load in lung tissue (Maggi et al., 2003b). Additionally, the prevalence of TTSuVs has been investigated in hepatitis that was previously linked to PCV2 and HEV infections. The study showed high prevalence of TTSuVs in the livers of pigs suffering from mild to moderate hepatitis caused by these other viruses (Savic et al., 2010).

Finally, in a recent study investigating the presence of TTSuVs in PCV2-infected postweaning pigs affected by kidney lesions or proliferative necrotizing pneumonia (PNP), it was observed that TTSuV2 infection was more prevalent in animals with those two pathological conditions. Such result was not found for TTSuV1 (Novosel et al., 2012).

Altogether, these studies highlighted the importance of investigating TTSuV prevalence and load in tissues as a potential marker of disease or pathogenicity. Despite these previous studies, to date, the results are inconclusive and TTSuVs have not been definitely proved as causing disease either directly or indirectly. There are a number of other scientific works that did not find correlation between TTSuVs infection and subsequent development of different swine diseases (Gauger et al., 2011; Lee et al., 2010; McMenamy et al., 2013; Taira et al., 2009). These authors suggested that TTSuVs might be opportunistic pathogens, and not a causative agent of disease in swine or a triggering factor in multifactorial diseases.

1.2.8 Laboratory techniques

The routine viral techniques used to detect anelloviruses include direct and indirect detection methods.

Direct detection methods comprise molecular techniques to detect viral DNA. Initial detection of TTV sequences was carried out with nested-PCR methods amplifying the

central part of the ORF1 region (Nishizawa et al., 1997; Okamoto et al., 1998b). Anelloviruses have a wide sequence divergence, higher in the coding than in the non-coding region of the genome (Hijikata et al., 1999; Okamoto et al., 1999b) and subsequent studies demonstrated that the part of the genome on which primers were designed influenced the detection of anelloviruses (Takahashi et al., 1998). Primers designed to detect anelloviruses for clinical research is of paramount importance. This situation resembles that of papillomaviruses or adenoviruses, viruses with high sequence variability and several genotypes identified, in which just certain genotypes are associated to disease (Howley and Lowy, 1996; Wold and Horowitz, 1996).

In pigs, different protocols have been used to detect TTSuVs. Leary et al. (1999) used primers deduced from the human UTR and were able to detect species-specific anelloviruses in a variety of mammalian species, including pigs. Subsequently, species-specific swine PCR and nested PCR methods were developed, amplifying fragments of the non-coding region (Bigarré et al., 2005; Kekarainen et al., 2006; McKeown et al., 2004). One of the disadvantages of the PCR, compared with other techniques, is that it gives information about presence or absence of the viral genome, but it gives no additional information.

Other authors developed real time quantitative PCR (qPCR) techniques, either using SYBR or Taqman probes, to detect anelloviruses in pigs (Brassard et al., 2010; Gallei et al., 2010; Huang et al., 2010a; Lee et al., 2010). The most powerful feature of qPCRs is the ability to quantify the amount of circulating virus. The viral load is of interest as it correlates with severity of clinical signs or prognosis of the disease in a number of other viral diseases, as is the case of PCV2, *Human papillomavirus*, *Simian immunodeficiency virus*, *Human influenza virus* type A, *Hepatitis B virus* and *Human immunodeficiency virus* (HIV) (Cozzi Lepri et al., 1998; de Jong et al., 2006; Grau-Roma et al., 2009; Ohkubo et al., 2002; Opriessnig et al., 2007; Sun et al., 2001; Zink et al., 1999).

PCR and qPCR give information about on-going infection, but not about a previous infection. In the last years, serological tests have been developed for TTSuVs in order to facilitate clinical and epidemiological studies, helping in the estimation of the true prevalence of TTSuVs infection. In humans, such studies have been carried out in a very

limited fashion (Handa et al., 2000; Kakkola et al., 2008; Ott et al., 2000). In pigs, serodiagnostic tools based on ELISA have recently been developed; these methods are based in the ORF1 recombinant protein of both TTSuVs, since this protein represents the predicted structural protein of the virus, and likely contains hydrophilic antigenic domains (Huang et al., 2011; Huang et al., 2012a; Jarosova and Celer, 2013).

Despite the current knowledge of the anelloviruses epidemiology, the mode of replication of anelloviruses is still poorly understood. The presence of viral intermediate replicative forms has been observed in several tissues, such as bone marrow, liver or, stimulated peripheral blood mononuclear cells (PBMC) (Mariscal et al., 2002; Okamoto et al., 2000b; Okamoto et al., 2000c). However, the advance in this field has been hampered by the lack of a suitable cell culture. Several studies have focused in a suitable *in vitro* system for replication and propagation of anelloviruses, but so far an inefficient TTV propagation in different cell lines of human origin has been achieved (de Villiers et al., 2011; Kakkola et al., 2007; Leppik et al., 2007). In pigs, the lack of knowledge is greater, and TTSuV propagation in cell culture has not been almost explored.

Apart from those methods, a multiple-primed rolling circle amplification (RCA) method has been also optimized (Dean et al., 2001). RCA amplify circular DNA molecules without knowing the nucleotide sequence to be amplified; the technique uses random hexamers primers for amplification. This technique allowed the identification of new isolates of TTSuVs (Cornelissen-Keijsers et al., 2012; Macera et al., 2011; Niel et al., 2005).

In situ hybridization (ISH) methods have also been developed (Cheng et al., 2000; Krakowka and Ellis, 2008; Lee et al., 2014; Rodríguez-Iñigo et al., 2000; Zhong et al., 2002), but their reliability is under debate.

Despite the different techniques developed, the diagnosis of anellovirus infection has been mainly dependent on PCR or qPCR detection of viral DNA using primers specific for the non-coding regions.

CHAPTER II: HYPOTHESIS AND OBJECTIVES

2 Hypothesis and objectives

The role played by TTSuVs, alone or in co-infection with other swine viral agents is still unknown or questionable. In previous years, a number of works have pointed out TTSuVs as cofactors in the development of PCV2-SD, although the role played by TTSuV1 or TTSuV2 in this process is still debatable. The study of anelloviruses in disease scenarios highlighted that tissue distribution and genetic variability of the virus are key factors that must be considered to clarify anelloviruses pathogenicity. Additionally, evidence of human anelloviruses demonstrated that serum and tissue viral loads are strongly related with disease development. Most of the reported studies used PCR to detect TTSuVs; however, one of the less studied fields, and consequently poorly understood, is the host immune response facing anelloviruses in acute and persistent infection.

The present PhD Thesis aimed to investigate the pathogenesis of TTSuVs in co-infection with PCV2 as well as to study the host humoral immune response during natural TTSuVs infection. The specific objectives were:

- To study the dynamics of TTSuV1 and TTSuV2 DNA loads in serum of healthy and PCV2-SD affected pigs
- To investigate TTSuV1 and TTSuV2 viral load and distribution in tissues of healthy and PCV2-SD affected pigs
- To study the humoral immune responses against TTSuV infections and its correlation with TTSuVs presence in serum of healthy pigs, along their productive life

CHAPTER III

Chapter III: Study I: Dynamics of *Torque teno sus virus* 1 (TTSuV1) and 2 (TTSuV2) DNA loads in serum of healthy and *Porcine circovirus types 2*-systemic disease (PCV2-SD) affected pigs

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3.1 Introduction

Anelloviruses are vertebrate infecting, non-enveloped, icosahedral viruses with a circular single-stranded DNA genome (Nishizawa et al., 1997). Swine infecting anelloviruses are grouped in two separated genus (http://www.ncbi.nlm.nih.gov/ICTVdb/): Iotatorquevirus, including TTSuV1a and 1b; and, Kappatorquevirus, including TTSuVk2a and k2b (Cornelissen-Keijsers et al., 2012; Huang et al., 2010b). TTSuVs have been found in swine serum worldwide in high prevalence. Infection rate increases with age and most animals get persistently infected (Bigarré et al., 2005; Gallei et al., 2010; Kekarainen et al., 2006; Martelli et al., 2006; Sibila et al., 2009a; Taira et al., 2009) and it is likely that both genus are ubiquitous in domestic pigs and wild boar (Kekarainen and Segalés, 2009). TTSuVs have been also found in biological fluids, indicating the occurrence of vertical and horizontal transmission (Aramouni et al., 2010; Kekarainen et al., 2007; Martínez-Guinó et al., 2009; Pozzuto et al., 2009; Sibila et al., 2009a). Also, tissues have been found PCR positive from the second third of gestation onwards (Aramouni et al., 2010).

Currently, the disease causing potential of anelloviruses is under debate. Human TTVs are apparently related to liver, respiratory diseases, haematological disorders and cancer (Okamoto, 2009). In pigs, it has been suggested that TTSuVs infection could be a factor of aggravation in co-infection with other pathogens, mainly PCV2. PCV2 is the essential but not sufficient cause of PCV2-SD, the economically most important porcine circovirus disease (PCVD). TTSuV2, but not TTSuV1, prevalence has been found to be significantly higher in PCV2-SD 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 PCV2-SD (Ellis et al., 2008). Combined infection of TTSuV1 and PRRSV has been linked to a 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 PCV2-SD and TTSuVs (Lee et al., 2010). Taking into account the potential relationship between TTSuVs and PCV2-SD, a quantitative approach was considered in a longitudinal study of pigs developing PCV2-SD. 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 PCV2-SD from their first week of age until the disease

outbreak. Such objective was accomplished by means of a newly developed qPCR based on The Light Upon eXtension (LUX™) technique.

3.2 Materials and methods

3.2.1 Animals and samples

Clinically healthy animals (n = 17) and PCV2-SD 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 PCV2-SD 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 ISH (Rosell et al., 1999). PCV2-SD pigs were chosen based on PCV2-SD like clinical signs confirmed subsequently by histopathological findings and amount of PCV2 measured by ISH (Segalés et al., 2005).

Included animals were from six different Spanish herds. Blood was taken at 1, 3, 7, 11 and around 15 weeks of age (time when the PCV2-SD 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 PCV2-SD 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 ºC until further processed.

3.2.2 Quantitative PCR (qPCR)

3.2.2.1 DNA extraction

DNA was extracted from 200 ml of serum using Nucleospin Blood and eluted in 100 ml of elution buffer (5 mM 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.

3.2.2.2 Primer design

GenBank entries AB076001 and AY823990 for TTSuV1 and TTSuV2 genomes, respectively, were used for the design of the corresponding primers. The UTR of the genome of both viruses was chosen for the primers design, since it is a highly conserved area of these viral genomes (Cortey et al., 2011; Okamoto et al., 2000a). TTSuV1 forward primer (TTSuV1F), TTSuV1 reverse primer (TTSuV1R), TTSuV2 forward primer (TTSuV2F) and TTSuV2 reverse primer (TTSuV2R) (table 3.1) 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, porcine kidney (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), using the BLAST software and in direct qPCR assays.

