Role of Torque teno sus viruses during co-infection with other swine pathogens

Mario Aramouni

Doctoral Thesis UAB - 2012

DIRECTORS

Tuija Kekarainen

Centre de Recerca en Sanitat Animal, IRTA-UAB, Barcelona

Joaquim Segalés

Departament de Sanitat i d'Anatomia Animals Universitat Autònoma de Barcelona

UAB Universitat Autònoma de Barcelona

CReSA Centre de Recerca en Sanitat Animal

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

Certifiquen:

Que la memòria titulada, "Role of Torque teno sus viruses during co-infection with other swine pathogens" presentada per Mario Aramouni per l'obtenció del grau de Doctor en Veterinària i Sanitat animal, s'ha realitzat sota la seva direcció al Centre de Recerca en Sanitat Animal (CReSA).

I per tal que consti als efectes oportuns, signen el present certificat a Bellaterra, a 25 de setembre 2012.

Dr. Tuija Kekarainen

Dr. Joaquim Segalés i Coma

PhD studies of Mr. Mario Aramouni were funded by a pre-doctoral AECI grant from the Spanish Ministry of foreign affairs.

This work was funded by grants AGL2006-02778/GAN, TRT2006-00018 and CONSOLIDER Ingenio 2010-PORCIVIR CSD2006-00007 from the Spanish Government.

A ma nièce

CONTENTS

ACKNOWLEDGEMENTS

THESIS ABSTRACT

Torque teno sus viruses (TTSuVs) belong to the family *Anelloviridae*, and are circular, single-stranded DNA viruses infecting pigs. So far, 4 different TTSuV species have been described: TTSuV1a and TTSuV1b members of the genus *Iotatorquevirus*, and TTSuV2 and TTSuVk2b, members of the genus *Kappatorquevirus*. In recent years, it has been suggested that TTSuVs could act together with other pathogens and participate as triggering factor for disease occurrence. Specifically, it has been described that they could be involved in the pathogenesis of porcine circovirus type 2 (PCV2) associated diseases (PCVDs). However, this subject remains controversial, thus the study of TTSuV infection in the pig has become a focus of scientific interest in recent years. The studies described in the following thesis go with the same line of previous studies with the aim to contribute with more clues on TTSuVs role in swine disease.

In the first study, TTSuV1 and TTSuV2 viral loads were quantified in serum of pigs affected by two PCVDs, postweaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS). Such study was carried out by means of a newly developed real-time quantitative PCR (qPCR) method. Results from this study showed that both TTSuVs were highly prevalent among studied pigs. TTSuV2 viral loads were significantly higher in PMWS affected animals, further supporting the previously suggested association between TTSuV2 and PMWS. On the contrary, TTSuV1 prevalence and loads were not related with the occurrence of studied PCVDs.

In the second study, TTSuVs prevalence and viral load were assessed in the context of an experimental infection with a highly virulent classical swine fever (CSF) virus (CSFV) isolate. Serum samples from 54 animals were analysed by means of a quantitative PCR (qPCR) for TTSuV1 and TTSuV2

before and after (between 6 and 13 days post-inoculation) the CSFV challenge. Based on the post-infection clinical evolution and immune responses against CSFV, the animals were divided into two groups: group I, with protecting immunity against CSFV and no clinical signs at the day of necropsy, and group II, with no detectable immune responses against CSFV and moderate to severe clinical signs. TTSuVs qPCR results indicated that TTSuV2, but not TTSuV1, load in serum increased significantly after challenge with CSFV in the group of pigs with clinical signs, specifically in those with a moderate course of the disease. Therefore, this study emphasizes the different behaviour of both TTSuVs, as already found in the previous work with PMWS, and further supports the association of TTSuV2 with disease occurrence.

In the third study, TTSuV1 and TTSuV2 loads and prevalence were evaluated in formalin-fixed, paraffin-embedded (FFPE) lung samples displaying different types of inflammatory lesions. Such measurements were made by means of a real time quantitative PCR technique optimised for its use on FFPE tissues. Results from the present study demonstrated that both TTSuVs were present in lung. However, TTSuV2 had higher viral load and prevalence in all the studied groups when compared to TTSuV1. TTSuV2 mean load was also higher in lungs with lesions attributed to a viral background when compared to normal lungs or to those with lesions of bacterial origin. This result suggests a possible role of TTSuV2s in the pathogenic mechanism of inflammatory lesions of lungs compatible with viral infection.

In light of these results, and taking into account that significant alterations of the immune system are recorded in most of the studied diseases, it has been speculated that TTSuV2 replication up-regulation would be controlled by the immune system. Therefore, it is considered that this virus, but not TTSuV1,

is likely a secondary agent in the context of various diseases that alter the immune response. The capabilities of TTSuV2 for causing pulmonary lesions by itself are apparently real, although of low severity.

RESUMEN DE LA TESIS

Los Torque teno sus virus (TTSuV) son virus ADN que pertenecen a la familia *Anelloviridae* y que infectan los cerdos. Son virus con genoma circular, de cadena simple, y hasta hoy se han descrito 4 especies diferentes: TTSuV1a y TTSuV1b, miembros del género *Iotatorquevirus*, y TTSuV2 y TTSuVk2b, miembros del género *Kappatorquevirus*. En los últimos años se ha sugerido que los TTSuVs podrían ser agentes que actuarían conjuntamente con otros patógenos y participar como posibles desencadenadores de enfermedad. En concreto, se ha descrito que podrían estar involucrados en la patogenia de enfermedades causadas por circovirus porcino tipo 2 (PCV2). No obstante, este tema continúa siendo controvertido, con lo cual el estudio de la infección por TTSuV en el cerdo se ha convertido en un foco de interés por parte de científicos durante los últimos años. Los estudios descritos en la siguiente tesis van en la línea de estudios previos con el objetivo de contribuir con más argumentos sobre el papel de los TTSuVs en enfermedades porcinas.

En el primer estudio se cuantificó la carga viral de TTSuV1 y TTSuV2 en suero de cerdos afectados por dos PCVDs, concretamente el síndrome multisistémico de emaciación post-destete o circovirosis porcina (PMWS) y el síndrome de dermatitis y nefropatía porcino (PDNS). Dicho estudio se llevó a cabo por medio del desarrollo de una nueva técnica de PCR cuantitativa a tiempo real (qPCR). Los resultados de este estudio mostraron que ambos TTSuVs fueron altamente prevalente entre los cerdos estudiados. La carga viral de TTSuV2 fue significativamente mayor en los animales afectados por PMWS, confirmando así la previa asociación sugerida entre este virus y PMWS. Por el contrario, la prevalencia y la carga en suero de TTSuV1 no se relacionaron con la ocurrencia de las PCVDs estudiadas.

En el segundo estudio, la prevalencia y la carga viral de los TTSuVs fueron evaluadas en el contexto de una infección experimental con un aislado de virus de la peste porcina clásica (VPPC) de alta virulencia. Las muestras de suero, procedentes de 54 animales, fueron analizadas por medio de una PCR cuantitativa (qPCR) para TTSuV1 y TTSuV2 antes y después (entre 6 y 13 días después) del desafío con el VPPC. Basándose en de la evolución post-infección de la sintomatología clínica y de la respuesta inmune frente el VPPC, los animales se dividieron en dos grupos: grupo I, incluyendo cerdos con una respuesta inmune adecuada contra el VPPC y sin signos clínicos en el día de la necropsia, y el grupo II, sin ninguna respuesta inmune detectable frente a VPPC y presencia de signos clínicos moderados a graves. Los resultados de la cuantificación de los TTSuVs indicaron que la carga viral del TTSuV2 aumentó significativamente después de la exposición con VPPC en el grupo de cerdos con signos clínicos, específicamente en aquellos con un curso moderado de la enfermedad. Esta situación no se dio para TTSuV1. Por tanto, este estudio pone de relieve el comportamiento diferente de ambos TTSuVs, como ya se había visto en el contexto de PMWS, y además apoya la asociación de TTSuV2 con la presentación de enfermedad.

En el tercer estudio, las cargas virales y la prevalencia de TTSuV1 y TTSuV2 se evaluaron en muestras de pulmón fijadas en formol y embebidas en parafina (FFPE) que mostraban diferentes tipos de lesiones inflamatorias. Para ello se utilizó una técnica cuantitativa en tiempo real PCR optimizada para su uso en tejidos FFPE. Los resultados demostraron que ambos TTSuVs estaban presentes en el pulmón. Sin embargo, TTSuV2 tuvo mayor carga viral y la prevalencia en todos los grupos estudiados en comparación con TTSuV1. La carga viral media de TTSuV2 también fue mayor en los pulmones con lesiones compatibles con un origen viral en comparación con los pulmones normales o aquellos con evidencia de infecciones causadas por bacterias. Este resultado sugiere un posible papel de TTSuV2 en el

mecanismo patogénico de lesiones inflamatorias de los pulmones compatibles con una infección viral.

A la luz de estos resultados, y teniendo en cuenta que en el transcurso de las enfermedades estudiadas existen alteraciones significativas del sistema inmunológico, se ha especulado que esta afectación favorezca una mayor capacidad de replicación de TTSuV2. Por tanto, se considera que este virus probablemente es un agente secundario en el contexto de distintas enfermedades que alteran la respuesta inmunitaria. Aparentemente el TTSuV2 sería capaz de causar lesiones pulmonares, aunque leves.

Still life flowers, by Johannes Bosschaert, 16th century, the J. Paul Getty Museum, Los Angeles, California.

> *"A pink carnation, a white rose, and a yellow tulip with red stripes lie in front of a basket of brilliantly coloured flowers. Various types of flowers appear together here: roses, forget-me-nots, lilies-of-the-valley, a cyclamen, a violet, a hyacinth, and tulips."*

Different flowers, from different seasons, united in the same painting. With joyful colours and rendering meticulous detail, Ambrosius Bosschaert the Elder conveyed the silky texture of the petals, the prickliness of the rose thorns, and the fragility of opening buds. But there is something that neither he nor his contemporaries knew. The rare bicoloured tulips, particularly popular and very expensive in the 17th century, were impressively striped by a virus.

List of abbreviations

aa	amino acid
ADV	Aujeszky's disease
ANOVA	analysis of variance
BIP	bronchointerstitial pneumonia
CAV	chicken anaemia virus
CPBP	catarrhal-purulent bronchopneumonia
CSF	classical swine fever
Ct	threshold cycle
CV	coefficient of variability
DNA	deoxyribonucleic acid
FAM	6-carboxy-fluorescein
FFPE	formalin-fixed paraffin-embedded
FNP	fibrinous-necrotizing pleuropneumonia
HEV	hepatitis E virus
HHV-6	human herpes virus 6
HIV	human immunodeficiency virus
HPV	human papilloma virus
IP	interstitial pneumonia
ICTV	International Committee for the Taxonomy of Viruses
ISH	in situ hybridization
JOE	6-carboxy-dichloro-dimethoxy-fluorescein
LUX	light upon extension
mRNA	messenger RNA
NLS	nuclear localization signal
NoLS	nucleolar localization signal
ORF	open reading frame
PASC	pairwise sequence comparison
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCV	Porcine circovirus
PCVD	porcine circovirus diseases
PDNS	porcine dermatitis and nephropathy syndrome
PMWS	postweaning multisystemic wasting syndrome
PRDC	porcine respiratory disease complex
PRRS	porcine reproductive and respiratory syndrome
PPV	porcine parvovirus
qPCR	quantitative PCR
R2	correlation coefficient
RCR	rolling circle replication

Rep	replication associated protein
RNA	ribonucleic acid
SIV	swine influenza virus
SD	standard deviation
TTV	Torque teno virus
TTSuV	Torque teno sus virus
UAB	Universitat Autònoma de Barcelona
UTR	untranslated region

PART I

INTRODUCTION AND AIMS

Chapter 1

Introduction

1.1 *Ubi virus, ibi pathogen?*

In the last 50 years, the field of virology has experienced an impressive development. The progress in molecular biology has converted virology into a molecular science, and since then, thousands of viruses have been discovered and many genomic strategies used by viruses have been described. The latest advances in virology evidenced new concepts such as ubiquity, latency, opportunism, multifactorial diseases and concurrent infections. They also showed that under certain conditions, ubiquitous viruses may turn into pathogenic ones or contribute in disease development. Lots of viruses, firstly catalogued as ubiquitous, have been linked later to diseases. For instance, latent Epstein-Barr virus infecting humans and discovered in 1964 (Epstein et al., 1964) was classified as a class I carcinogen by the International Agency for Research on Cancer in 1997 (World Health Organization [WHO], 1997; WHO, 2011). Human herpesvirus 6 (HHV-6) was discovered in 1986 and recognized as ubiquitous T-lymphotropic virus (Salahuddin et al., 1986) and later described at the origin of human diseases (De Bolle et al., 2005).

Torque teno viruses (TTVs) are not far from the actual scene of debate of the possible implication of a virus in the generation of a disease. Discovered in 1997 (Nishizawa et al., 1997) and described to infect more than 10 vertebrate species, their exact clinical implication and contribution to disease is still a conundrum. The better understanding of the virus life cycle, its molecular organization and evolution, and its possible *in vitro* replication, would be crucial for understanding and attributing any pathogenic role for TTVs. Although cumulative evidences link these viruses to human or pig diseases, to a large extent, these evidences are correlative and do not provide explanations regarding the causative role of TTVs in these diseases. This fact

does not deprive these studies from their veracity and their contribution to the approximation and whole understanding of the virus pathogenesis.

1.2 A history

In 1997, Nishizawa et al. discovered a novel DNA virus in a 58 year-old Japanese patient (initials T.T.) with post-transfusion hepatitis of unknown aetiology (Nishizawa et al., 1997). The virus was named "TT" virus (TTV) after the initials of the patient (Nishizawa et al., 1997). The International Committee on Taxonomy of Viruses (ICTV) proposed then that the "TT" of "TTV" should stand for "Torque teno", deriving from Latin terms, "torque" meaning "necklace" and "tenuis" meaning "thin", reflecting the circular form and small size of the virus.

Afterwards, TTV was found to infect several animal species showing host specificity and variable genomic length (the higher the species order, the longer the genome of the virus). Hosts able to be infected with TTV range from non-human primates, tupaia, domestic and wild suidae, chicken, cow, sheep, dog, cat to sea turtle (Abe et al., 2000; Brassard et al., 2008; Cong et al., 2000; Leary et al., 1999; Martínez et al., 2006; Ng et al., 2009; Okamoto et al., 2000a; Okamoto et al., 2001b; Okamoto et al., 2000b; Okamoto et al., 2002; Thom et al., 2003; Verschoor et al., 1999). TTV infecting pigs was discovered in 1999 (Leary et al., 1999) and recently, the ICTV has named it as Torque teno sus virus (TTSuV) (King et al., 2009).

1.3 A virus

1.3.1 Taxonomy and classification

After their discovery, TTVs were firstly classified in the group of viruses with circular, single-stranded DNA genomes and included in the family of *Circoviridae* (Mushahwar et al., 1999; Takahashi et al., 2000). Later on, in 2001, the ICTV, in charge of the official taxonomic nomenclature, created the genus *Anellovirus* unattached to any viral family (floating). The term *Anello* deriving from Latin and meaning "the ring", related to the circular nature of their genome. In 2009, the family *Anelloviridae*, with 9 different genera, was created based on the analysis of the entire ORF1 (King et al., 2009). According to the ICTV, the ORF1 nucleotide sequence divergence cut-off values to distinguish species is >35% and >56% for genus.

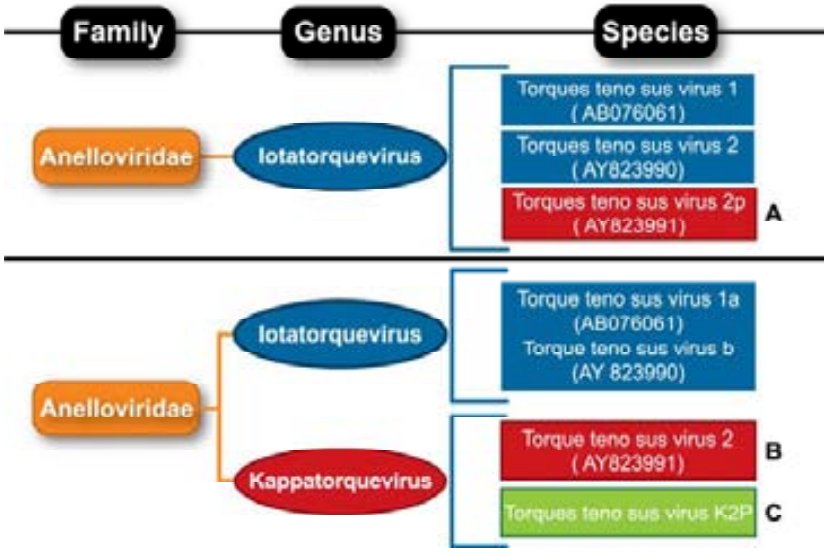

Figure 1.1: TTSuVs classification: **A**; classification proposed in the 9th ICTV report in 2009; **B**; classification modification proposed in 2010 and 2011 and ratified by the ICTV in 2012; **C**; new TTSuV species to be proposed for its classification in the *Kappatorquevirus* genus.

Therefore, due to the high degree of divergence and the low levels of nucleotide identity between discovered TTSuVs, four species have been so far described (figure 1.1): *Torque teno sus virus 1a and 1b* (TTSuV1a and TTSuV1b) classified into the *Iotatorquevirus* genus (King et al., 2009), *Torque teno sus virus 2* (TTSuV2) classified in the newly created genus *Kappatorquevirus* (Adams and Carstens, 2012) and a newly discovered species provisionally named TTSuVk2b and proposed to be classified as a species of the *Kappatorquevirus* genus (Cornelissen-Keijsers et al., 2012).

1.3.2 Genome organization

TTVs consist of small icosahedral, non-enveloped virions with a diameter of 30-32 nm (Mushahwar et al., 1999). The buoyant density in cesium chloride is 1.31–1.33 g/cm^3 for TTVs from serum and 1.33–1.35 g/cm^3 for TTV from faeces (Okamoto et al., 1998a). They have a circular single-stranded DNA genome of negative polarity with a size ranging between 2.1 to 3.9 kb, depending on the host species (Miyata et al., 1999; Mushahwar et al., 1999; Nishizawa et al., 1997; Okamoto et al., 1999; Peng et al., 2002). The smallest genome corresponds to the TTV infecting cats, of around 2.1 kb while those infecting humans and non-human primates range from 3.7 to 3.9 kb. TTSuV genome is approximately 2.8 kb (Okamoto et al., 2002). As all anelloviruses, TTSuVs contain three open reading frames, ORF1, ORF2 and ORF3 and a highly conserved untranslated region (UTR) (Okamoto, 2009b; Okamoto et al., 2000a). This UTR contains a G-C rich area (89-99% of GC content) (Okamoto et al., 2002) and includes a promoter and enhancer elements important for the replication and transcription activities (Kamada et al., 2004; Suzuki et al., 2004). By analogy with other single-stranded circular DNA viruses as chicken anaemia virus (CAV) and porcine circoviruses (PCV), the ORF1 of TTSuV is believed to encode a capsid and a replication-

associated protein (REP), ORF2 to be involved in the replication process and ORF3 to be associated with apoptosis (Maggi and Bendinelli, 2009).

