Description of a novel species of *Torque teno sus virus* (TTSuV) and first insights on immunization against TTSuVs in naturally infected pigs

Memòria presentada per l' **Alexandra Jiménez Melsió** per optar al grau de Doctora en el programa de Bioquímica, Biologia Molecular i Biomedicina del Departament de Bioquímica i Biologia Molecular de la Universitat Autònoma de Barcelona

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Certifiquen:

Que la memòria de tesi doctoral titulada: "***Description of a novel species of Torque teno sus virus (TTSuV) and first insights on immunization against TTSuVs in naturally infected pigs* "** presentada per l'Alexandra Jiménez Melsió s'ha realitzat sota la seva direcció en el CReSA i n'autoritzen la seva presentació per tal de ser avaluada per la comissió corresponent per l'obtenció del grau de Doctora del programa de Bioquímica, Biologia Molecular i Biomedicina del Departament de Bioquímica i Biologia Molecular de la Universitat Autònoma de Barcelona.

I per tal que consti, als efectes oportuns, signem el present certificat a Bellaterra, a 28 de setembre del 2015.

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A la meva mare i el meu pare

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"Petons, mitjons i americanes " dels *Els amics de les arts*

Contents

Summary ..... **III**

Resum ..... **V**

List of abbreviations ..... **VII**

1. Introduction **1**

1.1. History and discovery of *Torque teno virus* 3

1.2. Taxonomy and classification of anelloviruses 4

1.3. Molecular characteristics of anelloviruses 8

1.4. Epidemiology 10

1.5. Pathogenesis and immunology 12

1.6. TTSuV disease association 15

1.7. Prevention and control of TTSuV 16

2. Objectives **19**

3. Study I **23**

Discovery of a novel *Torque teno sus virus* species: genetic characterization, epidemiological assessment, and disease association

3.1. Introduction 25

3.2. Material and methods 26

3.3. Results 31

3.4. Discussion 39

4. Study II **43**

Expression of and pig immunization with *Torque teno sus virus* proteins

4.1. Introduction 45

4.2. Materials and methods 47

4.3. Results 55

4.4. Discussion 67

5. Study III 84

Vaccination of pigs reduces Torque teno sus virus viremia during natural infection

5.1. Introduction 73

5.2. Materials and methods 74

5.3. Results 79

5.4. Discussion 85

6. General discussion 84

7. Conclusions 104

8. References 105

Summary

Anelloviruses are a highly diverse group of circular single-stranded DNA viruses infecting vertebrates. *Torque teno sus viruses* (TTSuV) are ubiquitous pig-infecting anelloviruses. Three different viral species have been described, namely TTSuV1a and 1b within the genus *Iotatorquevirus* and TTSuVk2a, within the genus *Kappatorquevirus*. These viruses are genetically very distinct (>56% sequence diversity) but share similar genome organization and expression strategy. TTSuV infection in pigs is distributed worldwide, and is characterized by a persistent viremia. TTSuV themselves are considered non-pathogenic; however, it is believed that these viruses play a role in co-infection with other economically important viral porcine infections. Apparently, TTSuV infection can influence the development or may contribute to the pathogenesis of various porcine diseases during co-infection. The real impact of TTSuV on the pig health, if any, is still under debate.

The present Thesis aimed to characterize a novel TTSuV species and to explore possible ways of vaccination against TTSuVs.

The first study describes the discovery, genetic characterization and epidemiology of a novel TTSuV species, named *Torque teno sus virus k2b* (TTSuVk2b). According to phylogenetic analysis, this new virus belongs to the *Kappatorquevirus* genus, belonging to the same genus as TTSuVk2a. Quantitative PCR techniques based on SybrGreen technology were developed; one for quantification of total TTSuV load (TTSuV broad-spectrum qPCR) and others for quantification of each TTSuV species separately. These techniques were used for epidemiological studies and assess the geographical distribution. Moreover, prevalence and viral DNA load were determined in porcine circovirus type 2-systemic disease (PCV2-SD)-affected animals and healthy counterparts, since previous studies have associated another kappatorquevirus species, to the disease.
The epidemiological study revealed that TTSuVk2b is worldwide distributed, although less abundant and displaying lower viral DNA titres in serum than TTSuV1 and TTSuVk2a. TTSuVk2b was associated with PCV2-systemic disease (PCV2-SD), which revealed that the two kappatorquevirus species are both genetically and biologically related.

The second study contained two objectives. On one hand, TTSuV proteins were expressed in a baculovirus-based platform; on the other hand, the impact of a multivalent experimental vaccine was evaluated in a natural TTSuV infection model of pigs. The ORF1, ORF1-A, ORF2 and ORF3 recombinant proteins of all four known TTSuVs were successfully expressed in a baculovirus expression system. In additional, the multivalent experimental vaccine containing ORF1 and ORF3 proteins was administered by intramuscular and intradermal routes using two different vaccination schedules (twice or three times). Seroconversion and viral titres in blood were measured from 3 weeks until 15 weeks of age, using the indirect ELISA based on baculoviruses proteins and species-specific qPCRs, respectively. This study showed that vaccination induced anti-TTSuV antibodies; however the multivalent vaccine was not able to control viremia during TTSuV natural infection.

Finally, in the third study, the immunization against TTSuVk2a during natural infection was evaluated using a different approach. The immunizations consisted of a combination of DNA and protein to increase the possibilities of activating both cellular and humoral immune responses. Quantitative PCR techniques were used to detect and quantify the viremia levels of each TTSuV species, while the induction of specific antibodies was monitored by indirect ELISA. The vaccinated group showed a seroconversion and a significant reduction of the TTSuVk2a viral loads compared to the control group. This study demonstrated for the first time that TTSuV viremia can be controlled by a combined DNA and protein immunization.

Overall, the present Thesis contributes to increase the knowledge on TTSuV by means of describing a novel species, which may be involved in disease progression in co-infection with other pig pathogens. Moreover, TTSuV infection can be controlled by the administration of a combined DNA/protein immunization while a multivalent protein based vaccine was not efficient.

Resum

Els anellovirus són un grup de virus de cadena senzilla d'ADN amb una elevada diversitat genètica i que infecten a vertebrats. Els *Torque teno sus virus* (TTSuV) son ubics i és l'espècie d'anellovirus específica que infecta els porcs. S'han descrit 3 espècies víriques diferents, anomenades TTSuV1a i TTSuV1b dins del gènere *Iotatorquevirus* i TTSuVk2a, dins el gènere *Kappatorquevirus*. Aquests virus son genèticament molt diferents (>56% de diversitat de seqüencia nucleotídica) però comparteixen la mateixa organització del genoma i estratègia d'expressió. L 'infecció de TTSuV en porcs es caracteritza per una virèmia persistent. Els TTSuVs no es consideren patogènics, però es creu que juguen un paper en la co-infecció amb altres agents infecciosos que causen malalties porcines importants. L'impacte real dels TTSuVs sobre la salut dels porcs, si n'hi ha , segueix sent objecte de debat.

L'objectiu principal d'aquesta Tesi fou caracteritzar la variabilitat de TTSuV i explorar el desenvolupament de possibles prototipus vacunals enfront els TTSuVs.

El primer estudi descriu la caracterització genètica d'una nova espècie de TTSuV, anomenada *Torque teno sus virus* k2b (TTSuVk2b). Segons l'anàlisi filogenètica, aquest nou virus pertany al gènere *Kappatorquevirus*, el mateix al qual pertany TTSuVk2a. Es van desenvolupar tècniques quantitatives de PCR basades en la tecnologia de SybrGreen pels estudis epidemiològics i per l'estudi de la distribució geogràfica. En concret, es va desenvolupar una tècnica que quantificava la càrrega total de TTSuV (*TTSuV broad-spectrum* qPCR) i tres més que quantificaven la càrrega viral específica de cada espècie de TTSuV. D'altra banda, es va estudiar la prevalença i la càrrega d'ADN vírica en animals afectats amb per circovirosi porcina (CP). L'estudi epidemiològic va revelar que TTSuVk2b es distribueix a tot el món, encara que és menys abundant que TTSuV1 i TTSuVK2a. TTSuVk2b es va associar amb la CP, una malaltia porcina d'importància econòmica molt significativa.

El segon estudi contenia dos objectius: d'una banda, l'expressió de les proteïnes TTSuVs; per l'altra banda, l'avaluació de l'impacte d'una vacuna experimental multivalent enfront

les 4 espècies de TTSuVs conegudes, utilitzant com a model l'infecció natural de TTSuV en porcs. Les proteïnes recombinants ORF1, ORF1-A, ORF2 i ORF3 dels quatre TTSuVs coneguts es van expressar amb èxit mitjançant un sistema d'expressió de baculovirus. Per altra part, la vacuna experimental multivalent (formada per les proteïnes dels gens ORF1 i ORF3) va ser administrada per via intramuscular i intradèrmica utilitzant dos esquemes de vacunació diferents (dues o tres dosis). La seroconversió i els títols vírics en sang es van mesurar a partir de 3 fins a 15 setmanes d'edat, utilitzant respectivament l'ELISA indirecte basat en proteïnes del virus expressades en baculovirus i tècniques de PCR quantitativa per detectar i quantificar els nivells de virèmia de cada espècie TTSuV, respectivament. Aquest estudi va mostrar que la vacuna multivalent induïa anticossos anti-TTSuV, però aquest prototipus vacunal no era capaç de controlar la virèmia durant l'infecció natural de TTSuV.

Finalment, en el tercer estudi es va avaluar l'immunització enfront TTSuVk2a durant l'infecció natural en porcs. L'immunització va consistir en una combinació d'ADN i proteïnes víriques per augmentar les possibilitats d'activació de respostes immunitàries tant cel·lulars com humorals. Es van utilitzar tècniques de PCR quantitativa per detectar i quantificar els nivells de virèmia de cada espècie de TTSuV, mentre que l'inducció d'anticossos específics va ser monitoritzada mitjançant ELISA indirecte. El grup vacunat va mostrar una seroconversió i una reducció significativa de les càrregues víriques de TTSuVk2a en comparació amb el grup control. Aquest estudi va demostrar per primera vegada que la virèmia per TTSuV pot ser controlada a través de l'ús d'una combinació que inclou ADN i proteïnes recombinant.

En general, aquesta Tesi Doctoral contribueix a augmentar el coneixement dels TTSuVs mitjançant la descripció d'una nova espècie vírica, la qual pot estar associada al desenvolupament de malalties del porc, com s'ha indicat per TTSuVk2a. A més, l'infecció per TTSuV pot ser controlada mitjançant l'administració d'una vacuna d'ADN i proteïnes recombinats del virus, mentre que la vacuna basada en múltiples proteïnes (multivalent) no va ser eficient.

List of abbreviations

A	adenine
aa	amino acid
ASFV	*African swine fever virus*
BEI	binary ethylenimine
BFDV	Beak and feather disease virus
BSA	bovine serum albumin
C	Cytosine
CAV	*Chicken anaemia virus*
CMV	*Cytomegalovirus*
DNA	deoxyribonucleic acid
DENV	*Dengue virus*
Dpi	days post infection
ds	double stranded
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FIPV	*Feline infectious peritonitis virus*
FITC	fluorescein isothiocyanate
G	Guanine
GFP	green fluorescent protein
HCV	*Hepatitis C virus*
HEPE	N-(2-Hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid)
HPV	*Human papilloma virus*
ICTV	International Committee for the Taxonomy of Viruses
ID	intradermal
IFA	immunofluorescence assay
IM	intramuscular
IPTG	isopropyl ß-D-1-thiogalactopyranoide
Ig	immunoglobulin

Kb	kilobase
KDa	kilodalton
LUX	light upon extension
LOD	imit of deteccion
M	Molar
mAb	monoclonal antibody
min	minutes
MOI	multiplicity of infection
mM	milimolar
mRNA	messenger ribonucleic acid
MW	molecular weight
NLS	nuclear localization signal
OD	optical density
ORF	open reading frame
P	P-value statistics
PASC	pairwise sequence comparison
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCV	*Porcine circovirus*
PCV1	*Porcine circovirus type 1*
PCV2	*Porcine circovirus type 2*
PCVD	porcine circovirus diseases
PCV2-SD	*porcine circovirus type 2*-systemic disease
PDNS	porcine dermatitis and nephropathy syndrome
PFA	paraformaldehyde
PI	pairwise identity
PRRSV	*Porcine reproductive and respiratory syndrome virus*
qPCR	real time quantitative PCR
R^2	correlation coefficient
RCA	rolling-circle amplification

RCR	rolling-circle replication
RDA	representational difference assay
Rpm	revolution per minute
s	sedimentation coefficient
sec	seconds
ss	single stranded
SD	standard deviation
SDS-PAGE	Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis
Sf	*Spodoptera frugiperda*
SISPA	sequence-independent single primer amplification
SPSS	Statistical package for social sciences
T	Thymine
TBS-T	Tris-buffered saline –Tween 20
TMB	3,3’,5,5’-tetramethylbenzidine
TTV	*Torque teno virus*
TTSuV	*Torque teno sus virus*
U	enzyme unit
UTR	untranslated region
Vol	volume
VLP	virus like particles

1. Introduction

1.1. History and discovery of Torque teno virus

The first identification of the *Torque teno virus* (TTV) was in 1997, in the plasma of a Japanese patient with post-transfusion hepatitis of unknown aetiology (Nishizawa *et al.*, 1997). Later studies demonstrated that TTV was the first circular single-stranded DNA (ssDNA) virus found to infect humans (Miyata *et al.*, 1999; Mushahwar *et al.*, 1999). The novel virus was called 'TT' virus (TTV) in reference to the initials of the affected patient. The International Committee on Taxonomy of Viruses (ICTV), in charge of the official taxonomic nomenclature, proposed a new meaning for 'TT', namely "Torque" and "Teno", deriving from the Latin terms meaning "necklace" and "thin", respectively, reflecting the organisational arrangement of the TTV genome (Todd *et al.*, 2005).

A number of TTV-like viruses has also been identified in several vertebrates including pets (dogs and cats), farm animals (pigs, chicken, cow and sheep), wild animals (wild boar, tupaia, sea lions, sea turtle and rodents) and primates (chimpanzee, Japanese macaque, cotton-top tamarin and douroucouli) (Brassard *et al.*, 2008; Inami *et al.*, 2000; Leary *et al.*, 1999; Martínez *et al.*, 2006; Ng *et al.*, 2009a; Ng *et al.*, 2009b; Nishiyama *et al.*, 2014; Okamoto *et al.*, 2000a; Okamoto *et al.*, 2001; Okamoto *et al.*, 2000b; Okamoto *et al.*, 2002). These viruses, included nowadays in the family *Anelloviridae*, are considered species-specific (Okamoto *et al.*, 2000a). The initially designated swine TTV was discovered in 1999 (Leary *et al.*, 1999) and subsequently named as *Torque teno sus virus* (TTSuV) by the ICTV.

Most of the current research is done in human infecting anelloviruses, but swine could be an excellent model for the research due to its similarity to human metabolism and physiology. Therefore, the use of the TTSuV model might contribute to understand better the infection in humans as well (Kekarainen & Segalés, 2009). During the last years the knowledge about TTSuVs has significantly increased and it has become more important especially due to the co-infection with other pig pathogens as *Porcine circovirus type 2* (PCV2), an economically important viral agent of swine (Kekarainen & Segalés, 2009).

1.2. Taxonomy and classification of anelloviruses

After discovery, TTVs were initially proposed to be classified in the group of viruses with ssDNA genomes within the *Circoviridae* family (Miyata *et al.*, 1999; Takahashi *et al.*, 2000). *Circoviridae* family includes a number of small DNA viruses infecting several domesticated animals and bird species, such as *Porcine circovirus type 1* (PCV1) and type *2* (PCV2), *Chicken anemia virus* (CAV) and *Beak and feather disease virus* (BFDV). Although, the genome organization of TTV is similar to CAV, subsequent studies showed distinct biophysical and molecular characteristics exhibited by CAV that suggested their taxonomic grouping into a new genus or family different from *Circoviridae* family (Mushahwar *et al.*, 1999). At the end of 2001, the ICTV created the floating genus Anellovirus, unattached to any viral family. The term "Anello", deriving from Latin and meaning "the ring", was related to the circular nature of their genome. The description of the genus Anellovirus was effective in 2004 and corresponded to the first step in the official classification (Biagini *et al.*, 2005). Finally, in 2009, based on the molecular and structural characteristics of TTVs, a new viral family named *Anelloviridae* was created (Biagini, 2009).

Anelloviridae family (Figure 1.1) includes a number of viruses that are genetically highly variable, but share a common genomic organization (Biagini, 2009). However, these analyses also show different genome length and a great variability in the sequence identity. The taxonomic classification is currently based on the analysis of a genomic region corresponding to the entire open reading frame 1 (ORF1), which is representative of the whole genome identity as demonstrated by distance matrix comparison (Biagini, 2009). Actually, anelloviruses are divided in eleven genera depending on the host species, (http://www.ictvonline.org/virusTaxonomy.asp). TTSuVs are separated in two distinct genera namely *Iotatorquevirus* and *Kappatorquevirus (http://www.ictvonline.org/virus Taxonomy.asp?taxnode_id=20141494*). *Iotatorquevirus* genus includes two genetically distinct viral species *Torque teno sus virus 1a* (TTSuV 1a) (AB076061) and *Torque teno sus virus 1b* (TTSuV1b) (AY823990). Kappatorquevirus includes only one species, namely *Torque teno sus virus* k2a, TTSuVk2 (AY823991) (Niel *et al.*, 2005).