3.2.2.3 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 TG 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/ml and tested by qPCR to ensure that standard curve parameters are in accepted values (figure 3.1). Two ml of the standards ranging between 10^5 and 10 molecules/ml were used subsequently for the quantification of TTSuV1 and TTSuV2 in the studied samples.

Table 3.1: 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

3.2.2.4 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-dye in a total volume of 20 µl. Amplification and quantification were performed using ABI 17500 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 60 ºC.

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 of generated amplicon 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.

3.2.3 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 PCV2-SD 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.

3.3 Results

3.3.1 Quantitative PCR optimization

Only qPCR reactions with a SD <0.5 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 77 ºC for TTSuV1 and 82 ºC for TTSuV2 were accepted. Reactions not fulfilling those criteria or with contaminated negative controls were repeated.

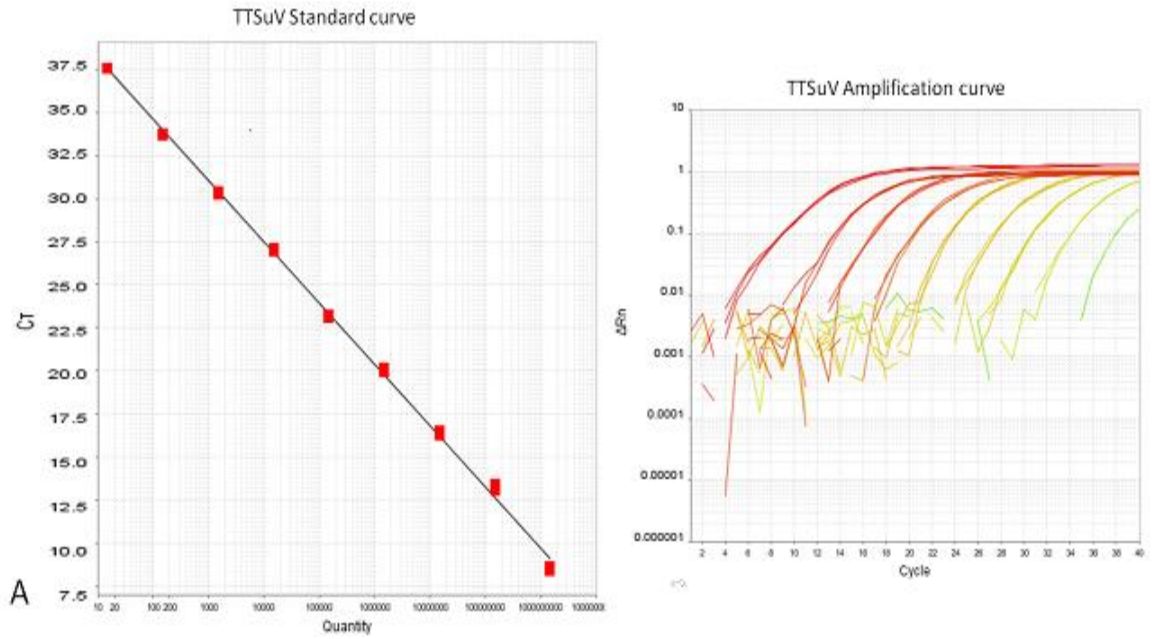

Figure. 3.1: Generation of a standard curve to assess reaction optimization using a 10-fold dilution of a quantified TTSuV2 template and amplified by ABI 17500 Fast Real Time PCR System (Applied Biosystems^{TM}). Each dilution was assayed in triplicate. (A) Standard curve with the CT plotted against the log of the starting quantity of template for each dilution. (B) Amplification curves of the dilution series.

3.3.2 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.

3.3.3 Prevalence of TTSuV1 and TTSuV2 in serum samples

Prevalence of TTSuVs in healthy and PCV2-SD affected pigs at different ages are shown in table 3.2. 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 PCV2-SD groups, while for TTSuV2 a tendency ($p < 0.1$) was observed between healthy and PCV2-SD affected pigs in the last two sampling points.

3.3.4 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 3.2). A similar pattern was observed for TTSuV2 in healthy animals. However, TTSuV2 loads in PCV2-SD affected animals increased until last sampling point corresponding to the clinical manifestation of the disease. At that point, PCV2-SD 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 3.3). For TTSuV2, profile A included 16 pigs (11 healthy, 5 PCV2-SD) on average with decreasing viral DNA loads throughout the study. At the last sampling point (necropsy time), all the animals had mean viral loads below 5 log10. In the profile B, 19 pigs (6 healthy, 13 PCV2-SD) were included, which showed increasing viral loads with mean viral load at necropsy above 6 log10. The percentage of healthy and PCV2-SD 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).

Table 3.2: 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
PCV2-SD	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

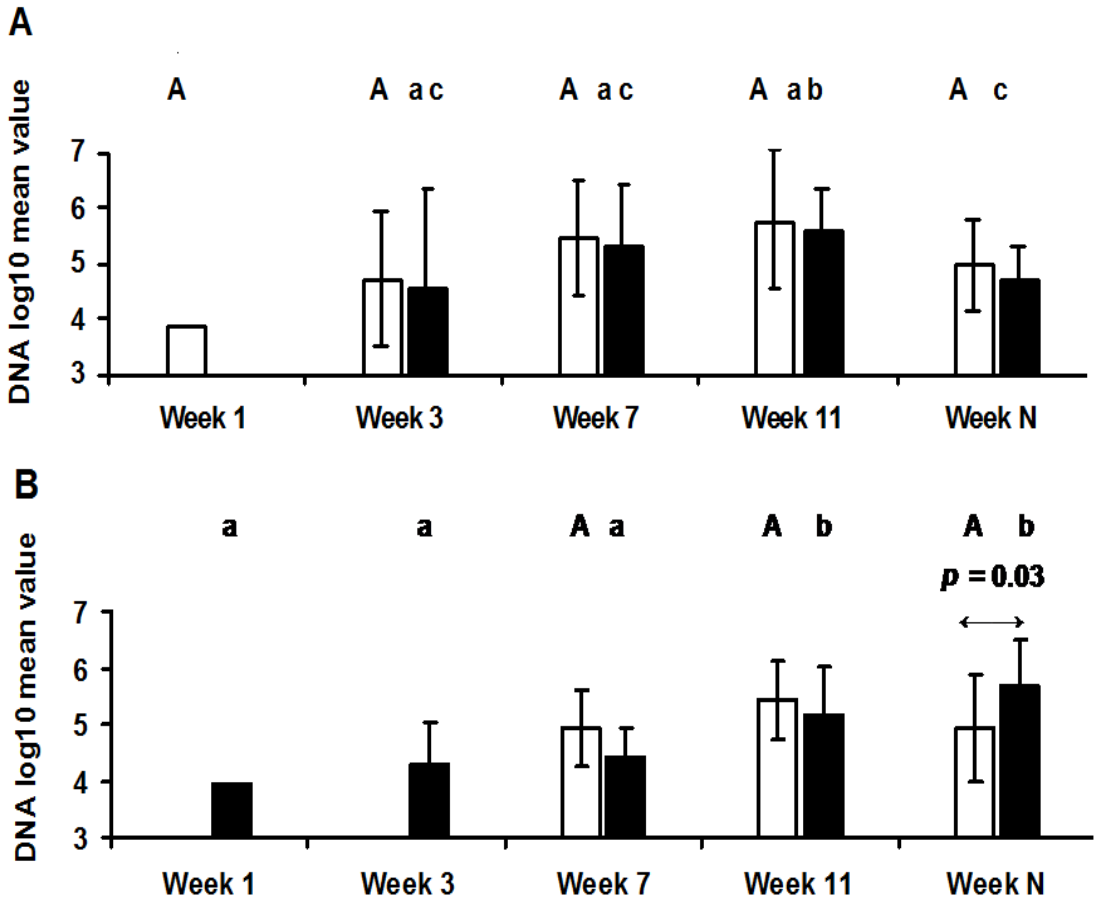

Figure 3.2: TTSuV1 (A) and TTSuV2 (B) viral load dynamics in healthy (white bars) and PCV2-SD (black bars) affected animals. Mean viral loads and standard deviation at different sampling times are represented in log10 scale. p-values for significant differences are shown. Different letters mean significant differences between ages within healthy (capital letters) or PCV2-SD (case letters) groups.

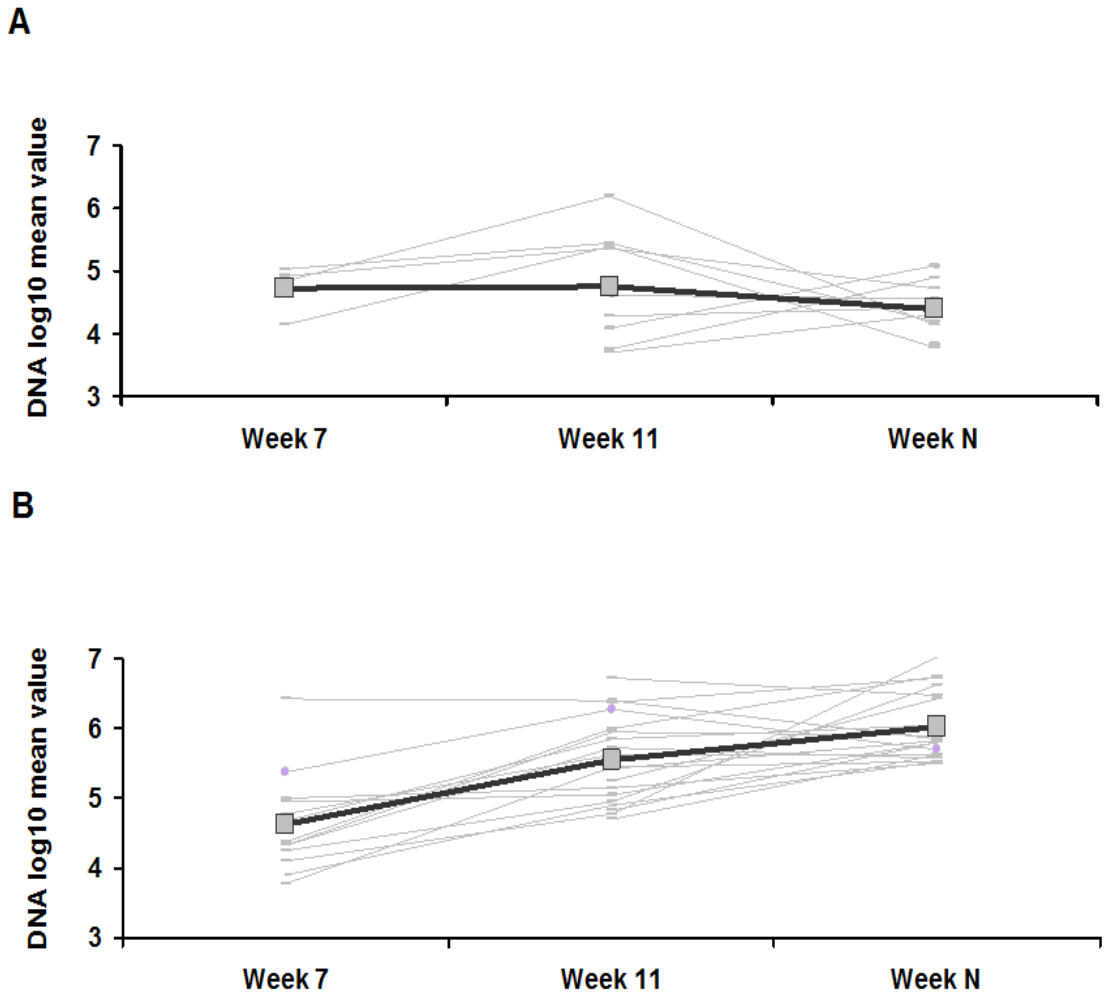

Figure 3.3: Profiles generated by the MeV software (A and B) grouping TTSuV2 infected animals according to individual viral load dynamics. Grey lines: viral load profile for each individual pig; black line: mean viral load of all pigs belonging to the same profile.