1.3.3 Genetic heterogeneity

In contrast to most DNA viruses, anelloviruses exhibit a high level of genetic heterogeneity. Comparison of the sequences available has revealed that the divergence is distributed throughout the genome (translated region: ORF1, ORF2 and ORF3), with a well conserved UTR. Studies reported high genetic p-distances within (>30%) and between species (>50%) of TTVs (Biagini et al., 2007; Cortey et al., 2011a; Huang et al., 2010; Jelcic et al., 2004).

TTSuVs inter-species nucleotide sequence identity was calculated to be around 56%. Mainly, genome variability is due to different groups of insertions-deletions, genomic mutations, transversions and transitions in the translated regions (Cortey et al., 2011a). Translated regions could also present more or less conserved regions. For instance, in a recent study comparing 5 TTSuV1 and 8 TTSuV2 genomes together with several already published genomic sequences, the pattern of mutation among ORFs was different: ORF1 was more conserved, while ORF2 and ORF3 were less conserved despite some conserved regions were also reported (Cortey et al., 2011a). Furthermore, pairwise sequence comparison (PASC) of 121 TTSuV sequences available at the GenBank database also defined the presence of two TTSuV1 species, (1a and 1b; with a nucleotide identity around 70%) and three possible species for TTSuV2 (2a, 2b and 2c; with around 90% of nucleotide sequence identity) (Huang et al., 2010).

It has been reported that for TTSuV1 a certain geographic structure may exist while for TTSuV2 definition remains unclear (Cortey et al., 2011a).

1.3.4 Biological properties and protein expression

Anelloviruses' biological properties are still poorly defined. This is in part, due to the lack of an efficient *in vitro* culture system that supports their replication (Mushahwar et al., 1999). Such fact has limited the possibility to investigate the cell types supporting the replication, the precise molecular pathways used by the virus during the replication, and the effects of the infection on the permissive cells.

Based on sequence analysis, it has been assumed that TTVs and TTSuVs, likewise the majority of small DNA viruses, do not encode a DNA polymerase for replication, but instead they use host cellular polymerase and replication machinery (Kakkola et al., 2007). Therefore, based on similarities with other circular ssDNA viruses, it is assumed that TTV could use the rolling circle mechanism (RCR) (Mushahwar et al., 1999). In this model after viral entry inside the cell, the single stranded viral genome is converted into a double stranded DNA replicative intermediate, with a mechanism that is still unknown, starting from the viral positive single-stranded DNA (Faurez et al., 2009). Based on studies on circoviruses, it is known that the viral encoded replication associated protein (REP) binds to the stem-loop structure, which is the origin of replication and initiates the replication by nicking the DNA (Mankertz et al., 1998; Niagro et al., 1998). Afterwards, cellular DNA polymerase initiates viral DNA replication from the free 3' OH extremity and the REP complex still binds the 5' end. After a round of replication, the REP complex closes the DNA and releases a single stranded DNA which can be used for new round of replication or can be encapsidated (Mankertz et al., 2004; Mankertz et al., 1998).

In vitro transcriptional analysis of TTSuVs indicated that these viruses use alternative splicing to generate ORF1 and ORF3 proteins using a common

mRNA 5'-end for mRNA translation (Huang et al., 2010; Martinez-Guinó et al., 2011). According to the results described in Martinez-Guinó et al., ORF1 full length mRNA was not found to be produced, instead, two spliced variants for TTSuV1 and three for TTSuV2 were observed. ORF3 was also found to generate different variants by splicing, with 3 variants for TTSuV2 and only 1 variant for TTSuV1 (table 1.1). ORF1 and ORF3 variants of both TTSuV1 and TTSuV2 were found to have a nuclear localization, specifically in the nucleolus, which may indicate their implication in basic viral processes.

1.4 An infection

1.4.1 Epidemiology

One of the well-known and documented aspects of anelloviruses is the ubiquity, the persistence and the extremely high spread of infection throughout the world (Bendinelli et al., 2001). It seems that anelloviruses are transmitted both vertically and horizontally. These facts are the attributes that permitted the extraordinary penetration of active TTVs infection into human and animal communities worldwide.

TTSuV DNA has been detected in swine serum worldwide and a recent study demonstrated the existence of TTSuVs since 1985 in Spanish herds (Segalés et al., 2009). Globalization and international trading of live animals has been demonstrated to play a crucial role in the propagation of TTSuV (Cortey et al., 2011b). As expected with newly discovered and unstudied viruses, TTSuVs attracted the interest of the research community and by the means of specific PCR and quantitative PCR (qPCR) methods, TTSuV1 and TTSuV2 have been found in sera of pigs from many countries (figure 1.3), confirming the active infection of TTSuVs and the high rate of co-infection (Aramouni et al., 2010; Bigarré et al., 2005; Blomström et al., 2010;

Cornelissen-Keijsers et al., 2012; Cortey et al., 2011b; Gallei et al., 2010a; Haramoto et al., 2005; Kekarainen et al., 2006; Lang et al., 2011; Leary et al., 1999; Lee et al., 2010; Liu et al., 2011; Martelli et al., 2006; McKeown et al., 2004; Niel et al., 2005; Okamoto et al., 2002; Perez et al., 2011; Segalés et al., 2009; Sibila et al., 2009a; Sibila et al., 2009b; Takacs et al., 2008; Xiao et al., 2012). Results from these studies showed that TTSuVs display different prevalences ranging from 17% to 100%, depending on the country, animals' age and animals' health status (Bigarré et al., 2005; Gallei et al., 2010a; Kekarainen et al., 2006; McKeown et al., 2004; Taira et al., 2009). Quantitative methods have shown also that both TTSuVs can be present in serum of healthy animals with high titers, reaching 10^7 DNA molecules per ml of sera in healthy animals (Lee et al., 2010; Nieto et al., 2011).

Figure 1.3: TTSuV1 and TTSuV2 have a worldwide distribution and they have been found in sera of pigs from Argentina, Australia, Austria, Brazil, Canada, Chile, China, Cuba, Czech Republic, Denmark, France, FYRO Macedonia, Germany, Hungary, Italy, Japan, Korea, Mozambique, Norway, Uganda, United Kindom, United States, Slovakia, Sweden, Spain, The Netherlands and Thailand

	Full protein Length	Spliced forms	Protein localization	Putative functions and properties
ORF1	TTSuV1 638 -650 aa	ORF1-1A	Nuclear	Encodes capsid protein. contains an arginine-rich region at the N-terminus, REP containing RCR motifs.
		ORF1-1B	Nuclear	
	TTSuV2 625 -628 aa	ORF1-2A ORF1-2B ORF1-2C		
	TTSuVk2b	ORF1-1	Nuclear	
ORF2	TTSuV1 73 -74 aa TTSuV2 69 aa TTSuVk2b		Cytoplasma and nuclear	Encodes a non-structural protein assumed to be involved in viral replication; Associated with the NF-Kβ pathway suppression; contains PTPase-like motif at the N-terminus. No functional nuclear localization signals (NLS).
ORF3	TTSuV1 221 -232 aa	ORF3-1A		Encodes a non-structural protein of unknown function. Contains NLS or genuine nuclear localization system (NoLs) signals that target it to the nucleolus. It has been associated with apoptosis.
	TTSuV2 200 -203 aa	ORF3-2A	Nuclear	
		ORF3-2B		
		ORF3-2C		
	TTSuVk2b			

Table 1.1: Summary of TTSuV ORFs and their putative functions

Although it has been assumed that the main route of TTSuV transmission is the fecal-oral route, other routes have been investigated and demonstrated to co-exist. It has been found that foetuses are already positive to TTSuV at the second third of gestation (Aramouni et al., 2010; Martinez-Guinó et al., 2010) and gnotobiotic pigs were positive to one or both TTSuV species with

high rates of foetal infection (Pozzuto et al., 2009). These data demonstrated that TTSuV may be transmitted to piglets by intrauterine or transplacental route. Other studies pointed out that the vertical infection was not the main route for TTSuV transmission, as foetuses did not show positivity to any of the studied TTSuVs (Xiao et al., 2012). TTSuV presence in boar semen indicates the possible sexual route of transmission (Kekarainen et al., 2007). Milk PCR positivity could also be a sign of vertical transmission in suckling piglets (Martínez-Guinó et al., 2009).

TTSuVs have been found in nasal and faecal samples starting from one week of age (Brassard et al., 2008; Sibila et al., 2009a) with prevalence increasing with the age, and higher nasal than faecal excretion (Sibila et al., 2009a). Most if not all pig tissues (brain, lung, liver, kidney, bone marrow, heart, spleen, mesenteric and mediastinal lymph nodes) were strongly positive at 5 weeks of age reaching the highest prevalence at slaughter age (about 24 weeks) (Aramouni et al., 2010). A recent study on the dynamics of TTSuV infection showed that serum viral titers of both species increased with age until 11 weeks and then showed a tendency to decline (Nieto et al., 2011). Although it has been showed that anti-ORF1 antibodies are produced and associated with a decreasing viral load in serum (Huang et al., 2011), these antibodies may not be able to totally clear TTSuVs, which might explain the persistence of the virus. It is to mention also that foetuses showed to be positive in the second third of gestation before the immunocompetence age, fact that would make TTSuV immunologically tolerated by the host immune system (Aramouni et al., 2010).

1.4.2 TTSuV and disease

TTSuVs have been studied in different pig disease scenarios and, as for human TTVs, its responsibility in disease development is controversial until

today. Even though TTSuVs are considered ubiquitous in the porcine population and widely spread, they have been associated to porcine circovirus diseases (PCVDs) occurrence (Ellis et al., 2008; Kekarainen et al., 2006). Post-weaning multi-systemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome, two of the most significant PCVDs (Segalés et al., 2005a), were the main diseases to be studied in association with TTSuVs. A first report showed that TTSuVs were highly prevalent in sera of animals with PMWS when compared to healthy animals,. Furthermore, TTSuV2 was significantly more prevalent than TTSuV1, in this group of diseased animals (Kekarainen et al., 2006).

Later on, TTSuV1 was found to contribute to the experimental induction of a porcine dermatitis and nephropathy syndrome (PDNS)-like condition, when co-infected with porcine reproductive and respiratory syndrome virus (PRRSV) (Krakowka et al., 2008a). Afterwards, an experimental co-infection with TTSuV1 and PCV2 resulted in the development of PMWS in gnotobiotic pigs (Ellis et al., 2008). TTSuV1 was subsequently demonstrated to be capable, by itself, to produce mild lesions in gnotobiotic pigs after their inoculation with TTSuV1 positive plasma (Krakowka and Ellis, 2008).

Furthermore, TTSuV infection in PMWS-affected pigs was also associated to the presence of other viruses such as bocavirus in addition to PCV2, suggesting that the development of this condition may include multiple viral infections and TTSuVs may play a role in its pathogenicity. In the same way, TTSuVs were associated to PMWS in Japan (Taira et al., 2009) and a Chinese study suggested TTSuV2 as responsible of lesion production in lung of animals after their inoculation with this virus (Mei et al., 2011). In a longitudinal study, Nieto and collaborators (2011) showed that TTSuV2 loads increased over time in PMWS-affected pigs' serum, which was not the case for healthy animals or for TTSuV1. In 2010, a study of TTSuV and

PCV2 in serum of naturally infected pigs with hepatitis E virus (HEV) demonstrated an association between TTSuVs and infectious hepatitis in concomitant infections with PCV2 and/or HEV, suggesting a role of TTVs in the pathogenesis of the disease (Savic et al., 2010). Very recently, an increased prevalence of TTSuVs was found in porcine respiratory disease complex (PRDC) affected pigs, and concluded that TTSuV1, but not TTSuV2, was strongly associated with this multiaetiological and multifactorial condition (Rammohan et al., 2011). These authors also indicated that their results may support the hypothesis that TTSuVs might act as co-factors in PRDC development.

Cited studies may point out a pathogenic role of TTSuVs or indicate that TTSuVs replication is up-regulated under disease scenarios. While abovementioned studies suggest TTSuVs involvement in disease development, other studies have not found a link between these viruses and PCVD development (Gauger et al., 2011; Lee et al., 2011).

1.4.3 TTV and human disease

In the few years elapsed since its discovery and due to the circumstances under which it has been unveiled (Nishizawa et al., 1997; Okamoto et al., 1998b), TTV was intensively investigated as possible cause of those forms of putative liver infections in which no causing agent could be identified (Berg et al., 1999; Charlton et al., 1998; Shang et al., 2000; Tuveri et al., 2000). Also, it was assessed whether TTVs may play a role (adverse, triggering or aggravating factor) in hepatitis and cirrhosis of known etiology (Moriyama et al., 2001; Tokita et al., 2002; Zein et al., 1999). Various authors showed that TTV prevalence and/or loads are associated with various hepatic disorders (Charlton et al., 1998; Ikeda et al., 1999; Ikeuchi et al., 2001; Kanda et al., 1999; Okamura et al., 2000; Tanaka et al., 1998; Tanaka et al., 2000; Tuveri et

al., 2000), and that the persistent infection with TTV contributed to cryptogenic hepatic failure in hemophiliacs (Takayama et al., 1999). Even though several investigations ruled out any important role of TTV in hepatic disease (Akiba et al., 2005; Hijikata et al., 1999; Hsieh et al., 1999; Kao et al., 2002; Kasirga et al., 2005; Naoumov et al., 1998; Niel et al., 1999; Prati et al., 1999; Schroter et al., 2003; Umemura et al., 2001; Viazov et al., 1998), several results associating enzymatic alterations and liver abnormalities to one or several TTV genotypes, are still appearing in the literature (Ishimura et al., 2010; Nobili et al., 2005; Piaggio et al., 2009b; Sagir et al., 2004). At present, despite the evidence of TTV replication in liver, it still does not fulfil the criteria for being considered an hepatitis inducing virus (Okamoto, 2009a; Shimizu et al., 2000).

TTV has been also suggested to have a potential role on respiratory disease in neonates and children (Bando et al., 2001; Biagini et al., 2003; Maggi et al., 2003; Okamoto et al., 2001a; Pifferi et al., 2005; Pifferi et al., 2006). Furthermore, association between TTV infection and the complication of lung cancer in patients with idiopathic pulmonary fibrosis has been also reported (Bando et al., 2008). Despite all these observations, it remains undetermined whether TTV is the cause or the result of the disease.

Several other potential associations have been described, including multiple sclerosis (Sospedra et al., 2005), systemic lupus erythematosus (Gergely et al., 2005), pancreatic cancer (Tomasiewicz et al., 2005), diabetes mellitus (Guney et al., 2005), laryngeal cancer (Szladek et al., 2005), cancer in general (Bando et al., 2008; Fehér et al., 2009; Figueiredo et al., 2007; Zhong et al., 2001; zur Hausen and de Villiers, 2005, 2009), periodontal disease (Rotundo et al., 2004) and certain rheumatic diseases (Maggi et al., 2007).

A possible contribution of TTV to the immune impairment of patients with AIDS or other immunocompromised conditions has also been suggested. Although it remains unclear which role the immune system plays in the natural course of TTV infection, TTVs may act as an opportunistic pathogen in immunocompromised hosts, analogous to human cytomegalovirus in AIDS patients. TTV viral loads in serum and tissues have been shown to increase in HIV infected individuals and other immunocompromised people. Also, a high TTV viral load has been associated with a low CD4 cell count, indicating a potential role of the immune system in controlling TTV replication (Christensen et al., 2000; Shibayama et al., 2001; Thom and Petrik, 2007; Touinssi et al., 2001; Zhong et al., 2002). HIV infected patients that were simply investigated qualitatively for the prevalence of TTV showed no major differences with the controls (Puig-Basagoiti et al., 2000; Sagir et al., 2005). Whereas, those in which the patients' loads of TTVs were also measured, with few exceptions (Moen et al., 2002), have produced evidence arguing for the existence of a correlation between severity of the patients' immunosuppression and burdens of TTVs carried. Recently, a study on the viral load kinetics of TTV in patients subjected to chemotherapy treatment for cancer showed that TTV viremia increased after high doses of the therapy. This finding paralleled with the increase of circulating CD8+CD57+ T lymphocytes; it has been suggested that this cell subset may represent an indirect marker of functional immune deficiency (Focosi et al., 2010a; Focosi et al., 2010b). Subsequently, within the period of some months, TTV levels returned to baseline values, being those constant over the time. This study suggested that monitoring TTV viremia could represent an opportunity to follow functional immune reconstitution in immunosuppressed patients, and could serve as guidance for the therapy course (Focosi et al., 2010b).

Chapter 2

Aims

TTSuVs have not been demonstrated to be pathogenic. However, their role during co-infection with other pathogens, mainly with PCV2, remains controversial. It is believed that TTVs might influence the development of some diseases, affect the outcome of a disease or, alternatively, their replication is up-regulated in a disease scenario. Considering the high variability of anelloviruses, it is likely that TTSuV species also differ in their pathogenetic capabilities.

The present thesis aimed to study TTSuV1 and TTSuV2 infections during co-infection with other swine pathogens. The specific objectives were:

- To evaluate and compare TTSuV1 and TTSuV2 prevalence and viral loads in serum of healthy animals and animals with PMWS and PDNS.

- To assess TTSuV1 and TTSuV2 prevalence and viral load in serum before and after an experimental infection with a highly virulent classical swine fever virus (CSFV) isolate and to correlate them with the different immune responses.

- To study the implication of TTSuV1 and TTSuV2 in pulmonary inflammatory lesions in the context of porcine respiratory disease complex (PRDC) by means of the analysis of viral loads in formalin-fixed, paraffin –embedded tissues.

PART II

STUDIES

Chapter 3

Torque teno sus virus 1 and 2 loads in postweaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS) affected pigs

Published at the journal Veterinary Microbiology. 2011 153:377-81.

ABSTRACT

TTVs are small, non-enveloped viruses with a circular single-stranded DNA genome, which are considered non-pathogenic. However, TTVs have been eventually linked to human diseases and TTVs infecting pigs, TTSuV1 and TTSuV2, have been recently associated to PCVD. To get more insights into such potential disease association, the aim of this study was to quantify TTSuV1 and TTSuV2 viral loads in serum of pigs affected by two PCVDs, PMWS and PDNS. Such study was carried out by means of a newly developed real time quantitative PCR (qPCR) method. Both TTSuVs were highly prevalent among studied pigs. TTSuV2 viral loads were significantly higher in PMWS affected animals, further supporting the previously suggested association between TTSuV2 and PMWS. On the contrary, TTSuV1 prevalence and loads were not related with the studied PCVDs.

INTRODUCTION

The role of TTV infection in disease occurrence in humans is controversial, even some species are assumed to be more virulent than others, either alone or co-infecting with other TTV species or other pathogens (Leppik et al., 2007; Okamoto, 2009a; Yokoyama et al., 2002). Therefore, it is believed that TTVs might influence the development of some diseases or even affect the outcome of disease by being present in blood or tissues (Okamoto, 2009a). Such controversy also affects TTSuVs. Even a clear-cut pathogenic role of TTSuVs has not been demonstrated to date, its role during co-infection with other pathogens is under debate, especially in regards to PCVDs (Kekarainen and Segalés, 2009).

PCV2 is a member of the family *Circoviridae*, genus *Circovirus*, and, as TTSuVs, is found ubiquitous in the pig population worldwide. PMWS is a multifactorial disease in which the infection with PCV2 is a necessary but not sufficient event to trigger the disease, while PDNS is considered an immuno-complex disease in which PCV2 might be the associated antigen (Segalés et al., 2005b). It has been postulated that a number of pathogens such as PRRSV, porcine parvovirus (PPV), *Mycoplasma hyopneumoniae* and others may act as potential triggers for PMWS occurrence (Grau-Roma et al., 2010) and PRRSV, *Pasteurella multocida* and others as potential PDNS-associated antigens (Segalés et al., 1998; Thomson et al., 2000).