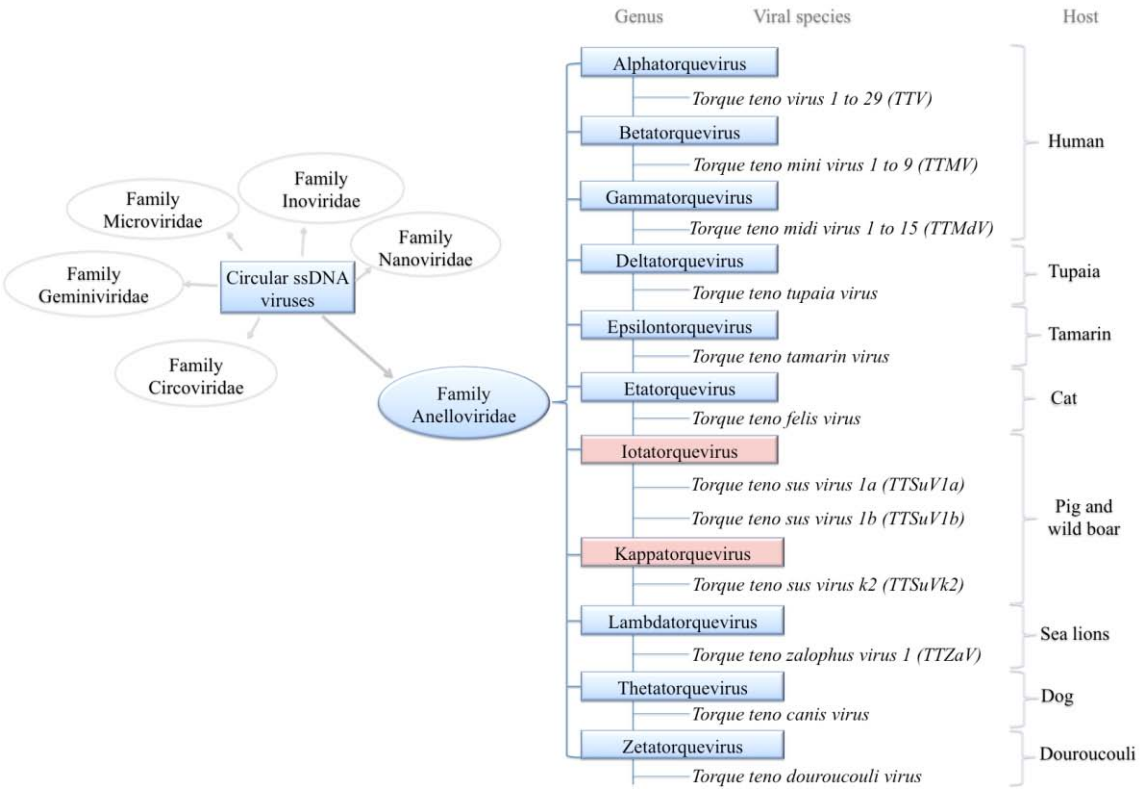

Figure 1.1 Taxonomical classification of anelloviruses according to ICTV (www.ictvonline.org).

1.2.1. Genetic variability

The extensive divergence described for anelloviruses has been attributed to different mechanisms of genomic variation, being mainly the genomic mutation, insertions-deletions, and transversions and transitions in the translated regions (Biagini *et al.*, 1999; Cortey *et al.*, 2011; de Villiers *et al.*, 2011; Nishizawa *et al.*, 1999). Pairwise sequence comparison (PASC) is a useful method that is used to plot the frequency distribution of pairwise nucleotide sequence identity percentage from all available genomic sequences of viruses in the same family (Bao *et al.*, 2008). Based on the phylogenetic and PASC analysis of anallovirus ORF1 nucleotide sequences, ICTV nowadays recommends to use the cut-off values for sequence divergence to distinguish different genera (>56%) and species (>35%). Is to be noted that ICTV is not responsible for classification and nomenclature of virus taxa below the rank of species and this task remains to research groups.

TTSuVs are genetically very distinct (>56% sequence diversity), but share similar genome organization and gene expression strategy (Martínez-Guinó *et al.*, 2011). Comparison of the sequences available and TTSuV genomes identified from Spain revealed that the number of variable nucleotide positions among genomes is lower in the untranslated region (UTR) and larger in the translated area (Cortey *et al.*, 2011). TTSuV1 was shown to contain more variability than TTSuVk2 (Cortey *et al.*, 2011).

TTSuV classification is not as accurate compared to that of human TTVs, since the number of complete TTSuV genomes available in the Genbank is not that high. However, the full-length genomes of TTSuVs have been increasing in the last years, and type and subtype definitions have been proposed for TTSuV species (Cortey *et al.*, 2011, 2012; Huang *et al.*, 2010b). Based on this, TTSuVs could be further classified to variants (>95% PI), subtypes (85-95 % PI), types (67-85% PI), species (55-67%), and genus (35-55%) based on several tentative pairwise identity (PI) thresholds (Huang *et al.*, 2010b). According to the suggested PI limits, each TTSuV species could be further divided from 3 to 4 types and subtypes. However, considering the high genetic divergence but lack of biological data of TTSuVs, classification beyond the species level should be postponed (Cortey *et al.*, 2011; Huang *et al.*, 2010b; Li *et al.*, 2013; Liu *et al.*, 2013)

1.2.2. Discovering novel anelloviruses

The prototype anellovirus, the human TTV, was discovered using the representational difference assay (RDA) (Nishizawa *et al.*, 1997). The novel DNA was detected amplifying a specific nucleotide sequence of 500 bp (named N22), which showed low homology to DNA sequences found in databases at that time. Following studies to detect novel anelloviruses were based on the detection of DNA by using primer specific for the short, highly conserved sequence in the non-coding region of the known TTV (Okamoto *et al.*, 2001; Okamoto *et al.*, 2002). Therefore, a number of new anelloviruses were discovered by means of PCR and sequencing, but this methodology allows only detection of close relatives of known viruses (Delwart, 2007).

New methodologies have been subsequently developed for the discovery of new viruses without prior knowledge on their genomic sequences such as virus-particle purification and shotgun sequencing, included within metagenomics packages (Delwart, 2007). Viral metagenomics allow identifying novel viruses from uncultured environments or clinical samples. Viruses have to be purified through filtrate processing and their nucleic acids randomly amplified prior to subcloning and sequencing. With this technology viruses have been characterized from samples with several origins like seawater, near shore sediments, mammal faeces, serum, plasma and respiratory secretions. Also, the viral metagenomic approach is useful in the case of viruses that cannot be cultivated in the laboratory or viruses unrelated to already known ones (Delwart, 2007; Edwards & Rohwer, 2005). Viral metagenomics has been also used to identify other pathogens as well as ssDNA viruses (Ng *et al.*, 2009a) and anelloviruses (Ng *et al.*, 2009b). A sequence-independent single primer amplification (SISPA) method has been also used in human plasma to identify two novel TTV species with a highly divergent circular sequence (Jones *et al.*, 2005). These methods, due to their simplicity, relative speed and lack of bias in identifying particular groups of viruses, have advantages to detect novel agents highly divergent from those already known (Delwart, 2007). The method is based on converting the target sequence to a double-stranded DNA, and use endonuclease restriction followed by non-specific linker ligation and PCR amplification (Allander *et al.*, 2001; Reyes & Kim, 1991)

The rolling-circle amplification (RCA) based sequence-independent approach has been also applied to identify TTVs (Niel *et al.*, 2005). RCA is a relatively novel technique based on the property of circular DNA molecules to replicate using the rolling circle mechanism (Dean *et al.*, 2001). The method utilizes the bacteriophage phi29 DNA polymerase, with strong strand-displacing capability, high processivity and proofreading activity (Esteban *et al.*, 1993). The amplification is done using random hexamer primers, without need of prior knowledge of the target nucleotide sequence. Indeed, nine anelloviruses have been detected in human plasma and cat saliva by means of a combination of RCA and SISPA (Biagini *et al.*, 2007) and virus sequences from pig and human sera have been detected by using a combination of RCA with a single-step-inverse-PCR (Macera *et al.*, 2011).

1.3. Molecular characteristics of anelloviruses

1.3.1. Genomic organization

Anelloviruses are small, non-enveloped viruses of 30-32 nm in diameter and icosahedral symmetry (Mushahwar *et al.*, 1999). The human TTV (prototype of *Anelloviridae* family) virions have a buoyant density of 1.31-1.33 g/cm^3 in cesium chloride for TTVs detected in serum and 1.22-1.35 g/cm^3 for TTVs from faeces (Mushahwar *et al.*, 1999; Okamoto *et al.*, 1998). The genome, of negative polarity, may have different size depending on the host species, ranging between 2.1 (cat) to 3.9 kilobases (kb) (human), being approximately 2.8 kb for TTSuV (Miyata *et al.*, 1999; Mushahwar *et al.*, 1999; Nishizawa *et al.*, 1997; Okamoto *et al.*, 2002; Okamoto *et al.*, 1999; Peng *et al.*, 2002).

TTSuVs have a similar genomic organization with human TTVs, but share less than 45% nt sequence identity (Niel *et al.*, 2005; Okamoto *et al.*, 2002). As all anlleoviruses, the TTSuV genome can be divided into an UTR and a coding region (Okamoto *et al.*, 2002) (Figure 1.3). The UTR in TTSuV is characterized as a short stretch of sequence with high GC content. It contains the most conserved region of the genome including the promoter and enhancer elements important for the virus replication and transcription (Kamada *et al.*, 2004; Suzuki *et al.*, 2004).

1.3.2. Gene transcription and protein translation

Despite of high divergence among TTV sequences, the splice sites and motifs between various sequences are well-conserved, which led to propose similar protein structure and functions (Peng *et al.*, 2002).

The TTSuV genome contains (Figure 1.2) two major ORFs; ORF1 and ORF2. Several analyses of TTSuV nucleotide sequences reveal the existence of additional mRNAs, all as result of splicing events. ORF3 mRNA has its 5'end identical to ORF2 and 3' end results after splicing event partially overlapping ORF1 encoding region. On the other hand,

alternative splicing of ORF1 results in several mRNAs (Huang *et al.*, 2012b; Martínez-Guinó *et al.*, 2011). Indeed, the predominant transcripts detected *in vitro* and *in vivo* of ORF1 are spliced and the full-length ORF1 transcript has not been detected so far (Huang *et al.*, 2012b; Martínez-Guinó *et al.*, 2011). Splicing of TTSuV1 ORF1 generates two isoforms (ORF1-A and B) of different sizes although the reading frame and the encoded amino acid (aa) sequence do not change (Martínez-Guinó *et al.*, 2011). In contrast, splicing of TTSuVk2a ORF1 results in three protein isoforms (ORF1-A, -B and -C) and, depending on the splicing site used, the aa composition between them varies (Huang *et al.*, 2012b; Martínez-Guinó *et al.*, 2011). TTSuVk2 ORF3 generates three protein isoforms, two of which altered the reading frame and TTSuV1 ORF3 only produces one isoform (Huang *et al.*, 2012b; Lu *et al.*, 2013; Martínez-Guinó *et al.*, 2011).

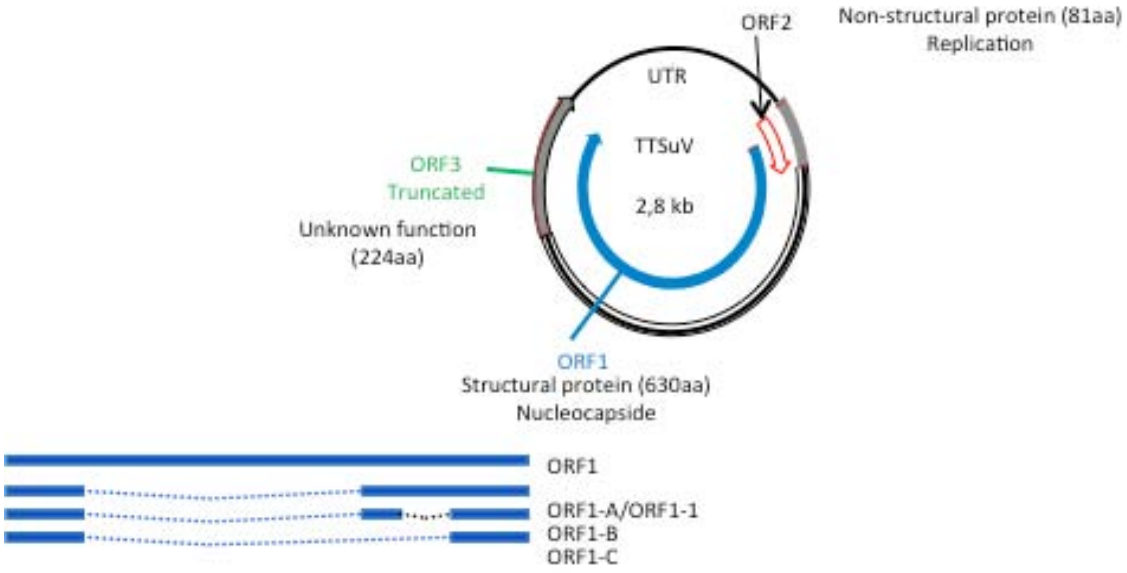

Figure 1.2 Schematic view of the TTSuV genome. UTR and encoding regions are shown. ORFs encode different TTSuV proteins

The ORF1 gene encodes the largest TTSuV protein with a variable length ranging from 638-650 aa in TTSuV1 and 625-628 aa in TTSuVk2). By analogy with CAV, PCV2 and human TTV, ORF1 encodes a predicted viral capsid domain at the N-terminal half of the protein and a putative replication-associated domain in its C-terminal half (Miyata *et al.*, 1999; Mushahwar *et al.*, 1999). ORF1 possesses a high number of arginine residues at the N-

terminus and rolling-circle replication (RCR) motifs, which were differently present in TTSuV species, but highly conserved within species (Huang *et al.*, 2010b). ORF2 smallest predicted gene, encode a non-structural protein and encodes protein of 73–74 aa in TTSuV1 and 69 aa in TTSuV2. It has been suggested that the ORF2 protein is involved in viral replication (Hijikata *et al.*, 1999; Huang *et al.*, 2010b). ORF2 protein conserved protein-tyrosine phosphatase (PTPase-like motife) at the N-terminal, typically found in anelloviruses (Huang *et al.*, 2010b). At present, the role of the ORF3 protein is unknown and is believed to encode non-structural protein that shares its N-terminus with ORF2 (Biagini *et al.*, 2001; Cortey *et al.*, 2011).

1.3.3. Replication mechanism

The exact mechanism of TTV replication is not fully understood. Based on similarities to other ssDNA-genome viruses, it is assumed that TTV uses the RCR mechanism (Mushahwar *et al.*, 1999). It is believed that TTSuVs and human TTVs use the cellular polymerase and replication machinery from the host, like the majority of small DNA viruses (Kakkola *et al.*, 2007). Due to the lack of a cell culture system to propagate anelloviruses, little is known regarding the molecular biology and pathogenesis of anelloviruses. Moreover, attempts to replicate both human TTV and TTSuV in cell cultures (free from TTVs) have not been successful (Huang *et al.*, 2012b; Kakkola *et al.*, 2007). To date, there is no available *in vitro* efficient replication model for anelloviruses.

1.4. Epidemiology

The prevalence and detection of TTSuVs DNA have been mainly based on species-specific nested PCR (Kekarainen *et al.*, 2006) and direct PCR (Brassard *et al.*, 2008; Segalés *et al.*, 2009) techniques detecting the UTR. Recently, quantitative (q) PCR techniques have been developed using different pairs of primers and probes (Brassard *et al.*, 2010; Gallei *et al.*, 2010; Huang *et al.*, 2010a; Lee *et al.*, 2010; Nieto *et al.*, 2011; Xiao *et al.*, 2012).

Detection and quantification of anelloviruses by molecular systems are constantly improving. The results obtained in different laboratories may be a source of confusion and results may not be comparable due to the differences on the applied techniques, since the molecular tools are highly dependent on the DNA fragment subjected to amplification and primers used in each laboratory. Therefore, effective diagnostic tools would also help to progress in the study of the TTSuV infections.

TTSuV DNA was detected in swine for the first time in 1999 (Leary *et al.*, 1999). However, a retrospective study from Segalés *et al*. (2009) revealed evidence of TTSuV infections in pigs as early as 1985 in Spain. Nowadays it is known that TTSuVs are ubiquitous in domestic pigs and wild boar (Kekarainen & Segalés, 2009) and co-infections of TTSuV species are often detected (Blois *et al.*, 2014; Gallei *et al.*, 2010; Huang *et al.*, 2010b). It is believed that anelloviruses are transmitted between members of the family *Suidae* (domestic pig, wild boar and bush pigs), since TTSuVs found in these hosts are highly genetically identical to each other (Blomström *et al.*, 2012; Leary *et al.*, 1999; Martínez *et al.*, 2006).