3.4 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 PCV2-SD, 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 PCV2-SD occurrence and TTSuV2.

PCV2-SD 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 PCV2-SD 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 PCV2-SD occurrence. It has been proposed that some porcine and human anelloviruses might be more disease-linked than others (Fehér et al., 2011; Kekarainen et al., 2006; Okamoto, 2009), and co-infection with other viruses could affect the outcome or progression of some diseases (Fehér et al., 2011). Papillomaviruses are one of the best known examples of different virulence depending on the viral species (Howley and Lowy, 1996). A closer example in pigs in regards different virulent capabilities comes from pathogenic PCV2 and non-pathogenic 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 range from 52-54% (Cortey et al., 2011; Huang et al., 2010b) while 60–70% in papillomaviral species (de Villiers 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., 2011). 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 (Aramouni et al., 2010; Martínez-Guinó et al., 2009; Pozzuto et al., 2009; Sibila et al., 2009b). 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 PCV2-SD 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., 2009b). On the other hand, in Japanese pigs with PCV2-SD like clinical signs (the disease was not confirmed by means of the classical laboratory criteria) or porcine respiratory disease complex, TTSuV2 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 PCV2-SD 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, 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 (Focosi et al., 2010; Maggi et al., 2010).

Although several recent studies have been reporting TTSuV viral loads (Brassard et al., 2010; Gallei et al., 2010; Lee 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 PCV2-SD (Lee et al., 2010). 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., 2010; Gallei et al., 2010) or a qPCR technique not able to differentiate the viral species (Brassard et al., 2010) 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.

CHAPTER IV

Chapter IV: Study II: *Torque teno sus virus* 1 and 2 distribution in tissues of *Porcine circovirus type 2*-sytemic disease affected and age-matched healthy pigs

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4.1 Introduction

TTVs (family *Anelloviridae*) have a circular ssDNA genome encapsidated into non-enveloped, icosahedral virions. TTSuVs are ubiquitous viruses distributed worldwide and have been circulating in pigs at least since 1985 (Segalés et al., 2009). Viral prevalence increases with age, leading to persistent infection in a proportion of adult animals (Kekarainen and Segalés, 2012). TTSuVs are transmitted by horizontal and vertical routes (Aramouni et al., 2010; Sibila et al., 2009a; Sibila et al., 2009b).

Anellovirus infections are believed to cause no disease, but it is suggested that they may act as co-factors in certain diseases (Kekarainen and Segalés, 2012). In pigs, TTSuVs have been associated with PCV2-SD (Aramouni et al., 2011; Ellis et al., 2008; Kekarainen et al., 2006). PCV2-SD is considered a multifactorial disease in which PCV2 infection is necessary but not sufficient (Segalés, 2012). From a diagnostic point of view, determination of viral load of ssDNA viruses is more important than assessing its prevalence. Such issue has been demonstrated in PCV2-SD affected pigs (Grau-Roma et al., 2009; Krakowka et al., 2005; Olvera et al., 2004) as well as in disease scenarios in humans (Maggi et al., 2003b). Latest authors have demonstrated that a high human TTV load in serum is linked with increased disease severity in association with other pathogens.

Only few studies have focused on anellovirus detection in tissues (Aramouni et al., 2010; Bigarré et al., 2005; Okamoto et al., 2001a). Therefore, the objective of this study was to determine TTSuV1 and TTSuV2 DNA loads and prevalence in different tissues of PCV2-SD and age-matched healthy pigs.

4.2 Material and methods

4.2.1 Animal selection

A total of 20 pigs were selected from four herds in northeastern Spain from a previous epidemiological study (Grau-Roma et al., 2009). Criteria accomplishing PCV2-SD (P) and healthy (H) groups are described in detail by Grau-Roma and collaborators (2009). All

treatments, housing, husbandry and slaughtering conditions were conformed to the European Union Guidelines and Good Clinical Practices.

4.2.2 Tissues

When the PCV2-SD outbreak took place, both healthy and diseased animals were necropsied and tissue and serum samples stored at -80 ºC until further processing. Seven tissues of each pig were analyzed, including bone marrow (BM), ileum (IL), kidney (K), liver (LI), lung (LU), and mesenteric (LMES, one lymph node from a PCV2-SD affected pig was missing) and mediastinal (LMED) lymph nodes.

4.2.3 DNA extraction and TTSuVs quantification

DNA was extracted from 25 mg of frozen tissues, using NucleoSpin® Tissue kit (Macherey-Nagel) as recommended and quantified using NanoDrop. DNA samples were then adjusted to 125 ng/ml using autoclaved bi-distilled water. In few cases, DNA quantification was below 125 ng/ml and therefore, such sample was not further diluted.

For qPCR, 250 ng (2 µl) DNA sample was used as a template following the published protocol in chapter III. The average log10 copies per microgram of extracted DNA were used to compare data. PCV2 and TTSuVs loads per ml of serum were assessed in all studied samples as described (Olvera et al., 2004) and (chapter III).

4.2.4 Statistical analysis

The Chi-square test was used to compare the proportion of positive qPCR results between the studied tissues and groups of animals. Viral load differences among tissues and animal status (PCV2-SD versus healthy) were analyzed by means of ANOVA (using SAS software; SAS Institute Inc.,Cary, North Carolina, USA). Statistical significance level was set at $p < 0.05$).

4.3 Results

TTSuV1 and TTSuV2 prevalence in different tissues and serum from healthy and PCV2-SD affected pigs are displayed in figure 4.1. The highest prevalence for TTSuV1 was observed in BM of both H and P animals. For TTSuV2, the highest prevalence was observed in LU and BM in healthy pigs and BM in PCV2-SD affected ones. Both viruses were detected in all studied tissue types, and co-infection was commonly observed. The number of tissues infected by one or the other TTSuV in a given animal was variable (table 4.1). Only 3 pigs had all tissues qPCR negative for TTSuV1 or TTSuV2.

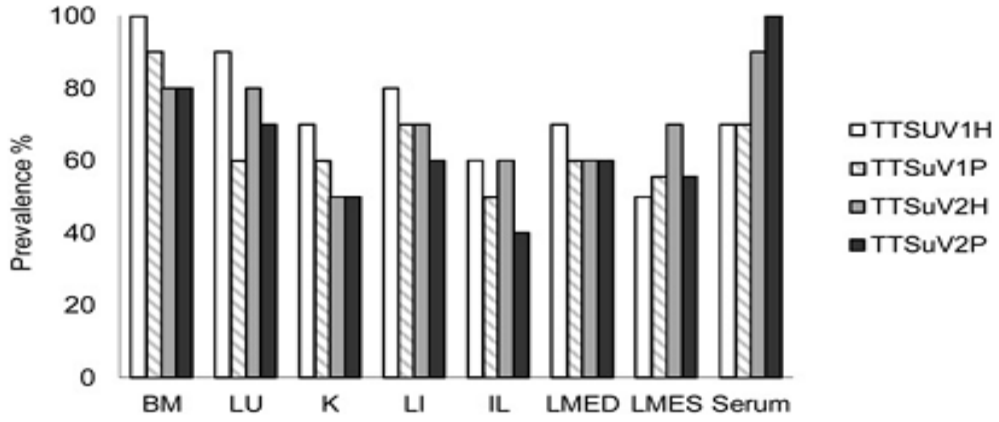

Figure 4.1: TTSuV1 and TTSuV2 prevalences observed in the studied tissues and serum of healthy (H) and PCV2-SD (P) affected pigs. BM, bone marrow; LU, Lung; K, Kidney; LI, Liver; IL, Ileum; LMED, mediastinal lymph node; LMES, mesenteric lymph node.

A correlation between number of tissues infected of a given animal and the viral load for the corresponding TTSuV in serum was observed. In consequence, pigs with all tissues qPCR positive had also the highest viral loads in serum, except for TTSuV1 H pigs. Five out of 7 pigs with serum viral loads under the detection limit yielded positive results in tissues.

Overall, TTSuV1 (6.43 log10 copies/mg of total DNA, standard deviation, SD 1.11 log10) and TTSuV2 load in tissues (7.72 log10, SD 1.08 log10) were statistically different. In case of TTSuV2, significant differences in mean tissue viral load were observed between healthy (7.47 log10, SD 0.63 log10 copies/mg of total DNA) and PCV2-SD (8.00 log10,

SD 1.39 log10) groups. Such difference was not significant comparing TTSuV1 loads in healthy (6.32 log10, SD 0.82 log10) with those of diseased pigs (6.56 log10, SD 1.36 log10).

Table 4.1. Number of qPCR positive tissues for TTSuV1 and TTSuV2 and mean viral load in serum (log10 viral DNA copies/ml) per each individual pig within the healthy and PCV2-SD groups.

	TTSuV1		TTSuV2	
Pig*	**No. tissues infected/total tissues**	**Viral load in serum**	**No. tissues infected/total tissues**	**Viral load in serum**
1-H	6/7	6.44	7/7	6.23
2-H	7/7	5.82	7/7	5.65
3-H	7/7	5.65	7/7	5.97
4-H	1/7	5.58	0/7	UDL
5-H	6/7	5.31	6/7	5.54
6-H	7/7	4.71	0/7	4.28
7-H	7/7	4.15	7/7	5.93
8-H	5/7	UDL	6/7	4.60
9-H	4/7	UDL	3/7	3.71
10-H	2/7	UDL	4/7	4.81
1-P	7/7	7.93	3/7	3.75
2-P	7/7	6.09	7/7	10.04
3-P	7/7	5.73	7/7	6.49
4-P	7/7	4.98	7/7	7.01
5-P	6/7	4.86	2/7	4.59
6-P	6/7	4.77	1/7	5.13
7-P	1/7	3.98	5/7	5.88
8-P	0/6	UDL	2/6	4.93
9-P	1/7	UDL	1/7	4.32
10-P	2/7	UDL	7/7	6.66

* Individual pigs numbered from 1 to 10 within each animal group: healthy (H) or PCV2-SD affected (P). UDL = under the detection limit of the qPCR technique.