Within this complex framework, the objective of the present study was to further insight in the potential participation of TTSuVs on the occurrence of PMWS and PDNS affected pigs under natural conditions. To pursue such aim, TTSuV1 and TTSuV2 viral loads were quantified in serum of PMWS, PDNS and healthy control animals using a newly optimized real-time qPCR, based on The Light Upon Extension ™ (LUX ™) technique.

MATERIALS AND METHODS

Animals and samples

Serum samples from 45 PMWS affected pigs, 34 pigs suffering from PDNS and 29 healthy pigs aging between 11 and 21 weeks were used in the present study. Thirty one PMWS affected animals and 29 age-matched healthy ones corresponded to the Spanish part of a previously published longitudinal case-control study in PMWS affected farms (Grau-Roma et al., 2009). The remaining 14 PMWS cases and all the PDNS affected animals corresponded to case submissions to the Pathology Veterinary Diagnostic Service at the Veterinary School of Barcelona (Spain). Diagnosis of PDNS was based on clinical signs and gross lesions, together with the presence of characteristic histopathological lesions (fibrino-necrotizing glomerulonephritis and systemic necrotizing vasculitis), as described elsewhere (Segalés et al., 2005b). PMWS cases were diagnosed based on the internationally accepted case definition of the disease (Segalés et al., 2005b), including clinical signs, histopathological lymphoid lesions and PCV2 presence in tissues by *in situ* hybridization.

TTSuV1 and TTSuV2 quantitative real time PCR

DNA extraction

DNA was extracted from 200 µl of serum samples using a DNA extraction kit (Nucleospin® Blood, Macherey-Nagel GmbH & Co KG, Düren, Germany) according to the manufacturer's instructions. A negative control (PBS) was included with each set of samples to test any contamination of the extracted DNA during the extraction process.

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 (Okamoto et al., 2000b). 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 TTSuV2R primers were labelled at the 3' with JOE ™ (6-carboxy-dichloro-dimethoxy-fluorescein) and FAM ™ (6-carboxy-fluorescein), respectively. Amplicon sizes of TTSuV1 and TTSuV2 were 86 bp and 67 bp, respectively.

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

PRIMER	Tm (°C)	GC%	bp	Sequence (5' to 3')	Location in genome
TTSuV1F	71	50	26	CGA CCG GAG TCA AAT CTG ATT GGT [JOE] G	195-211
TTSuV1R	62	50	22	TAC TGG GAA CGC CCT AAT TCT G	259-281
TTSuV2F	69	50	28	CGG TTG AAC AGA GCT GAG TGT CTA AC[FAM] G	281-309
TTSuV2R	65	65	20	CCC TTG ACT CCG CTC TCA GG	329-348

Table 3.1: TTV1 and TTV2 LUX primer characteristics

Standards

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

Quantitative PCR reaction

Reactions were carried out in 96-well plates. Each sample and standards were run in triplicate and a negative control was added between each three wells, using autoclaved bi-distilled water instead of sample DNA. After optimization, each reaction contained 2 µl of sample or standard DNA, 200 nM of each primer, 10 µl of Express qPCR Supermix Universal™ (Invitrogen), 0.04 µl of Rox in a total volume of 20 µl. Amplification and quantification were perfomed using ABI®7500 Fast Real Time PCR System (Applied Biosystems™) under universal conditions: 10 min at 95ºC, 2 min at 50ºC and 40 cycles of 15 s at 95ºC, 1 min at 60ºC.

PCV2 quantitative PCR reaction

PCV2 viral loads per ml of serum were determined in all studied samples by a previously described PCV2 qPCR (Olvera et al., 2004).

Data analyses and statistics

The number of copies of TTSuV1 and TTSuV2 in each reaction was determined using the ABI ® 7500 detection software. For each viral species, a standard curve was created with the threshold cycle (Ct) values of the 10-fold dilutions of corresponding standards ranging from $2x10^5$ to 20 molecules per reaction. Standard deviation of triplicates (SD) was calculated and a correlation coefficient of the standard curve (R2) was determined. The melting curve for each pair of primers was also analysed to check qPCR reactions for primer-dimer artifacts and contamination, and to ensure reaction specificity and accurate quantification.

The reproducibility of the qPCR was assessed by the inter-assay and intra-assay coefficients of variability (CV), measured as the mean of the standards Ct's in each experiment (intra-assay) and between the different experiments (inter-assay).

TTSuV1, TTSuV2 and PCV2 quantifications per ml of serum sample were calculated and the average log10 copies per ml of serum were compared globally, and between healthy, PMWS and PDNS affected pigs with an ANOVA statistical test. Normality of data was assessed using Shapiro-Wilk test. Correlation between TTSuVs and PCV2 viral loads was also performed. The number of positive and negative cases was also compared between healthy animals, PMWS and PDNS affected animals by the mean of absolute Fisher tests. All statistical analysis was made using SPSS Statistics for

Windows, 137 Rel. 17.0.0. 2008. Chicago: SPSS Inc. Significance was set at $P<0.05$ for all tests.

RESULTS

Quantitative PCR optimization

Only qPCR reactions with a CV<0.05 between triplicates (intra-assay variability), standard curve with an accuracy of $R^2 > 0.97$, a slope measuring the efficiency between - 3.2 and - 3.7 and a melting temperature of 77ºC for TTSuV1 and 82ºC for TTSuV2 were accepted. Reactions not fulfilling those criteria or with contaminated negative controls were repeated.

Reproducibility, specificity and sensitivity of the method

The reproducibility of the method was established with the inter-assay CVs of the threshold cycle of the standard curves generated in the different quantification assays. Inter-assay variations of detecting TTSuV standards in the range from 10 to 10^9 genome equivalents were calculated through 6 experiments and the values were below 5.1% for TTSuV1 and below 5.5% 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.

PCV2, TTSuV1 and TTSuV2 detection and quantification

PCV2, TTSuV1 and TTSuV2 prevalence in the three groups of studied pigs are summarized in table 3.2. Globally, TTSuV2 prevalence (94%) was significantly higher ($P<0.05$) than the TTSuV1 one (64%). TTSuV2 was highly prevalent in the three groups of studied pigs with no significant differences between them. On the contrary, TTSuV1 prevalence in healthy and PMWS affected animals was similar and significantly higher than that of PDNS affected pigs. PCV2 was highly prevalent in PMWS and PDNS affected animals, while prevalence in healthy pigs was significantly lower. Also, TTSuV1 prevalence was significantly lower than that of TTSuV2 and PCV2 in PCVD affected animals (PMWS and PDNS). Finally, PCV2 prevalence was significantly lower than that of TTSuV1 or TTSuV2 in healthy animals.

	Total	TTSuV1+	TTSuV2+	TTSuV1+ Or TTSuV2+	TTSuV1+ TTSuV2+	TTSuV1+ TTSuV2+	TTSuV1- TTSuV2 +	TTSuV1- TTSuV2 +
Healthy	29	21 (72)	26 (90)	29 (100)	18 (62)	3 (10)	8 (28)	12 (41)
PMWS	45	34 (76)	45 (100)	45 (100)	34 (76)	0 (0)	11 (24)	45 (100)
PDNS	34	14 (41)	31 (91)	32 (94)	13 (38)	1 (3)	18 (53)	31 (91)
Total	108	69 (64)	102 (94)	106 (98)	65 (60)	4 (4)	37 (34)	88 (81)

Table 3.2: Prevalence values for both TTSuV species and PCV2. Percentage is shown in parentheses.

The mean TTSuV1, TTSuV2 and PCV2 loads were quantified and $\log_{10}$ mean loads of DNA copies per ml were compared between the three groups of pigs (figure 3.1). A significant difference ($P<0.05$) was found on average TTSuV2 load between PMWS affected and healthy animals. TTSuV2 viral load mean of PDNS affected pigs was numerically higher than that of healthy animals, even such difference did not reach the level of significance

(P=0.06). Such differences were not observed for TTSuV1. PCV2 viral loads were significantly higher in PMWS affected pigs compared to healthy and PDNS affected ones; no significant differences were observed among the latter groups. Moreover, no correlation was found between the individual quantifications of TTSuV1, TTSuV2 and PCV2 in any of the three groups of pigs.

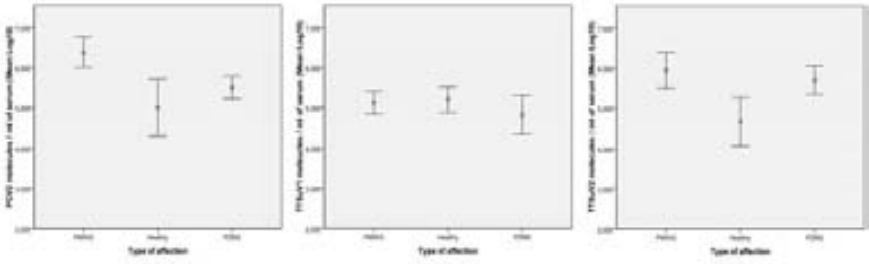

Figure 3.1: Log10 viral genomic loads per ml of serum, for TTSuV1, TTSuV2 and PCV2 in the different tested groups.

Considering that the mean value of viral loads for both TTSuV species in healthy animals was lower than 10^6 DNA copies/ml of serum, animals were grouped in three categories based on viral load: higher than 10^6, between $10^{3.69}$ and 10^6, and less than $10^{3.69}$ (detection limit of the technique) DNA copies/ml of serum (table 3.3). For TTSuV1, 9% (4/45) of the PMWS affected animals, 9% (3/34) of the PDNS affected animals and 7% (2/29) of the healthy animals had viral loads higher than 10^6 DNA copies/ml. In contrast, 24% (11/45), 28% (8/29) and 59% (20/34) of the PMWS, PDNS and healthy animals, respectively, had no detectable TTSuV1 viral loads. For TTSuV2, 42% (19/45) of the PMWS affected and 32% (13/34) of the PDNS affected animals had viral loads in serum higher than 10^6 DNA copies/ml, while only 7% (2/29) of the healthy animals had such high viral load. On the other hand, 10% (3/29) of the healthy animals and 9% (3/34) of PDNS affected animals were below the detection threshold for TTSuV2; all PMWS affected animals were positive for this TTSuV species.

	Healthy		PMWS		PDNS	
Total (molecules/ml of serum)	TTSuV1 29	TTSuV2 29	TTSuV1 45	TTSuV2 45	TTSuV1 34	TTSuV2 34
$>10^6$	2 (7)	2 (7)	4 (9)	19 (42)	3 (9)	13 (32)
$>10^{3.69}$, $<10^6$	19 (66)	24 (83)	30(67)	26 (58)	11 (32)	18 (53)
$\leq 10^{3.69}$	8 (28)	3 (10)	11 (24)	-	20 (59)	3 (9)

Table 3.3: Distribution of Viral loads for TTSuV1 and TTSuV2 among healthy and clinically affected pigs. Percentages are presented in parenthesis.

DISCUSSION

Until recently, all studies performed on TTSuV infections were based on the use of an end-point PCR for both viral species (Bigarré et al., 2005; Kekarainen et al., 2006; Martelli et al., 2006; Martínez-Guinó et al., 2010; Martínez-Guinó et al., 2009; Pozzuto et al., 2009; Segalés et al., 2009; Sibila et al., 2009a; Sibila et al., 2009b). Therefore, all available results were qualitative (positive versus negative) and only permitted to assess prevalence of infection. However, recent studies described different qPCR techniques for the quantification of TTSuV1 and TTSuV2 (Brassard et al., 2010; Gallei et al., 2010b; Lee et al., 2010). In addition, a semi-quantitative PCR approach has been recently established by means of a comparative PCR (Aramouni et al., 2010). 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 a relatively high scale epidemiological study of TTSuVs in the context of PCVDs.

The potential pathogenic role of TTSuV infections has been investigated in a number of studies by means of standard PCR and qPCR, yielding controversial results. The objective of the present work was to further insight into the potential association between TTSuV and PCVDs by means of a newly developed quantification technique. The most relevant result of the present study was that TTSuV2 load in serum was significantly higher in pigs suffering from PMWS, which in turn had significantly higher PCV2 loads in serum, as expected (Grau-Roma et al., 2009; McIntosh et al., 2009). Such results point out the possibility that TTSuV2, but not TTSuV1, may play a role on the development of this multifactorial disease or, alternatively, that immunosuppression associated to PMWS up-regulates its replication (Kekarainen et al., 2010). The precise mechanism by which these putative scenarios may take place remains to be elucidated. Assuming a potential association between PCV2 and TTSuV2 to trigger PMWS, it is important to mention that no correlation was found between the individual quantifications of both viruses. This result may reflect different viral loads of these viruses depending on the timing of disease evolution among studied animals. In addition to these data, all PCV2 qPCR negative animals were positive to one or both TTSuV species, indicating that TTSuV prevalence in healthy or diseased animals is independent from PCV2 presence.

Obtained results also indicated a trend of higher TTSuV2 load in serum of PDNS affected pigs. It is difficult to assess if significance would have been achieved or not with a higher number of studied animals. PDNS is considered an immune-complex disease (Segalés et al., 2005b) and the specific triggering antigen has not been determined yet. It has been suggested that excessive PCV2 antibody titres may trigger the disease (Wellenberg et al., 2004) but this hypothesis still awaits experimental confirmation. Therefore, the potential higher TTSuV2 loads in pigs already affected by PDNS would difficultly explain the pathogenesis of disease occurrence.

TTSuV1 showed significantly lower prevalence when compared to TTSuV2 in PMWS and PDNS affected animals, as it was already described for PMWS (Kekarainen et al., 2006). However, the significant difference in prevalence for TTSuV2 between PMWS and healthy pigs described in the mentioned work (Kekarainen et al., 2006) was not confirmed here. Reasons for these differences can be related with the sensitivity of the techniques used in both studies (more sensitive in the present one) and the different source and number of animals used. In fact, a higher sensitivity of qPCR has been already described in comparison to standard PCR for both TTSuVs (Gallei et al., 2010b). In any case, the present study showed also numerical differences in prevalence (90% in healthy pigs and 100% in PMWS affected animals), even the very high percentages would have prevented to find any significance. It is noteworthy that prevalence values obtained for both TTSuV species are comparable to those obtained by other qPCR methods (Gallei et al., 2010b; Lee et al., 2010).

Interestingly, TTSuV1 was significantly more prevalent in healthy animals and PMWS affected animals than in PDNS affected pigs. Based on these results, it seems that this species is not apparently related to PDNS occurrence, as it has been previously suggested in an experimental reproduction of a PDNS-like condition in gnotobiotic pigs (Krakowka et al., 2008). In this latter work, TTSuV1 was suggested to interact with PRRSV to cause such condition. To elucidate the potential role of PRRSV in the studied PDNS affected pigs, an RT-PCR technique to detect the virus (Mateu et al., 2003) was applied to available sera and only 9 out of 34 PDNS cases were positive. In addition, the amount of TTSuV1 was not related to the PRRSV infection status (data not shown). On the other hand, the abovementioned study (Krakowka et al., 2008) described disseminated intravascular coagulation as the mechanism underlying the PDNS-like condition, which is rather different from the pathological descriptions of

classical PDNS included in the present work, which showed systemic necrotizing vasculitis and fibrino-necrotizing glomerulonephritis (Ramos-Vara et al., 1997; Segalés et al., 1998). Therefore, based on accumulated data, the role of TTSuV1 and/or PRRSV in classical PDNS occurrence is at least debatable and not supported by the present study. On the other hand, TTSuV1 viral load and prevalence were not significantly different between healthy and PMWS affected animals. Those results do not support TTSuV1 as a possible factor inducing PMWS development in conventional pigs, even this association has been found in gnotobiotic pigs when co-infected with TTSuV1 and PCV2 (Ellis et al., 2008).

In summary, the present study describes a newly developed specific and sensitive qPCR method to detect TTSuVs in swine sera. Moreover, its application on serum of PMWS affected pigs indicated that TTSuV2 titers were higher in those pigs but not in healthy or PDNS affected pig. Such differences on viral loads were not evident on TTSuV1. Therefore these viruses, specifically TTSuV2, might play a role in disease manifestation in combination with another pathogen.

Chapter 4

Increased viral load and prevalence of Torque teno sus virus 2 (TTSuV2) in pigs experimentally infected with classical swine fever virus (CSFV)

Submitted for publication

ABSTRACT

TTSuVs are considered non-pathogenic viruses, although lately they have been linked to PCVDs, mainly with PMWS. These associations point out a possible pathogenic role of TTSuVs or, alternatively, that TTSuV replication is up-regulated under disease conditions. In order to further explore the association of TTSuVs with disease occurrence, TTSuVs prevalence and viral load were assessed before and after an experimental infection with a highly virulent CSFV isolate. Serum samples from 54 animals were analysed by means of a qPCR for TTSuV1 and TTSuV2 before and after (between 6 and 13 days post-inoculation) the CSFV challenge. Based on the post-infection clinical evolution and immune response against CSFV, the animals were divided into two groups: group I, with protecting immunity against CSFV and no clinical signs at the day of necropsy, and group II, with no detectable immune response against CSFV and moderate to severe clinical signs. TTSuVs qPCR results indicated that TTSuV2 and not TTSuV1 load in serum increased significantly after challenge with CSFV in the group of pigs with clinical signs, specifically in those with a moderate course of the disease. Therefore, this study emphasizes the different behaviour of both TTSuVs, as already found in the PMWS background, and further supports the association of TTSuV2 with disease occurrence.

INTRODUCTION

TTSuV infection in pigs is distributed worldwide (Cortey et al., 2011b), and it is characterized by a persistent viremia with high prevalence of both species (Sibila et al., 2009a) and circulating levels of up to 10^5 DNA copies per ml of sera for TTSuV1 and up to 10^6 for TTSuV2 in healthy animals (Nieto et al., 2011).

TTSuVs have not been demonstrated to be pathogenic; however, their role during co-infection with other pathogens remains controversial. Several studies associated TTSuVs to a number of pig diseases; especially with regards to PCVDs. In the gnotobiotic pig model, TTSuV1 apparently acted as a triggering factor for the occurrence of PMWS (Ellis et al., 2008), the most economically important PCVD (Segalés et al., 2005a). In addition, a PDNS-like condition was observed in gnotobiotic animals infected with TTSuV1 and PRRSV (Krakowka et al., 2008). Moreover, and as previously described in chapter 3, TTSuV2 was found to be in higher prevalence and viral loads in serum of PMWS-affected pigs (Kekarainen et al., 2006). Also, very recently, an increased prevalence of TTSuVs was found in PRDC affected pigs, and concluded that TTSuV1, but not TTSuV2, was strongly associated with this multietiological and multifactorial condition (Rammohan et al., 2011). These authors also indicated that their results may support the hypothesis that TTSuVs might act as co-factors in PRDC development. Cited studies may point out a pathogenic role of TTSuVs or indicate that TTSuVs replication is up-regulated under disease scenarios. While abovementioned studies suggest TTSuVs involvement in disease development, other studies have not found a link between these viruses and PCVD development (Gauger et al., 2011; Lee et al., 2011).