TTSuV infection is distributed worldwide. They have been found in swine serum from many different countries as Canada, China, France, Italy, Korea, Spain, Thailand and the United Sates with prevalence rates ranging from 24% to 100% (Bigarré *et al.*, 2005; Gallei *et al.*, 2010; Kekarainen *et al.*, 2006; Martelli *et al.*, 2006; McKeown *et al.*, 2004; Taira *et al.*, 2009). Globalization and international trading of live animals is considered to play a crucial role in the worldwide propagation of TTSuV in pig population (Cortey *et al.*, 2012).

TTSuV infections occur early in life, as they have been found in foetuses (Martinez-Guino *et al.*, 2010; Xiao *et al.*, 2012). In addition, viral prevalence increases with age and it looks like that a high proportion of animals get persistently infected (Aramouni *et al.*, 2010; Blois *et al.*, 2014; Nieto *et al.*, 2011; Sibila *et al.*, 2009a; Sibila *et al.*, 2009b; Taira *et al.*, 2009; Xiao *et al.*, 2012). TTSuV1 and TTSuVk2 seem to display similar dynamics of infection, with a prevalence of viraemic piglets increasing progressively over time, and with a maximum rate of TTSuV1 detection at 11 weeks of age and at 15 weeks of age for TTSuVk2 (Nieto *et al.*, 2011; Sibila *et al.*, 2009a).

Several investigations have noted a higher prevalence of TTSuV1 DNA compared to that of TTSuVk2 DNA in different countries (Gallei *et al.*, 2010; Martínez-Guinó *et al.*, 2009; Perez *et al.*, 2011; Sibila *et al.*, 2009a; Sibila *et al.*, 2009b). In contrast, one study from the Czech Republic (Jarosova et al., 2011) and another from China (Li *et al.*, 2013) detected higher TTSuVk2 prevalences Since the outcome of a given study is highly dependent on the PCR method used, it is expectable that considerable discrepancies and variations have occurred.

TTSuV DNAs in tissues have also been detected by a semi-quantitative method. Prevalence increased over age, from foetal period until 15 weeks of age, and then maintained until slaughter age (Aramouni *et al.*, 2010). TTSuVs have been also found in semen, colostrum, nasal cavity and faeces (Kekarainen *et al.*, 2007; Martínez-Guinó *et al.*, 2009; Sibila *et al.*, 2009b), indicating the occurrence of both vertical and horizontal transmission (Kekarainen & Segalés, 2012a).

Moreover, commercial pigs vaccines, human drugs with components of pig origin, trypsin derived from pigs used for cell culturing, as well as in cell lineages derived from different species have been found contaminated with TTSuV DNA (Kekarainen *et al.*, 2009; Kulcsar *et al.*, 2010; Teixeira *et al.*, 2011)

In summary, TTSuVs natural infections are characterized for being ubiquitous, increasing viral prevalence with age, and establishing long-lasting to persistent infections, such as it has been observed in human TTVs (Maggi *et al.*, 2001; Moen *et al.*, 2003).

1.5. Pathogenesis and immunology

To date, the pathogenic potential of anelloviruses is still controversial and they are considered non-pathogenic by themselves. Anelloviruses are characterized by high prevalence in the general human and pig populations that are apparently healthy (Kekarainen & Segalés, 2012b; Okamoto, 2009a). So far, there are no clinical manifestations that can be directly attributed to anellovirus infections. Experimental

studies of gnotobiotic pigs demonstrated mild lesions after their inoculation with TTSuV1-containing tissue homogenate (Krakowka and Ellis, 2008) or TTSuVk2-containing tissue homogenate (Mei *et al.*, 2011). In another study, mild pathological lesions in brain, kidney and liver were observed in colostrum-deprived (CD) pigs after inoculation with an infectious TTSuVk2 clone (Huang *et al.*, 2012b). Inoculated gnotobiotic pigs did not show any clinical sign during the experiments. However, it is believed that TTSuVs might influence the development or even modulate some diseases and play a pathogenic role during co-infection with other pathogens (Kekarainen & Segalés, 2012b; Okamoto, 2009a).

The immunology of anelloviruses is poorly understood (Maggi & Bendinelli, 2009) and little is known about the interaction that anelloviruses establish with their specific host. It is assumed that these viruses generate a persistent and life-long infection in the human and pig populations. Moreover, the high genetic variability of anelloviruses has been considered as an efficient mechanism of immune evasion to establish persistent infections (Jelcic *et al.*, 2004). It is possible that only certain genotypes/ genogroups/species of human TTVs could be more pathogenic than others, as it is well known for human papillomaviruses (Okamoto, 2009a).

Recently serodiagnostic tools have permitted to detect the humoral immune response produced by natural TTSuV infections and provided new knowledge about TTSuV. Western blot and indirect ELISA assay based on the recombinant C-terminal portion of ORF1 antigen have been described for TTSuV1a and 1b and TTSuVk2 (Huang *et al.*, 2011; Huang *et al.*, 2012a). Also, an indirect ELISA using peptides based on antigenic regions in the C-terminal part of ORF1 (Jarosova & Celer, 2013) has been described to detect antibodies against TTSuVs.

In conventional healthy pigs the antibody levels during natural TTSuV infection increase with age; specific antibodies can be detected TTSuV in all age categories (Huang *et al.*, 2011; Jarosova & Celer, 2013; Nieto *et al.*, 2015). TTSuV-specific antibodies usually start to appear when the animals are around 10 weeks of age and peak around week 20. TTSuV1- and TTSuVk2-specific antibodies have been detected at 4 weeks and decreased afterwards (Jarosova & Celer, 2013). The authors of this work suggested that the antibody

detection at 4 weeks of age could be residual maternally derived antibodies, which could be normally present in weaned piglets for several weeks. The levels of anti-TTSuVk2 antibodies were low or absent in young animals and in the foetuses in a study based on the recombinant C-terminal portion of ORF1 antigen (Xiao *et al.*, 2012).
Recently, an ELISA assay using the ORF1-A (splicing variant) against TTSuV1 and TTSuVk2a has been developed (Nieto *et al.*, 2015). All tested pigs in this study developed antibodies against TTSuV1 and TTSuVk2 along their productive life (from nursery to slaughter), which fits with previous studies using truncated ORF1 protein (Huang *et al.*, 2011; Huang *et al.*, 2012a; Xiao *et al.*, 2012) or peptides (Jarosova & Celer, 2013).

Huang and co-workers analysed TTSuV1 (Huang *et al.*, 2012a) and TTSuVk2 (Huang *et al.*, 2011) antibodies in a two-point longitudinal study of 10 conventional pigs and showed that the antibodies increased with the age. They also found that higher level of ORF1 antibodies correlated with low levels of viral DNA in serum (Jarosova & Celer, 2013). The decreased viremia in adult animals could be due to gradual virus neutralization by virus-specific antibodies together with the activation of cell-mediated immunity (Huang *et al.*, 2011; Jarosova & Celer, 2013). However, these antibodies may not be able to completely clear TTSuVs, which might explain the persistence of the virus (Jarosova & Celer, 2013; Nieto *et al.*, 2015). Considering that TTSuV infection has been detected in foetuses, in the second third of gestation, before the immunocompetence age, it would not be surprising that TTSuV may be immunologically tolerated by the host immune system (Aramouni *et al.*, 2010).

Moreover, antigenic regions have been identified at the C-terminus portion of ORF1 protein (the putative capsid), so it is a theoretically appropriate target for development of serodiagnostic assays (Huang *et al.*, 2011; Huang *et al.*, 2012a; Jarosova & Celer, 2013)**.** Additionally, a most recent study identified new epitopes on the ORF1 protein of TTSuV1 using monoclonal antibodies (mAbs) from immunized mice (Liu J, 2013). Although the presence of immune-dominant epitope has been revealed in the ORF1 of TTSuVs further studies are required to clarify if this region induces the production of neutralizing antibodies.

Anti-TTSuV antibodies are not recognizing other anelloviruses (aa identity about 20%). TTSuVs from different genus (aa identity about 25%) do not cross-react as well (Huang *et al.*, 2012a). However, cross-reactive antibodies can be detected against species within the same genus, like TTSuV1a and TTSuV1b (aa identity about 50%) (Huang *et al.*, 2012a).

1.6. TTSuV disease association.

Anellovirus infections are believed to influence the development or modulate disease outcome during co-infection with other pathogens. Human TTVs have been linked to a number of inflammatory diseases, liver pathologies and neoplasia (Okamoto, 2009a). Regarding TTSuV, it is believed that it might play a collaborative role in co-infection scenarios, as a potential disease triggering factor or, alternatively, as opportunistic pathogen (Kekarainen & Segales, 2012).

Several reports have associated both TTSuVs species with clinical disease manifestations in pigs affected by porcine circovirus diseases (PCVD) (Aramouni *et al.*, 2011; Kekarainen *et al.*, 2006; Nieto *et al.*, 2011). TTSuV is often present in pigs with PCV2- PCV2-systemic disease (SD), the most significant PCVD (Aramouni *et al.*, 2011; Blomström *et al.*, 2010; Kekarainen *et al.*, 2006; Nieto *et al.*, 2011). In fact, higher prevalence and load of TTSuVk2 have been detected in pig sera with PCV2-SD compared to healthy animals; such difference was not observed in the case of TTSuV1 (Aramouni *et al.*, 2011; Kekarainen *et al.*, 2006; Nieto *et al.*, 2011). It has been speculated that TTSuVk2 is up-regulated in the framework of immunosuppression associated with PCV2-SD (Kekarainen *et al.*, 2010). Furthermore, PCV2-SD affected pigs have been found with high TTSuVk2 DNA loads but significantly lower TTSuVk2 antibody levels than non-affected animals (Huang *et al.*, 2011). However, the association of TTSuVs with this disease is not entirely clear, since some preliminary studies of virus load in PCV2-SD affected and non-affected pigs suggested no association (Lee *et al.*, 2010; Taira *et al.*, 2009).

PDNS is considered an immune-complex disease in which PCV2 is suspected to be the associated antigen (Segalés *et al.*, 2005). TTSuVs are often present in pigs with PDNS; it

has been observed that TTSuVk2 load in serum was higher in PDNS affected pigs than in healthy pigs, while no differences were observed in the case of TTSuV1 (Aramouni *et al.*, 2011). On the contrary, concomitant inoculation of PRRSV and a PRRSV-negative tissue homogenate containing TTSuV1 were considered to contribute to the experimental induction of a porcine dermatitis and nephropathy syndrome (PDNS)-like condition in gnotobiotic pigs (Krakowka *et al.*, 2008). However, the appropriate examination of tissues of such experimentally inoculated animals did not confirm the classical form of PDNS. Also, a study revealed that natural TTSuV1 infection could reduce the immune response of the host induced by PRRSV vaccine and may aggravate clinical signs associated to PRRS (Zhang *et al.*, 2012). Again, this interpretation is highly speculative and difficult to demonstrate, since it is not really known if TTSuV infection suppresses the immune responses of the pig.

Another study found association between TTSuVs and porcine respiratory disease complex (PRDC). It was found that TTSuV1, and not TTSuVk2, was highly prevalent in pigs affected by this clinical multi-aetiological and multifactorial condition (Rammohan *et al.*, 2012). The authors of the study suggested the hypothesis of role of TTSuV as co-factor in PRDC development.

The fact of not getting a cell culture system to propagate anellovirus complicates the research on pathogenicity and TTSuV disease associations. Moreover, in TTSuV investigations, their extreme ubiquity further complicates the studies since it is almost impossible to select uninfected animals.

1.7. Prevention and control of TTSuV

Nowadays, the impact of TTSuV infections and their consequence in the health of the pig have not yet been established. To study the characteristics and the pathogenicity of TTSuV or their relation with other pig pathogens would be crucial to prevent or control the TTSuV infection.

Animal viral diseases can be controlled and prevented using vaccines. Many factors can influence whether or not a vaccine will be efficient. One of them is the composition of the vaccine to stimulate the humoral and cellular responses. Currently, several classes and types of vaccines are used to prevent and control viral pathogens, such as DNA vaccines, recombinant vaccines and classical inactivated (killed) or modified live (attenuated) products. Vaccine studies should cover several aspects such as administration routes or the efficient vaccine dose, and to select the proper strategy to induce the protective immune responses in the host to prevent the infection.

Although TTSuVs are currently considered non-pathogenic, it is hard to assume that a viral infection would not have any consequences to its host. Indeed, it has been shown that a similar viruses, such as PCV2, is ubiquitous in the swine population and is necessary but not sufficient for the onset of PCV2-SD (Segalés *et al.*, 2005).

For this particular virus, PCV2, its capsid protein (encoded by ORF2) is the principal immunogenic protein (Blanchard *et al.*, 2003; Mahé *et al.*, 2000) and consequently has been used to develop vaccines and serodiagnostic assays for tracking PCV2-specific immune responses (Beach & Meng, 2012). Currently, efficient commercial vaccines against PCV2 are available to the pig market worldwide (Opriessnig *et al.*, 2007), which are efficient in reducing the serum viral titres, preventing the disease and improving the performance of the farm (Beach & Meng, 2012). Current vaccines, however, do not completely prevent infection or spread of PCV2 (Beach & Meng, 2012). Moreover, PCV2 infection causes clinical signs and subclinical effects (PCVD) in the pig population. The decreased average daily weight gain without any evidence of clinical signs has been associated to PCV2 infection in healthy animals with relatively high virus load in serum (Segalés, 2012). In fact, even in PCV2 subclinical infection scenarios PCV2 vaccination is able to improve productive parameters (average daily gain, percentage of runts, body condition and carcass weight) in pig farms (Young *et al.*, 2011). Therefore, a likely scenario should not be discarded for TTSuVs.

2. Objectives

The first anelloviruses were detected in humans and subsequent studies demonstrated that these viruses are able to infect several vertebrate species, including farm animals, pets, and wild animals including non-human primates. Currently, new anelloviruses are constantly found by applying novel discovery methods. TTSuVs are the anelloviruses able to infect swine, and represent an excellent model to study the infection by anelloviruses. TTSuV infection is characterized by high viral loads and persistent infections that apparently do not have evident clinical consequences to its host. However, it is difficult to believe that such long lasting infection would not have consequences to such infected host. Furthermore, there is increasing interest in their association in co-infection with economically important pig pathogens. As known in other single-stranded DNA viruses, subclinical infections can also negatively affect pig production; if such would be the case for TTSuVs, still remains unsolved. It is also known that anelloviruses display high genetic variability, which may account as a mechanism to counteract the immune system responses from the host.

With the main objective to gain further insights in TTSuV biology, this PhD Thesis aimed to characterize TTSuV variability and explore possible ways of vaccination against TTSuVs. Therefore, the specific objectives of this Thesis were:

1. To identify, characterize and study disease association of a novel pig anellovirus using molecular and epidemiological studies.
2. To evaluate the impact of pig immunization against all known TTSuV species using a cocktail of ORF1 and ORF3 recombinant proteins expressed in a baculovirus system.
3. To evaluate the impact of pig immunization against TTSuVk2a in a natural infection model using a combination of DNA and proteins.

3. Study I

Discovery of a novel *Torque teno sus virus* species: genetic characterization, epidemiological assessment, and disease association

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3.1. Introduction

Anelloviruses are a highly diverse group of circular ssDNA viruses infecting vertebrates. *Torque teno sus virus* 1 (TTSuV1, genus *Iotatorquevirus*) and k2 (TTSuVk2, genus *Kappatorquevirus*) are ubiquitous pig-infecting anelloviruses (Kekarainen & Segales, 2012). These viruses are genetically very distinct (>56% sequence diversity) but share similar genome organization and expression strategy (Martínez-Guinó *et al.*, 2011). Indeed, torque teno virus (TTV) strains are considered species and genera if ORF1 nucleotide sequences differ more than 35% and 56%, respectively (Biagini, 2009; Biagini *et al.*, 2011; Biagini *et al.*, 2005). TTSuVs are widespread and transmitted by vertical and horizontal routes (Martinez-Guino *et al.*, 2010; Martínez-Guinó *et al.*, 2009; Pozzuto *et al.*, 2009). Lately, TTSuVk2a has been associated with PCV2-SD (Aramouni *et al.*, 2011; Blomström *et al.*, 2010; Kekarainen *et al.*, 2006; Nieto *et al.*, 2011), an economically important multifactorial disease in pig production (Segalés *et al.*, 2005).

After the first anellovirus was described in 1997 in human, novel anelloviruses have been detected by using primers specific for the short, highly conserved sequences in the non-coding region of the genome (Okamoto *et al.*, 2001; Okamoto *et al.*, 2002). Recently, the RCA-based sequence-independent approach has been applied to identify TTVs (Biagini *et al.*, 2007; Macera *et al.*, 2011; Niel *et al.*, 2005).

In this study, a novel TTSuV species, tentatively named *Torque teno sus virus* k2b (TTSuVk2b), originally found in pig sera by applying the RCA technique is described. Molecular and epidemiological studies were performed to further characterize this novel pig virus. For such purpose, the full-length genomic sequences of three TTSuVk2b strains were obtained. Furthermore, qPCR techniques were developed to detect and quantify all TTSuVs species together and separately. For epidemiological studies, the geographic distribution and viral loads of TTSuVk2b were determined and its association to PCV2-SD assessed.

3.2. Material and methods

3.2.1. Samples

Commercial porcine sera were used to identify a novel TTSuV species applying the RCA technique. These sera were purchased from Hyclone (New Zealand origin, FSD29672), Kraeber (German origin, 9071172-G50, 9050957-G50, SS02/06-1, SS03/06) and Sigma (USA origin, 026k8453, 064K8451), which are pools from a large number of pigs.