Table 4.2 summarizes the mean viral loads per tissue type and group of studied pigs. BM was the tissue with the highest mean viral load in either healthy or PCV2-SD affected pigs for both TTSuVs; tissues with the highest mean viral load were always found in the PCV2-SD group for TTSuV2. Statistical differences in viral load among studied tissues were observed only within healthy animals, for both TTSuV species (table 4.2).

Table 4.2: Mean TTSuV DNA loads (log 10 viral load/mg of total DNA) in different tissues in healthy (H) and PCV2-SD (P) affected pigs. BM, bone marrow; LU, Lung; K, Kidney; LI, Liver; IL, Ileum; LMED, mediastinal lymph node; LMES, mesenteric lymph node; TL, Mean tissue DNA load; SD, Mean serum DNA load (log10 viral load/ml of serum).

Tissue/ Group	TTSuV1H	TTSuV1P	TTSuV2H	TTSuV2P
BM	6.92 (a)	6.87 (a)	8.18 (a)	8.47 (a)
LU	6.57 (a,b)	6.74 (a)	7.61 (a,b)	7.62 (a)
K	6.39 (a,b)	6.33 (a)	7.14 (b)	7.61 (a)
LI	6.25 (a,b)	6.49 (a)	7.32 (b)	8.08 (a)
IL	6.04 (a,b)	6.50 (a)	6.92 (b)	7.96 (a)
LMED	5.85 (a,b)	6.44 (a)	7.68 (a,b)	8.07 (a)
LMES	5.67 (b)	6.34 (a)	7.20 (b)	8.10 (a)
TL	6.32	6.56	7.47*	8.00*
SD	5.38	5.48	5.19	5.88

Different letters in brackets mean statistically significant differences within the same column. * Significant difference in mean TL between healthy and PCV2-SD affected pigs

4.4 Discussion

Recently, scientific interest in TTSuVs has risen due to its possible disease association or its consideration as a co-factor of diseases (Kekarainen and Segalés, 2012). Co-infection with TTSuV2 has been debated as a factor for PCV2-SD aggravation (Aramouni et al., 2011; Kekarainen et al., 2006), but other studies linked PCV2-SD with TTSuV1 (Ellis et al., 2008) or did not find any association (Lee et al., 2010).

In studied pigs, prevalence of both TTSuVs was similar in healthy and PCV2-SD affected pigs, and co-infection was commonly observed. Although pig infecting anelloviruses are genetically distinct, tissue distribution was fairly similar for both viral species. Opposed to some other studies in humans, where different tissue distribution has been described depending on the TTV species considered (Maggi et al., 2003a; Okamoto et al., 2001a).

In the present study, TTSuVs were mostly prevalent in BM, LU and LI, similarly to other works performed in humans and pigs (Bigarré et al., 2005; Okamoto et al., 2001a). The highest viral load was systematically detected in BM, in both groups of studied pigs. In

humans, BM has been suggested as a site of viral replication as double stranded intermediate replicative form of human TTV has been detected in BM (Okamoto et al., 2000b).

In 5 out of 7 non-viremic pigs for TTSuV1 or 2, viral DNA was detected in tissues of these animals. This indicates that replicating virus was detected rather than virus trapped in the tissue from the circulating blood. In those animals, BM was always qPCR positive, suggesting a main role of this tissue in virus replication, although the participation of other tissues/organs cannot be ruled out. It can be speculated as well that the immune response may have cleared out the virus from the serum, while those tissues may act as a persistent source of viral replication. Moreover, these findings further demonstrate that TTSuVs prevalence based on serum detection definitively underestimates the true prevalence of infection (Aramouni et al., 2010).

The study of TTSuVs load in tissues was considered of interest because many studies indicate that high loads of pathogens can be related with disease occurrence, as is the case of PCV2-SD (Olvera et al., 2004) and CAV infection. In the latter one, the extent of clinical signs had been positively linked with viral load in particular organs (Tan and Tannock, 2005). Such correlation has also been observed in TTV in human, where high viral load is related with severity of disease induced by other agents (Maggi et al., 2003b).

The present work showed differences between TTSuV1 and TTSuV2 loads within healthy or diseased groups of pigs, and also between both animals groups for TTSuV2. When studying differences between both viral species, it was observed that TTSuV2 loads in PCV2-SD affected pigs reached values above 8 log10 DNA viral copies/mg of total DNA in LMES, LMED, LI and BM. Those high viral loads were not observed in any other tissue types except for TTSuV2 in BM of healthy pigs. The diagnostic value of viral load has been fairly well established in the case of PCV2-SD, where above 10^7 genomes of PCV2/ml of sera has been related with PCV2-SD diagnosis (Olvera et al., 2004). At this point, high TTSuVs amounts are linked with disease occurrence, but its diagnostic value, if any, is to be established.

The direct causality of disease by TTVs has been suggested. High TTV loads in human have been related with the development of lymphoproliferative disorders like B-cell

lymphoma and Hodgkin's disease (Figueiredo et al., 2007; Garbuglia et al., 2003). Interestingly, a recent in vivo experiment using caesarean derived pigs inoculated with an infectious TTSuV2 clone developed lesions in brain, kidney and liver (Huang et al., 2012). Furthermore, higher TTSuV2 loads have been found in lungs with interstitial pneumonia (viral-like lesion) when compared with healthy lungs or lungs with bacterial-like lesions (Aramouni et al., 2013b). In the present case, however, it can be hypothesized that high loads of TTSuV2 in tissues promote replication of PCV2 and subsequent development of PCV2-SD. However, it would not be surprising that the immunosuppression characteristic of PCV2-SD affected pigs might have allowed TTSuV2 to increase tissue replication, as it has been previously suggested in chapter III of this thesis. In summary, the present study describes the tissue distribution of both TTSuV in healthy and PCV2-SD affected animals. BM was the tissue yielding the higher viral loads, and TTSuV2 load in tissues was significantly higher than that of TTSuV1.

CHAPTER V

Chapter V: Study III: Development of an indirect ELISA assay for the detection of IgG antibodies against the ORF1 of *Torque teno sus viruses* 1 and 2 in conventional pigs

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5.1 Introduction

Anelloviridae is a family of single stranded circular DNA viruses infecting mammals (Okamoto et al., 1998b). Anelloviruses are species specific, characterized by a high degree of genetic divergence, and similar genome organization and gene expression strategy (Martínez-Guinó et al., 2011; Okamoto et al., 2001b). Swine infecting anelloviruses are grouped in two separated genera: *Iotatorquevirus* and *Kappatorquevirus* (Cornelissen-Keijsers et al., 2012; Huang et al., 2010b).

Prevalence of infection increases with age, with early infection occurring during the lactation period, although a percentage of animals are born already infected (Martínez-Guinó et al., 2010; Pozzuto et al., 2009; Sibila et al., 2009b). Once infection is established, TTSuVs are commonly detected in sera and several tissues (Aramouni et al., 2010; Bigarré et al., 2005; Gallei et al., 2010; Xiao et al., 2012).

Up to date, there is no known disease caused exclusively by anelloviruses, but it has been suggested that in the background of other well-known swine pathogens, like PCV2, PRRSV or in the framework of PRDC, TTSuV could trigger the development of disease or to aggravate it (Ellis et al., 2008; Krakowka et al., 2008; Rammohan et al., 2012).

Currently, little is known about swine immune responses against TTSuV infections. Just recently, the first swine serological assays against TTSuV1 and TTSuV2 have been developed (Giménez-Lirola et al., 2014; Huang et al., 2011; Huang et al., 2012a; Jarosova and Celer, 2013). To better understand the dynamics of the host immune response, an ELISA assay was developed using the ORF1-A of both TTSuVs as antigen (Martínez-Guinó et al., 2011). With the developed ELISA assay, and in combination with the qPCR developed in chapter III, the dynamics of the host immune response and its correlation with TTSuVs infection were studied in 37 healthy pigs, from weaning until they reached the slaughter age, as well as in their dams.

5.2 Material and methods

5.2.1 Expression and production of TTSuV1 and 2 ORF1 splice variants using a prokaryotic expression system

ORF1-A splicing variants of TTSuV1 and TTSuV2 were subcloned into pET24a vector in frame with polyhistidine tag (Novagen) (Martínez-Guinó et al., 2011). Bacterial colonies containing the pET-ORF1-A of TTSuV1 or TTSuV2 were scraped and inoculated on agar plates containing Kanamycin/Cloramphenicol (Kan/Cam), and left them grow overnight (o/n) at 37 ºC. The day after, colonies containing the pET-ORF1-A of TTSuV1 or TTSuV2 were inoculated into 3 ml Luria-Bertani (LB) broth Kan/Cam starter cultures and incubated for 8 h at 37 ºC. Starter cultures were subsequently inoculated into 100 ml of LB broth Kan/Cam, and left them grow at 37 ºC until optical density (OD) 600 reached an absorbance of 0.5. At this point, β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM was added to induce protein expression, and cultures were incubated for another 4 h at 37 ºC. After 4 h, cells were pelleted by centrifugation at 3,220 x g for 30 min at 4 ºC, the supernatant discarded, and the pellets were stored at -80 ºC prior to protein purification.

5.2.2 Purification and quantification of TTSuVs recombinant proteins

Purification of insoluble TTSuVs ORF1-A proteins were done under denaturing conditions, with the presence of urea 8 M, according to recommendation of the manufacturer. Briefly, binding buffer (0.1 M NaH_2PO_4 , 10 mM Tris-HCl , 8 M urea, pH 8) was added to the pellet together with a protease inhibitor cocktail pefabloc 1 mM (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride). After mixing, samples were sonicated for 15 min, 95% amplitude and 0.5 cycles. Once finished, all samples were centrifuged at 10,000 x g for 30 min at 4 ºC, the supernatant recovered. Purification of the protein was carried out using histidine (His) SpinTrap columns containing Ni Sepharose™ High Performance (GE Healthcare), the previously described binding buffer, washing buffer (TrisHCl 20 nM, NaCl 500 nm, Imidazol 40 nM, β-mercaptoethanol 98%, urea 8 M)

and elution buffer (TrisHCl 20 nM, NaCl 500 nM, Imidazol 500 nM, β-mercaptoethanol 98%, Urea 8 M). The eluted protein was kept at -80 ºC.

After protein purification, samples were dialysed using dialysis cassettes with 20.000-Da molecular weight cut off (Pierce). A gradient of 4 dialysis buffers were used for dialysis (Huang et al., 2011). Proteins were dialyzed in each dialysis buffer for at least 6 h at 4 ºC. Purified recombinant ORF1-A proteins were quantified using BCA™ Protein Assay Kit (Pierce) following manufacturer's instructions.