CSFV, the etiological agent of CSF, is an icosahedral, enveloped, positive strand RNA virus that belongs to the *Pestivirus* genus of the *Flaviviridae* family (Van Regenmortel et al., 2000). Clinical presentation and severity of CSF are diverse, depending largely on the virulence of the strain and the immunological status of the host. In its natural host, CSFV infection results in generalized haemorrhages, leukocytopenia and immunosuppression (Le Poitier et al., 2006; Susa et al., 1992). The virus displays tropism towards immune cells such as dendritic and monocyte/macrophage lineage cells and infection of such cells is assumed to play a key role in immunosuppression, dissemination and/or viral persistence (Carrasco et al., 2004).

In order to further explore the association of natural occurring TTSuV infections with disease, the aim of this work was to evaluate TTSuVs prevalence and viral load in serum before and after an experimental infection with a highly virulent CSFV isolate.

MATERIALS AND METHODS

Animals and samples

Fifty four animals from previous CSFV vaccination experimental trials (Tarradas et al., 2011a; Tarradas et al., 2010; Tarradas et al., 2011b)were selected for the present study. Animals in those experiments were challenged with 10^5 $TCID_{50}$ of a highly virulent CSFV isolate (strain Margarita). Based on the post-infection clinical evolution and several laboratory parameters (cellular and humoral immune responses against CSFV and CSFV RNA load determined by RT-PCR), the animals were grouped into 2 categories (table 4.1). Group I (18 animals): animals with protecting immune response against CSFV and no clinical signs at the day of necropsy (13 d.p.i.); some of those animals experienced pyrexia and mild clinical signs in the course of the

experiment. Group II (38 animals): animals with no detectable immune response against CSFV and moderate to severe clinical signs at the day of necropsy (6 to 13 d.p.i); all animals developed the classical neurological picture.

	Group I (n=18)	Group II (n=38)
CSF clinical score1	0	3 to 6
Mean Ct for qPCR	No RNA detection	RNA detection at Ct from 10 to 28
Antibodies (ELISA)	From 80% to 90% blocking at 7 and 13 dpi	Up to 40% of blocking at necropsy
INF-γ specific response (ELISPOT)*	≥ 25 spots	Absence of spots
Survival	Until end of experiment	¥ between 6 and 13 dpi

Table 4.1: Groups of animals based on CSFV infection course (based on Tarradas et al., 2011b).

Group II was further sub-divided in two subgroups: group IIa (n=24), including animals with moderate CSF signs and survival until 9 to 13 days d.p.i., and group IIb (n=14), composed by animals with severe CSF signs that had to be sacrificed between days 6 and 8 post-infection.

All 56 selected pigs were bled before the CSFV challenge (T0) and at the moment of necropsy (TN), which varied from 6 to 13 days post-challenge depending on the CSF clinical outcome. Sera were collected and frozen at -80°C until their use.

DNA extraction and TTSuV1 and TTSuV2 qPCR

DNA was extracted from 200 µl of serum samples using a DNA extraction kit (Nucleospin® Blood, Macherey-Nagel GmbH & Co KG, Düren,

Germany) according to the manufacturer's instructions. A negative control (PBS) was included with each set of samples to test any contamination of the extracted DNA.

TTSuV1 and TTSuV2 LUX™ (Invitrogen) real time qPCR methods were performed according to previously described techniques (Nieto et al., 2011). Briefly, 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 double-distilled water instead of sample DNA. Amplification and quantification were performed using ABI®7500 Fast Real Time PCR System (Applied Biosystems™) under universal conditions: 10 min at 95ºC, 2 min at 50ºC and 40 cycles of 15 s at 95ºC, 1 min at 60ºC.

Statistical studies

TTSuV1 and TTSuV2 quantifications were expressed as $\log_{10}$ DNA copies per ml of serum. Viral loads were compared intra-groups at T0 and TN by means of a t-student statistical test for two correlated samples. Mean viral loads were compared inter-groups at TN using an ANOVA statistical test. Normality of data was assessed using the Shapiro-Wilk test; the chi-square test was used to compare prevalence among groups and studied time points (T0 and TN). All statistical analyses were performed using SPSS Statistics for Windows, 137 Rel. 17.0.0. 2008. Chicago: SPSS Inc. Significance was set at $P<0.05$ for all tests.

RESULTS

TTSuV1 and TTSuV2 prevalences and mean log10 viral loads per ml of sera in both pig groups are summarised in table 4.2. In both groups and globally,

TTSuV2 was significantly more prevalent than TTSuV1 at T0 and TN. TTSuV1 prevalence increased even though not significantly, in group I and group II(a), while decreasing in the other groups. TTSuV2 prevalence increased overall and in group II generally and II(a) and II(b) specifically. Analysis of individual samples at T0 and TN shows that for TTSuV1, 3 positive samples at T0 are negative at TN in group II(a) and 2 in group II(b) while for TTSuV2 only 1 positive sample at T0 was negative at TN in group I To assess the viral load homogeneity in the chosen groups, TTSuV1 and TTSuV2 loads were compared at T0 among all the groups and subgroups, results showing no significant differences between any of the viral species in any group.

			TTSuV1		TTSuV2	
			T0	TN	T0	TN
	Overall(56)	VL	4.410	4.403	5.049	5.443
		P	31 (55)	29 (52)	50 (89)	52 (93)
Healthy animals	Group I (18)	VL	4.562	4.528	4.944	5.153
		P	11 (61)	12 (67)	17 (94)	16 (89)
Diseased animals	Group II (38)	VL	4.337	4.256	5.098	5.554
		P	20 (53)	17 (45)	33 (87)	36 (95)
	Group IIa (24)	VL	4.402	4.446	5.300	5.805
		P	13 (54)	14 (58)	22 (92)	24 (100)
	Group IIb (14)	VL	4.226	3.937	4.753	5.123
		P	7 (50)	3 (21)	11 (79)	12 (86)

Table 4.2: Prevalence and mean log10 viral loads of TTSuV1 and TTSuV2 at T0 (time of CSFV challenge) and TN (time of necropsy).

TTSuV1 and TTSuV2 mean $\log_{10}$ viral loads per ml of serum were compared between the different animal groups (figure 4.1). A significant increase of

TTSuV2 mean viral load from T0 to TN was observed globally (log_{10} 5.049 DNA copies/ml vs. log_{10} 5.443; p<0.01) and in the group of diseased animals, group II (log_{10} 5.098 vs. log_{10} 5.554; p<0.01). TTSuV1 did not show any significant increase in viral load from T0 to TN in any of both studied groups or overall (group I, log_{10} 4.562 vs. log_{10} 4.528; and group II, log_{10} 4.337 vs. log_{10} 4.258). When comparing TTSuVs mean viral load at TN, only a statistical tendency was observed for TTSuV2 between groups of non-diseased (group I) and diseased animals (group II) (log_{10} 5.153/ log_{10} 5.554; p=0.1). However, when considering group II subgroups, viral TTSuV2 loads of subgroup IIa (log_{10} 5.805) were significantly higher at TN when compared to those of groups I (log_{10} 5.153; p=0.033 and IIb (log_{10} 5.123; p=0.040) (figure 4.2).

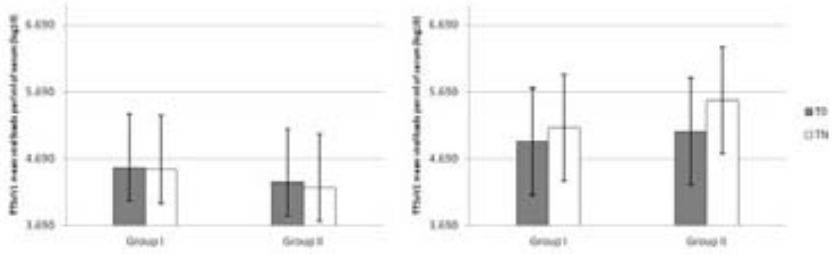

Figure 4.1: Mean (and standard deviation) of TTSuV1 and TTSuV2 loads per ml of serum in each studied group before CSFV challenge (T0) and at the time of necropsy (TN).

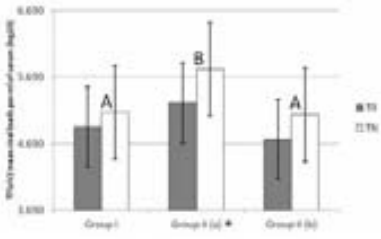

Figure 4.2: Mean (and standard deviation) of TTSuV2 loads per ml of serum comparison between group I and subgroup IIa and IIb. * means significant differences in TTSuV2 load in serum between T0 and TN. Different letters mean significant differences at TN between the three groups.

DISCUSSION

The involvement of TTSuVs on disease occurrence has been one focus of swine viral research in recent years, mainly in regards multifactorial conditions such as PCVDs and PRDC (Blomström et al., 2010; Ellis et al., 2008; Gauger et al., 2011; Kekarainen et al., 2006; Krakowka et al., 2008; Lee et al., 2011; Lee et al., 2010; Perez et al., 2011; Rammohan et al., 2011; Taira et al., 2009) In chapter 3, it was demonstrated that, under field conditions, TTSuV2 was highly prevalent and present to higher viral loads in PMWS-affected pigs in comparison to healthy animals. TTSuV1 infection was also present, but no differences between diseased and non-diseased pigs were found. These findings lead to think about a possible role of TTSuV2 in the development of this multifactorial disease and/or, alternatively, that immunosuppression associated to PMWS may up-regulate its replication (Nieto et al., 2011).

The relationship between immunosuppression and TTV infections has mainly been studied in humans. It has been shown that chemical and viral induced immunosuppression can lead to an increase in TTV viral load and prevalence (Christensen et al., 2000; Madsen et al., 2002; Moen et al., 2003; Thom and Petrik, 2007; Touinssi et al., 2001). TTVs were also shown to be capable of producing viral-induced hepatitis after liver transplantation (Piaggio et al., 2009a) and higher TTV prevalence and load have been found in cirrhotic patients after liver transplantation (Burra et al., 2008). Indeed, viral load increase depended on the amount of immunosuppressive drugs administered (Burra et al., 2008). Interestingly, TTV prevalence and load in plasma of human immunodeficiency virus (HIV) affected patients were significantly higher than in healthy blood donors. Moreover, an association between high TTV loads and low CD4 T cell counts, high HIV viral loads and overt acquired immunodeficiency syndrome was found (Shibayama et

al., 2001). Finally, a recent publication suggested that human TTV could be used to follow functional immune reconstitution in immunosuppressed patients; in that sense, TTV viremia was considered of help in the assessment of the dose and the time interval of an immunosuppressive treatments (Focosi et al., 2010b).

In the present study it was investigated whether the scenario previously described in PMWS-affected pigs was also present in animals with CSFV, a well-known immunosuppressive viral infection (Le Poitier et al., 2006). For this, TTSuV1 and TTSuV2 loads were determined and compared in animals with different CSFV infection outcomes. Specific CSFV immune responses developed after virus infection correlated with protection or not against challenge, therefore generating groups of healthy and diseased status at the time of necropsy. The group of pigs with a deficient immune response against CSFV accounted for the ones suffering from moderate to severe CSF clinical signs. Such variety of immunological responses against CSFV was paramount to assess TTSuV loads in different scenarios of immunocompetence. In line with previous studies in the PMWS context, TTSuV2 but not TTSuV1 mean loads in serum increased globally in the diseased animal group. Pigs suffering from CSF are known to be immunocompromised and when clinical signs appear, leukopenia and high viral RNA loads of CSFV and low levels of CSFV specific antibodies develop (Knoetig et al., 1999). Taking into account the different immunological outcome against CSFV of studied pigs, it appeared that TTSuV2 viremia did not show any increase after challenge in the animals with good neutralizing response to CSFV at the time of necropsy. The highest TTSuV2 viremia rate was found in pigs showing the moderate-severe course of clinical disease, which showed laboratorial evidence of immune suppression. Interestingly, animals surviving until the end of the experiment and suffering from moderate course of CSF disease, showed TTSuV2 loads

significantly higher after the challenge in comparison to the group of healthy animals or the group of animals that were sacrificed and/or died very early in the course of the experiment. This result might be explained by the fact that TTSuV2 should probably need a sufficient time-frame to benefit from the compromise of the immune system in order to increase viral production and release in blood.

While TTSuV2 loads were inversely correlated with immunological responses of the animals against CSFV, TTSuV1 loads were similar in all animals at both sampling times. This difference between both viruses is difficult to establish since this latter virus was also under the same immunological circumstances. However, one potential reasoning to explain the different TTSuV outcome may rely on the fact that both TTSuVs are genetically very different and may display a differentiated biological behaviour as it has been suggested (Cortey et al., 2011a). For instance, within another ssDNA virus family such as *Circoviridae*, PCV2 is known to be pathogenic and contributing to the development of PCVDs while PCV1 has been demonstrated to be non-pathogenic for pigs (Allan and Ellis, 2000). Furthermore, it has been proposed that some porcine and human anelloviruses might be more disease-linked than others (Kekarainen et al., 2006; Okamoto, 2009a), and co-infection with other viruses could variably affect the outcome or progression of some diseases (Fehér et al., 2009).

Results from chapter 3, altogether with previous studies tried to link TTSuVs to the occurrence of disease, mainly as potential triggering factors or contributors in a multifactorial environment, resulting in controversial results (Ellis et al., 2008; Gauger et al., 2011; Kekarainen et al., 2006; Krakowka et al., 2008; Lee et al., 2010; Rammohan et al., 2011; Taira et al., 2009). Alternatively, and based on the results of the present study, it would not be surprising that TTSuVs, and specifically TTSuV2, might be considered as a

potential indicator of immunosuppression in pigs, as it has been already proposed for human TTVs under the effect of immunosuppressive treatments (Focosi et al., 2010).

Chapter 5

Exploratory study on Torque teno sus virus in pulmonary lesions in pigs

Published at the journal Veterinary Microbiology. 2012. doi: j.vetmic.2012.09.011

ABSTRACT

TTSuVs have been linked to PCVDs and described as cause of mild respiratory lesions in gnotobiotic pig; moreover, an increased TTSuV prevalence was found in PRDC affected pigs. In the present study, TTSuV1 and TTSuV2 loads and their prevalence were evaluated in formalin-fixed, paraffin-embedded (FFPE) lung samples displaying different types of inflammatory lesions. Such measurements were made by means of a real time quantitative PCR technique optimised for its use on FFPE tissues. Selection criteria included negativity against PCV2, PRRSV, Aujeszky's disease virus (ADV) and swine influenza virus (SIV). Results from the present study demonstrated that both TTSuVs were present in lung. However, TTSuV2 had higher viral load and prevalence in all the studied groups when compared to TTSuV1. TTSuV2 mean load was also higher in lungs with viral background when compared to normal lungs or to those with bacterial background. This result suggests a possible role of TTSuV2s in the pathogenic mechanism of inflammatory lesions of lungs compatible with viral infection.

INTRODUCTION

TTVs are considered non-pathogenic, although their role during co-infection with other pathogens is under debate. Human TTVs have been linked to a number of inflammatory diseases and tumours (Okamoto, 2009a) and TTSuVs have been related with the occurrence of PCVDs such as PMWS (chapter 3) and (Kekarainen et al., 2006; Nieto et al., 2011) and a PDNS-like condition (Ellis et al., 2008; Krakowka and Ellis, 2008). In contrast, a recent study suggested that TTSuVs were not involved in the occurrence of diseases associated to PCV2 infection, the essential agent of PCVDs (Lee et al., 2010). Therefore, the pathogenic role of TTSuVs remains to be elucidated.

The capability of TTSuV1 and TTSuV2 for producing lesions in pigs is also controversial. Krakowka and Ellis (2008) reported mild lesions in gnotobiotic pigs after inoculation with TTSuV1. They consisted of transient thymic atrophy, membranous glomerulonephropathy, modest lympho-histiocytic infiltrates in the liver and mild interstitial pneumonia. Very similar results have been obtained with TTSuV2 by means of inoculation of a liver homogenate containing the virus in specific-pathogen free pigs (Mei et al., 2011). Also, very recently, an increased prevalence of TTSuVs was found in PRDC affected pigs, and concluded that TTSuV1, but not TTSuV2, was strongly associated with this clinical multiaetiological and multifactorial condition (Rammohan et al., 2011). These authors also indicated that their results may support the hypothesis that TTSuVs might act as co-factors in PRDC development.

The precise role of TTSuVs in disease pathogenesis during co-infection has not been yet elucidated, but most of the data available, including that presented in chapters 3 and 4 of the present thesis, suggest they may act as potential disease triggering factors, or alternatively, as opportunistic

pathogens in disease complex scenarios (Blomström et al., 2010). Therefore, the objective of the present work was to investigate the involvement of TTSuVs in the occurrence of different pulmonary inflammatory lesions. A parallel objective included the validation of the previously described real time qPCR to detect TTSuVs (chapter 3) for its use on formalin-fixed, paraffin-embedded tissues.

MATERIALS AND METHODS

Tissue samples

Forty-five FFPE lung tissues with different inflammatory lesion types were selected from a total of 2401 pigs submitted for necropsy to the Veterinary Pathology Diagnostic Service of the *Universitat Autònoma de Barcelona* (Spain), between 1998 and 2010. Paraffin blocks were selected on the base of the following criteria: a) pigs of 8 (±3) weeks of age, b) clinical history and pulmonary lesions indicative of respiratory disease, c) negative *in situ* hybridization (ISH) for PCV2, and negative immunohistochemistry for PRRSV, ADV and SIV, and d) presence of only one type of microscopic lesion in the lung. As negative controls, FFPE lung tissues with no lesions from 18 pigs of similar age were selected.

To evaluate the effect of formalin-fixing and paraffin-embedding on TTSuV2 quantification, 13 frozen (-80ºC) lung tissues from the negative control pigs, were also selected for DNA extraction and viral quantification.

Histopathological studies

Tissue samples from each block were previously maintained in formalin for 24 to 48 hours, and subsequently dehydrated and embedded in paraffin wax.

Five consecutive 4 μm thick sections were cut. One section was processed for histopathology, one for PCV2 nucleic acid detection by ISH, and the remaining were processed to detect PRRSV, SIV and ADV antigens by the corresponding immunohistochemical methods, respectively.

Microscopic pulmonary lesions were classified following previously proposed criteria (Caswell and Williams, 2007; Grau-Roma and Segales, 2007). Catarrhal-purulent bronchopneumonia (CPBP) consisted of mucous, neutrophils and macrophages filling the bronchioles and alveoli. Pleuritis was considered when fibrinous or fibrino-purulent exudates were observed in or over the pleura, with or without mesothelial proliferation or fibrosis, and lack of lung parenchyma inflammation. Bronchointerstitial pneumonia (BIP) was assessed when bronchiolar necrosis with thickening of septa in the surrounding alveoli were observed. Interstitial pneumonia (IP) was assessed when lymphohistiocytic interstitial inflammation of the alveolar walls was accompanied with eventual hypertrophy and proliferation of type 2 pneumocytes. Fibrinous-necrotizing pleuropneumonia (FNP) consisted of extensive areas of necrosis in the lung parenchyma with pleura, alveoli and terminal bronchioles filled by fibrin, oedema, neutrophils, fewer macrophages, many necrotic leukocytes and occasional intravascular fibrinous thrombi. Severity of CPBP, pleuritis and FNP was assessed macroscopically in 3 grades regarding the percentage of lung parenchyma or pleura affected by pneumonia or pleuritis (Straw et al., 1986). Thus, grade 1: <30% of extension, grade 2: 30-50% and grade 3: >50%. Severity of IP and BIP (as these lesions had a multifocal to diffuse distribution) was also classified in 3 grades, but following a microscopic criterion consisting of amount of inflammatory cells in the alveolar walls: mild (grade 1), moderate (grade 2) and severe (grade 3).