For a preliminary epidemiological assessment, 224 pig serum samples were collected from commercial farms located in 17 different countries: Greece, the Netherlands, Brazil, Vietnam, China, Philippines, Lithuania, Mexico, Thailand, Russia, Belarus, Ukraine, Venezuela, South Africa, Mozambique, Canada and Spain. Furthermore, sera from 34 PCV2-SD affected pigs and 29 age-matched healthy pigs aged between 11 and 21 weeks from a previously published study (Aramouni *et al.*, 2011; Nieto *et al.*, 2011) were also tested. It has to be noted that in this study the 29 healthy pig sera were also used as the Spanish sera in the epidemiological study and are therefore the same.

3.2.2. DNA Extraction

DNA extraction from individual pig serum samples were performed on the MagNA Pure 96 Instrument using the MagNA Pure 96 DNA and Viral Nucleic Acid Small Volume Kit (Roche Diagnostic). This procedure was performed automatically using the Viral NA Universal SV 2.0 protocol. Serum from PCV2-SD affected pigs, as well as pig serum from Spain and Mozambique were processed as described previously Aramouni *et al.*, 2011. The commercial porcine sera were processed manually by the QIAamp® MinElute® Virus Spin Kit according to the manufacturer's instructions (Qiagen).

3.2.3. Rolling circle amplification

The RCA-based sequence-independent approach was to identify a novel TTSuV species. For RCA, 10 µl of extracted viral DNA was denaturated for 5 min at 95°C. After cooling on ice, 10 µl of a mixture was added containing 10 U phi29 DNA Polymerase (New England Biolabs), 150 pmol exo-resistant random primers (Fermentas), 48 nmol dNTP (HT Biotechnology LTD), 40 ng BSA and 2 µl of 10x phi29 DNA Polymerase Reaction Buffer (New England Biolabs). Amplification was done for 18h at 30°C followed by 10 min at 65°C to inactivate phi29 DNA polymerase. Subsequently, 5 µl of the amplification product was digested with 10 U *Bam*HI or *Eco*RI. Restriction digests were separated by 0.8 % agarose gel electrophoresis. Restriction fragments of approximately 2.9 kb were purified from gel and inserted into the *Bam*HI or *Eco*RI sites of vector pSC-A-amp/kan (Agilent). Inserts were sequenced using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems).

3.2.4. Sequence and phylogenetic analyses

The phylogenetic analyses of the obtained nucleotide sequences [(GenBank accession numbers: JQ406844 (TTSuVk2b-38E05), JQ406845 (TTSuVk2b-38E19), and JQ406846 (TTSuVk2b-38E23)] were done using MEGA version 5 (Tamura *et al.*, 2011) including TTSuV1 and TTSuVk2a sequences obtained from GenBank (Cortey *et al.*, 2011; Huang *et al.*, 2010b). The alignments were performed using a ClustalW multiple alignment tool with a gap creation penalty of 10 and a gap extension penalty of 5. Phylogenies were inferred from p-distance matrices using the neighbour-joining method (Saitou & Nei, 1987). Statistical significance of the branching was estimated using bootstrap with 1000 replications and from this a consensus phylogenetic tree was built. Pairwise sequences comparison (PASC; http://www.ncbi.nlm.nih.gov/sutils/pasc/) was performed using available anellovirus sequences from GenBank (Bao *et al.*, 2008)

The annotation of the TTSuVk2b genomes was based on the available animal TTV genomes already characterized (Okamoto, 2009b; Okamoto *et al.*, 2001). For gene

predictions, the Open Reading Frame Finder program (http://www.ncbi.nlm.nih.gov/projects/gorf/) was used. The NetGene2 program (http://www.cbs.dtu.dk/services/NetGene2/) (Brunak *et al.*, 1991) was used to predict splice sites in TTSuVk2b genes and PSORT II (http://psort.hgc.jp) (Nakai & Horton, 1999) was used for protein localization predictions as described previously (Martínez-Guinó *et al.*, 2011). The promoter region was predicted using the Neural Network Promoter Prediction program version 2.2 (http://www.fruitfly.org/seq_tools) with a score cut-off of 0.80.

3.2.5. q PCR specific for TTSuV1, TTSuVk2a, and TTSuVk2b

Species-specific qPCRs were developed to detect TTSuV1 (detecting both species a and b), TTSuVk2a, and TTSuVk2b in porcine serum. The sequence of the species-specific primers and their location in the genome are listed in Table 3.1. All primers were tested to be species specific by BLAST analysis and challenging each primer set with using full-length TTSuV clones of each species as a template in qPCR (data not shown). The 25μl PCR reaction contained 12.5 μl of iQ-SYBR Green Supermix (Bio-rad), 10 pmol forward primer, 10 pmol reverse primer and 2 μl of DNA. The qPCR was performed on a CFX 96 Real-Time System (Bio-rad) and the program consisted of an initial denaturation step of 5 min at 95°C, followed by 40 cycles of 30 sec at 95°C, 15 sec at 56°C (for TTSuV1 and TTSuVk2a) or 62°C (for TTSuVk2b and broad spectrum TTSuV qPCR), and 30 sec at 68°C. The specificity of each PCR was determined by melting curve analysis: After an extra extension step of 7 min at 68°C, the temperature was raised from 70°C to 95°C with 0.5°C increments. Fluorescence was measured during each extension step and melting curve analysis. As standard curve a dilution series of plasmids TTV001, TTV008, or 38E05 was used. The qPCR amplification efficiency was 94.6% for TTSuV1, 93.0% for TTSuVk2a, and 90.2% for TTSuVk2b; the inter-assay variation was 2.3% for TTSuV1, 1.7% for TTSuVk2a, and 1.8% for TTSuVk2b; the limit of detection (LOD) was 10 copies reaction^{-1} for each qPCR, which corresponded to 1250 copies ml serum^{-1}.

Table 3.1 Primer sequences used in this study. * The primer locations are based on sequences available in GenBank; accession numbers are given in parentheses.

Primers for generation of PCR standards		
Name	**Sequence**	**Location***
TTV1-1F	TACACTTCCGGGTTCAGGAGGCT	001-023 (AY076001)
TTV1-390R	TTGACTCCGGATCAGGGATCCTC	368-390 (AY823990)
TTV2-106F	AGTTACACATAACCACCAAACC	106-127 (AY823991)
TTV2-425R	GCAGTACGCTACCGTCAGCCATC	403-425 (AY823991)
TTSuV1-specific primers		
Name	**Sequence**	**Location**
TTV1-240F	TGCATAGGGTGTAACCAATC	240-259 (AY823990)
TTV1-390R	TTGACTCCGGATCAGGGATCCTC	368-390 (AY823990)
TTSuVk2a-specific primers		
Name	**Sequence**	**Location**
TTV2-215F	ACTTCCTCTTTTAGAGTATATAAGTAAGT	215-243 (AY823991)
TTV2-360R	GATTACCGCCTGCCCGATAG	351-370 (AY823991)
TTSuVk2b-specific primers		
Name	**Sequence**	**Location**
TTSuV-all-F1	CGAATGGCTGAGTTTATGCCGC	306-327 (AY823990)
TTV3-361R	TTCGCTGTGACTGGCGTCTC	332-351 (JQ406844)
Primers for broad spectrum qPCR		
Name	**Sequence**	**Location**
TTSuV-all-F1	CGAATGGCTGAGTTTATGCCGC	306-327 (AY823990)
TTSuV-all-R4	CCTGCCCGATAGGCCCCTTG	388-407 (AY823990)

3.2.6. TTSuV broad spectrum qPCR

The TTSuV broad spectrum qPCR was designed to detect and quantify total TTSuV load (including TTSuV1, TTSuVk2a and TTSuVk2b) in serum samples. The sequence of the primers and their location in the genome are listed in Table 3.1. The PCR mixture contained: 12.5 μl iQ-SYBR Green Supermix (Bio-Rad), 10 pmol of the forward primer TTSuV-all-F1, 10 pmol of the reverse primer TTSuV-all-R4 and 2 μl of extracted DNA. The real time qPCR was performed on the CFX 96 Real-Time System (Bio-rad) and the programme started with an initial denaturation step for 5 min at 95 °C, followed by 40 cycles of 30 sec at 95 °C, 15 sec at 62 °C and 30 sec extension at 68 °C. After an extra extension step of 7 min at 68 °C, a melting curve was made by raising the temperature from 70 to 95 °C with an increment 0.5 °C per 5sec. Fluorescence data were acquired at

each extension step and during the melt curve. As standard curve a dilution series of plasmid TTV008 was used. The qPCR amplification efficiency was 102.8%; the inter-assay variation was 1.6%; the LOD was 10 copies reaction^{-1}, which corresponded to 1250 copies ml serum^{-1}.

3.2.7. Generation of PCR standard curves and viral DNA load calculation

The TTSuV1 PCR standard, TTV001, was generated by PCR amplification of a 390 bp sequence from DNA isolated from Kreaber porcine serum (9071172-G50) using primers TTV1-1F and TTV1-390R (Table 3.1). For the generation of the TTSuVk2a and broad spectrum TTSuV qPCR standard, TTV008, a 320 bp PCR product was obtained from DNA isolated from Hyclone porcine serum (FSD29672) using primers TTV2-106F and TTV2-425R (Table 3.1). Both PCR products were cloned in vector pSC-A-amp/kan following the instruction manual of the StrataClone PCR cloning kit (Stratagene). A 10-fold dilution series of plasmids TTV001 and TTV008 was used to generate PCR standard curves corresponding to $1x10^7$ - $1x10^1$ copies of DNA per reaction. To calculate the amount of TTSuVk2b DNA in sera, a 10-fold dilution series was used corresponding to $3.3x10^5$ - $3.3x10^0$ copies reaction^{-1} of plasmid 38E05 that contained the full-length sequence of clone 38E05 in vector pSC-A-amp/kan.
Finally, the average $\log_{10}$ DNA copies ml^{-1} of serum was used to compare data.

3.2.8. Statistical analyses

The correlation test was used to compare the prevalence of viruses. Normality of data was assessed using the Shapiro-Wilk test. In order to assess differences of viral loads between healthy and PCV2-SD groups for each TTSuV, Student's t-test was used for normally distributed samples (TTSuV1 and TTSuVk2b) and Mann–Whitney U test was used for non-parametric data (TTSuVk2a). All statistical analysis were made using SPSS Statistics for Windows (SPSS Inc). Significance was set at $P<0.05$ for all tests.

3.3. Results

3.3.1. A novel pig-infecting Anellovirus identified using RCA technique

Commercial sera are generally pools of serum from several pigs and therefore a useful source for detecting viruses. To determine which porcine Anelloviruses were present in commercial sera, the RCA technique with random primers was applied on extracted serum DNA. Amplicons of approximately 2.9 kb corresponding to the size of the full-length TTSuV genome were cloned resulting in 21 clones of which 11 contained a full-length TTSuV genome and 10 a partial TTSuV sequence of 2.6-2.7 kb in length.

All clones were sequenced and compared with the sequence data available in GenBank to annotate the genomes. Eleven clones had an insertion that was closely related to TTSuV1. In Table 1 these clones were further divided in four different subtypes (Cortey *et al.*, 2011). Five clones were annotated as TTSuVk2 subtypes. Five clones contained similar inserts that were identified as TTSuV sequences, but were considerably different from either TTSuV1 or TTSuVk2 sequences, suggesting that a novel TTSuV species was identified (Table 3.2).

Table 3.2 Characterization of 21 TTSuV clones derived from Hyclone Porcine Serum

		Subtype					
	Total	1a	1b	1c	1d	2a	2b
TTSuV1 (complete genome)	3	-	-	1	2	-	-
TTSuV1 (partial genome)	8	1	7	-	-	-	-
TTSuVk2 (complete genome)	5	-	-	-	-	5	-
TTSuVk2 (complete genome)	3	-	-	-	-	-	3
TTSuVk2 (partial genome)	2	-	-	-	-	-	2

3.3.2. Novel TTSuV sequences represented a new anellovirus species

The International Committee on Taxonomy of Viruses (ICTV) has classified TTVs into the family *Anelloviridae* based on the DNA sequence of the largest open reading frame, ORF1 (Biagini, 2009; Biagini *et al.*, 2011; Biagini *et al.*, 2005). According to the ICTV the ORF1

nucleotide sequence divergence cut-off values to distinguish species is >35% and >56% for genera. To further classify the novel sequences, phylogenetic and pairwise sequence comparison (PASC) analyses were performed. The mean group distance of the ORF1 sequences with TTSuV1 isolates was 63.1 (61.9-65.6%) and 41.7% (40.26-43.59%) with TTSuVk2, while the phylogenetic distance between TTSuV1 and TTSuVk2 species is 59.5% (56.6-62%) (Figure 3.1). Phylogenetic tree constructions using nucleotide sequence of full-length genomes resulted in similar tree topology as obtained with ORF1. Therefore, the unclassified viral sequences were considered to represent a novel pig anellovirus species within the genus *Kappatorquevirus*. The novel virus was provisionally designated as TTSuVk2b. Logically, and following the naming of *Iotatorqueviruses*, TTSuVk2 should be renamed as TTSuVk2a.

Next, the three obtained full length TTSuVk2b sequences, 38E05, 38E19, and 38E23, were annotated and the deduced genomic features are presented in Table 3.2. Genomes 38E19 and 38E23 were 99% identical on the nucleotide sequence level, whereas genome 38E05 was only 95-96% identical to them. The TTSuVk2a genomes contained the characteristic GC-rich region and conserved sequences in the non-coding region In addition, ORF1 and its splice variant ORF1/1, ORF2 and ORF3 were identified. The deduced aa sequences of ORF1 contained a typical arginine-rich N-terminus and nuclear localization signals. Rolling-circle replication motif III located at aa position 473 (YxxK) (Huang *et al.*, 2010b; Martínez-Guinó *et al.*, 2011; Müeller *et al.*, 2008; Mushahwar *et al.*, 1999) while motif II (HxQ) (Huang *et al.*, 2010b; Mankertz *et al.*, 2004) could not be identified. This domain has only been detected in TTSuVk2a, but not in TTSuV1 (Huang *et al.*, 2010b; Martínez-Guinó *et al.*, 2011). Motif I (FTL) described in human TTV studies (Müeller *et al.*, 2008) was not detected in any TTSuV species. TTSuVk2b-ORF2 contained a protein-tyrosine phosphatase motif (aa position 21-41) typically found in anelloviruses (Martínez-Guinó *et al.*, 2011; Müeller *et al.*, 2008). The TTSuVk2b3 ORF1, ORF1/1 and ORF3 proteins were predicted to be nuclear with more than 90% reliability, all of them containing bipartite, pat4 and pat7 nuclear localization signals (NLS). No NLS were detected in ORF2, which was predicted to be cytoplasmic with the reliability of 94%. The predicted promoter region (5'-CTTTTTAAGTATATAAGTAAGTGCACTGGCGAATGGCTGAGTTTATGCCG-3') was highly

conserved between TTSuVk2b and TTSuVk2a. The splicing site for ORF1/1 and ORF3 was similar to the one described in TTSuVk2a ORF1 (Martínez-Guinó *et al.*, 2011). However, other splice sites mapped for TTSuVk2a in transfection experiments (Martínez-Guinó *et al.*, 2011) could not be annotated for TTSuVk2b.