5.2.3 Western blot analysis

Western blot (WB) analysis was performed to confirm if purified proteins corresponded with the desired TTSuVs ORF1-A proteins. So far, 15 µl of each ORF1-A TTSuV1 or TTSuV2 proteins were used as antigens for the WB analysis. At the same time, a pre-stained protein ladder (Benchmark) and pre-stained his-tag protein (Benchmark) were also run. All samples were run under denaturing conditions in NuPAGE® (4-2%) Bis-tris gels (Invitrogen) for 2 h, and then electroblotted to a Hybond® ECL™ nitrocellulose membrane (GE Healthcare) during 4 h. The membrane was blocked with casein 2% overnight at 4 ºC, and subsequently, incubated with anti-his (C-term) horseradish peroxidase (HRP) monoclonal antibody, 1:100.000 (Invitrogen) in casein 2% for 2 h at room temperature. Protein detection was done by incubating the membrane with Lumigen A + B working solution prepared by 1:1 mixing, in darkness. Between each incubation step, the membrane was washed 3 times with T-TBS (Tris buffered saline with tween 20) and visualized by means of a Fluorochem HD2 chemiluminescent workstation (Alpha Innotech).

Expressed protein (about 30 kilo (k) dalton (Da)) was highly pure as assessed by comassie brilliant blue staining. Only the bands corresponding to the recombinant ORF1-A recombinant protein were observed (data not shown). The specificity of the purified protein was confirmed by Western blotting using anti His-tag monoclonal antibody. ORF1-A purified protein from TTSuV1 and TTSuV2 were correctly expressed in Escherichia coli transfected cells.

5.2.4 Sources of porcine sera

Porcine sera used for detecting IgG antibodies against ORF1-A from TTSuV1 or TTSuV2 come from conventional healthy pigs (n=37) and their dams (n=15), selected from a larger PCV2 vaccination study (Fraile et al., 2012b). All piglets were clinically monitored from weaning (4 weeks of age) until slaughter; blood samples were taken at 4, 8, 12, 16, 21 and 25 weeks of age. Sows were bled one week before farrowing. All samples were individually identified and transported in refrigeration to the laboratory. Then, sera were collected and stored at -80 ºC until testing. Treatments, housing and husbandry conditions conformed to the European Union Guidelines and Good Clinical Practices.

5.2.5 Real-time qPCR assays for quantitation of TTSuV1 and 2

To quantify TTSuV1 and TTSuV2 DNA loads, 2 µl of sample DNA were used and an already described qPCR was applied (chapter III). The average $\log_{10}$ copies per ml of serum was used to compare samples; all samples and controls were tested per triplicate.

5.2.6 Indirect TTSuV1 and TTSuV2-specific ELISA

The purified recombinant ORF1-A proteins were used to develop indirect ELISAs to detect the presence of antibodies against TTSuV1 or TTSuV2 in swine serum. Optimal conditions and concentration of reagents for ELISA tests (antigen concentration, sera and secondary antibody dilution) were determined by check board titration. Conditions with the greatest differences between the positive and the negative controls, combined with the lowest background, were considered optimal. Then, the ELISA 96-well plates were coated with 100 µl/well of carbonate/bicarbonate coating buffer (pH 9.6) and left incubating o/n at 4 ºC. After 24 h, plates were washed 3 times with 200 µl of T-TBS and then blocked with 100 µl phosphate-buffered saline (PBS) 1x + casein 1% for 1 hour at 37 ºC. After, plates were washed 3 times with 200 µl of T-TBS and subsequently incubated with 100 µl serum sample diluted 1/100 in casein 1% for 2 h at 37 ºC. Then, plates were washed three times with 200 µl of T-TBS and incubated with 100 µl of the HRP conjugated mouse anti-swine

IgG, diluted 1/20.000 in casein 1%. After, wells were 3 times washed with 200 µl T-TBS. Next, the enzymatic reaction was developed by adding 100 µl of tetramethylbenzine (TMB) substrate (Sigma-Aldrich) to each well. Finally, after 15 min at room temperature, the enzymatic reaction was stopped by adding 100 µl of H_2SO_4 per well. Absorbance was measured at 450 nm using a spectrophotometer.

5.2.7 Source of positive and negative controls

To detect positive and negative serum samples to be used subsequently in the ELISA assay, a WB was developed. Following purification, ORF1-A recombinant TTSuV1 was transferred onto the Hybond® ECL™ nitrocellulose membrane and subsequently blocked with casein 2%. The membrane was cut into strips, each individual strip was incubated with sera of porcine origin at final dilution of 1:100 in casein 2%, and after a round of three washing with T-TBS, each strip was incubated with mouse anti-pig IgG at 1:20.000 at room temperature, after, the membrane was visualized by means of a Fluorochem HD2 chemiluminescent workstation (Alpha Innotech). In case of TTSuV2, negative and positive controls were identified based on a published ELISA test (Huang et al., 2011) and recombinant TTSuV2-ORF1 protein, kindly provided by Dr. X.J. Meng (College of Veterinary Medicine, Virginia Tech, Blacksburg, VA, USA).

5.2.8 Statistical analyses

Due to the lack of reference sera for TTSuVs, the ELISA cut-off was calculated as the mean OD of negative controls, plus 3 times the standard deviation. To analyze data, all sample results were transformed as sample to positive ratio (S/P), calculated as: (OD mean serum sample – OD mean negative value)/(OD mean positive values – OD mean negative value). All S/P ratio values were converted into presence or absence of antibodies using a cut-off point calculated as previously mentioned. Differences of antibody levels for each group were compared using a 2-sample t-test. The chi-square test was used to compare the

proportion of positive qPCR and seroprevalence results between the studied pigs. The significant level (α) was set at 0.05.

5.3 Results

5.3.1 Development of an indirect ELISA to detect anti ORF1-A IgG in swine serum

ORF1-A-His-tagged proteins of TTSuV1 and TTSuV2 were bacterially expressed, purified and subsequently used as antigens in the optimization of ELISA assays. The optimal conditions for the ELISA tests were the following: optimal protein concentration of 68 ng/well, optimal swine serum dilution 1:100 and optimal secondary antibody dilution 1:20.000.

Since, there are no reference sera for TTSuVs. Fifteen serum samples were WB and ELISA tested using ORF1-A TTSuV1 as antigen. The results showed that 6/6 serum samples that were negative to the ELISA assay were also negative when WB tested. At the same time, from 9 serum samples tested positive in ELISA assay, a total of 7 sera samples were also confirmed positive when WB tested.

In case of TTSuV2 sera samples were ELISA tested twice, first based on the published ELISA test (Huang et al., 2011) and recombinant TTSuV2-ORF1 protein, kindly provided by Dr. X.J. Meng (College of Veterinary Medicine, Virginia Tech, Blacksburg, VA, USA) and secondly using our ELISA test, samples giving positive results and negatives results in both techniques were considered positive and negative respectively.

Finally, the positive and negative controls used in all ELISAs were two sera being positive (used as a positive control) and negative (used as a negative control) for both TTSuVs.

5.3.2 Antibody and infection dynamics of TTSuV1

Samples were considered positive when the S/P ratio was above the cut-off limit, which values ranged from 0.13 to 0.17, depending upon the plate.

Sow sera were not tested by real time quantitative PCR (qPCR), but all of them (n=15) were seropositive to ORF1-A with a mean S/P ratio of 0.51 ± 0.14 (figure 5.1).

Regarding the piglets, 37 were included in the study but qPCR was only available in 32 of them. Twenty eight out of 32 (88%) pigs were qPCR positive for TTSuV1 at one or more time points during the study. At week 8, 59% (19/32) of pigs were TTSuV1 viremic, while at week 21 the number of qPCR positives increased to 81% (26/32) (figure 5.1). Overall, the analyses showed that infection occurred early in life and prevalence increased progressively until slaughter (figure 5.1).

The viral loads in the TTSuV1 qPCR positive pigs showed that mean DNA viral load was very stable along the trial, with an average $\log_{10}$ DNA copies per ml of serum of 4.38, 4.27 and 4.47 at weeks 8, 16 and 21, respectively. The statistical analysis did not observe differences in viral load or prevalence between any of the study sampling point ($p > 0.05$).

From a total of 37 ELISA analyzed pigs, 36 seroconverted during the follow-up period; the remaining pig did not seroconvert, but was viremic on week 21. The ELISA test showed that anti TTSuV1 ORF1-A antibodies were detectable in pigs at 4 weeks of age, 65% of piglets (24/37) with a mean S/P ratio of 0.24, suggesting the maternal origin of the antibodies. Thereafter, a decrease in seroprevalence was observed on week 8, 30% (11/37) with an average S/P ratio of 0.08, suggesting that maternal immunity was waning. Statistically significant differences were observed between both weeks ($p < 0.01$). A progressive increase of seroprevalence was seen from week 12 (38%, 14/37) until end of the study, (68%, 25/37). Average S/P ratio reached maximum at week 16 (S/P ratio 0.24) such value was statistically significant from week 12 ($p < 0.01$) and maintained thereafter (figure 5.1).

The observed 4 non-viremic pigs, as assessed by qPCR were ELISA positive sometime during the study, although for two of them, the positivity was apparently due to the

presence of maternally derived antibodies (MDA) (positive results obtained at 4 and 8 weeks of age).

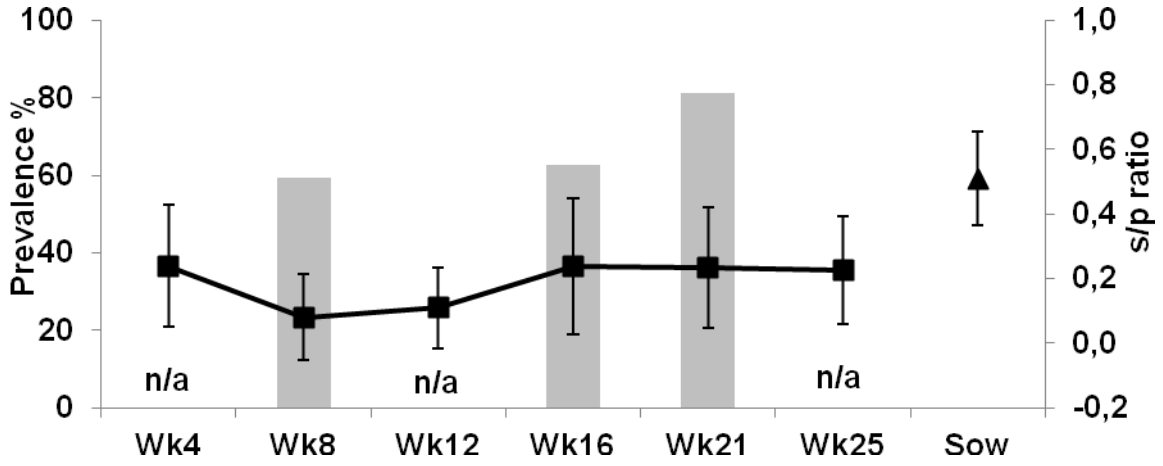

Figure 5.1: Prevalence of TTSuV1 in serum by qPCR at 8, 16 and 21 weeks of age (bars). At weeks 4, 12 and 25, pigs were not qPCR tested, not neither the sows, and average S/P ratios of anti-ORF1-1A IgG, as determined by ELISA test (black line), of pigs from 4 to 25 weeks of age and for sows. n/a, means: prevalence not available at this week.