In situ hybridization (ISH) and immunohistochemistry

A previously described ISH technique to detect PCV2 (Rosell et al., 1999) was performed using a 41 pb digoxigenin labelled DNA probe corresponding to ORF1 of PCV2 (DIG-CCT TCC TCA TTA CCC TCC TCG CCA ACA ATA AAA TAA TCA AA).

For immunohistochemistry, three avidin–biotin–peroxidase assays using the corresponding monoclonal antibodies to detect PRRSV, ADV and SIV were performed. Techniques were based on previous published procedures (Grau-Roma and Segales, 2007) . Monoclonal antibodies used to detect PRRSV, SIV and ADV were SDOW17 (Rural Technologies, USA), C65331 M (Tib Molbiol, Germany) and ICII (kindly donated by Dr. Hans Nauwynck, University of Ghent, Belgium), respectively, with corresponding dilutions of 1:500, 1:200 and 1:100 in TBS.

DNA extraction

Paraffin was removed by melting at 60ºC, and a piece of 20-mg of lung tissue was cut with a blade. In order to prevent carryover of contaminating DNA, a fresh blade was used for each sample and all material was cleaned with xylene and 100% ethanol. Tissues were then rehydrated, cut in small pieces and collected in 1.5 ml microcentrifuge tube. Paraffin was further removed by adding 1.5 ml of xylene, vortexing, and incubating the mixture at room temperature for 30 min followed by centrifugation at 13.000 g for 5 min. The supernatant was removed and discarded. A further 1.5 ml of xylene was added to the pellet and the procedure was repeated. To facilitate pelleting and hydration of the samples, 1.5 ml of 96% ethanol was added. After vortexing, the samples were pelleted by centrifugation at 13.000 g for 5 min

and the supernatant was removed. The pellet was then air dried, being ready for the DNA extraction process.

DNA was extracted from all 63 FFPE and 13 frozen lung tissues using NucleoSpin® Tissue kit (Macherey-Nagel) according to manufacturer instructions. Extraction process was made by batches of 19 samples together with a negative control for extraction consisting of 200 µl PBS. Extracted DNA was eluted in 100 µl of elution buffer (5 mM Tris/HCl, pH 8.5) and quantified using the Nanodrop ND-1000 Spectrophotometer. DNA purity was assessed with an A260/280 values higher than 1.6. DNA integrity and quality for PCR amplification was tested amplifying the porcine housekeeping gene β-lactoglobuline (Ballester et al., 2005), with a pair of primers that produce a 237 bp product. Samples with low DNA quantity (less than 125 ng/µl), purity or quality, were extracted again.

Real time qPCR to detect TTSuVs

TTSuV1 and TTSuV2 load quantification in FFPE and frozen tissues was made by optimizing the previously described D-Lux based qPCR technique (chapter 3). 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. Each reaction contained 2 µl of sample (total of 250ng DNA) or standard DNA, 200 nM of each primer, 10 µl of Express qPCR Supermix Universal™ (Invitrogen) and 0.04 µl of Rox in a total volume of 20 µl. Amplification and quantification were performed using the ABI®7500 Fast Real Time PCR System (Applied Biosystems™) under universal conditions: 10 min at 95ºC, 2 min at 50ºC, 40 cycles of 15 s at 95ºC and 1 min at 60ºC. The same frozen and FFPE tissues were investigated to determine the effect of formalin

fixation and paraffin embedding on the viral load and compare qPCR efficiencies on the same tissues conserved by different methods.

The qPCR primers specificity was studied previously and a series of conditions were applied for the acceptance of each reaction: (1) standard deviation of sample triplicates under 0.5, (2) standards curves with an accuracy of R2 > 0.97, and (3) the reaction efficiency (standard curve's slope) between 3.2 and 3.7. Also, if any of the negative controls was positive the run was repeated.
TTSuV viral loads were obtained in 250 ng of DNA. To calculate the genomic load per milligram of DNA, individual results from qPCR were multiplied by 4000. Finally, average $\log_{10}$ copies per milligram of DNA were used for mean viral load comparisons.

TTSuV1 and TTSuV2 qPCR detection limit was determined to be 20 copies per 250 ng of DNA (1.301 $\log_{10}$) which is equivalent to $8x10^4$ copies per milligram of DNA (4.903 $\log_{10}$).

Statistical analyses

Statistical analyses were done using IBM SPSS® 19. Lesions were grouped in two categories in regards of the possible causative agent. Hence, CPBP, Pleuritis and FNP were considered to be potentially caused by bacterial pathogens, and BIP and IP were assumed to be putatively originated by viral infections. Selection criteria included negativity against usual viral pathogens (PCV2, PRRSV, SIV and ADV) that may cause these pulmonary lesion types. Based on the established classification (bacterial versus viral) and on lesion groups, TTSuV prevalences and mean viral loads were assessed and a series of comparisons between the different groups were made using the analysis of variance (ANOVA) and t-student test. Moreover, relationships

between lesion types/groups/severity and TTSuV viral loads were performed using the Pearson correlation "r". Linear regression was used to discard any correlation between animal age and TTSuV1 and TTSuV2 viral loads.

RESULTS

Histopathology

The number and percentage of cases for each pulmonary lesion was as follows: CPBP, 17 (37.8 %); pleuritis, 8 (17.8 %); FNP, 6 (13.3 %); IP, 12 (26.6 %) and BIP, 2 (4.4 %). The average and standard deviation for severity was: CPBP, 2.1 (0.8); pleuritis, 2.4 (0.7); FNP, 2.8 (0.4); IP, 1.5 (0.5) and BIP, 1.5 (0.7). Thirty-one (68.9 %) cases were included in the group of bacterial-like origin and 14 (31.1 %) in the viral-like group.

qPCR optimization

A difference of an average of 0.793 (±0.316) and 0.887 (±0.228) $\log_{10}$ viral load was observed for TTSuV1 and TTSuV2 respectively, when comparing quantifications made on FFPE and fresh tissues (figure 5.1).

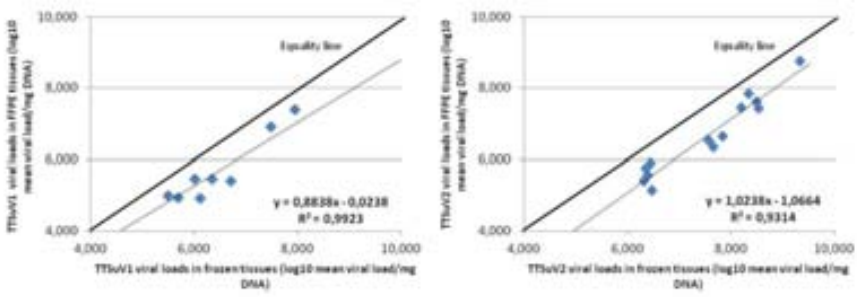

Figure 5.1: Linear regression of Log10 loads per milligram of DNA for TTSuV1 and TTSuV2 in frozen and FFPE tissues.

Standard curves generated in the different runs of quantification of the present study did not show significant variation, remaining the coefficient of variation inter-assay in 4%. The amplification efficiency (*E*) was between 96.5% and 98.5%.

TTSuV loads and lung lesions

TTSuV loads studied in relation to each described pulmonary inflammatory lesion and the putative viral or bacterial origin of the lesions are summarized in table 5.1. Results of TTSuV1 and TTSuV2 prevalences and detection either as single infection or co-infection in all studied samples are shown in table 5.2.

	Origin	Bacterial (31)			Viral (14)		
	Lesion	CPBP (17)	P(8)	FNP(6)	IP(12)	BIP(2)	Control (18)
VLL	TTSuV1 TTSuV2	5.544 6.102[a]	5.876 6.257	und 7.219	5.026 7.198[b]	und 7.102	5.987 6.155[a]
VLO	TTSuV1 TTSuV2	5.697 6.333[a]			5.026 7.179[b]		5.987 6.155[a]

VLL: Mean viral load by lesion type; VLO: Mean viral load when grouping by lesion origin (bacterial versus viral).

Table 5.1: TTSuVs log10 mean load of positive samples in different lung inflammatory lesions of potential bacterial and viral origin. Different letters within a row mean significant differences ($p<0.05$); und means "under detection limit"

A total of 41 out of 45(91%) of the tested samples with lung lesion were TTSuV positive. From 45 samples with pulmonary lesions, 17 (38%) were qPCR TTSuV1 positive while 34 (78%) were qPCR TTSuV2 positive with a

co-infection rate of 24% (11/45). From 31 samples of bacterial origin, TTSuV1 was detected in 13 (42%) and TTSuV2 in 24 (77%) with a co-infection rate of 9 (29%). From 14 samples of viral origin, only 4 (29%) were qPCR TTSuV1 positive and 10 (71%) were qPCR TTSuV2 positive with a co-infection rate of 2 (14%). From 18 studied samples with normal lung histology, 11 (61%) were qPCR TTSuV1 positive while all of them (100%) were qPCR TTSuV2 positive with a co-infection rate of 11 (61%). From all 63 studied samples, only 4 (6%) were negative for both TTSuV species, 6 (10%) were only qPCR positive for TTSuV1 and 30 (48%) were only qPCR positive for TTSuV2. Statistically, TTSuV2 was significantly more prevalent than TTSuV1 in all studied groupings. No difference in prevalence was observed for any TTSuV species in relation to presence or absence of lesions, as well as in the type, severity and origin of the lesions.

Lesion	Lesion severity			TTSuV prevalence		TTV1+ or	TTV1+ and	TTV1- and	TTV1+ and	TTV1- and
Category	Mild	Moderate	Severe	TTSuV1+	TTSuV2+	TTV2+	TTV2+	TTV2-	TTV2-	TTV2+
CPBP (n=17)	4(24)	7(41)	6(35)	7 (41)*	13 (76)*	17 (100)	4 (24)	0	3 (18)	9 (53)
P (n=8)	1(13)	3(38)	4(50)	6(75)	7 (88)	8 (100)	5 (63)	0	1 (13)	2 (25)
FNP (n=6)	0	1(17)	5(83)	0*	4 (67)*	4 (67)	0	2 (33)	0	4 (67)
Total Bacterial (n=31)	5(16)	11(35)	15(48)	13(42)*	24 (77)*	29 (94)	9 (29)	2 (6)	4 (13)	15 (48)
BIP (n=2)	1(50)	1(50)	0	0*	2 (100)*	2 (100)	0	0	0	2 (100)
IP (n=12)	6(50)	6(50)	0	4(33)*	8 (67)*	10 (83)	2 (17)	2 (17)	2(17)	6 (50)
Total Viral (n=14)	7(50)	15 (33)	0	4(29)*	10 (71)*	12 (86)	2 (14)	2 (14)	2 (14)	8 (57)
Total Lesions (n=45)	12(27)	18 (40)	15 (33)	17(38)*	34 (76)*	41 (91)	11 (24)	4 (9)	6 (13)	23 (51)
Controls (n=18)	0	0	0	11(61)*	18 (100)*	18 (100)	11 (61)	0	0	7 (39)
TOTAL (n=63)				28(44)*	52(83)*	59 (94)	22 (35)	4 (6)	6 (10)	30 (48)

Table 5.2: Results of lung microscopic lesions and TTSuV detection in the studied pulmonary tissues. Percentages within lesion categories are presented in parenthesis.

* means significant differences between TTSuV1 and TTSuV2 prevalences.

Globally, TTSuV2 mean viral load was significantly higher than TTSuV1 mean viral load in all studied samples independently from their

histopathological characteristics (6.592 log_{10}/mg DNA for TTSuV2 and 5.715 log_{10}/mg DNA for TTSuV1; $p<0.01$). TTSuV1 mean log_{10} loads did not differ in relation to the type, origin or severity of lesions, although all samples within FNP and BIP were found to be under the detection limit of the technique. TTSuV2 mean log_{10} loads were significantly different only when comparing samples with CPBP (6.102 ± 0.9 log_{10}/mg DNA) to those with IP (7.198 ± 1.3 log_{10}/mg DNA) ($p=0.038$) and those with IP to the group of samples with no lesions (6.155 ± 0.8 log_{10}/mg DNA) ($p=0.019$). When considering the generic potential causative agent, samples with lesions of a viral background had significantly higher viral load (7.179 ± 1.25 log_{10}/mg DNA) when compared to the control group (6.155 ± 0.8 log_{10}/mg DNA) ($p=0.014$) and to those of bacterial origin (6.333 ± 1.0 log_{10}/mg DNA) ($p=0.048$). TTSuV2 mean log_{10} loads from lung lesions considered of bacterial background (6.333 ± 1.0 log_{10}/mg DNA) did not significantly differ from those of the control group.

At the same time that all observed viral loads were highly variable, there was no correlation between TTSuV1 or TTSuV2 individual viral loads with lesion origin, lesion type or lesion severity (Figure 5.2).

DISCUSSION

Huge amounts of FFPE tissues are being stored in pathology departments and hospitals archives all over the world as this is the standard preservation method for pathological studies. These samples represent a highly valuable DNA bank for many studies. In spite of this advantage, formalin fixation of tissue samples can affect the integrity of the DNA due to a time-dependent decrease of pH (Gilbert et al., 2007). Moreover, formalin may inhibit PCR and other molecular analysis (Stanta and Schneider, 1991). Therefore, effective methods for DNA extraction involving minimum manipulation are

required for securing pure and high quality DNA to support trustable results in quantitative assays on FFPE tissues. Several protocols for genomic DNA extraction from this sample type had been published. Variations of xylene/ethanol based methods are the most used (Goelz et al., 1985; Santos et al., 2008; Shi et al., 2004) and had shown to be effective to conserve DNA fragment of up to 1.18 kb (Santos et al., 2009). In the present study, a DNA extraction method based on xylene/ethanol deparaffinization is described. DNA quantity (ng) and purity were in acceptable values as well as those from frozen tissues and the amplification of the housekeeping gene demonstrated the conservation of the genomic DNA and the removal of PCR inhibitors from the samples. Nevertheless, the quantitative assay on FFPE-extracted DNA showed a decrease in the viral quantification in comparison to frozen samples. That may be attributed to DNA degradation associated to storage time as it was previously described (Ludyga et al., 2012). However, such decrease in the quantification showed not to be correlated to the time of conservation in the present samples (data not shown).

Prevalence and viral loads of both TTSuV species were assessed in FFPE lung tissues using the previously described qPCR (chapter 3) here optimised for its use on DNA from FFPE tissues. Several lung pathological conditions were taken into consideration and lesions were grouped based on a putative involvement of generic infectious agents. In this way, three conditions (CPBP, pleuritis and FNP) were associated with bacterial aetiology and two others (IP and BIP) were associated with viral aetiology, although for the latter one, the presence of PRRSV, ADV and SIV antigens and PCV2 genome were ruled out. This selection criterion was important to avoid the direct attribution of these lesions to one or several of these well known viruses in pigs.

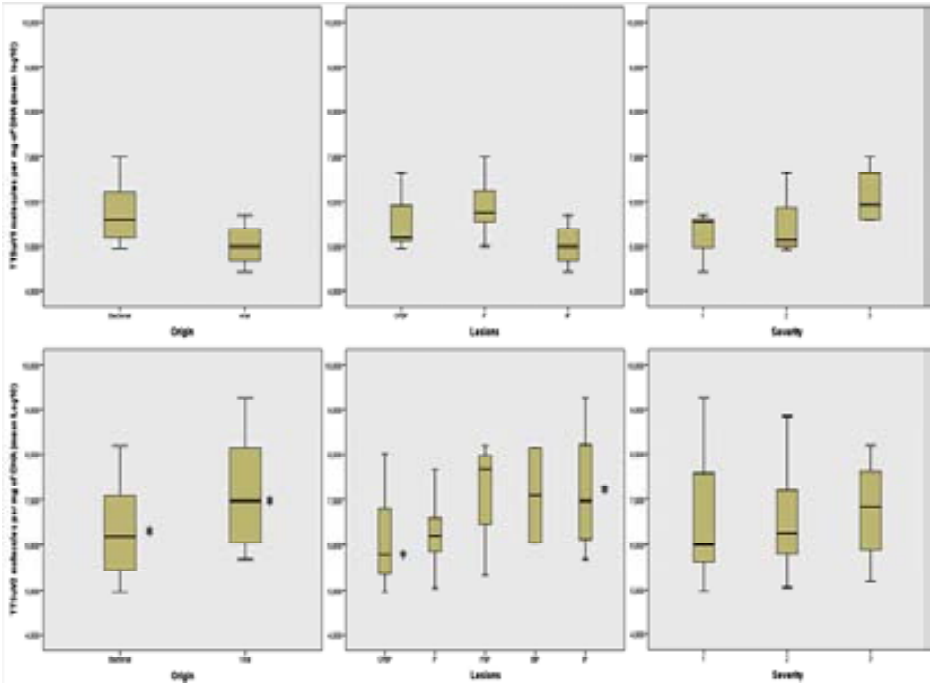

Figure 5.2: Box plots for log10 TTSuVs viral loads corresponding to origin, lesion type and severity. *significant differences in mean viral loads.

Therefore, an objective of this work was to assess TTSuV as potential agents involved in respiratory disease as has been suggested elsewhere. The first evidence of TTSuVs detection in pig respiratory disease was reported in Japan where both TTSuVs were found to be widespread in sera of PRDC affected pigs (Taira et al., 2009). Afterwards, TTSuV1 was found to be highly prevalent in lungs of PRDC-affected pigs (Rammohan et al., 2011). Results from the present study demonstrated that both TTSuVs are present in lung tissues with a special predominance of TTSuV2 among tested samples. Statistically, this species showed to have higher viral load and higher prevalence in all the studied groups when compared to TTSuV1. TTSuV2 mean load was also higher in lungs with viral background when compared to normal lungs or to those with bacterial background. This result suggests a

possible role of this TTSuV species in the pathogenic mechanism of inflammatory lesions of lungs compatible with viral infection.

The pathogenic role of TTSuVs has been discussed previously in several studies leading to controversial conclusions. As presented in chapter 3 of the present thesis, TTSuV2 was more frequently related to PMWS when compared to TTSuV1 under field conditions in Spain (Kekarainen et al., 2006) while TTSuV1 was linked with occurrence of PMWS and a PDNS-like condition in gnotobiotic pigs experimentally infected with PCV2 and PRRSV, respectively (Ellis et al., 2008; Krakowka et al., 2008). Moreover, PMWS-affected pigs also had higher viral DNA loads and lower anti-TTSuV2 antibody values than healthy animals (Huang et al., 2011). Such situation did not occur with TTSuV1, since viral loads and antibody levels were not signicantly different between PCVD-affected and non-affected pigs (Huang et al., 2012). In addition, TTSuV2 has been also associated to CSF disease outcome (chapter 4). While positive associations with diseases associated to PCV2 infection were described, other studies indicated that TTSuVs may not play any role in the exacerbation of swine viral diseases (Gauger et al., 2011; Lee et al., 2010).