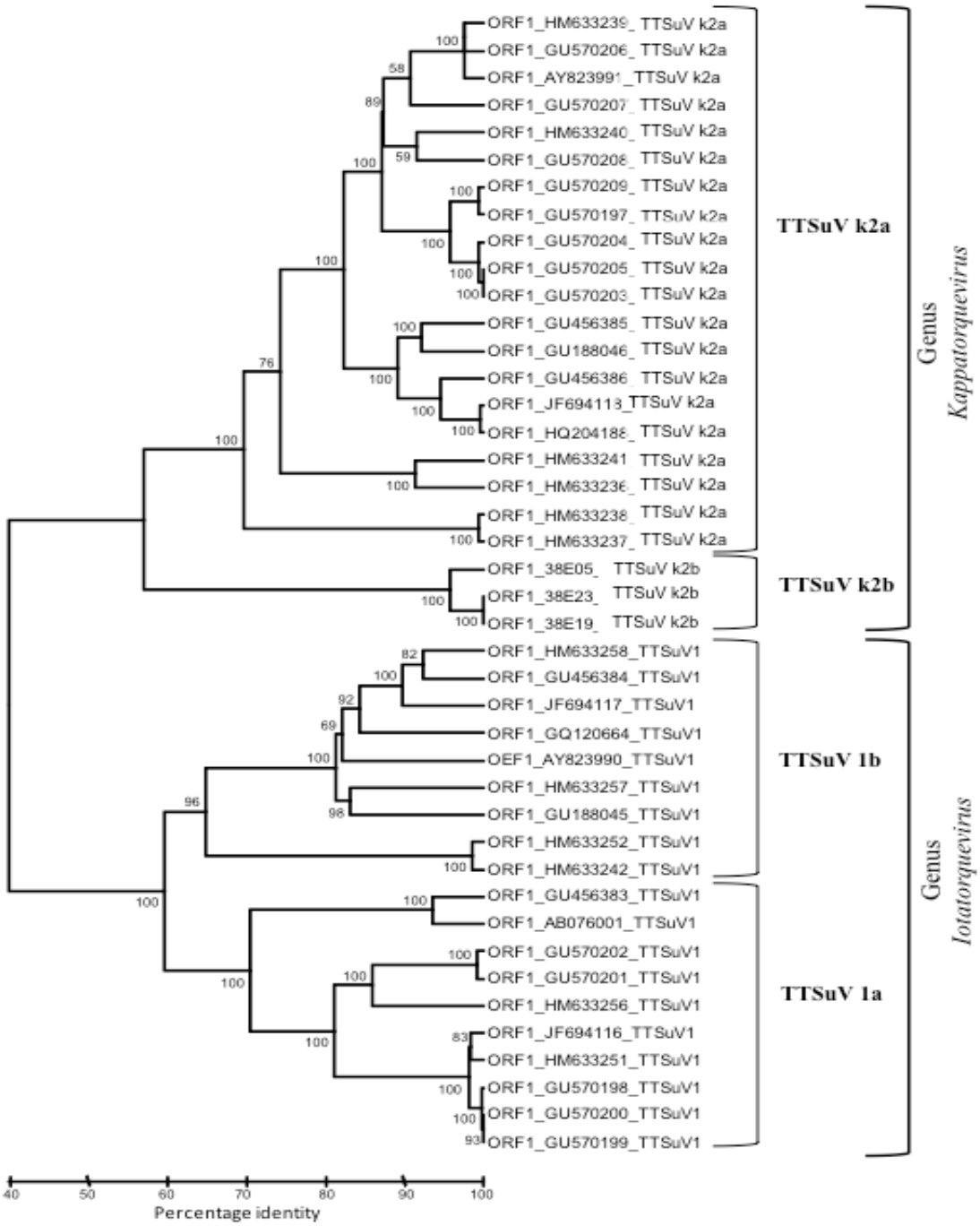

Figure.3.1. Neighbour-joining phylogenetic tree based on the percentage identity of TTSuV ORF1 sequence available in GenBank and from this study. Confidence bootstrap values higher than 50 % are shown at the nodes.

Table 3.3. Summary of the TTSuV genomic features. Genome and ORF lengths in base pairs are shown. Data for TTSuV1 and k2 were taken from previous studies (Cortey *et al.*, 2011;Martínez-Guinó *et al.*, 2011).

	Species		
	TTSuV1	**TTSuVk2a**	**TTSuVk2b**
Genomic length	2863-2913	2735-2803	2899-2901
ORF1 nt (aa)	1914-1950 (638-650)	1875-1884 (625-628)	1890-1893 (629-630)
ORF1/1 nt (aa)	550 (181)	535 (176)	577 (188)
ORF2 nt (aa)	219-222 (73-74)	207 (69)	243 (81)
ORF3 nt (aa)	663-696 (221-232)	600-609 (200-203)	673 (224)

3.3.3. TTSuV species from different origins are found in commercial porcine sera of different origin

Quantitative broad spectrum and species specific qPCR techniques were applied to screen seven commercial sera originating from either USA, New Zealand or Germany for the presence of TTSuVs (Table 3.3). All tested commercial sera, which are pools from a large number of pigs, contained each of the three viral species. The amount of total TTSuV varied between 5.93 and 6.65 $\log_{10}$ DNA copies ml^{-1} serum. In each batch a similar pattern of prevalence of the three TTSuVs was found and the prevalence of TTSuVk2b was much lower than that of the other two viruses. This can be confidently concluded since the three species-specific qPCRs (TTSuV1, TTSuVk2a and k2b) had the same limit of detection and very similar amplification efficiencies (see 3.2 section). The relative percentage of TTSuVk2b detected was 0.7-2.1%, while those of TTSuV1 and TTSuVk2a were 18.0-55.3% and 42.8-81.4%, respectively (Table 3.3)

Table 3.4. TTSuV loads (log_{10} DNA copies ml^{-1} serum) in commercial batches of porcine serum determined by the broad spectrum and TTSuV-specific qPCRs. The percentage of TTSuV species is indicated between brackets.

Porcine serum	Broad spectrum	TTSuV1	TTSuVk2a	TTSuVk2b
Hyclone 0000066666	6.48	5.84 (20.8)	6.41 (77.2)	4.84 (2.1)
Sigma 026k8453	6.39	5.81 (24.6)	6.28 (73.6)	4.69 (1.9)
Sigma 064k8451	6.08	5.98 (55.3)	5.87 (42.8)	4.52 (1.9)
Kreaber 9050957-G50	6.65	5.92 (20.1)	6.52 (79.3)	4.45 (0.7)
Kreaber 9071172-G50	6.47	5.66 (18.0)	6.32 (81.4)	4.23 (1.7)
Kreaber SS02/06-1	6.09	5.63 (27.2)	6.04 (71.1)	4.41 (1.7)
Kreaber SS03/06	5.93	5.70 (42.6)	5.81 (55.2)	4.40 (2.1)
Average	6.30	5.79 (26.3)	6.18 (72.2)	4.50 (1.4)

3.3.4. Global prevalence and viral DNA loads of TTSuV species in conventional pigs

Once the presence of TTSuVk2b3 in commercial pools of porcine sera was confirmed we aimed to determine the global distribution of it and its relation to the already well characterized TTSuV1 and k2a (Cortey *et al.*, 2012; McKeown *et al.*, 2004). For this a total of 244 sera samples collected from farms located in 17 different countries were analysed by the different qPCRs.

In general, the total prevalence of TTSuV was 92.6 (226/244) and 74.6 % for TTSuV1 (182/244), 61.1 % for TTSuVk2a (149/244), and 41.0 % for TTSuVk2b (100/244) (Table 3.5). While TTSuV1 could be detected in sera from all countries analysed, TTSuVk2a was not found in the samples originating from Brazil and Vietnam, and TTSuVk2b was not present in sera from Brazil, Ukraine and Vietnam (Figure.3.2 and Table 3.5). TTSuVk2a and TTSuVk2b global prevalences were correlated ($P<0.05$), while the prevalence of TTSuV1 did not correlate with that of the other two.

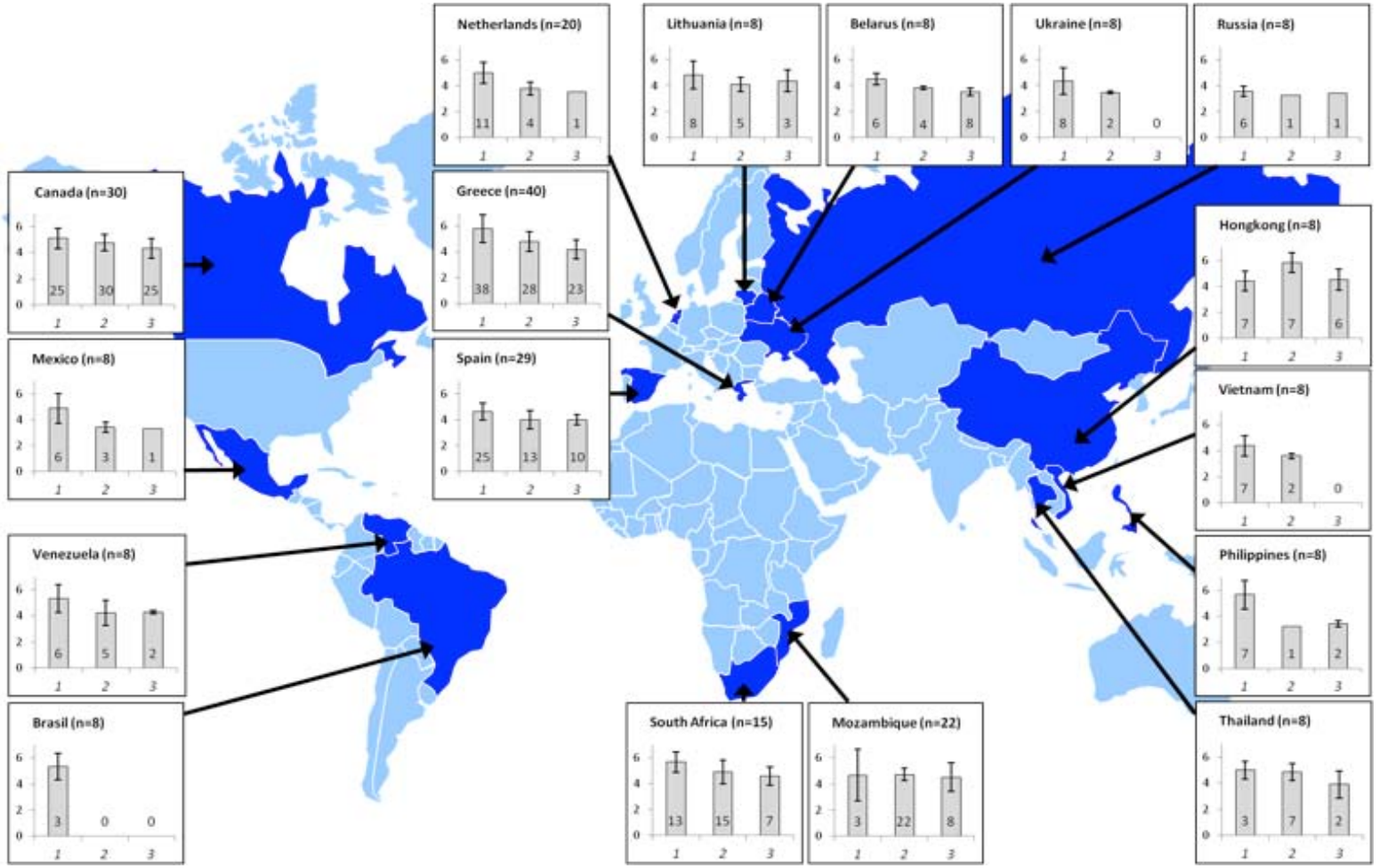

Figure 3.2. TTSuV loads and prevalences in pig sera collected from pig farms from several countries. TTSuV1, TTSuVk2a and TTSuVk2b $\log_{10}$ DNA copies ml-1 serum and error bars represent SD. Total number of analysed sera is shown after the name of the country and number of positive samples in the corresponding bar

There was in general a very good correlation between the total viral loads determined by the broad spectrum qPCR and the sum of the viral loads determined by the TTSuVq-TTSuVk2a- and TTSuVk2b-specific qPCRs: of 224 samples analysed, only 21 showed a greater than fivefold difference (data not shown). In three cases the broad spectrum qPCR detected TTSuV, while the specific qPCRs failed to detect TTSuV species. It was attempted by RCA to isolate TTSuV from the corresponding three sera, but this was unsuccessful probably due to the low levels of TTSuV in these sera.

Table 3.5 TTSuV prevalence (positives samples/total number of samples) in pig sera from 17 different countries determined by qPCR. Percentage of the positive sera is shown in parenthesis.

Country	TTSuV1	TTSuVk2a	TTSuVk2b	Borad spectrum
Belarus	6/8 (75.0)	4/8 (50.0)	8/8 (100)	8/8 (100)
Brazil	3/8 (37.5)	0/8 (0.0)	0/8 (0.0)	3/8 (0.0)
Canada	25/30 (83.3)	30/30 (100)	25/30 (83.3)	30/30 (83.3)
Greece	38/40 (95.0)	28/40 (70.0)	23/40 (57.5)	40/40 (57.5)
China	7/8 (87.5)	7/8 (87.5)	6/8 (75.0)	8/8 (75.0)
Lithuania	8/8 (100)	5/8 (62.5)	3/8 (37.5)	8/8 (37.5)
Mexico	6/8 (75.0)	3/8 (37.5)	1/8 (12.5)	5/8 (12.5)
Mozambique	3/22 (13.6)	22/22 (100)	9/22 (40.9)	22/22 (40.9)
Netherlands	11/20 (55.0)	4/20 (20.0)	1/20 (5.0)	18/20 (5.0)
Philippines	7/8 (87.5)	1/8 (12.5)	2/8 (25.0)	7/8 (25.0)
Russia	6/8 (75.0)	1/8 (12.5)	1/8 (12.5)	5/8 (12.5)
South Africa	13/15 (86.6)	15/15 (100)	7/15 (46.6)	15/15 (46.6)
Spain	25/29 (86.2)	13/29 (44.8)	10/29 (34.4)	27/29 (34.4)
Thailand	3/8 (37.5)	7/8 (87.5)	2/8 (25.0)	8/8 (25.0)
Ukraine	8/8 (100)	2/8 (25.0)	0/8 (0.0)	7/8 (0.0)
Venezuela	6/8 (75.0)	5/8 (62.5)	2/8 (25.0)	8/8 (25.0)
Vietnam	7/8 (87.5)	2/8 (25.0)	0/8(0.0)	7/8(0.0)
Total	182/244 (74.6)	149/244 (61.1)	100/244 (41.0)	226/244 (92.6)

Viral DNA loads were in the range of 3.12 to 7.90 (average 5.79) log_{10} DNA copies ml^{-1} serum for TTSuV1, 3.18 to 8.41 (average 4.58) log_{10} DNA copies ml^{-1} serum for TTSuVk2a, and 3.13 to 6.87 (average 4.14) log_{10} DNA copies ml^{-1} serum for TTSuVk2b in conventional pigs. There was no correlation of viral loads among the three TTSuV species. The average TTSuV1 and TTSuVk2b loads in sera of individual pigs were similar to the loads observed in commercial sera (Table.3.3). However, the average TTSuVk2a loads in individual sera were approximately 1.5 log_{10} lower than that in commercial sera.

3.3.5. High TTSuVk2b loads in serum of PCV2-SD-affected pigs

TTSuVk2a has been associated in previous studies with porcine circovirus associated diseases (Huang *et al.*, 2011) and especially to PCV2-SD occurrence (Aramouni *et al.*, 2011; Nieto *et al.*, 2011). Considering the molecular similarity of TTSuVk2a with TTSuVk2b, and the correlation of their prevalence in geographical samples, the possible association of TTSuVk2b with PCV2-SD was explored. TTSuV1, k2a and k2b viral DNA loads were measured by the three TTSuV-specific qPCRs in 34 serum samples of PCV2-SD-affected pigs and 29 healthy control animals. The average TTSuVk2a and TTSuVk2b loads were significantly higher ($P<0.05$) in PCV2-SD-affected pigs than in healthy animals (Figure 3.2). Such difference was not observed for TTSuV1.

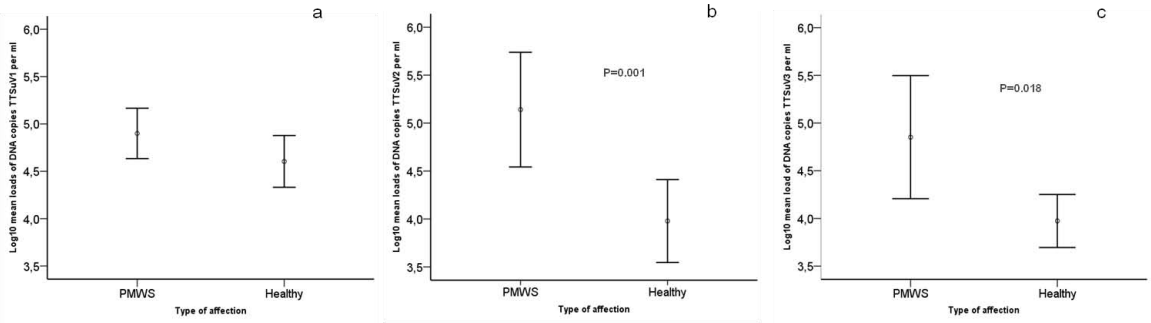

Figure 3.3. TTSuV1 (a), TTSuVk2a (b) and TTSuVk2b (c) log $_{10}$ mean viral DNA loads in healthy and PCV2-SD-affected animals P-values are shown.

3.4. Discussion

Three species were so far known to infect pigs before the present study, namely TTSuV1a and 1b within the genus *Iotatorquevirus* and TTSuVk2a within the genus *Kappatorquevirus*. Here, a novel anellovirus was identified in porcine sera, which, according to the degree of divergence with already known TTSuVs is sufficient to warrant classification of this virus as a new species in the genus *Kappatorquevirus*. Considering the current taxonomic organization of anelloviruses, this novel virus species was tentatively named TTSuVk2b.

Although there have been attempts to discover novel pig anelloviruses (Macera *et al.*, 2011), TTSuVk2b was not detected before. Primers used in published TTSuV1- or TTSuVk2a-specific PCRs (Brassard *et al.*, 2010; Gallei *et al.*, 2010; Huang *et al.*, 2010a; Nieto *et al.*, 2011) are unlikely to amplify TTSuVk2b given the mismatch of these primers with TTSuVk2b sequences. Furthermore, sequencing of these amplicons is necessary to identify possible new species.

Different qPCR techniques were developed in this study. The wide spectrum qPCR was shown to be very useful in determining the overall TTSuV load (TTSuV1, TTSuVk2a and TTSuVk2b) in pig serum. This technique could be useful for diagnostic purposes and, when necessary, the species-specific qPCRs can be useful to determine the loads of each TTSuV species.