In the present study, we also investigated the influence of MDA on the development of humoral immune response following waning of maternal antibodies, as it is well known that MDA may interfere with the host immune response development upon infection at early ages. For TTSuV1, differences in the development of the humoral immune response in piglets were investigated. Piglets showing positive serology at week 4 (interpreted as MDA), were included in one group namely, MDA positive piglets (MDA+), and the rest of the piglets were included in the group MDA negative piglets (MDA-). It was observed that in the absence of MDA, seroconvertion occurred quickly, since 77% (10/13) of MDA- pigs had anti TTSuV1 ORF1-A antibodies at week 16 compared with the 50% (12/24) of MDA+ pigs, although differences were not statistically significant. Then, in the MDA- group, the seroprevalence was maintained, while in the MDA+ group increased to 58% (14/24) and 63% (15/24) at weeks 21 and 25, respectively. Mean antibody responses were

significantly higher in MDA- than in MDA+ pigs ($p < 0.05$), specifically at weeks 16 and 21 (figure 5.2).

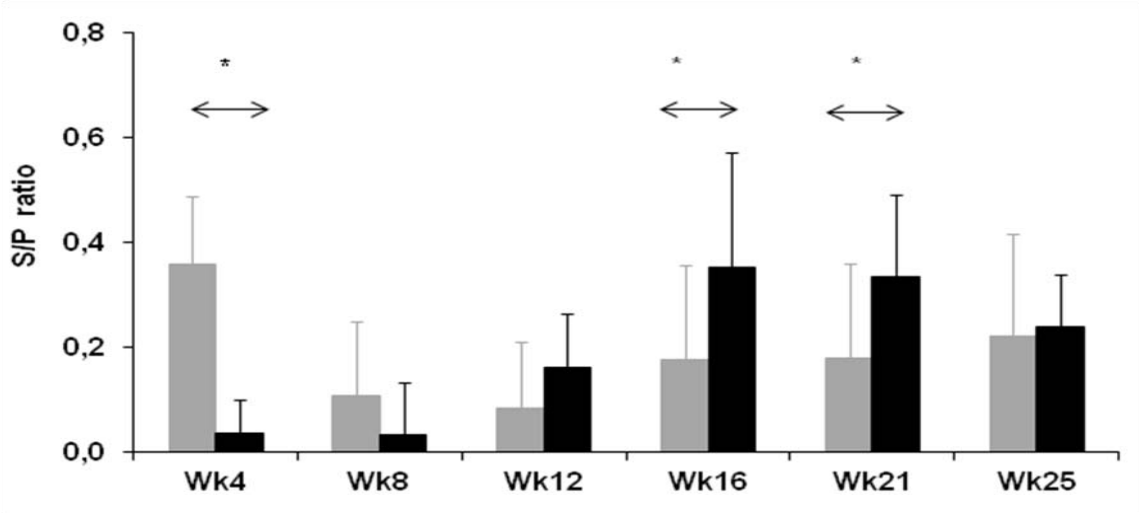

Figure 5.2: Antibody leves of pigs with (grey bars) and without(black bars) maternal antibodies (MDA) * statistically significant differences between the analyzed groups, S/P, sample to positive ratio.

5.3.3 Antibody and infection dynamics of TTSuV2

Samples with S/P ratio above cut-off were considered positive (cut-off ranged from 0.15 to 0.2 depending on the plate).

Sow sera were not tested by qPCR, but thirteen serum samples from a total of 15 sows (87%) were positive to ORF1-A IgG, with a mean S/P ratio of 0.46 ± 0.24 (figure 5.3).

The number of viremic pigs at weeks 8, 16 and 21 were 18/37 (49%), 19/37 (51%) and 34/37 (92%), respectively. All tested pigs were TTSuV2 viremic at least once during the trial. The average $\log_{10}$ copy number increased with time from 4.94 (week 8) to 5.58 (week 21) DNA copies/ml of serum. Differences between weeks 8 and 16 when compared with week 21 were statistically significant for, viral load and prevalence ($p < 0.01$).

Regarding ELISA results, from 37 analyzed pigs, 36 had anti ORF1-A IgGs at least in one sampling point during the trial. The remaining pig was seronegative during all the study, but viremic on week 21. The seroprevalence of anti TTSuV2 ORF1-A antibodies increased with age (figure 5.3). Two pigs out of 37 were seropositive on week 4 (5%), while 31/37 were seropositive on week 21 (84%). Regarding the S/P ratio, a progressive increase was

observed from week 4 until week 25, time when the maximum mean S/P ratio was observed (figure 5.3). Due to the low number of pig having MDA against TTSuV2, their influence on the development of humoral immune response following weaning of maternal antibodies was not assessed.

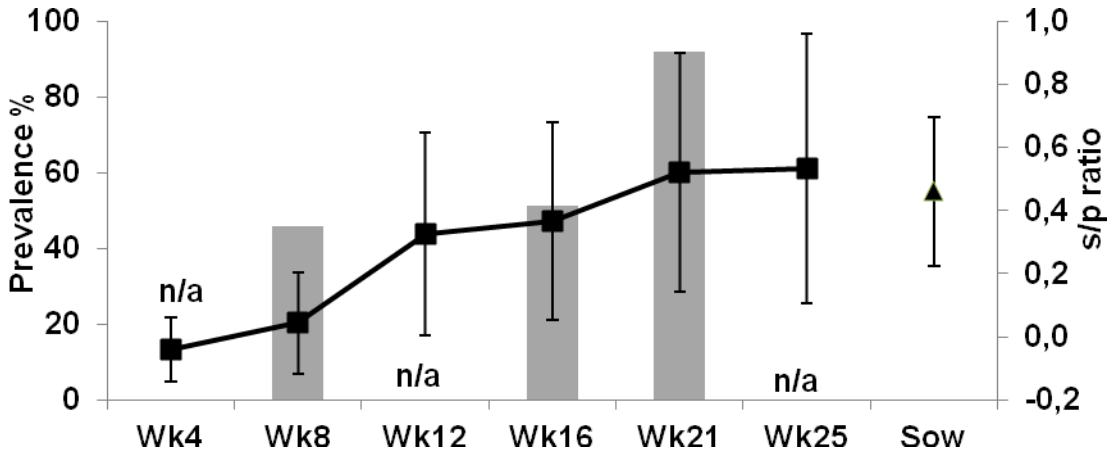

Figure 5.3: Prevalence of TTSuV2, in serum by qPCR at 8, 16 and 21 weeks of age (bars). At weeks 4, 12 and 25, piglets were not qPCR tested, not neither the sows, and average S/P ratios of anti-ORF1-A IgG, as determined by ELISA test (black line), of pigs from 4 to 25 weeks of age and for sows. n/a, means: prevalence not available at this week.

5.4 Discussion

The epidemiology of anelloviruses has been mainly studied by detecting viral DNA. Just few studies have assessed immune responses against anelloviruses in humans and pigs (Chen et al., 2013; Handa et al., 2000; Huang et al., 2011; Huang et al., 2012a; Jarosova and Celer, 2013; Kakkola et al., 2008; Ott et al., 2000). Most of these studies have used different fragments of ORF1 as antigens: ORF1 C-terminus (Huang et al., 2011; Huang et

al., 2012a; Ott et al., 2000), ORF1 N-terminus (Handa et al., 2000) or their splice variant (Kakkola et al., 2008). The structural protein of the virus is believed to be encoded by the ORF1 gene, and thus, representing the major viral antigen.

In the present study, ORF1-A proteins of both TTSuVs were expressed and used as antigens in ELISA assays. This ORF1-A isoform was chosen as the antigen based on previous results of the characterization of alternative splicing of ORF1 *in vitro* and its detection in tissues (Martínez-Guinó et al., 2011). Contrary to the full-length ORF1 protein, ORF1-A product was efficiently expressed in bacteria and it shares its N-terminus with the full-length ORF1 protein. Furthermore, *in vitro* experiments have demonstrated the presence of full size ORF1, but expression of the protein has not been achieved in humans neither in pigs (Kakkola et al., 2002; Martínez-Guinó et al., 2011; Qiu et al., 2005). In addition, the presence of full length mRNA transcript has been detected *in vivo* (Kakkola et al., 2008), suggesting that the full size ORF1 mRNA is expressed, but it might be too unstable or susceptible to degradation (Kakkola et al., 2002; Qiu et al., 2005).

Furthermore, the ORF1-A protein is unlikely to cause cross-reaction between TTSuV1 and TTSuV2 due to their low amino acid identity of about 22% to 26% (Huang et al., 2010b). Indeed, sera from mice immunized against TTSuV1 or TTSuV2 ORF1-A proteins were tested by ELISA and no cross-reactivity was observed (data not shown). Moreover, cross-reactivity was not observed in the present study as shown by distinct antibody dynamics for TTSuV1 and TTSuV2, and presence of detectable MDA mainly against TTSuV1.

Regarding studied pigs, anti-ORF1-A IgG antibodies were observed in sera of conventional pigs, suggesting that the host immune system is producing anti-TTSuV1 and TTSuV2 antibodies against ORF1 or their splice variants. The production of anti-TTSuV antibodies was expected based on previous studies in swine populations, but also in humans (Handa et al., 2000; Huang et al., 2011; Huang et al., 2012a; Ott et al., 2000). Although pigs developed antibodies against TTSuVs, they were not clearing out the viral infection since until end of the study these viruses were detected in sera by qPCR. It is known that anelloviruses cause persistent infections and may use different mechanisms to evade immune system, like high mutation rate, presence of multiple variant infecting single host or recombination (Cortey et al., 2011; Jelcic et al., 2004; Manni et al., 2002).

In the present study, one piglet did not seroconvert although it was TTSuV2 infected. A previous study on TTSuVs showed that a proportion of animals may become infected before the immune system matures (Aramouni et al., 2010). Such animals are likely to get immunotolerant to TTSuV. However, more work on this field would be needed in order to confirm such hypothesis.