It is important to mention that TTSuVs have also been several times described as responsible of producing pulmonary lesions, specifically IP, as a primary and unique infecting agents or co-infecting with other viral agents (PCV2 and PRRSV). The first evidence of this involvement was described when gnotobiotic pigs, exposed to TTSuV1, developed mild IP (Ellis et al., 2008; Krakowka and Ellis, 2008; Krakowka et al., 2008). Later on, TTSuV2 was also suggested to produce IP when pigs were inoculated with a liver homogenate containing TTSuV2 (Mei et al., 2011). Although the animals of the latter studies showed lung and other organs lesions, they did not show any clinical symptom at the moment of necropsy. Based on these studies and

the results presented in the present one, TTSuV2 may be an agent able to produce, solely or in combination, lesions of IP. Based on results of this study, it seems that this ability would not be shared by TTSuV1. TTSuV2 presence in lesions of bacterial causality was not significantly different from control animals, suggesting that the detected viral load in lungs might be expectable in subclinically infected pigs.

No correlation could be established between TTSuV2 viral loads and any of the studied lesions by separate or with their severity. This could indicate that the specific effect of TTSuV2 on lesion generation would be relatively limited, and probably a higher number of available samples would have been needed to assess such potential correlation.

In conclusion, the present study further emphasizes that TTSuV2, but not TTSuV1, might display a potential pathogenic effect in pigs, specifically in regards the causality of IP.

TTSuV1 and TTSuV2 are genetically different viruses. Under field conditions, the co-infection with both viruses is very high, with no cross-reactivity in their antigenic activity (Huang et al., 2012). This could explain their differentiated ability to produce lesions and the different potential association to other swine diseases.

PART III

GENERAL DISCUSSION AND CONCLUSIONS

Chapter 6

General discussion

TTSuV infection is worldwide distributed. These viruses seem to be well-adapted to the host (pig and wild boar), as evidenced by the systematic occurrence of persistent infections and relatively low genetic change over time (Segalés et al., 2009). TTSuV infection has been found to be highly prevalent in both healthy and diseased animals, further complicating the debate on the pathogenic capabilities of these viruses. Since their discovery, researchers around the world tried to understand their role in swine disease occurrence. First evidence for TTSuVs association with disease was established with PCVDs (Ellis et al., 2008; Kekarainen et al., 2006). Those starting works paved the way for further field and experimental studies aimed to clarify their role in triggering or aggravating disease (Blomström et al., 2010; Gauger et al., 2011; Huang et al., 2011; Krakowka et al., 2008; Lee et al., 2010; Mei et al., 2011; Nieto et al., 2011; Rammohan et al., 2012; Savic et al., 2010; Taira et al., 2009).

In their nature as multifactorial diseases, PCVDs are subject to continuous studies that may reveal other agents involved in their development. PCV2, the essential etiological agent for PCVDs, is an ubiquitous virus that under certain circumstance, is capable to produce disease. Therefore, it is not unlikely that TTSuVs may be part of the triggering factors for PCVDs or be, by themselves, another factor able to produce disease under the same conditions. Ellis and co-workers (2008) showed that after an experimental infection with TTSuV1 and PCV2 in gnotobiotic pigs, neither PCV2 nor TTSuV1 were able to produce PMWS by themselves. A co-infection with both of them was needed to reproduce this condition (Ellis et al., 2008). The same research group confirmed later that under experimental conditions, TTSuV1 was involved in the production of a PDNS-like condition when co-infecting with PRRSV, also in gnotobiotic pigs (Krakowka et al., 2008). These studies referred principally to the role of TTSuV1 under experimental conditions, being considered a cofactor in the triggering of the condition.

Nevertheless, these conditions observed in gnotobiotic pigs are not likely to be natural in the field where commercial pigs are normally in contact with TTSuVs and these other agents, and even foetuses are found positive with TTSuVs before the age of immune-competence (Aramouni et al., 2010; Martinez-Guinó et al., 2010). Furthermore, in field studies, healthy animals and PMWS-affected animals are both TTSuV and PCV2 positive (Nieto et al., 2011; Sibila et al., 2009a), with the difference that PMWS-affected pigs had a higher TTSuV2 prevalence than healthy ones (Kekarainen et al., 2006). Results in naturally PMWS-affected animals (chapter 3) showed that TTSuV2 was highly prevalent and had higher viral load in affected animals when compared to the healthy ones. The fact that PMWS-affected animals are immunocompromised (Darwich and Mateu, 2012; Kekarainen et al., 2010), might open the debate about a role of the immune system in the up-regulation of viruses that could contribute in triggering or aggravating disease. In fact, the mechanisms of viral up-regulation may not be clear, but the immunological panorama of PCVD-affected animals, involving a depletion of B-cells, T-cells and NK cells (Darwich and Mateu, 2012), allows the speculation about the involvement of the adaptive and/or innate immune responses. Indeed, the recent study of Huang and collaborators (2011) confirmed that PCVD-affected animals had lower level of TTSuV2 specific antibodies when compared to healthy ones. Such scenario would imply a reduced/inefficient humoral response against TTSuV2 in the PCVD context, and the subsequent expectable higher viral loads.

To get further insights into the investigation on the role of TTSuV in disease and in a way to elucidate the up-regulation of these viruses in an immunocompromised scenario, the second study (chapter 4) allowed the study of TTSuVs' loads in animals experimentally infected with CSFV, another immunosuppressive virus. Here, different outputs of disease were obtained, allowing the analysis before and after the infection in animals with

good immunological response against CSFV and others with no detectable response. Interestingly the results of the study showed, as expected, higher TTSuV2 loads after challenge in the animals that showed CSF clinical signs and laboratory evidence of immunosuppression. Pigs suffering from CSF show a marked leukopenia with affection of T-cells, B-cells, granulocytes and monocytes (Le Poitier et al., 2006). These data further allow speculating about the possible involvement of the adaptive and/or innate immune response in the up-regulation of TTSuV2.

Another important point in the possible pathogenic role of TTSuVs could be correlated with its capacity of producing specific lesions in the organs supporting its replication. In fact, TTSuVs have been several times described as responsible of producing pulmonary lesions, specifically mild IP, as a primary and unique infecting agents or co-infecting with other viral agents (PCV2 and PRRSV) (Ellis et al., 2008; Krakowka and Ellis, 2008; Krakowka et al., 2008; Mei et al., 2011). Additionally, an increased prevalence of TTSuV1 was found in PRDC affected pigs, and concluded that this virus was strongly associated with this clinical multiaetiological and multifactorial condition (Rammohan et al., 2011). Therefore, the aim of the third study of this thesis was to investigate the involvement of TTSuVs in the occurrence of different pulmonary inflammatory lesions of potential bacterial or viral causality. The main results of this study showed that TTSuV2 mean load was higher in lungs with viral lesion background when compared to normal lungs or to those with bacterial lesion background. A correlation was also found between high TTSuV2 loads and IP lesions. The findings in this study are in the line with the previously obtained in chapters 3 and 4: is TTSuV2 able to produce lesions, favoured by the presence of another viral agent? Or the systemic effect of other viruses induces TTSuV2 to up-regulate its reaplication and allow producing lesions?

In the three studies, unlike TTSuV2, TTSuV1 did not appear to be affected by the health/immunological status of the animals, the nature of the disease or the co-infecting agent. It is known that TTSuV1 and TTSuV2 are genetically different viruses belonging to different genus and under field conditions, the co-infection with both viruses is very high, with no cross-reactivity in their antigenic activity (Huang et al., 2012). This could explain their differentiated ability to produce lesions and the different potential association to other swine diseases. In the literature, such differences can be found in other closely related viruses as well. For instance, while PCV2 is found to be involved in the pathogenesis of PCVD, this is not the situation for PCV1, which is considered non-pathogenic (Segalés et al., 2005a). In poultry, three herpes viruses belonging to *Mardivirus* genus within the *Alphaherpesvirinae* subfamily, are genetically and antigenically very similar but only one of them, the *Gallid herpesvirus 2*, is demonstrated to be pathogenic producing Marek's disease in domestic chickens (Biggs and Nair, 2012).

While the results found in this thesis are in accordance with many studies finding TTSuV2 correlated with disease (Huang et al., 2012; Kekarainen et al., 2006; Mei et al., 2011; Nieto et al., 2011), they are in contrast with a list of studies that suggested TTSuV1 association with disease (Ellis et al., 2008; Krakowka and Ellis, 2008; Krakowka et al., 2008; Rammohan et al., 2012) or no correlation between any TTSuVs and PCVDs (Gauger et al., 2011; Lee et al., 2010). It is important to note that a number of differences may apply among these studies, including animal material used (serum, tissues, age of pigs (age variability), TTSuVs detection techniques (quantitative versus non-quantitative, sensitivity, etc.) and variability in the specific diagnostic methods used to characterize the studied pathological conditions. Therefore, it is very likely that the role of TTSuVs in pig disease occurrence guarantees further research in the future, as it is happening with TTVs in humans.

This thesis represents the biggest effort so far to extensively study TTSuVs in the background of swine diseases using the same diagnostic technology. It showed that TTSuVs are linked to pig disease occurrence, apparently in a way in which TTSuV2 is taking advantage of the disease outcome and therefore contribute to its complications and damage. Therefore it is speculated that TTSuV2 viraemia up-regulation seems to be associated with the level of immunocompetence of the animals.

Chapter 7

Conclusions

1. TTSuVs were identified and quantified in serum of pigs by means of a qPCR technique. This technique was set up also for TTSuVs quantification in formalin-fixed paraffin-embedded tissues.

2. TTSuVs were found to be highly prevalent in both healthy and sick animals. Overall, TTSuV2 serum loads were higher than those of TTSuV1.

3. TTSuV2, but not TTSuV1, showed higher loads in serum of naturally occurring PMWS affected pigs compared to age-matched controls. Moreover, pigs suffering from a moderate clinical course of experimentally-induced CSF had also increased serum loads of TTSuV2. Therefore, TTSuV2 replication is apparently up-regulated in the context of immunosuppressive pig diseases.

4. TTSuV2 was detected in lungs from healthy pigs as well as in lungs with different inflammatory lesions. Higher loads of TTSuV2 were found in lung lesions compatible with viral origin, especially IP. These loads were significantly lower in lesions of bacterial origin and normal lungs. Such differences were not observed for TTSuV1 loads in pulmonary tissue.

PART IV
REFERENCES

References

Abe, K., Inami, T., Ishikawa, K., Nakamura, S., and Goto, S. (2000). TT Virus Infection in Nonhuman Primates and Characterization of the Viral Genome: Identification of Simian TT Virus Isolates. J Virol *74*, 1549-1553.

Adams, M.J., and Carstens, E.B. (2012). Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2012). Arch Virol.

Akiba, J., Umemura, T., Alter, H.J., Kojiro, M., and Tabor, E. (2005). SEN virus: epidemiology and characteristics of a transfusion-transmitted virus. Transfusion *45*, 1084-1088.

Allan, G.M., and Ellis, J.A. (2000). Porcine circoviruses: a review. J Vet Diagn Invest *12*, 3-14.

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

Ballester, M., Sanchez, A., and Folch, J.M. (2005). Assignment of the beta-lactoglobulin (BLG) gene to porcine chromosome 1. Anim Genet *36*, 356-358.

Bando, M., Ohno, S., Oshikawa, K., Takahashi, M., Okamoto, H., and Sugiyama, Y. (2001). Infection of TT virus in patients with idiopathic pulmonary fibrosis. Respiratory medicine *95*, 935-942.

Bando, M., Takahashi, M., Ohno, S., Hosono, T., Hironaka, M., Okamoto, H., and Sugiyama, Y. (2008). Torque teno virus DNA titre elevated in idiopathic pulmonary fibrosis with primary lung cancer. Respirology *13*, 263-269.

Bendinelli, M., Pistello, M., Maggi, F., Fornai, C., Freer, G., and Vatteroni, M.L. (2001). Molecular properties, biology, and clinical implications of TT Virus, a recently identified widespread infectious agent of humans. Clin Microbiol Rev *14*, 98-113.

Berg, T., Schreier, E., Heuft, H.G., Hohne, M., Bechstein, W.O., Leder, K., Hopf, U., Neuhaus, P., and Wiedenmann, B. (1999). Occurrence of a novel DNA virus (TTV) infection in patients with liver diseases and its frequency in blood donors. J Med Virol *59*, 117-121.

Biagini, P., Charrel, R.N., de Micco, P., and de Lamballerie, X. (2003). Association of TT virus primary infection with rhinitis in a newborn. Clin Infect Dis *36*, 128-129.

Biagini, P., Uch, R., Belhouchet, M., Attoui, H., Cantaloube, J.-F., Brisbarre, N., and de Micco, P. (2007). Circular genomes related to anelloviruses identified in human and animal samples by using a combined rolling-circle amplification/sequence-independent single primer amplification approach. J Gen Virol *88*, 2696-2701.

Bigarré, L., Beven, V., de Boisseson, C., Grasland, B., Rose, N., Biagini, P., and Jestin, A. (2005). Pig anelloviruses are highly prevalent in swine herds in France. J Gen Virol *86*, 631-635.

Biggs, P.M., and Nair, V. (2012). The long view: 40 years of Marek's disease research and Avian Pathology. Avian pathology : journal of the WVPA *41*, 3-9.

Blomström, A.L., Belak, S., Fossum, C., Fuxler, L., Wallgren, P., and Berg, M. (2010). Studies of porcine circovirus type 2, porcine boca-like virus and torque teno virus indicate the presence of multiple viral infections in postweaning multisystemic wasting syndrome pigs. Virus Res *152*, 59-64.

Brassard, J., Gagne, M.J., Houde, A., Poitras, E., and Ward, P. (2010). Development of a real-time TaqMan PCR assay for the detection of porcine and bovine Torque teno virus. J Appl Microbiol (in press).

Brassard, J., Gagne, M.J., Lamoureux, L., Inglis, G.D., Leblanc, D., and Houde, A. (2008). Molecular detection of bovine and porcine Torque teno virus in plasma and feces. Vet Microbiol *126*, 271-276.

Burra, P., Masier, A., Boldrin, C., Calistri, A., Andreoli, E., Senzolo, M., Zorzi, M., Sgarabotto, D., Guido, M., Cillo, U.*, et al.* (2008). Torque Teno virus: any pathological role in liver transplanted patients? Transpl Int.

Carrasco, C.P., Rigden, R.C., Vincent, I.E., Balmelli, C., Ceppi, M., Bauhofer, O., Tache, V., Hjertner, B., McNeilly, F., van Gennip, H.G.*, et al.* (2004). Interaction of classical swine fever virus with dendritic cells. J Gen Virol *85*, 1633-1641.

Caswell, J., and Williams, K. (2007). Respiratory system. Pathology of domestic animals *5th ed., Vol. 2* 555-575.

Charlton, M., Adjei, P., Poterucha, J., Zein, N., Moore, B., Therneau, T., Krom, R., and Wiesner, R. (1998). TT-virus infection in North American

blood donors, patients with fulminant hepatic failure, and cryptogenic cirrhosis. Hepatology *28*, 839-842.

Christensen, J.K., Eugen-Olsen, J., M, S.L., Ullum, H., Gjedde, S.B., Pedersen, B.K., Nielsen, J.O., and Krogsgaard, K. (2000). Prevalence and prognostic significance of infection with TT virus in patients infected with human immunodeficiency virus. J Infect Dis *181*, 1796-1799.

Cong, M.E., Nichols, B., Dou, X.G., Spelbring, J.E., Krawczynski, K., Fields, H.A., and Khudyakov, Y.E. (2000). Related TT viruses in chimpanzees. Virology *274*, 343-355.

Cornelissen-Keijsers, V., Jimenez-Melsio, A., Cortey, M., Segales, J., van den Born, E., and Kekarainen, T. (2012). Discovery of a novel Torque teno sus virus species: genetic characterization, epidemiological assessment, and disease association. J Gen Virol.

Cortey, M., Macera, L., Segales, J., and Kekarainen, T. (2011a). Genetic variability and phylogeny of Torque teno sus virus 1 (TTSuV1) and 2 (TTSuV2) based on complete genomes. Vet Microbiol *148*, 125-131.

Cortey, M., Pileri, E., Segales, J., and Kekarainen, T. (2011b). Globalisation and global trade influence molecular viral population genetics of Torque Teno Sus Viruses 1 and 2 in pigs. Vet Microbiol.

Darwich, L., and Mateu, E. (2012). Immunology of porcine circovirus type 2 (PCV2). Virus Res *164*, 61-67.

De Bolle, L., Naesens, L., and De Clercq, E. (2005). Update on human herpesvirus 6 biology, clinical features, and therapy. Clinical microbiology reviews *18*, 217-245.

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

Epstein, M.A., Achong, B.G., and Barr, Y.M. (1964). Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma. Lancet *1*, 702-703.

Faurez, F., Dory, D., Grasland, B., and Jestin, A. (2009). Replication of porcine circoviruses. Virol J *6*, 60.

Fehér, E., Gall, T., Murvai, M., Kis, A., Boda, R., Sapy, T., Tar, I., Gergely, L., and Szarka, K. (2009). Investigation of the occurrence of torque tenovirus

in malignant and potentially malignant disorders associated with human papillomavirus. J Med Virol *81*, 1975-1981.

Figueiredo, C.P., Franz-Vasconcelos, H.C., Giunta, G., Mazzuco, T.L., Caon, T., Fernandes, A.L., Simoes, C.M., Antunes, V.L., Niel, C., and Barardi, C.R. (2007). Detection of Torque teno virus in Epstein-Barr virus positive and negative lymph nodes of patients with Hodgkin lymphoma. Leukemia & lymphoma *48*, 731-735.

Focosi, D., Bestagno, M., Burrone, O., and Petrini, M. (2010a). CD57+ T lymphocytes and functional immune deficiency. Journal of leukocyte biology *87*, 107-116.

Focosi, D., Maggi, F., Albani, M., Macera, L., Ricci, V., Gragnani, S., Di Beo, S., Ghimenti, M., Antonelli, G., Bendinelli, M.*, et al.* (2010b). Torquetenovirus viremia kinetics after autologous stem cell transplantation are predictable and may serve as a surrogate marker of functional immune reconstitution. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology *47*, 189-192.

Gallei, A., Pesch, S., Esking, W.S., Keller, C., and Ohlinger, V.F. (2010a). Porcine Torque teno virus: determination of viral genomic loads by genogroup-specific multiplex rt-PCR, detection of frequent multiple infections with genogroups 1 or 2, and establishment of viral full-length sequences. Vet Microbiol *143*, 202-212.

Gallei, A., Pesch, S., Esking, W.S., Keller, C., and Ohlinger, V.F. (2010b). Porcine Torque teno virus: Determination of viral genomic loads by genogroup-specific multiplex rt-PCR, detection of frequent multiple infections with genogroups 1 or 2, and establishment of viral full-length sequences. Vet Microbiol (in press).

Gauger, P.C., Lager, K.M., Vincent, A.L., Opriessnig, T., Kehrli, M.E., Jr., and Cheung, A.K. (2011). Postweaning multisystemic wasting syndrome produced in gnotobiotic pigs following exposure to various amounts of porcine circovirus type 2a or type 2b. Vet Microbiol *153*, 229-239.

Gergely, J., Peter, Pullmann, R., Stancato, C., Otvos, J., Laszlo, Koncz, A., Blazsek, A., Poor, G., and Brown, K.E. (2005). Increased prevalence of transfusion-transmitted virus and cross-reactivity with immunodominant epitopes of the HRES-1/p28 endogenous retroviral autoantigen in patients with systemic lupus erythematosus. Clinical Immunology *116*, 124-134.

Gilbert, M.T., Haselkorn, T., Bunce, M., Sánchez, J.J., Lucas, S.B., Jewell, L.D., Van Marck, E., and Worobey, M. (2007). The isolation of nucleic acids

from fixed, paraffin-embedded tissues-which methods are useful when? PloS one *2*, e537.