Bioinformatics analysis of full length TTSuVk2b sequences showed that these viruses are rather distinct from, already known pig anelloviruses. However, a high similarity with TTSuVk2a was observed on genomic regions like splice sites and promoter elements (Martínez-Guinó *et al.*, 2011). These predictions may not reflect the reality, sine a short intron of 91 bp could not be predicted from any genome of TTSuVk2 but has been shown to exist in genomes of TTSUVk2a by in vitro transfection experiments(Huang *et al.*, 2012b; Martínez-Guinó *et al.*, 2011), and therefore further experiments are necessary to characterize TTSuVk2b transcription and protein expression strategies. The relatedness to TTSuVk2a was supported by phylogenetic analysis that grouped TTSuVk2b closer to TTSuVk2a than TTSuV1. Considering the current criteria by the ICTV, TTSuVk2b would be

the second species in the genus *Kappatorquevirus*

In the epidemiological study, TTSuVk2b infection was confirmed in pigs from most of the countries. However, for some countries where TTSuVk2b appeared to be absent the number of serum samples analysed was low, so in order to conclude the presence of TTSuVk2b in these countries, more samples and farms should be analysed. There is also a possibility that the TTSuVk2b-specific primers do not detect all TTSuVk2b variants, since the primer binding sites all TTSuVk2b variants, since the primer binding sites were determined based only on the five TTSuVk2b sequences that were obtained in this study (see Table 3.2). Therefore, more viral genomic sequences should be obtained to confirm that the annealing sites of the used primers are highly conserved. Considering the global trading and its influence on the distribution of TTSuVs (Cortey *et al.*, 2012), it is likely that each TTSuV species would be found in any given country.

Overall, it was seen for each TTSuV that the higher the prevalence the higher the viral load in serum. It is well documented that TTSuV1 and TTSuVk2 viral loads and prevalences increase with the age of the animals (Aramouni *et al.*, 2010; Nieto *et al.*, 2011; Xiao *et al.*, 2012). This is probably due to the efficient viral transmission by vertical and horizontal routes and the persistent nature of TTSuV infection (Martínez-Guinó *et al.*, 2009; Pozzuto *et al.*, 2009) in pig and wild boar (Martínez *et al.*, 2006). Therefore, the observed differences between countries in TTSuV prevalence and viral loads may reflect the age of the tested animals, which was not known in this study.

Due to the genetic similarity between TTSuVk2a and TTSuVk2b and the reported association of TTSuVk2a, but not TTSuV1, with PCV2-SD (Aramouni *et al.*, 2011; Kekarainen *et al.*, 2006; Nieto *et al.*, 2011), it was also assessed whether TTSuVk2b was also associated with this disease. Indeed, both TTSuVk2a and TTSuVk2b DNA loads were significantly higher in PCV2-SD-affected animals compared to healthy counterparts. No significant difference was seen in the case of TTSuV1, which confirmed previous findings. It has to be mentioned that the serum samples of PCV2-SD and healthy animals tested in this study were the same as analysed before with different qPCR methods (Aramouni *et al.*, 2011; Kekarainen *et al.*, 2006; Nieto *et al.*, 2011). However, in this study the samples

were analysed with a SYBR Green-based qPCR technique, while in the earlier studies Light Upon Extension (LUX) qPCRs were used. With both assays association of TTSuVk2a loads with PCV2-SD was confirmed, but the viral loads were quantified about 10-fold higher with the LUX assay. This is not surprising considering that the techniques use different quantification methods and primer pairs.

With the description here of a novel TTSuV species, provisionally named TTSuVk2b, this group of pig-infecting viruses has become even more intriguing. These viruses are ubiquitous in pig and it has been demonstrated that the three species, TTSuV1, TTSuVk2a, and -TTSuVk2b, are often found with high loads in serum of the same animal. This may be due to lack of cross-protection between species. Indeed, antigenic cross-reactivity has been demonstrated to exist between different genotypes (aa identity about 50%) but are lacking between species (aa identity about 25%) (Huang *et al.*, 2012a). The aa identity of ORF1 between TTSuVk2a and TTSuVk2b is about 58.3% and therefore cross-reactivity is likely to exist between these species. To get further insight into the possible cross protection, the infection dynamics of and antibody development against TTSuVk2a and TTSuVk2b should be investigated using longitudinal serum samples.

Since TTSuVs are known to cause persistent infections, important questions are raised about the role of these viruses in modulating the immune status of the animals. Although considered non-pathogenic by themselves, TTSuV is often present in pigs with diseases like PCV2-SD, PRDS (Rammohan *et al.*, 2012), and PDNS (Aramouni *et al.*, 2011; Krakowka *et al.*, 2008). Studies are undertaken in our laboratory to further address the disease association of TTSuV.

4. Study II

Expression of and pig immunization with *Torque teno sus virus* proteins

4.1. Introduction

Anelloviruses are species-specific and the ones infecting swine are classified into four species, namely *Torque teno sus virus* 1a and 1b (TTSuV1a, TTSuV1b; genus *Iotatorquevirus*), and TTSuVk2a and TTSuVk2b (described study I) (genus *Kappatorquevirus*). These genera are genetically very distinct (>56% sequence diversity), but share similar genome organization and gene expression strategy (Martínez-Guinó *et al.*, 2011). TTSuV infection in pigs is distributed worldwide and characterized by a persistent viremia with high prevalence of TTSuV1 and TTSuVk2a species (Sibila *et al.*, 2009a) while prevalence of TTSuVk2b is lower (according study I). TTSuVs have been found in serum, plasma, faeces, semen, colostrum and nasal cavity, suggesting the transmission can be both vertical and horizontal (Kekarainen & Segalés, 2012a). A single pig can be co-infected with different TTSuV species or subtypes such as confirmed in study I and previous reported (Gallei *et al.*, 2010; Huang *et al.*, 2010b)

The length of the TTSuV genome is approximately 2.8 kb (Okamoto *et al.*, 2002), consisting of at least four putative open reading frames (ORF), namely ORF1, ORF1-A (also known as ORF1/1), ORF2, and ORF3 (also known as ORF2/2) (Huang *et al.*, 2012b; Lu *et al.*, 2013; Martínez-Guinó *et al.*, 2011). ORF1 gene encodes the largest protein (~75 kDa) with a predicted viral capsid domain in the N-terminal half of the protein and a putative replication-associated domain in its C-terminal half. ORF2 gene encodes a non-structural protein, ORF2 protein, assumed to be involved in viral replication (Hijikata *et al.*, 1999; Huang *et al.*, 2012b). ORF3 can only be translated after splicing of a mRNA precursor (Huang *et al.*, 2012b; Martínez-Guinó *et al.*, 2011). The ORF3 protein (~25 kDa) shares its N-terminus with the ORF2 protein (~8 kDa) and its role is unknown at present. The predominant transcripts (i.e. mRNA) detected *in vitro* and *in vivo* of ORF1 gene are spliced and full-length ORF1 transcripts have not been detected so far (Huang *et al.*, 2012b; Martínez-Guinó *et al.*, 2011). Splicing of TTSuV1 ORF1 gene results in two protein isoforms (ORF1-A and -B) of different sizes (~20 and ~15 kDa, respectively) (Martínez-Guinó *et al.*, 2011). In contrast, splicing of TTSuVk2a ORF1 gene results in three protein isoforms (ORF1-A, -B, and -C) of different sizes (~20, ~15 and ~11 kDa, respectively); the

aa composition between them varies, depending on the splicing site used (Huang *et al.*, 2012b; Martínez-Guinó *et al.*, 2011).

TTSuVs persistently infect a high proportion of animals that are apparently healthy (Sibila *et al.*, 2009b). TTSuV infection by itself does not cause immediate disease but it is believed that TTSuVs influence the development of some economically important viral diseases or even affect their outcome. Evidence for TTSuV disease association has been accumulated, especially with regard to *Porcine circovirus type 2* (PCV2) infection and its associated conditions, namely PCVDs (Aramouni *et al.*, 2011; Ellis *et al.*, 2008; Kekarainen *et al.*, 2006; Nieto *et al.*, 2011).

Although TTSuVs are currently considered non-pathogenic, it is hard to believe that a viral infection characterized by high viral loads and virus persistence would not have any consequence to its host. Indeed, farm performance is improved when PCV2 (also a single stranded circular DNA virus) infection is controlled by vaccination even in farms without disease, displaying only a subclinical infection (Beach & Meng, 2012).

This study had two main objectives. On one hand, the four TTSuV proteins for all known TTSuV species (i.e. TTSuV1a, 1b, k2a, and k2b) were expressed in a baculovirus system. On the other hand, a multivalent vaccine prototype was designed against currently circulating TTSuV species to be used to control viremia in naturally infected pigs. For this latter purpose, the vaccine was designed containing baculovirus expressed ORF1 and ORF3 proteins of the four known TTSuV species. Subsequently, the vaccine prototype efficacy was evaluated by immunizing pigs; humoral immunity and viremia during natural TTSuV infection were monitored by ELISA and species-specific quantitative PCRs (qPCR), respectively. In addition, specific antibodies against the multivalent vaccine prototype where followed by indirect ELISA.

4.2. Material and methods

4.2.1. TTSuV protein selection for expression in a baculovirus expression system

A selection of available ORF1, ORF1-A, ORF2 and ORF3 sequences of the four TTSuV species were downloaded from GenBank and aligned using MEGA version 5 (Tamura *et al.*, 2011). Representative sequences from each of the 4 TTSuV groups were selected (Table 4.1), which conserve a nucleotide sequence of each gene with the majority of the sequence analysed for each species. Clones containing each gene in the pFastBac/CT-TOPO vector were ordered from GenScript. All genes contained a Strep-tag coding sequence at their 3'end with the exception of the ORF1 constructs of TTSuV1b and TTSuVk2a, which were already available in our laboratory with a 6xHis-tag sequence. As a control in expression experiments, a GFP gene containing a Strep-tag at its 3' end in pFastBac/CT-TOPO was ordered from GenScript (Table 4.1).

4.2.2. Production of TTSuV baculovirus stocks

Baculovirus production was done according to the manufacturer's instructions (Invitrogen). Briefly, the pFastBac constructs were transformed into *Escherichia (E.) coli* DH10BacΔcc (Kaba *et al.*, 2004). PCR was used to confirm the correctness of recombinant Bacmids. Bacmid DNA was subsequently isolated from *E.coli* cultures and used to transfect *Spodoptera frugiperda* (Sf9) insect cells using Cellfectin II reagent (Invitrogen). The cultures were incubated at 27 °C for 5 days, and supernatants were harvested as the first passage (P1) viral stocks. P1 stocks were used to infect insect cell cultures at a multiplicity of infection (MOI) of 0.1 to obtain high virus titre stocks (P2). To quantify the viral stock titre, TTSuV baculovirus stocks were added at 10-fold serial dilutions to Sf9 cells in 96-well microtitre plates and after 6 days incubation at 27°C, an immunofluorescence assay (IFA) was performed to detect the baculoviruses.

Table 4.1 Overview of the generated Bacmids, the size of the TTSuV insert and corresponding proteins when expressed in Sf9 cells. The size of the PCR fragment to confirm the correct insertion of the TTSuV gene into the Bacmid is also indicated. Control bacmids, dccGFP and dcc_empty, or also listed. GenBank accession numbers for each TTSuV are indicated.

	Name construct	Accession numbers	Gene	PCR product (DNA Bacmid)	Insert (nt)	Protein (aa)
TTSuV1a	dccTTSuV1a_ORF1	GU570202	ORF1	4291	1991	73
	dccTTSuV1a_ORF1-1	GU570202	ORF1-1	2896	596	22
	dccTTSuV1a_ORF2	GU570202	ORF2	2563	263	10
	dccTTSuV1a_ORF3	GU570202	ORF3	3040	740	27
TTSuV1b	dccTTSuV1b_ORF1	AY823990	ORF1	4213	1913	70
	dccTTSuV1b_ORF1-1	AY823990	ORF1-1	2893	593	22
	dccTTSuV1b_ORF2	AY823990	ORF2	2563	263	10
	dccTTSuV1b_ORF3	AY823990	ORF3	3031	731	27
TTSuVk2a	dccTTSuVk2a_ORF1	AY823991	ORF1	4174	1874	69
	dccTTSuVk2a_ORF1-1	GU570205	ORF1-1	2890	590	22
	dccTTSuVk2a_ORF2	GU570205	ORF2	2551	251	9
	dccTTSuVk2a_ORF3	GU570205	ORF3	2953	653	24
TTSuVk2b	dccTTSuVk2b_ORF1	JQ406845	ORF1	4237	1937	71
	dccTTSuVk2b_ORF1-1	JQ406845	ORF1-1	2911	611	22
	dccTTSuVk2b_ORF2	JQ406845	ORF2	2587	287	11
	dccTTSuVk2b_ORF3	JQ406845	ORF3	3019	719	26
	dcc_GFP		GFP	3064	764	25
	dcc_empty		-	2300	-	-

For IFA procedure, Sf9 cells were fixed with 4 % paraformaldehyde for 10 min at room temperature. Next, cells were washed three times with 1x phosphate buffered saline (1x PBS) and permeabilized with 0.5 % Triton X-100 in 1x PBS for 15 min at room temperature and finally washed with 1x PBS for 5 min. Then, the cells were incubated at 37ºC for 1 h with anti-Strep (Qiagen) or anti-His (in house; INT-HIS-O1-O3) monoclonal antibodies diluted 1:1000 in 1x PBS. Then, the cells were washed three times with sterile water and incubated with a FITC-conjugated goat anti-mouse IgG (whole molecule) antibody (Sigma) diluted 1:100 in 1x PBS with Evans Blue at 37ºC for 1 h. Finally, cells were covered with 1x PBS/glycerol (1:1) before observing with a fluorescence microscope at a wavelength of 488 nm.

4.2.3. Expression of TTSuV proteins in insect cells

ORF1, ORF1-A, ORF2, and ORF3 baculovirus stocks were used to infect Sf9 insect cells at a MOI of 0.1 to produce TTSuV recombinant proteins. In addition, a GFP and an empty baculovirus were used as controls. The cultures were incubated at 27ºC and harvested at 4 days post infection (dpi) and then centrifuged at 1,000xg for 5 min at 4ºC. The resulting supernatants and cell pellets were analysed by Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. For this, samples were diluted 1:1 in 2x Laemmli sample buffer (Bio-Rad), heated to 95°C for 5 min and loaded onto 4-15% precasted polyacrylamide gels (Criterion TGX, Bio-Rad) followed by SDS-PAGE. Coomassie blue dye (Bio-Rad) was used to stain the proteins present in the SDS-PAGE gels. Alternatively, the gels were not stained and proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad), which were blocked in 1% non-fat milk in TBS-T (Tris-buffered saline, 0.1% Tween20) blocking buffer. PVDF membranes were then incubated with anti-Strep (Qiagen) or anti-His (in house; INT-HIS-O1-O3) antibody, both diluted to 1:1000 in blocking buffer at room temperature for 1 h. Next, membranes were incubated with a horseradish peroxidase conjugated goat anti-mouse antibody (Nordic) at a 1:1000 dilution in blocking buffer at room temperature for 1 h. Peroxidase (Vector Laboratories) was used as substrate and colour development was stopped by rinsing the membranes with water and air drying.

4.2.4. Purification of the recombinant ORF1, ORF2, ORF1-A and ORF3 proteins

Pelleted baculovirus-infected insect cells were used to prepare crude cellular lysates. Solubility tests were performed to find the optimal lysis buffer for each expressed protein. All lysis buffers were supplemented with a protease inhibitor cocktail (Complete, EDTA-free; Roche). The optimal lysis buffer for the ORF1 protein was Buffer 5O, pH 8 (50 mM Na_2HPO_4, 600 mM NaCl, 0.05% Tween 20, 1 U/µl Benzonase). For the ORF2 protein it was Buffer NP-T, pH8 (50 mM NaH_2PO_4, 300 mM NaCl, 0.05% Tween 20, 1 U/µl Benzonase) and finally for the ORF1-A and ORF3 proteins it was Buffer 4O, pH 8 (50 mM Na_2HPO_4, 300 mM NaCl, 10% glycerol, 1 U/µl Benzonase). After lysis, suspensions were sonicated three times on ice at 1 min cycles with 10 s intervals at 20% amplitude. The

sonicated lysates were centrifuged at 16,000xg at 4ºC for 10 min. Resulting supernatants were used to purify the proteins of interest.

Strep- and His-tagged recombinant TTSuV ORF1 proteins were purified using the sedimentation coefficient (s) by Beckman Coulter ultracentrifugation. The ultracentrifugation was done using the SW41Ti rotor with a K factor of 123.6 at maximum speed (41,000 rpm) at 4ºC. In total, 3 steps were used and the step(s) that yielded the best protein yield in the pellet and highest purity was/were selected: s200: 37min, s100: 1h 14min, and s50: 2h 28min. Equilibration buffer (250 mM $MgCl_2$ and 50 mM HEPES) was used to adjust volume up to 12 ml.

Strep-tagged recombinant TTSuV ORF2 proteins were purified using Strep-Tactin Magnetic beads (Qiagen) according to the manufacture's protocol using washing buffer Buffer NP-T, pH 8 (without protease inhibitor and Benzonase) and elution buffer NPB-T (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM biotin and 0.05% Tween 20) at pH 8. Strep-tagged recombinant TTSuV ORF1-A and ORF3 proteins were purified using Strep-Tactin Magnetic beads (Qiagen) according to the recommended protocol using washing buffer 4O, pH 8 (without protease inhibitor and Benzonase) and elution buffer Buffer 4-B, pH 8 (50 mM Na_2HPO_4, 300 mM NaCl, 10 mM biotin, 10% glycerol). Purified proteins were analysed by SDS-PAGE and Western blotting as described above.