TTSuVs are producing subclinical infections, but one of the main concerns is their role in combination with other swine pathogens. It has been suggested that TTSuV2 may trigger PCV2-SD when co-infected with PCV2 (Aramouni et al., 2011; Ellis et al., 2008; Kekarainen et al., 2006). PCV2-SD is a multifactorial disease that mainly affects 2–4-month-old pigs (Segalés et al., 2005). In chapter III it was observed that pigs early infected with TTSuV2 were more prone to suffer from PCV2-SD. One possible explanation is that piglets lacking those MDA and infected early in life predispose pigs to suffer from PCV2-SD when subsequent PCV2 infections occur, as MDA are known to play a protective role during first weeks of life (McKeown et al., 2005; Pravieux et al., 2007; Ward et al., 1996). In the present study, MDA were almost not found for TTSuV2, and hypothetically might affect the outcome of PCV2 infection towards PCV2-SD development.

In case of TTSuV1, MDA against TTSuV1 ORF1-A protein were commonly observed in piglets. The antibody profile indicates that MDA adversely affected the development of the humoral immune response against TTSuV1, in the sense that evidence of seroconversion in TTSuV1 MDA+ pigs took place later in time compared to TTSuV1 MDA- animals. This result should not be considered surprising since the inhibitory effect of MDA on the development of the humoral immune response upon infection has been well documented for other swine pathogens, as is the case of *Erysipelothrix rhusiopathiae*, H1N1 influenza virus or even in case of other ssDNA virus like PCV2. In these examples, development of the host immune response following vaccination or experimental infection was significantly influenced by the MDA status of the piglet at the time of vaccination or infection (Fraile et al., 2012a; Fraile et al., 2012b; Loeffen et al., 2003; Pomorska-Mól et al., 2012). In conclusion, the current study was aimed to develop specific immune assays to detect antibodies against two TTSuV species. Almost all tested pigs developed antibodies against both viruses along their productive life (from nursery to slaughter), but presence of antibodies at early ages (4 week-old pigs) were mainly detected for TTSuV1. This

difference raised the question if lack of MDAs could play a role during the development of diseases linked to TTSuV2.

CHAPTER VI: GENERAL DISCUSSION

To date, there is no doubt that TTSuVs show high genetic variability (Cornelissen-Keijsers et al., 2012; Cortey et al., 2011; Huang et al., 2010b; Zhai et al., 2013) and are worldwide distributed in healthy and diseased pigs (Cortey et al., 2012; Gallei et al., 2010; Taira et al., 2009; Teixeira et al., 2013; Xiao et al., 2012; Zhai et al., 2013). Besides the development of new laboratory tools, including qPCR and ELISA tests (Gallei et al., 2010; Giménez-Lirola et al., 2014; Huang et al., 2010a; Huang et al., 2011; Huang et al., 2012a; Jarosova and Celer, 2013; Lee et al., 2010; Taira et al., 2009), there are still many gaps in the knowledge on TTSuVs.

Following their discovery in pigs (Leary et al., 1999), some authors focused on the potential pathogenic role of TTSuVs in the swine population (Aramouni et al., 2011; Blomström et al., 2010; Ellis et al., 2008; Kekarainen et al., 2006; Krakowka and Ellis, 2008; Krakowka et al., 2008; Mei et al., 2011; Rammohan et al., 2012; Taira et al., 2009). Similar scenario has been described in humans. Anelloviruses had already been linked with several hepatics and extra-hepatic diseases according to some authors (Bando et al., 2001; Maggi et al., 2003b; Miyamoto et al., 2000; Moriyama et al., 2001; Pifferi et al., 2005; Sugiyama et al., 2000; Zhong et al., 2001), but others have been unsuccessful to demonstrate any link between TTV infection and specific pathologic conditions (Chan et al., 2001; Das et al., 2004; Hu et al., 2002; Mi et al., 2014; Suzuki et al., 2001).

To date, it looks like TTSuVs are not responsible of a particular swine disease. In fact, it is speculated that anelloviruses are acting secondarily in the development or aggravation of well-known swine diseases, including PCV2-SD, PRRS or PRDC (Aramouni et al., 2011; Kekarainen et al., 2006; Krakowka et al., 2008; Rammohan et al., 2012).

Based on previous research in humans, several factors must be taken into account regarding the consideration of TTSuVs as infectious agents able to contribute to disease. First, it has been hypothesized that the high genetic variability of TTSuVs (Cortey et al., 2011; Huang et al., 2010b; Zhai et al., 2013), with new variants and subtypes being continuously described (Cornelissen-Keijsers et al., 2012; Wang et al., 2012b), may account for some of them being able to cause or participate in disease scenarios (Aramouni et al., 2011; Cornelissen-Keijsers et al., 2012; Ellis et al., 2008; Kekarainen et al., 2006; Krakowka et al., 2008). Such hypothesis has been pointed out for human TTVs (Fehér et

al., 2011; Szládek et al., 2005; Tokita et al., 2002). Second, the high prevalence commonly observed in the swine population (Bigarré et al., 2005; Gallei et al., 2010; Kekarainen et al., 2006; Martelli et al., 2006; Martínez et al., 2006; Sibila et al., 2009a; Taira et al., 2009) may hamper the establishment of any consistent disease association. Additionally, the virtual lack of specific techniques to detect the target cell or cells where TTSuVs replicate also complicates the investigations for a potential unequivocal link with disease association. Although an ISH technique has been recently developed (Lee et al., 2014), a number of groups worldwide tried to do the same without success. Moreover, there is still no availability of a cell culture to replicate the virus (de Villiers et al., 2011; Kakkola et al., 2007; Leppik et al., 2007), impeding the possible generation of viral inocula.

In the swine field, the TTSuV role has been investigated in the background of multifactorial diseases, mainly in case of PCV2-SD. It has been demonstrated that PCV2 is the pivotal infectious agent of PCV2-SD (Allan and Ellis, 2000; Rose et al., 2012; Segalés et al., 2013); however, PCV2-SD needs other factors, including infectious (different from PCV2 infection) and non-infectious ones, to switch from infection to disease (Rose et al., 2012; Segalés et al., 2013; Tomás et al., 2008; Woodbine et al., 2007). Considering infectious agents, it has been proved that those co-infecting agents increase PCV2-SD clinical and pathological severity under experimental and field conditions (Allan et al., 2000; Rose et al., 2003; Rose et al., 2012).

In this sense, the role played by TTSuVs in the development of PCV2-SD has also been suggested. TTSuV is found commonly in pigs suffering from PCV2-SD; in fact, before the starting of this Thesis, other authors had already suggested the implication of TTSuVs in the development of PCV2-SD. PCV2-SD was experimentally reproduced in gnotobiotic pigs by inoculating TTSuV1 prior to PCV2 infection (Ellis et al., 2008). Under natural infection conditions, it was suggested that TTSuV2 could be linked to PCV2-SD development (Aramouni et al., 2011; Kekarainen et al., 2006; Taira et al., 2009).

The present Thesis was designed to gain further insights on the natural infection of TTSuVs, and its relation with their potential role in pig disease occurrence. Naturally infected pigs are subjected to different conditions, even in the same farms, that in case of multifactorial disease might affect (in favour or against) the outcome of the disease:

genetic factors, environment, management or pathogens co-infecting pigs. Under experimentally infection conditions all those factors can be controlled and might provide a better understanding of TTSuVs role. For example, an experiment carried out in gnotobiotics pigs linked TTSuV1 with PCV2-SD occurrence when TTSuV1 infection was carried out prior to PCV2 infection (Ellis et al., 2008). Anyway, other TTSuV1 infection timings were not studied, which implies no knowledge on other potential outcomes. Of course, under field conditions such timing of infection is usually unknown.

In chapter III, naturally infected pigs were monitored in a longitudinal study, and prevalence and viral load were determined in healthy and PCV2-SD affected pigs. On one hand, the amount of circulating virus is considered as a threshold factor between disease and subclinical infection, as it has been demonstrated in case of PCV2-SD (Grau-Roma et al., 2009; Olvera et al., 2004; Rose et al., 2012). Viral load is also related with the extent of clinical signs for CAV (Tan and Tannock, 2005). In case of human TTV, viral load has been suggested as a factor linked with pathogenicity, as well as a useful prognostic marker in case of other human diseases (Bando et al., 2008; de Jong et al., 2006; Maggi et al., 2003a; Maggi et al., 2003b; Ohkubo et al., 2002; Zhong et al., 2001; Zink et al., 1999). On the other hand, the longitudinal study was considered interesting since allowed correlating the timing of infection with the sequence of events observed in the farm (in the present Thesis, exposure to suspected causes of PCV2-SD outcome).

Chapter III results indicated that pigs developing PCV2-SD had a progressive increment in TTSuV2 prevalence, reaching 100% prevalence at the time of PCV2-SD outbreak; its viral load was also maximum at the last sampling point. These results were not observed for TTSuV1, in which similar prevalence and viral load in healthy and PCV2-SD pigs were observed at the same sampling point. Moreover, it was observed that pigs infected early in life with TTSuV2 were more prone to develop PCV2-SD than the rest of the pigs, as all TTSuV2 infected pigs at 3 weeks of life or earlier developed PCV2-SD subsequently. As PCV2-SD affected pigs are assumed to be immunocompromised (Kekarainen et al., 2010; Opriessnig et al., 2007), it would be expectable that TTSuV2 load would not be counteracted by diseased pigs, while healthy animals were capable of limiting the viremia load due to normal functioning of the immune system. In fact, such scenario has already been investigated by means of an ELISA assay, in which antibody levels against TTSuV2

were found significantly lower in clinically PCV2-SD affected pigs compared to healthy counterparts (Huang et al., 2011). Interestingly, this effect was not observed for TTSuV1 in the same presumably immunocompromised pigs, suggesting that other unknown mechanisms may affect TTSuVs load following infection. Recently, a similar scenario was found with TTSuV2 in *Classical swine fever* (CSF) *virus* (CSFV) experimentally infected pigs, in which up-regulation of TTSuV2 was found in animals suffering from a moderate course of CSF, but not observed for TTSuV1 (Aramouni et al., 2013a). Such correlation between immunosuppression and rise in viral load has been observed in humans, as TTV load raised following liver transplantation, in a immunosuppressant dose dependent manner (Burra et al., 2008). Curiously, the same authors suggested that human TTV genogroup 5 was more sensitive to immunosuppressive conditions than the rest of the TTV studied. Based on the results of this Thesis, it might be speculated a similar situation in which TTSuV2 would be more sensitive to immunosuppression than TTSuV1.

Another field of almost naïve research about TTSuVs is related with their pathogenesis. In that sense, distribution and load of virus in different tissues was the main objective of Chapter IV of this Thesis. So far, just few studies had focused on TTSuV infection in tissues (Aramouni et al., 2010; Bigarré et al., 2005; Lee et al., 2014; Teixeira et al., 2013). In the study of Chapter IV, TTSuVs were found in similar prevalence in all studied tissue types, suggesting no particular target organ/tissue or differences in tissue tropism for TTSuVs belonging to different genera. Importantly, BM was the tissue harbouring the highest viral load for both TTSuVs. This result was somewhat expectable, since intermediate replicative forms of human TTVs have been found in BM and, hence, this tissue has been suggested as one of the main sites of anellovirus replication (Kanda et al., 1999; Okamoto et al., 2000b; Zhong et al., 2002). Nevertheless, replication of human TTVs has been proved in other tissues/cell types, as is the case of liver or PBMC (Okamoto et al., 2000c; Zhong et al., 2002). Regarding differences between different TTSuV species, TTSuV2 load in BM was found statistically higher than that of TTSuV1.