Goelz, S.E., Hamilton, S.R., and Vogelstein, B. (1985). Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. Biochem Biophys Res Commun *130*, 118-126.

Grau-Roma, L., Fraile, L., and Segales, J. (2010). Recent advances in the epidemiology, diagnosis and control of diseases caused by porcine circovirus type 2. Vet J (in press).

Grau-Roma, L., Hjulsager, C.K., Sibila, M., Kristensen, C.S., Lopez-Soria, S., Enoe, C., Casal, J., Botner, A., Nofrarias, M., Bille-Hansen, V.*, et al.* (2009). Infection, excretion and seroconversion dynamics of porcine circovirus type 2 (PCV2) in pigs from post-weaning multisystemic wasting syndrome (PMWS) affected farms in Spain and Denmark. Vet Microbiol *135*, 272-282.

Grau-Roma, L., and Segales, J. (2007). Detection of porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, swine influenza virus and Aujeszky's disease virus in cases of porcine proliferative and necrotizing pneumonia (PNP) in Spain. Vet Microbiol *119*, 144-151.

Guney, C., Kadayifci, A., Savas, M.C., Uygun, A., Balkan, A., and Kubar, A. (2005). Frequency of hepatitis G virus and transfusion-transmitted virus infection in type II diabetes mellitus. Int J Clin Pract *59*, 206-209.

Haramoto, E., Katayama, H., Oguma, K., Yamashita, H., Nakajima, E., and Ohgaki, S. (2005). One-year monthly monitoring of Torque teno virus (TTV) in wastewater treatment plants in Japan. Water Res *39*, 2008-2013.

Hijikata, M., Iwata, K., Ohta, Y., Nakao, K., Matsumoto, M., Matsumoto, H., Kanai, K., Baba, K., Samokhvalov, E.I., and Mishiro, S. (1999). Genotypes of TT virus (TTV) compared between liver disease patients and healthy individuals using a new PCR system capable of differentiating 1a and 1b types from others*. Arch Virol *144*, 2345-2354.

Hsieh, S.Y., Wu, Y.H., Ho, Y.P., Tsao, K.C., Yeh, C.T., and Liaw, Y.F. (1999). High prevalence of TT virus infection in healthy children and adults and in patients with liver disease in Taiwan. J Clin Microbiol *37*, 1829-1831.

Huang, Y.W., Harrall, K.K., Dryman, B.A., Beach, N.M., Kenney, S.P., Opriessnig, T., Vaughn, E.M., Roof, M.B., and Meng, X.J. (2011). Expression of the putative ORF1 capsid protein of Torque teno sus virus 2 (TTSuV2) and development of Western blot and ELISA serodiagnostic assays: correlation between TTSuV2 viral load and IgG antibody level in pigs. Virus Res *158*, 79-88.

Huang, Y.W., Harrall, K.K., Dryman, B.A., Opriessnig, T., Vaughn, E.M., Roof, M.B., and Meng, X.J. (2012). Serological Profile of Torque Teno Sus Virus Species 1 (TTSuV1) in Pigs and Antigenic Relationships between Two TTSuV1 Genotypes (1a and 1b), between Two Species (TTSuV1 and -2), and between Porcine and Human Anelloviruses. J Virol *86*, 10628-10639.

Huang, Y.W., Ni, Y.Y., Dryman, B.A., and Meng, X.J. (2010). Multiple infection of porcine Torque teno virus in a single pig and characterization of the full-length genomic sequences of four U.S. prototype PTTV strains: implication for genotyping of PTTV. Virology *396*, 289-297.

Ikeda, H., Takasu, M., Inoue, K., Okamoto, H., Miyakawa, Y., and Mayumi, M. (1999). Infection with an unenveloped DNA virus (TTV) in patients with acute or chronic liver disease of unknown etiology and in those positive for hepatitis C virus RNA. Journal of hepatology *30*, 205-212.

Ikeuchi, T., Yokosuka, O., Kanda, T., Imazeki, F., Seta, T., and Saisho, H. (2001). Roles of TT virus infection in various types of chronic hepatitis. Intervirology *44*, 219-223.

Ishimura, M., Ohga, S., Ichiyama, M., Kusuhara, K., Takada, H., Hara, T., Takahashi, M., and Okamoto, H. (2010). Hepatitis-associated aplastic anemia during a primary infection of genotype 1a torque teno virus. European journal of pediatrics *169*, 899-902.

Jelcic, I., Hotz-Wagenblatt, A., Hunziker, A., zur Hausen, H., and de Villiers, E.-M. (2004). Isolation of Multiple TT Virus Genotypes from Spleen Biopsy Tissue from a Hodgkin's Disease Patient: Genome Reorganization and Diversity in the Hypervariable Region. J Virol *78*, 7498-7507.

Kakkola, L., Tommiska, J., Boele, L., Miettinen, S., Blom, T., Kekarainen, T., Qiu, J., Pintel, D., Hoeben, R., Hedman, K.*, et al.* (2007). Construction and biological activity of a full-length molecular clone of human Torque teno virus (TTV) genotype 6. FEBS Journal *accepted*.

Kamada, K., Kamahora, T., Kabat, P., and Hino, S. (2004). Transcriptional regulation of TT virus: promoter and enhancer regions in the 1.2-kb noncoding region. Virology *321*, 341-348.

Kanda, T., Yokosuka, O., Ikeuchi, T., Seta, T., Kawai, S., Imazeki, F., and Saisho, H. (1999). The role of TT virus infection in acute viral hepatitis. Hepatology *29*, 1905-1908.

Kao, J.H., Chen, W., Chen, P.J., Lai, M.Y., and Chen, D.S. (2002). Prevalence and implication of a newly identified infectious agent (SEN virus) in Taiwan. J Infect Dis *185*, 389-392.

Kasirga, E., Sanlidag, T., Akcali, S., Keskin, S., Aktas, E., Karakoc, Z., Helvaci, M., Sozen, G., and Kuzu, M. (2005). Clinical significance of TT virus infection in children with chronic hepatitis B. Pediatrics international : official journal of the Japan Pediatric Society *47*, 300-304.

Kekarainen, T., López-Soria, S., and Segalés, J. (2007). Detection of swine Torque teno virus genogroups 1 and 2 in boar sera and semen. Theriogenology *68*, 966-971.

Kekarainen, T., McCullough, K., Fort, M., Fossum, C., Segales, J., and Allan, G.M. (2010). Immune responses and vaccine-induced immunity against Porcine circovirus type 2. Vet Immunol Immunopathol *136*, 185-193.

Kekarainen, T., and Segalés, J. (2009). Torque teno virus infection in the pig and its potential role as a model of human infection. Vet J *180*, 163-168.

Kekarainen, T., Sibila, M., and Segalés, J. (2006). Prevalence of swine Torque teno virus in post-weaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs in Spain. J Gen Virol *87*, 833-837.

King, A., Lefkowitz, E., Adams, M.J., and Carstens, E.B. (2009). Virus Taxonomy. IXth report of the International Committee on Taxonomy of Viruses *Academic press*.

Knoetig, S.M., Summerfield, A., Spagnuolo-Weaver, M., and McCullough, K.C. (1999). Immunopathogenesis of classical swine fever: role of monocytic cells. Immunology *97*, 359-366.

Krakowka, S., and Ellis, J.A. (2008). Evaluation of the effects of porcine genogroup 1 torque teno virus in gnotobiotic swine. Am J Vet Res *69*, 1623-1629.

Krakowka, S., Hartunian, C., Hamberg, A., Shoup, D., Rings, M., Zhang, Y., Allan, G., and Ellis, J.A. (2008). Evaluation of induction of porcine dermatitis and nephropathy syndrome in gnotobiotic pigs with negative results for porcine circovirus type 2. Am J Vet Res *69*, 1615-1622.

Lang, C., Sollner, H., Barz, A., Ladinig, A., Langhoff, R., Weissenbock, H., Kekarainen, T., Segales, J., and Ritzmann, M. (2011). Investigation of the prevalence of swine torque teno virus in Austria. Berl Munch Tierarztl Wochenschr *124*, 142-147.

Le Poitier, M.-F., Mesplède, A., and Vannier, P. (2006). Classical swine fever and other pestiviruses. in Diseases of Swine; *Ames, Iowa, Blackwell Publishing (9th edn)*, pp. 309-322.

Leary, T.P., Erker, J.C., Chalmers, M.L., Desai, S.M., and Mushahwar, I.K. (1999). Improved detection systems for TT virus reveal high prevalence in humans, non-human primates and farm animals. J Gen Virol *80*, 2115-2120.

Lee, S., Shin, J., Kim, C., and Lyoo, Y. (2011). Comparison of Torque Teno Sus Virus (TTSuV) viral load in Porcine Circovirus Type 2 vaccinated and non-vaccinated pig herds. Research in veterinary science.

Lee, S.S., Sunyoung, S., Jung, H., Shin, J., and Lyoo, Y.S. (2010). Quantitative detection of porcine Torque teno virus in Porcine circovirus-2-negative and Porcine circovirus-associated disease-affected pigs. J Vet Diagn Invest *22*, 261-264.

Leppik, L., Gunst, K., Lehtinen, M., Dillner, J., Streker, K., and de Villiers, E.-M. (2007). In Vivo and in Vitro Intragenomic Rearrangement of TT Viruses. J Virol, JVI.00781-00707.

Liu, X., Gu, W., Guo, X., Ge, X., Chen, Y., and Yang, H. (2011). Prevalence of torque teno virus infection in pig herds in China. Vet Rec *168*, 410.

Ludyga, N., Grunwald, B., Azimzadeh, O., Englert, S., Hofler, H., Tapio, S., and Aubele, M. (2012). Nucleic acids from long-term preserved FFPE tissues are suitable for downstream analyses. Virchows Arch.

Madsen, C.D., Eugen-Olsen, J., Kirk, O., Parner, J., Kaae Christensen, J., Brasholt, M.S., Ole Nielsen, J., and Krogsgaard, K. (2002). TTV viral load as a marker for immune reconstitution after initiation of HAART in HIV-infected patients. HIV clinical trials *3*, 287-295.

Maggi, F., Andreoli, E., Riente, L., Meschi, S., Rocchi, J., Delle Sedie, A., Vatteroni, M.L., Ceccherini-Nelli, L., Specter, S., and Bendinelli, M. (2007). Torquetenovirus in patients with arthritis. Rheumatology (Oxford) *46*, 885-886.

Maggi, F., and Bendinelli, M. (2009). Immunobiology of the Torque teno viruses and other anelloviruses. Curr Top Microbiol Immunol *331*, 65-90.

Maggi, F., Pifferi, M., Fornai, C., Andreoli, E., Tempestini, E., Vatteroni, M., Presciuttini, S., Marchi, S., Pietrobelli, A., Boner, A.*, et al.* (2003). TT Virus in the Nasal Secretions of Children with Acute Respiratory Diseases: Relations to Viremia and Disease Severity. J Virol *77*, 2418-2425.

Mankertz, A., Caliskan, R., Hattermann, K., Hillenbrand, B., Kurzendoerfer, P., Mueller, B., Schmitt, C., Steinfeldt, T., and Finsterbusch, T. (2004). Molecular biology of Porcine circovirus: analyses of gene expression and viral replication. Vet Microbiol *98*, 81-88.

Mankertz, A., Mankertz, J., Wolf, K., and Buhk, H.J. (1998). Identification of a protein essential for replication of porcine circovirus. J Gen Virol *79 (Pt 2)*, 381-384.

Martelli, F., Caprioli, A., Di Bartolo, I., Cibin, V., Pezzotti, G., Ruggeri, F.M., and Ostanello, F. (2006). Detection of swine Torque Teno virus in Italian pig herds. Journal of Veterinary Medicine Series B *53*, 234-238.

Martinez-Guinó, L., Ballester, M., Segales, J., and Kekarainen, T. (2011). Expression profile and subcellular localization of Torque teno sus virus proteins. J Gen Virol *92*, 2446-2457.

Martinez-Guinó, L., Kekarainen, T., Maldonado, J., Aramouni, M., Llorens, A., and Segales, J. (2010). Torque teno sus virus (TTV) detection in aborted and slaughterhouse collected foetuses. Theriogenology *74*, 277-281.

Martínez-Guinó, L., Kekarainen, T., Maldonado, J., Aramouni, M., Llorens, A., and Segalés, J. (2010). Torque teno virus (TTV) detection in aborted and slaughterhouse collected foetuses. Theriogenology, (in press).

Martínez-Guinó, L., Kekarainen, T., and Segalés, J. (2009). Evidence of Torque teno virus (TTV) vertical transmission in swine. Theriogenology *71*, 1390-1395.

Martínez, L., Kekarainen, T., Sibila, M., Ruiz-Fons, F., Vidal, D., Gortazar, C., and Segalés, J. (2006). Torque teno virus (TTV) is highly prevalent in the European wild boar (Sus scrofa). Veterinary Microbiology *118*, 223-229.

Mateu, E., Martin, M., and Vidal, D. (2003). Genetic diversity and phylogenetic analysis of glycoprotein 5 of European-type porcine reproductive and respiratory virus strains in Spain. J Gen Virol *84*, 529-534.

McIntosh, K.A., Tumber, A., Harding, J.C., Krakowka, S., Ellis, J.A., and Hill, J.E. (2009). Development and validation of a SYBR green real-time PCR for the quantification of porcine circovirus type 2 in serum, buffy coat, feces, and multiple tissues. Vet Microbiol *133*, 23-33.

McKeown, N.E., Fenaux, M., Halbur, P.G., and Meng, X.J. (2004). Molecular characterization of porcine TT virus, an orphan virus, in pigs from six different countries. Vet Microbiol *104*, 113-117.

Mei, M., Zhu, L., Wang, Y., Xu, Z., Zhao, L., Peng, X., Wu, Y., Li, S., and Guo, W. (2011). Histopathological investigation in porcine infected with torque teno sus virus type 2 by inoculation. Virol J *8*, 545.

Miyata, H., Tsunoda, H., Kazi, A., Yamada, A., Khan, M.A., Murakami, J., Kamahora, T., Shiraki, K., and Hino, S. (1999). Identification of a novel GC-rich 113-nucleotide region to complete the circular, single-stranded DNA genome of TT virus, the first human circovirus. J Virol *73*, 3582-3586.

Moen, E.M., Sagedal, S., Bjoro, K., Degre, M., Opstad, P.K., and Grinde, B. (2003). Effect of immune modulation on TT virus (TTV) and TTV-like-mini-virus (TLMV) viremia. J Med Virol *70*, 177-182.

Moen, E.M., Sleboda, J., and Grinde, B. (2002). Serum concentrations of TT virus and TT virus-like mini virus in patients developing AIDS. AIDS *16*, 1679-1682.

Moriyama, M., Matsumura, H., Shimizu, T., Shioda, A., Kaneko, M., Miyazawa, K., Miyata, H., Tanaka, N., Uchida, T., and Arakawa, Y. (2001). Histopathologic impact of TT virus infection on the liver of type C chronic hepatitis and liver cirrhosis in Japan. J Med Virol *64*, 74-81.

Mushahwar, I.K., Erker, J.C., Muerhoff, A.S., Leary, T.P., Simons, J.N., Birkenmeyer, L.G., Chalmers, M.L., Pilot-Matias, T.J., and Dexai, S.M. (1999). Molecular and biophysical characterization of TT virus: Evidence for a new virus family infecting humans. Proceedings of the National Academy of Sciences *96*, 3177-3182.

Naoumov, N.V., Petrova, E.P., Thomas, M.G., and Williams, R. (1998). Presence of a newly described human DNA virus (TTV) in patients with liver disease. Lancet *352*, 195-197.

Ng, T.F.F., Manire, C., Borrowman, K., Langer, T., Ehrhart, L., and Breitbart, M. (2009). Discovery of a Novel Single-Stranded DNA Virus from a Sea Turtle Fibropapilloma by Using Viral Metagenomics. J Virol *83*, 2500-2509.

Niagro, F.D., Forsthoefel, A.N., Lawther, R.P., Kamalanathan, L., Ritchie, B.W., Latimer, K.S., and Lukert, P.D. (1998). Beak and feather disease virus and porcine circovirus genomes: intermediates between the geminiviruses and plant circoviruses. Arch Virol *143*, 1723-1744.

Niel, C., de Oliveira, J.M., Ross, R.S., Gomes, S.A., Roggendorf, M., and Viazov, S. (1999). High prevalence of TT virus infection in Brazilian blood donors. J Med Virol *57*, 259-263.

Niel, C., Diniz-Mendes, L., and Devalle, S. (2005). Rolling-circle amplification of Torque teno virus (TTV) complete genomes from human and swine sera and identification of a novel swine TTV genogroup. J Gen Virol *86*, 1343-1347.

Nieto, D., Aramouni, M., Grau-Roma, L., Segalés, J., and Kekarainen, T. (2011). Dynamics of Torque teno sus virus 1 (TTSuV1) and 2 (TTSuV2) DNA loads in serum of healthy and postweaning multisystemic wasting syndrome (PMWS) affected pigs. Vet Microbiol *152*, 284-290.

Nishizawa, T., Okamoto, H., Konishi, K., Yoshizawa, H., Miyakawa, Y., and Mayumi, M. (1997). A Novel DNA Virus (TTV) Associated with Elevated Transaminase Levels in Posttransfusion Hepatitis of Unknown Etiology. Biochemical and Biophysical Research Communications *241*, 92-97.

Nobili, V., Maggi, F., Diciommo, V., Caione, D., Marcellini, M., and Bendinelli, M. (2005). Is torquetenovirus a potential cause of liver damage in children? The Journal of infection *50*, 368-369.

Okamoto, H. (2009a). History of discoveries and pathogenicity of TT viruses. Curr Top Microbiol Immunol *331*, 1-20.

Okamoto, H. (2009b). TT viruses in animals. Curr Top Microbiol Immunol *331*, 35-52.

Okamoto, H., Akahane, Y., Ukita, M., Fukuda, M., Tsuda, Y., Miyakawa, Y., and Mayumi, M. (1998a). Fecal excretion of a nonenveloped DNA virus (TTV) associated with posttransfusion non-A-G hepatitis. Journal of Medical Virology *56*, 128-132.

Okamoto, H., Fukuda, M., Tawara, A., Nishizawa, T., Itoh, Y., Hayasaka, I., Tsuda, F., Tanaka, T., Miyakawa, Y., and Mayumi, M. (2000a). Species-Specific TT Viruses and Cross-Species Infection in Nonhuman Primates. J Virol *74*, 1132-1139.

Okamoto, H., Nishizawa, T., and Kato, N. (1998b). Molecular cloning and characterization of novel DNA virus (TTV) associated with post-transfusion hepatitis of unknown etiology. hepatol Res *10*, 1-16.

Okamoto, H., Nishizawa, T., Takahashi, M., Asabe, S., Tsuda, F., and Yoshikawa, A. (2001a). Heterogeneous Distribution of TT Virus of Distinct Genotypes in Multiple Tissues from Infected Humans. Virology *288*, 358-368.

Okamoto, H., Nishizawa, T., Takahashi, M., Tawara, A., Peng, Y., Kishimoto, J., and Wang, Y. (2001b). Genomic and evolutionary characterization of TT virus (TTV) in tupaias and comparison with species-specific TTVs in humans and non-human primates. J Gen Virol *82*, 2041-2050.