4.2.5. Pig immunization with multivalent TTSuV vaccine

A multivalent vaccine prototype was designed including His- and Strep-tagged recombinant TTSuV ORF1 and Strep-tagged ORF3 proteins produced as described above. However, Strep-Tactin Superflow Plus (Qiagen) was used to purify the Strep-tagged ORF3 protein following the recommended protocol (with the same changes to protocol as above: Buffer 4O, pH8 used in the wash step and Buffer 4O-B, pH8 used in the elution step). Recombinant baculoviruses present in the purified protein preparations were inactivated by binary ethylenimine (BEI) inactivation using a treatment of 30 mM BEI for the ORF1 preparation and 5 mM for the ORF3 preparation. Protein concentrations were

estimated by SDS-PAGeS and comparison of band intensities to that of a Bovine Serum Albumin (BSA) standard. Preparation of diluted BSA standards was done following the manufacturer Pierce™ BCA Protein Assay Kit (Life Technologies). To prepare the multivalent vaccine, ORF1 and ORF3 purified proteins of each TTSuV species were pooled and mixed 1:1 with an oil-in-water adjuvant (X-Solve, MSD Animal Health). Intramuscular (IM) and intradermal (ID) vaccines contained in total 20 µg of protein per dose. The volume per injection for IM administration was 2 ml and 0.2 ml for ID administration. The first administration was given in the right side of the neck, alternating left and right sides in subsequent immunizations.

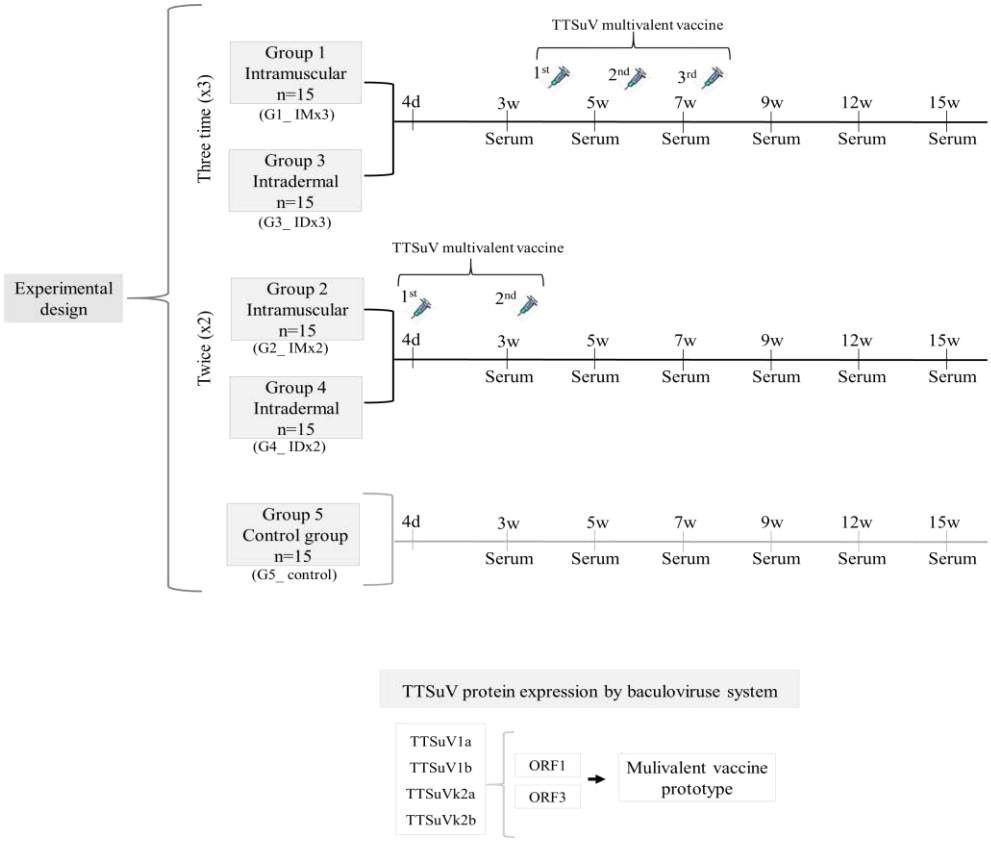

Figure 4.1. Experimental design of the animal trial. The administration route, intramuscular (IM) or intradermal (ID), and the vaccination schedule (twice or three times) are shown. Control group did not receive the multivalent vaccine. Date of serum collection is also indicated.

An overview of the experimental design of the animal trial is seen in Figure 4.1. Seventy-five (75) 4 day-old piglets (Large White x Landrace x Duroc) were selected from a conventional Dutch farm, randomized and divided into five groups (n=15 per group). The farm was selected based on the prevalence of TTSuV species (Figure 4.2) determined by species-specific qPCRs performed on sera from piglets and sows (described in study I). Pigs were immunized using the developed multivalent vaccine prototype. Intramuscular (IM; groups 1 and 2) and intradermal (ID; groups 3 and 4) routes of administration were tested based on the vaccination schedule shown in Figure 4.1. Thus, animals in groups 1 (IMx3) and 3 (IDx3) were vaccinated three times at 3, 5, and 7 weeks of age. Groups 2 (IMx2) and 4 (IDx2) received the multivalent vaccine twice, the first administration at 4 days and the second at 3 weeks of age. In addition, unvaccinated pigs were used as control animals (group 5). Animals from different groups were housed together throughout the study. Treatment, housing and husbandry conditions conformed to the guidelines of the European Union for animal welfare and the study was performed according to Good Experimental Practices. Serum samples were collected when the animals were 3 weeks of age and every two weeks thereafter until the pigs reached 15 weeks of age. During the study, 3 animals died, being 1 animal from group 2 at 12 weeks of age and two animals from group 4, one at 3 and the other at 12 weeks of age.

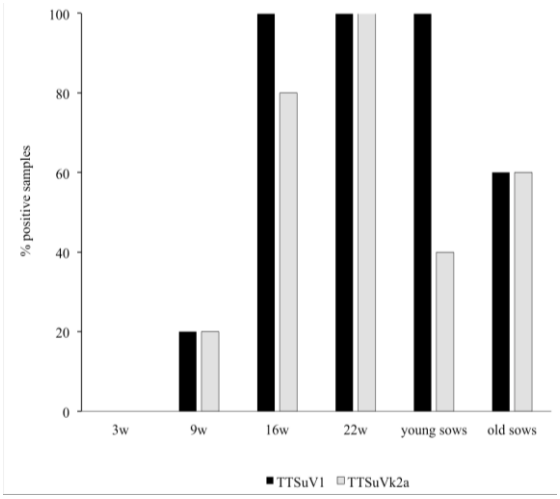

Figure 4.2. Dynamics of TTSuV infection in the selected farm where the animal trial was carried out. TTSuV species were determined by species-specific qPCRs performed on sera from piglets at different weeks of age and sows. The figure shows the percentage of positive sample of TTSuV1 and TTSuVk2a. In total, five animals per each group of age were tested.

4.2.6. Determination of viral DNA load

TTSuV DNA was extracted from 200 µl of sera using the MagNA Pure 96 DNA and Viral Nucleic Acid Small Volume Kit (Roche Diagnostic) and using the Viral NA Universal SV 2.0 protocol. Next, TTSuV1 (detecting both species a and b), TTSuVk2a and TTSuVk2b DNA loads were determined by species-specific qPCRs as described in the study I.

4.2.7. Antibody detection by ELISA and Western Blot analysis.

Seroconversion of animals was tested by ELISA using purified ORF1 or ORF3 TTSuV1a and k2a baculovirus proteins (the same ones included in the vaccine) as antigens. In addition, purified ORF1-A or ORF2 TTSuV1a and k2a proteins generated by the *E.coli* expression system (Nieto *et al.*, 2015) were used as antigens in a second ELISA also to detect TTSuV-specific antibodies.

For both ELISA methods (based on baculovirus and *E. coli* proteins), low binding 96-well microtitre plates were coated with 50 µl per well containing 680 ng/ml protein in carbonate-bicarbonate buffer pH 9.6. After overnight incubation at 4ºC, plates were washed 3 times with TBS-T and then blocked with blocking buffer (1x phosphate-buffered saline, 1% casein, 0.05% Tween 20). After incubation at 37°C for 1 h, each well was washed 3 times with TBS-T. Serum samples were diluted 1:200 in blocking buffer and 50 µl was transferred to the corresponding well. After incubation at 37ºC for 2 h, plates were washed 3 times with TBS-T. Then 50 µl per well the diluted HRP-conjugated anti-swine whole IgG (1:40,000 in blocking buffer; Sigma) was added and plates were incubated at 37ºC for 1 h. Finally, 50 µl of TMB substrate (3,3,5,5-Tetramethylbenzidine liquid, supersensitive, Sigma) was added and the plates were incubated in the dark at room temperature until the OD_{650} of the negative controls reached about 0.05. At this time-point the reaction was stopped by adding 50 µl/well of 2M H_2SO_4, and the OD_{450} of each sample was determined. All serum samples were run in duplicate. Each plate contained one positive and one negative control serum.

The cut-off value of the ELISA based on baculovirus proteins was calculated as the mean OD at 450 nm of the serum samples from control animals plus three times the standard deviation, and the positive cut-off value was set at 0.2 for ORF3 and 0.6 for ORF1. For the ELISA based on *E. coli* proteins, the cut-off value was calculated as the mean OD at 450 nm of the negative sample from each ELISA plate plus three times the standard deviation. The relative optical density was calculated by dividing the mean OD of a sample by the mean OD of the negative control, and the positive cut-off value was set at 2.

A Western blot assay was used to confirm the seroconversion results obtained by ELISA based on baculovirus proteins. For this, purified TTSuV1a or TTSuVk2a ORF1 and ORF3 proteins were run under denaturing conditions in a NuPageNovex 4-12% Bis-Tris polyacrylamide Gel (Invitrogen) and subsequently transferred to Hybond ECL nitrocellulose membranes (GE Healthcare). The membranes were blocked with blocking buffer (1x phosphate-buffered saline, 2% casein, 0.05% Tween 20) and incubated with porcine sera diluted 1:100 in blocking buffer for 1 h at room temperature, followed by addition of anti-swine whole IgG (Sigma) at a 1:150,000 dilution in blocking buffer for 1 h at room temperature. Captured antibodies were detected by Western Lightning ultra (PerkinElmer) and visualized by using a FluoroChem HD2 chemiluminescent workstation (Alpha Innotech). An Strep-Tactin® horseradish peroxidase (HRP) conjugate (IBA, Solution for life) monoclonal antibody (Invitrogen) was used as positive control to detect TTSuV1a or TTSuVk2a ORF1 and ORF3 proteins.

4.2.8. Statistical analyses

Statistical analysis was done with IBM SPSS Statistics software, version 20. Normal distribution was assessed using the Shapiro-Wilk test. In order to assess differences in viral loads between groups, the student's t-test was used for normally distributed samples and Mann-Whitney U test was used for non-parametric data. Correlations between antibody levels (represented by OD values) and $\log_{10}$ viral titres were determined by the Pearson's correlation coefficients. P-values <0.05 were considered statistically significant.

4.3. Results

4.3.1. Recombinant baculovirus construction and TTSuV protein expression and distribution in transfected insect cells

In order to express TTSuV proteins using the baculovirus system, recombinant baculoviruses were generated containing the ORF1, ORF1-A, ORF2 and ORF3 genes from each TTSuV species. For that, the corresponding pFastBac vectors were transformed into *E.coli* DH10BacΔcc to generate Bacmid DNA. The presence of the ORF1, ORF1-A, ORF2 and ORF3 genes in the Bacmid DNA was verified by PCR (Figure. 4.3, panel A). The expected size of each gene is shown in Table 4.1. Bacmid DNA was subsequently transfected into Sf9 insect cells to yield recombinant baculoviruses. The immunofluorescence assay (IFA) demonstrated that transfected Sf9 cells expressed TTSuV proteins as demonstrated in Figure 4.2, panel B. The ORF1 and ORF1-A proteins were located in the cell nucleus (Figure 4.3a and b, panel B) while the ORF2 protein was located in the cytoplasm (Figure 4.3c, panel B). The ORF3 protein was detected in both cytoplasm and nucleus (Figure 4.3d, panel B).

4.3.2. Successful expression of and purification of TTSuV proteins from infected insect cells.

Sf9 cells were infected using the respective TTSuV baculovirus stocks (dccBacORF1, dccBaccORF1-A, dccBaccORF2 and dccBacORF3 for each of the four TTSuV species) and SDS-PAGE analysis revealed the successful expression of four TTSuV proteins in the cell fraction (data no shown). The ORF2 protein appeared also to be present in the cell culture supernatant (Figure 4.4). While the predicted molecular weights (MW) of the ORF1-A and ORF3 proteins for all TTSuVs are ~22 kDa and ~26 kDa, respectively (Table 4.1), the observed MWs were approximately ~8kDa larger (Figure 4.3 a and b). The ORF1 and ORF2 proteins were observed approximately at the predicted size (~72 kDa and ~10 kDa, respectively), except the ORF2 protein of TTSuV1a that was ~5 kDa larger than predicted.

The observed higher MWs were not due to glycosylation of proteins and/or their binding to DNA or RNA according to glycosylation and DNase/RNase treatment experiments (data not shown).

The ORF1 protein was purified according to the sedimentation coefficient (s). ORF1 purified protein was observed in cell pellets from s200 and s100 centrifugation steps. The s100 pellets were selected to obtain a more purified protein. Strep-tagged ORF3 was purified using Magnetic Beads (Qiagen) (data not show) and Strep-Tactin Superflow (Qiagen). It was also attempted to purify the Strep-tagged ORF2 and ORF1-A proteins using Magnetic Beads (Qiagen) (data no shown). Purified ORF2 protein could be obtained; however, Strep-tagged ORF1-A could be not purified. ORF1 and ORF3 proteins were finally used to produce the multivalent vaccine as shown in Figure 4.4.

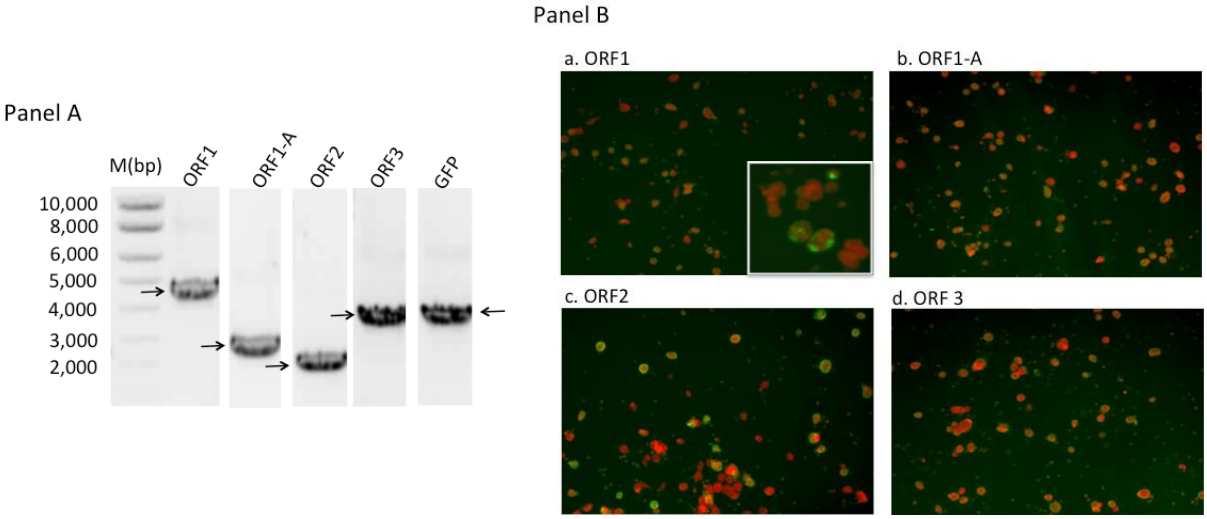

Figure 4.3. PCR an IF assay were used to confirm that Bacmid DNA contained the correct insertion and that it can express the TTSuV proteins in transfected insect cells. **Panel A,** the arrow indicate the amplification product obtained by PCR of the ORF1, ORF1-A, ORF2 ORF3 and GFP gene inserted into the Bacmid on a 2% of agarose gel. M: DNA size marker, bp (SmartLadder, Eurogentec). **Panel B**, the ORF1 (a), ORF1-A(b) , ORF2(c) and ORF3 (d) protein expressed in Sf9 cells by IFA. Baculovirus TTSuV proteins were detected by the primary Strep-tag antibody, except for th both were recognized with a secondary antibody conjugated to FITC. The red signal shows nuclei of insect cells. The green signal indicate TTSuV proteins.