Main outcome of Chapter IV showed higher TTSuV2 load in some tissues compared to TTSuV1, specifically from PCV2-SD affected pigs, but future studies are needed to clarify tissue tropism or correlation with specific disease conditions. In a recent publication, lung lesions in swine compatible with viral origin, TTSuV2 load was found significantly higher

when compared with TTSuV1 or when compared with TTSuV2 loads in lung lesions of non-viral origin (Aramouni et al., 2013b). However, results on viral load may vary among different published studies. For example, TTSuV1 prevalence was found more elevated in lung tissues in the background of the PRDC, compared to TTSuV2 (Rammohan et al., 2012). In humans, where the number of viral tropism studies in tissues is larger, it was suggested that viral load in a specific tissue might be more informative in the prognosis of the disease than the mere presence of TTVs (Bando et al., 2008; Maggi et al., 2003a; Zhong et al., 2001). In that sense, TTV load was found elevated in the PBMC of cancer patients (Zhong et al., 2001), higher TTV titres were found in stomach of patients with gastritis (Maggi et al., 2003a) and elevated TTV load was found in patients with BP when compared with children suffering from acute respiratory disease. Therefore, as pointed out by these and other studies, TTV viremia correlated with disease severity, independently of the cause of such disease (Maggi et al., 2003b).

TTSuVs produce long lasting infection in the host, demonstrated by a persistent presence of TTSuVs in blood and high prevalence of TTSuVs observed in adults, sows and boars (Kekarainen et al., 2007; Martelli et al., 2006; Pozzuto et al., 2009; Sibila et al., 2009b; Xiao et al., 2012). Continuous exposure to the virus should trigger an immune response to the virus, but this information has been traditionally missing, since serological studies in pigs were not carried out until 2011. From that year onwards, different research groups have developed several serological assays, all of them based on the ELISA technique (Giménez-Lirola et al., 2014; Huang et al., 2011; Huang et al., 2012a; Jarosova and Celer, 2013). TTSuVs seem to evade the immune response, and so far, several mechanisms have been proposed for such evasion.

First, genetic variation; TTSuVs are characterized by a high degree of genetic variability (Cornelissen-Keijsers et al., 2012; Cortey et al., 2011; Huang et al., 2010b), with hypervariable regions identified in the central part of ORF1 (Cortey et al., 2011; Huang et al., 2010b; Wang et al., 2012b). As ORF1 protein is a structural protein of anelloviruses (Bendinelli et al., 2001), it represents the major viral antigen and, hence, highly variable regions could help the virus to escape immune surveillance (Nishizawa et al., 1999). This hypothesis has also been suggested for other viruses like HCV or HIV (Bowen and Walker, 2005; Letvin, 2005). In case of HCV, it has been suggested that mutations generate minor

viral variants resulting ultimately in evasion of recognition or impaired binding to major histocompatibility complex (Bowen and Walker, 2005). In case of HIV, mutations help the virus to escape from neutralizing antibodies but also from cytotoxic T lymphocytes (Letvin, 2005). Both viruses have been suggested to circulate as quasi-species in the bloodstream (Letvin, 2005; Martell et al., 1992). This finding resembles that of human anelloviruses, which have been also suggested to circulate as quasispecies (Nishizawa et al., 1999). Therefore, mechanisms used for HCV and HIV to evade the immune response might apply for anelloviruses.

Second, viral recombination among closely related strains has been also suggested as a mechanism of immune escaping, contributing to the large genetic variability observed among TTVs (Manni et al., 2002).

Finally, a third mechanism has been suggested for immune evasion; although not proven and, probably, of less importance, it would be the development of immunotolerance to TTSuVs. This hypothesis was based on the presence of TTSuVs PCR positives in pig foetal samples before the immune systems maturation (Redman, 1979), during the second half of gestation (Aramouni et al., 2010). Certainly, further studies are required in all these fields in order to prove proposed hypotheses.

Chapter V describes the development of an indirect ELISA assay to detect anti ORF1-A IgGs. It was demonstrated that pigs were able to produce IgG antibodies against TTSuV infections. Almost all tested pigs developed antibodies against both viruses along their productive life (from nursery to slaughter). TTSuVs were detected to a high prevalence in blood of pigs. Detection of antibodies at early ages (4 week-old pigs) was mainly against TTSuV1. Such IgG antibodies were not clearing out the virus or protecting against re-infection, suggesting non-protective immune responses. These results were expectable since similar findings have been described for human TTVs (Handa et al., 2000; Kakkola et al., 2002; Ott et al., 2000), and may fit with the previously proposed hypotheses on immune system evading.

The most remarkable finding in chapter V was the high prevalence of MDA against TTSuV1, compared with the limited presence of MDA against TTSuV2. After birth, pigs intake colostrum to get protection against infectious pathogens present in the environment.

In swine there is no transplacental transfer of immunoglobulins from the sow to piglet during gestation (Pravieux et al., 2007). It is well known that MDA play a role in disease protection for most of the pathogens as long as MDA are still present (Loeffen et al., 2003; McKeown et al., 2005; Parreño et al., 1999; Ward et al., 1996; Yuasa et al., 1980). In consequence, pigs deprived of colostrum are highly susceptible to environmental infectious agents. It is unknown if TTSuV2 may exert a detrimental effect in pigs since young pigs are almost devoid of MDA against this pathogen. Obviously, this adverse consequence would be exerted in the first stages of life, as host immune response developed around 8 weeks of age. In case of TTSuV1, MDAs would protect pigs against TTSuV1 infection and its pathological effects (if any). Such differences in terms of MDA among TTSuVs may allow speculating about the differences observed in the involvement of these viruses in disease occurrence; earlier infection of TTSuV2 or lack of immune control at early ages might favour co-infection with other pathogens towards disease outcome.

Although evidence of direct disease association has not been found with TTSuVs, several findings may suggest a kind of indirect association:

1. Chapter III describes that early TTSuV2 infected piglets are more prone to suffer from PCV2-SD. This fits with Chapter V, since most of the 4 week-old piglets did not have IgG against TTSuV2 infection.

2. The main tissue target of TTSuV2 seems to be BM, as observed in Chapter IV, fitting with obtained results for human TTVs (Okamoto et al., 2000b; Zhong et al., 2002). BM contains the myeloid progenitors, producing haemocytoblasts, as well as lymphoid progenitors. In consequence, it can be speculated that replication of TTSuV2 in stem cells of BM may produce their destruction and, consequently, depletion of haemocytoblasts and/or T and B lymphocyte precursors. Although this has not demonstrated for TTSuV2, it has been shown for CAV, a very similar genetically organized ssDNA pathogen (Adair, 2000). In fact, CAV infection predisposes chickens to secondary infections and lack of proper responsiveness to vaccines, suggesting an immunosuppressive effect (Adair, 2000). TTSuVs target cell replication at the BM has not been determined, but based on human TTV studies they may replicate in hematopoietic cells. In fact, reduction to undetectable TTV levels in blood was observed in human patients during a myelosuppressive period after BM transplantation (Kanda et al., 1999). Indeed, thrombocytopenia and aplastic

anaemia have already been linked to TTV (Miyamoto et al., 2000; Tokita et al., 2001). Theoretically, TTSuVs replication in BM of young piglets would affect haemocytoblasts and/or T and B lymphocyte genesis, producing a certain degree of immunosuppression. In any case, such effect is not producing overt disease and probably the effect is subclinical, but it may be speculated that, at individual level, may act as a disease trigger factor.

3. Chapters III and IV pointed out the different behaviour of TTSuVs in the background of PCV2-SD, with TTSuV2 closely linked to PCV2-SD occurrence. It could be possible that the immune status of pigs suffering from PCV2-SD may favour replication of TTSuVs, although why such effect was just observed for TTSuV2 is not well understood at present.

Contemporary studies have demonstrated that TTSuV loads increase in the background of other swine viral diseases or pathological conditions (Aramouni et al., 2013a; Aramouni et al., 2013b; Rammohan et al., 2012). However, a link between TTSuVs infection and triggering multifactorial disease is difficult to be established, especially due to the high viral prevalence observed in healthy animals and the myriad of factors that might contribute to the outcome of multifactorial diseases. Moreover, to confirm if TTSuVs infection affects the outcome of any swine disease it would be necessary to develop an experimental infection model where all variables influencing the outcome of a multifactorial disease could be controlled. Interesting variables to take into account would include genetics and immunological status of the pigs, age of the animals at inoculation, presence of concomitant pathogens, TTSuV strains, infectious dose, and route of administration. Of course, viral propagation in cell culture or other biological supports would be compulsory to generate the inocula needed for such experimental model.

In conclusion, two new laboratory techniques to study TTSuVs were developed in this Thesis, which allowed establishing that TTSuV infections behave differently under the same environmental conditions. TTSuV2 seems to be more closely linked to PCV2-SD occurrence, although it is still not clear if such correlation is due to a higher pathogenic capacity of TTSuV2 or due to the impaired immune status of PCV2-SD affected pigs. Moreover, both TTSuVs seem to evade the immune response, although specific mechanisms of such immune evasion are not well understood yet

CHAPTER VII: CONCLUSIONS

1. New laboratory techniques were developed to study TTSuVs. Specifically, a new qPCR was developed to detect and quantify both TTSuVs in serum and tissues of porcine origin, and an indirect ELISA was developed to detect antibodies against ORF1-A protein of these viruses.

2. TTSuV2 prevalence and loads were higher in serum and tissues of PCV2-SD affected animals. Increments on both parameters were observed in serum until last sampling points of diseased animals, which was not the case for healthy pigs. For TTSuV1, no differences were observed for any of the parameters studied between both groups of studied pigs.

3. Early TTSuV2 infection was linked with the subsequent development of PCV2-SD in PCV2 infected pigs, and coincided with a very limited presence of anti-ORF1-A TTSuV2 MDA. In contrast, TTSuV1 infection at early ages, as well as antibodies of maternal origin, was detected in most of studied pigs.

4. Tissue distribution was fairly similar for both TTSuV1 and TTSuV2. All tissues studied were qPCR positive to both TTSuVs. The highest viral load was systematically detected in bone marrow, in both groups of studied pigs.

5. Almost all pigs produce antibodies against both TTSuV species throughout their life. However, these antibodies did not clear the viral infection from the body, demonstrating the persistent nature of these viral infections in the natural host.

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