Okamoto, H., Nishizawa, T., Tawara, A., Peng, Y., Takahashi, M., Kishimoto, J., Tanaka, T., Miyakawa, Y., and Mayumi, M. (2000b). Species-Specific TT Viruses in Humans and Nonhuman Primates and Their Phylogenetic Relatedness. Virology *277*, 368-378.

Okamoto, H., Nishizawa, T., and Ukita, M. (1999). A novel unenveloped DNA virus (TT virus) associated with acute and chronic non-A to G hepatitis. Intervirology *42*, 196-204.

Okamoto, H., Takahashi, M., Nishizawa, T., Tawara, A., Fukai, K., Muramatsu, U., Naito, Y., and Yoshikawa, A. (2002). Genomic characterization of TT viruses (TTVs) in pigs, cats and dogs and their relatedness with species-specific TTVs in primates and tupaias. J Gen Virol *83*, 1291-1297.

Okamura, A., Yoshioka, M., Kikuta, H., Kubota, M., Ma, X., Hayashi, A., Ishiko, H., and Kobayashi, K. (2000). Detection of TT virus sequences in children with liver disease of unknown etiology. J Med Virol *62*, 104-108.

Olvera, A., Sibila, M., Calsamiglia, M., Segales, J., and Domingo, M. (2004). Comparison of porcine circovirus type 2 load in serum quantified by a real time PCR in postweaning multisystemic wasting syndrome and porcine dermatitis and nephropathy syndrome naturally affected pigs. J Virol Methods *117*, 75-80.

Peng, Y.H., Nishizawa, T., Takahashi, M., Ishikawa, T., Yoshikawa, A., and Okamoto, H. (2002). Analysis of the entire genomes of thirteen TT virus variants classifiable into the fourth and fifth genetic groups, isolated from viremic infants. Arch Virol *147*, 21-41.

Perez, L.J., de Arce, H.D., Frias, M.T., Perera, C.L., Ganges, L., and Nunez, J.I. (2011). Molecular detection of Torque teno sus virus in lymphoid tissues in concomitant infections with other porcine viral pathogens. Res Vet Sci *91*, e154-157.

Piaggio, F., Dodi, F., Bottino, G., Andorno, E., Gentile, R., Ferrari, C., Barabino, G., Giannone, A., Immordino, G., Miggino, M*., et al.* (2009a). Torque Teno Virus--cause of viral liver disease following liver transplantation: a case report. Transplant Proc *41*, 1378-1379.

Piaggio, F., Dodi, F., Bottino, G., Andorno, E., Gentile, R., Ferrari, C., Barabino, G., Giannone, A., Immordino, G., Miggino, M*., et al.* (2009b). Torque Teno Virus-cause of viral liver disease following liver transplantation: a case report. Transplant Proc *41*, 1378-1379.

Pifferi, M., Maggi, F., Andreoli, E., Lanini, L., Marco, E.D., Fornai, C., Vatteroni, M.L., Pistello, M., Ragazzo, V., Macchia, P.*, et al.* (2005). Associations between Nasal Torquetenovirus Load and Spirometric Indices in Children with Asthma. J Infect Dis *192*, 1141-1148.

Pifferi, M., Maggi, F., Caramella, D., De Marco, E., Andreoli, E., Meschi, S., Macchia, P., Bendinelli, M., and Boner, A.L. (2006). High torquetenovirus loads are correlated with bronchiectasis and peripheral airflow limitation in children. Pediatr Infect Dis J *25*, 804-808.

Pozzuto, T., Mueller, B., Meehan, B., Ringler, S.S., McIntosh, K.A., Ellis, J.A., Mankertz, A., and Krakowka, S. (2009). In utero transmission of porcine torque teno viruses. Vet Microbiol *137*, 375-379.

Prati, D., Lin, Y.H., De Mattei, C., Liu, J.K., Farma, E., Ramaswamy, L., Zanella, A., Lee, H., Rebulla, P., Allain, J.P.*, et al.* (1999). A prospective study on TT virus infection in transfusion-dependent patients with beta-thalassemia. Blood *93*, 1502-1505.

Puig-Basagoiti, F., Cabana, M., Guilera, M., Gimenez-Barcons, M., Sirera, G., Tural, C., Clotet, B., Sanchez-Tapias, J.M., Rodes, J., Saiz, J.C.*, et al.* (2000). Prevalence and route of transmission of infection with a novel DNA virus (TTV), hepatitis C virus, and hepatitis G virus in patients infected with HIV. J Acquir Immune Defic Syndr *23*, 89-94.

Rammohan, L., Xue, L., Wang, C., Chittick, W., Ganesan, S., and Ramamoorthy, S. (2011). Increased prevalence of torque teno viruses in porcine respiratory disease complex affected pigs. Vet Microbiol.

Rammohan, L., Xue, L., Wang, C., Chittick, W., Ganesan, S., and Ramamoorthy, S. (2012). Increased prevalence of torque teno viruses in porcine respiratory disease complex affected pigs. Vet Microbiol *157*, 61-68.

Ramos-Vara, J.A., Duran, O., Render, J.A., and Craft, D. (1997). Porcine dermatitis and nephropathy syndrome in the USA. Vet Rec *141*, 479-480.

Rosell, C., Segalés, J., Plana-Durán, J., Balasch, M., Rodríguez-Arrioja, G.M., Kennedy, S., Allan, G.M., McNeilly, F., Latimer, K.S., and Domingo, M. (1999). Pathological, immunohistochemical, and in-situ hybridization studies of natural cases of postweaning multisystemic wasting syndrome (PMWS) in pigs. J Comp Pathol *120*, 59-78.

Rotundo, R., Maggi, F., Nieri, M., Muzzi, L., Bendinelli, M., and Prato, G.P. (2004). TT virus infection of periodontal tissues: a controlled clinical and laboratory pilot study. Journal of periodontology *75*, 1216-1220.

Sagir, A., Adams, O., Antakyali, M., Oette, M., Erhardt, A., Heintges, T., and Haussinger, D. (2005). SEN virus has an adverse effect on the survival of HIV-positive patients. AIDS *19*, 1091-1096.

Sagir, A., Kirschberg, O., Heintges, T., Erhardt, A., and Haussinger, D. (2004). SEN virus infection. Rev Med Virol *14*, 141-148.

Salahuddin, S.Z., Ablashi, D.V., Markham, P.D., Josephs, S.F., Sturzenegger, S., Kaplan, M., Halligan, G., Biberfeld, P., Wong-Staal, F., Kramarsky, B.*, et al.* (1986). Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. Science *234*, 596-601.

Santos, M.C., Saito, C.P., and Line, S.R. (2008). Extraction of genomic DNA from paraffin-embedded tissue sections of human fetuses fixed and stored in formalin for long periods. Pathol Res Pract *204*, 633-636.

Santos, S., Sa, D., Bastos, E., Guedes-Pinto, H., Gut, I., Gartner, F., and Chaves, R. (2009). An efficient protocol for genomic DNA extraction from formalin-fixed paraffin-embedded tissues. Res Vet Sci *86*, 421-426.

Savic, B., Milicevic, V., Bojkovski, J., Kureljusic, B., Ivetic, V., and Pavlovic, I. (2010). Detection rates of the swine torque teno viruses (TTVs), porcine circovirus type 2 (PCV2) and hepatitis E virus (HEV) in the livers of pigs with hepatitis. Vet Res Commun *34*, 641-648.

Schroter, M., Laufs, R., Zollner, B., Knodler, B., Schafer, P., and Feucht, H.H. (2003). A novel DNA virus (SEN) among patients on maintenance hemodialysis: prevalence and clinical importance. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology *27*, 69-73.

Segalés, J., Allan, G.M., and Domingo, M. (2005a). Porcine circovirus diseases. Anim Health Res Rev *6*, 119-142.

Segalés, J., Allan, G.M., and Domingo, M. (2005b). Porcine circovirus diseases. Animal Health Research Reviews *6*, 119-142.

Segalés, J., Martínez-Guinó, L., Cortey, M., Navarro, N., Huerta, E., Sibila, M., Pujols, J., and Kekarainen, T. (2009). Retrospective study on swine Torque teno virus genogroups 1 and 2 infection from 1985 to 2005 in Spain. Veterinary Microbiology *134*, 199-207.

Segalés, J., Piella, J., Marco, E., Mateu-de-Antonio, E.M., Espuna, E., and Domingo, M. (1998). Porcine dermatitis and nephropathy syndrome in Spain. Vet Rec *142*, 483-486.

Shang, D., Lin, Y.H., Rigopoulou, I., Chen, B., Alexander, G.J., and Allain, J.P. (2000). Detection of TT virus DNA in patients with liver disease and recipients of liver transplant. J Med Virol *61*, 455-461.

Shi, S.R., Datar, R., Liu, C., Wu, L., Zhang, Z., Cote, R.J., and Taylor, C.R. (2004). DNA extraction from archival formalin-fixed, paraffin-embedded tissues: heat-induced retrieval in alkaline solution. Histochem Cell Biol *122*, 211-218.

Shibayama, T., Masuda, G., Ajisawa, A., Takahashi, M., Nishizawa, T., Tsuda, F., and Okamoto, H. (2001). Inverse relationship between the titre of TT virus DNA and the CD4 cell count in patients infected with HIV. AIDS *15*, 563-570.

Shimizu, T., Moriyama, M., Tanaka, N., and Arakawa, Y. (2000). Persistent TT virus infection does not contribute to the development of non-A to -G hepatocellular carcinoma. A case-control study of 19 patients in Japan. Intervirology *43*, 80-86.

Sibila, M., Martínez-Guinó, L., Huerta, E., Llorens, A., Mora, M., Grau-Roma, L., Kekarainen, T., and Segalés, J. (2009a). Swine torque teno virus (TTV) infection and excretion dynamics in conventional pig farms. Veterinary Microbiology *139*, 213-218.

Sibila, M., Martínez-Guinó, L., Huerta, E., Mora, M., Grau-Roma, L., Kekarainen, T., and Segalés, J. (2009b). Torque teno virus (TTV) infection in sows and suckling piglets. Veterinary Microbiology *137*, 354-358.

Sospedra, M., Zhao, Y., zur Hausen, H., Muraro, P.A., Hamashin, C., de Villiers, E.M., Pinilla, C., and Martin, R. (2005). Recognition of conserved amino acid motifs of common viruses and its role in autoimmunity. PLoS Pathog *1*, e41.

Stanta, G., and Schneider, C. (1991). RNA extracted from paraffin-embedded human tissues is amenable to analysis by PCR amplification. Biotechniques *11*, 304, 306, 308.

Straw, B.E., Backstrom, L., and Leman, A.D. (1986). Examination of swine at slaughter. Part II. Findings at slaughter and their significance. Comp. Cont. Edu. Pract. Veter. 8, 106–112.

Susa, M., Konig, M., Saalmuller, A., Reddehase, M.J., and Thiel, H.J. (1992). Pathogenesis of classical swine fever: B-lymphocyte deficiency caused by hog cholera virus. Journal of virology *66*, 1171-1175.

Suzuki, T., Suzuki, R., Li, J., Hijikata, M., Matsuda, M., Li, T.-C., Matsuura, Y., Mishiro, S., and Miyamura, T. (2004). Identification of Basal Promoter and Enhancer Elements in an Untranslated Region of the TT Virus Genome. J Virol *78*, 10820-10824.

Szladek, G., Juhasz, A., Kardos, G., Szoke, K., Major, T., Sziklai, I., Tar, I., Marton, I., Konya, J., Gergely, L.*, et al.* (2005). High co-prevalence of genogroup 1 TT virus and human papillomavirus is associated with poor clinical outcome of laryngeal carcinoma. Journal of clinical pathology *58*, 402-405.

Taira, O., Ogawa, H., Nagao, A., Tuchiya, K., Nunoya, T., and Ueda, S. (2009). Prevalence of swine Torque teno virus genogroups 1 and 2 in Japanese swine with suspected post-weaning multisystemic wasting syndrome and porcine respiratory disease complex. Vet Microbiol *139*, 347-350.

Takacs, M., Dencs, A., Csiszar, C., Hettmann, A., Rusvai, E., Szomor, K.N., Palfi, V., and Nagy, B. (2008). First description of swine Torque teno virus (TTV) and detection of a new genogroup in Hungary: short communication. Acta Vet Hung *56*, 547-553.

Takahashi, K., Iwasa, Y., Hijikata, M., and Mishiro, S. (2000). Identification of a new human DNA virus (TTV-like mini virus, TLMV) intermediately related to TT virus and chicken anemia virus. Archives of Virology *145*, 979-993.

Takayama, S., Miura, T., Matsuo, S., Taki, M., and Sugii, S. (1999). Prevalence and persistence of a novel DNA TT virus (TTV) infection in Japanese haemophiliacs. British journal of haematology *104*, 626-629.

Tanaka, H., Okamoto, H., Luengrojanakul, P., Chainuvati, T., Tsuda, F., Tanaka, T., Miyakawa, Y., and Mayumi, M. (1998). Infection with an unenveloped DNA virus (TTV) associated with posttransfusion non-A to G hepatitis in hepatitis patients and healthy blood donors in Thailand. J Med Virol *56*, 234-238.

Tanaka, Y., Hayashi, J., Ariyama, I., Furusyo, N., Etoh, Y., and Kashiwagi, S. (2000). Seroepidemiology of TT virus infection and relationship between genotype and liver damage. Dig Dis Sci *45*, 2214-2220.

Tarradas, J., Alvarez, B., Fraile, L., Rosell, R., Munoz, M., Galindo-Cardiel, I., Domingo, M., Dominguez, J., Ezquerra, A., Sobrino, F.*, et al.* (2011a). Immunomodulatory effect of swine CCL20 chemokine in DNA vaccination against CSFV. Vet Immunol Immunopathol *142*, 243-251.

Tarradas, J., Argilaguet, J.M., Rosell, R., Nofrarias, M., Crisci, E., Cordoba, L., Perez-Martin, E., Diaz, I., Rodriguez, F., Domingo, M., *et al.* (2010). Interferon-gamma induction correlates with protection by DNA vaccine expressing E2 glycoprotein against classical swine fever virus infection in domestic pigs. Vet Microbiol *142*, 51-58.

Tarradas, J., Monso, M., Munoz, M., Rosell, R., Fraile, L., Frias, M.T., Domingo, M., Andreu, D., Sobrino, F., and Ganges, L. (2011b). Partial protection against classical swine fever virus elicited by dendrimeric vaccine-candidate peptides in domestic pigs. Vaccine *29*, 4422-4429.

Thom, K., Morrison, C., Lewis, J.C.M., and Simmonds, P. (2003). Distribution of TT virus (TTV), TTV-like minivirus, and related viruses in humans and nonhuman primates. Virology *306*, 324-333.

Thom, K., and Petrik, J. (2007). Progression towards AIDS leads to increased Torque teno virus and Torque teno minivirus titers in tissues of HIV infected individuals. J Med Virol *79*, 1-7.

Thomson, J., Smith, B., Allan, G., McNeilly, F., and McVicar, C. (2000). PDNS, PMWS and porcine circovirus type 2 in Scotland. Porcine dermatitis and nephropathy syndrome. Post-weaning multisystemic wasting syndrome. Vet Rec *146*, 651-652.

Tokita, H., Murai, S., Kamitsukasa, H., Yagura, M., Harada, H., Takahashi, M., and Okamoto, H. (2002). TT virus of certain genotypes may reduce the platelet count in patients who achieve a sustained virologic response to interferon treatment for chronic hepatitis C. Hepatol Res *23*, 105-114.

Tomasiewicz, K., Modrzewska, R., Lyczak, A., and Krawczuk, G. (2005). TT virus infection and pancreatic cancer: relationship or accidental coexistence. World J Gastroenterol *11*, 2847-2849.

Touinssi, M., Gallian, P., Biagini, P., Attoui, H., Vialettes, B., Berland, Y., Tamalet, C., Dhiver, C., Ravaux, I., De Micco, P., *et al.* (2001). TT virus infection: prevalence of elevated viraemia and arguments for the immune control of viral load. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology *21*, 135-141.

Tuveri, R., Jaffredo, F., Lunel, F., Nalpa, B., Pol, S., Feray, C., Marcellin, P., Thibault, V., Delagneau, J.F., Opolon, P., *et al.* (2000). Impact of TT virus infection in acute and chronic, viral- and non viral-related liver diseases. Journal of hepatology *33*, 121-127.

Umemura, T., Yeo, A.E., Sottini, A., Moratto, D., Tanaka, Y., Wang, R.Y., Shih, J.W., Donahue, P., Primi, D., and Alter, H.J. (2001). SEN virus

infection and its relationship to transfusion-associated hepatitis. Hepatology *33*, 1303-1311.

Van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R.*, et al.* (2000). Flaviviridae. The Classification and Nomenclature of Viruses The Seventh Report of the International Committee on Taxonomy of Viruses *Academic Press, San Diego.*

Verschoor, E.J., Langenhuijzen, S., and Heeney, J.L. (1999). TT viruses (TTV) of non-human primates and their relationship to the human TTV genotypes. J Gen Virol *80*, 2491-2499.

Viazov, S., Ross, R.S., Varenholz, C., Lange, R., Holtmann, M., Niel, C., and Roggendorf, M. (1998). Lack of evidence for an association between TTV infection and severe liver disease. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology *11*, 183-187.

Wellenberg, G.J., Stockhofe-Zurwieden, N., de Jong, M.F., Boersma, W.J., and Elbers, A.R. (2004). Excessive porcine circovirus type 2 antibody titres may trigger the development of porcine dermatitis and nephropathy syndrome: a case-control study. Vet Microbiol *99*, 203-214.

Xiao, C.T., Gimenez-Lirola, L., Huang, Y.W., Meng, X.J., Halbur, P.G., and Opriessnig, T. (2012). The prevalence of Torque teno sus virus (TTSuV) is common and increases with the age of growing pigs in the United States. J Virol Methods *183*, 40-44.

Yokoyama, H., Yasuda, J., Okamoto, H., and Iwakura, Y. (2002). Pathological changes of renal epithelial cells in mice transgenic for the TT virus ORF1 gene. J Gen Virol *83*, 141-150.

Zein, N.N., Arslan, M., Li, H., Charlton, M.R., Gross, J.B., Jr., Poterucha, J.J., Therneau, T.M., Kolbert, C.P., and Persing, D.H. (1999). Clinical significance of TT virus infection in patients with chronic hepatitis C. Am J Gastroenterol *94*, 3020-3027.

Zhong, S., Yeo, W., Tang, M., Liu, C., Lin, X.-r., Ho, W.M., Hui, P., and Johnson, P.J. (2002). Frequent detection of the replicative form of TT virus DNA in peripheral blood mononuclear cells and bone marrow cells in cancer patients. Journal of Medical Virology *66*, 428-434.

Zhong, S., Yeo, W., Tang, M.W., Lin, X.R., Mo, F., Ho, W.M., Hui, P., and Johnson, P.J. (2001). Gross elevation of TT virus genome load in the peripheral blood mononuclear cells of cancer patients. Ann N Y Acad Sci *945*, 84-92.

zur Hausen, H., and de Villiers, E.M. (2005). Virus target cell conditioning model to explain some epidemiologic characteristics of childhood leukemias and lymphomas. International journal of cancer Journal international du cancer *115*, 1-5.

zur Hausen, H., and de Villiers, E.M. (2009). TT viruses: oncogenic or tumor-suppressive properties? Curr Top Microbiol Immunol *331*, 109-116.