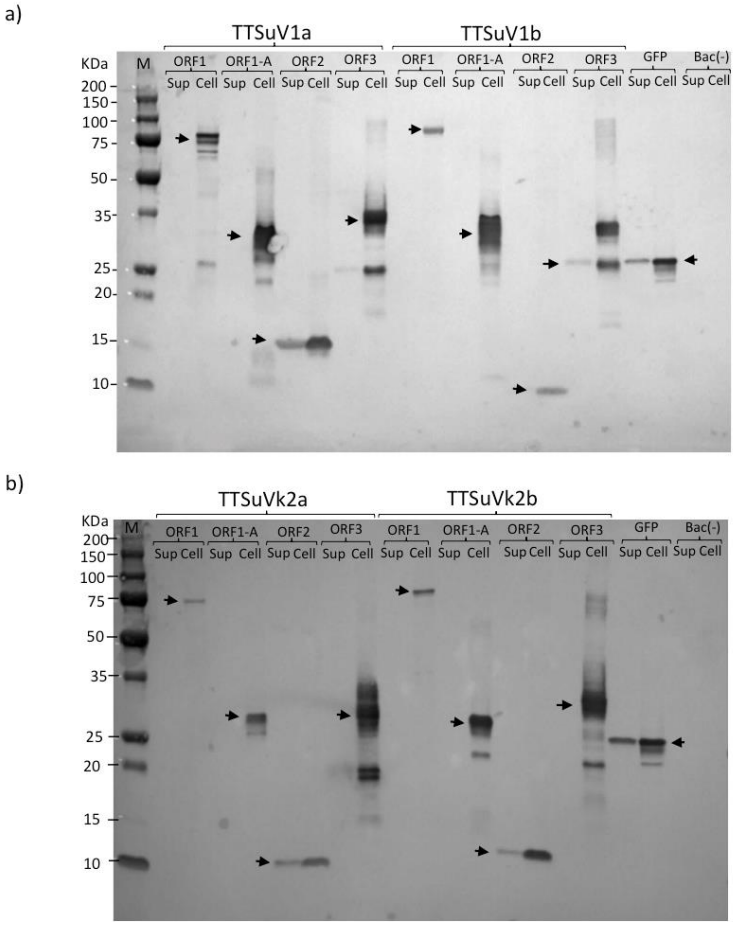

Figure 4.4 Analysis of protein expression (ORF1, ORF1-A, ORF2 and ORF3) of four TTSuV species by the baculovirus expression system. Western blot (a, b) analysis of protein that is released to the supernatant (sup) and protein that is intracellular (cell, pellet cell). Black arrow heads indicate the TTSuV proteins, expressed in the cell pellet. GFP and empty baculoviruses were used as positive and negative expression controls, respectively

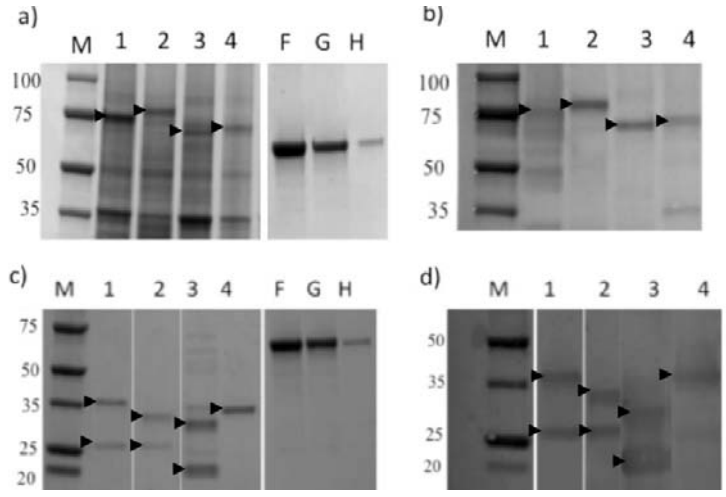

Figure 4.5 Analysis of protein expression (ORF1, ORF1-A, ORF2 and ORF3) of four TTSuV species by the baculovirus expression system. Western blot (a, b) analysis of protein that is released to the supernatant (sup) and protein that is intracellular (cell, pellet cell). Black arrow heads indicate the TTSuV proteins, expressed in the cell pellet. GFP and empty baculoviruses were used as positive and negative expression controls, respectively

4.3.3. Vaccination did not lower the TTSuV loads

The vaccination against TTSuVs was done using a multivalent vaccine applied either intramuscularly (IM; groups 1 and 2) or intradermally (ID; groups 3 and 4) following the schedule shown in Figure 4.1. Viral loads in vaccinated and control groups throughout the experiment were monitored by qPCR.

TTSuV1 was detected in 7% (1/15) of control group (group 5) animals at 3 weeks of age and reached 53% (8/15) at 15 weeks of age (Figure 4.5a, panel A). Similar prevalence was observed in vaccinated groups, reaching to 60% (9/15) at the end of the study (Figure 4.5a, panel A). The average TTSuV1 load in control group was below 5 $\log_{10}$ mean DNA copies ml^{-1} throughout the study, similar to those of the ID inoculated groups; however, group 4 pigs (IDx2) vaccinated first time at 4 days of age showed a higher but statistically not significant, viral load at 12 weeks of age (Figure 4.6a, panel B). Although IM inoculated groups (groups 1 and 2) had viral loads above 5 $\log_{10}$ mean DNA copies ml^{-1} at 7 weeks of age, being 5.16 $\log_{10}$ (group 1) and 5.45 $\log_{10}$ (group 2) mean DNA copies ml^{-1}, it was followed by a viral load reduction (4.78 $\log_{10}$ and 4.50 $\log_{10}$ mean DNA copies ml^{-1}, respectively) until the end of the study (Figure 4.6a, panel B).

ID and IM inoculated groups showed non-significant higher TTSuVk2a prevalence compared to the control group throughout the study, reaching 80-100% at 15 weeks of age (Figure. 4.6b, panel A). TTSuVk2a loads observed in groups 2 (IMx2) and 4 (IDx2), vaccinated twice, were non-significance higher than the control group and the other vaccinated groups, being 5 $\log_{10}$ mean DNA copies ml^{-1} at 12 weeks of age. Finally, the TTSuVk2b prevalence reached to 40-43% for IM groups (groups 1 and 2) and 33-38% for ID groups (groups 3 and 4) compared to 33% for the control group (Figure 4.6c, panel A). The average TTSuVk2b loads animals were below 4.5 $\log_{10}$ mean DNA copies ml^{-1}. In fact, the viral titre in the control group was non-significantly lower than those of the vaccinated groups (Figure. 4.6c, panel B).

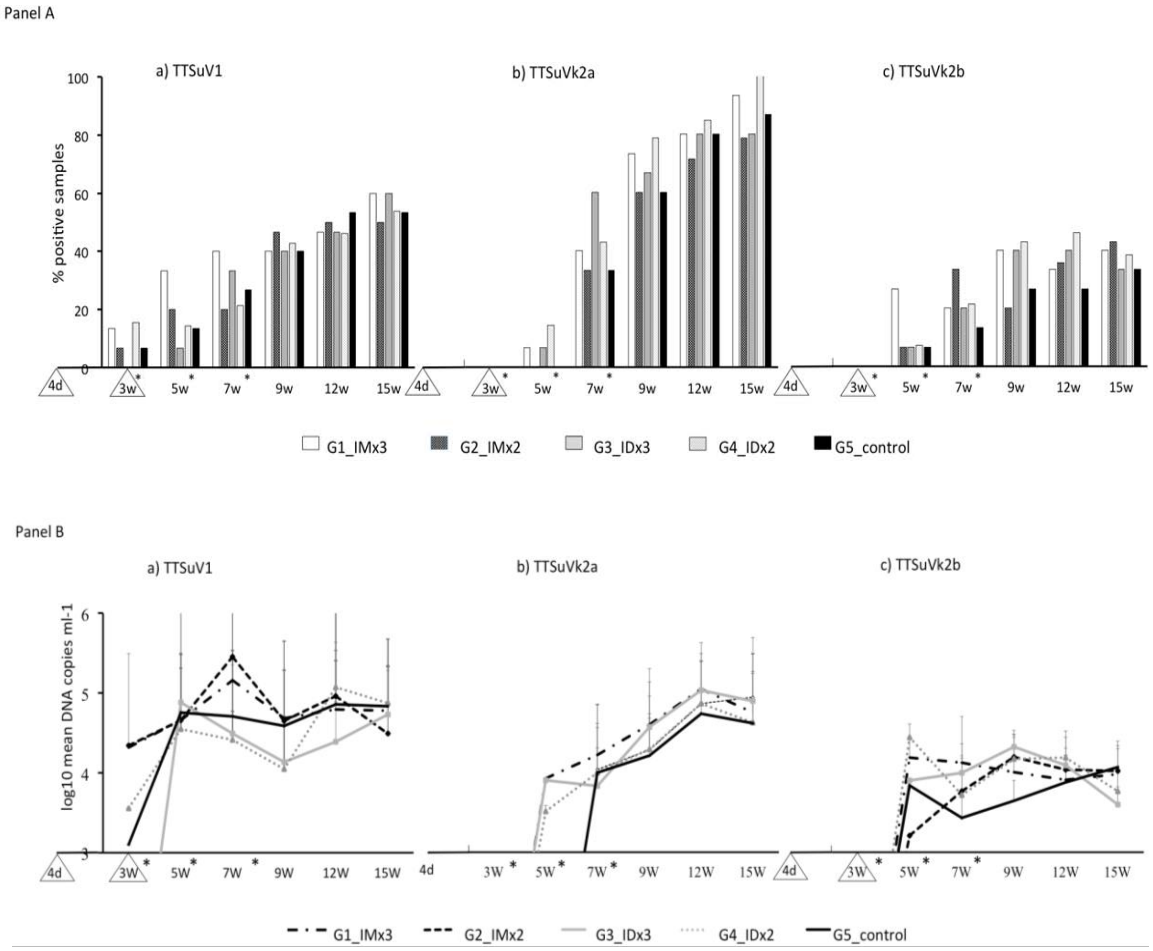

Figure 4.6 TTSuV1 (a), TTSuVk2a (b) and TTSuVk2b (c) viral load dynamics in intramuscularly inoculated groups (G1 and G2), intradermally inoculated groups (G3 and G4) and control group (G5), as determined by qPCR. The triangle indicates vaccination at 4 days (4d) and 3 weeks (w) of animals of groups 2 and 4. The asterisk indicates vaccination at 3w, 5w and 7w of animals of groups 1 and 3. IM, intramuscular group. ID, intradermal group. X2 indicates twice vaccinated and X3 three times. Panel A. Percentage of positive TTSuV1 (a), TTSuVk2a (b) and TTSuVk2b (c) in vaccinated groups (1, 2, 3 and 4) and control group (group 5) by qPCR. Panel B, TTSuV1 (a), TTSuVk2a (b) and TTSuVk2b (c) viral load dynamics in vaccinated groups (1, 2, 3 and 4) and control group (group 5). Average viral loads and standard deviations at different sampling times are represented in log10 scale.

4.3.4. Seroconversion and specific antibodies against TTSuVs were detected

From the previous section it can be concluded that no differences in viremia (measured by qPCR) were observed between vaccinated and control pigs. For that reason, ELISAs based on baculovirus proteins were used to see if vaccinated animals produced specific antibodies against the multivalent vaccine prototype that included ORF1 and ORF3 recombinant TTSuVs proteins.

All pigs (15/15) from group 1 (IMx3) seroconverted against the ORF1 protein of TTSuV1a by 7 weeks of age (28 days after the first vaccination) and remained positive until 15 weeks of age (Figure 4.7a). A total of 93% (14/15) of pigs from group 2 (IMx2) seroconverted against the ORF1 protein of TTSuV1a by 5 weeks of age (31 days after the first vaccination), but reached 100% (15/15) by 7 weeks of age. Group 1 (IMx3) animals produced higher antibody titres than group 2 (IMx2). However, antibody titres in group 2 (IMx2) started to decrease from 9 weeks onwards (Figure 4.7 a). Similar results were observed when TTSuVk2a anti-ORF1 antibodies were measured (Figure 4.8 a). Regarding ORF3 protein, group 1 (IMx3) reached 80% (12/15) seroconversion at 12 weeks of age for TTSuV1a and 87% (13/15) at 9 weeks of age for TTSuVk2a. However, in group 2, 20% (3/15) of pigs seroconverted by 9 weeks of age against TTSuV1a (Figure 4.7 c) and 14% (3/14) by 12 weeks for TTSuVk2a (Figure 4.8 c).

In pigs vaccinated ID (groups 3 and 4), the level of TTSuVk2a anti-ORF1 antibodies was comparable to those of pigs immunized intramuscularly (groups 1 and 2) (Figure 4.8b). However, antibodies against ORF1 TTSuV1a were lower (Figure 4.7b). Group 3 (IDx3) pigs reached 93% (14/15) seroconversion at 7 weeks of age and remained two weeks later (9 weeks of age). Group 4 (IDx2) pigs reached the maximum percentage of seropositivity against the TTSuV1a ORF1 protein by 5 weeks of age (71%, 10/14). Antibody prevalence against the ORF3 protein in group 3 reached 87% (13/15) for TTSuV1a and 60% (9/15) for TTSuVk2a at 9 weeks of age. None of the animals in group 4 (IDx2) seroconverted against the ORF3 protein.

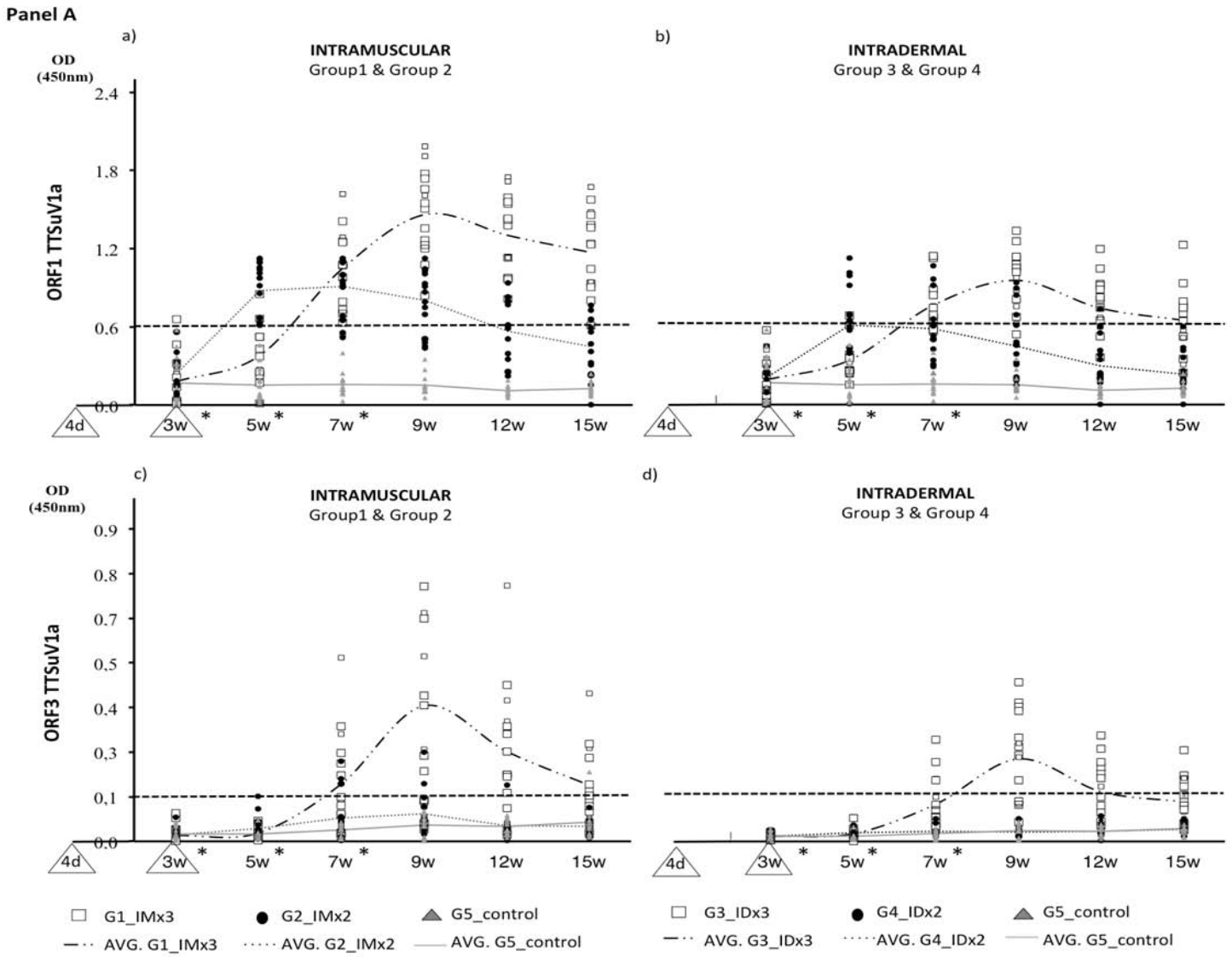

Figure 4.7. Specific antibodies against multivalent vaccine detected by ELISA based on baculovirus expressed proteins. Antibody dynamics against ORF1 (a and b) and ORF3 (c and d) proteins at different ages is displayed in both panels for TTSuVk2a. Panels display intramuscular (groups 1 and 2) (a and c) and intradermal groups (group 3 and 4) (b, d). The triangle 4 days (4d) and 3 weeks (w) indicate vaccination of animals of group 2 and 4. Asterisk drawn at 3w, 5w and 7w indicate vaccination of animals of group 1 and 3. Values were obtained by reading the ELISA plates at OD 450 nm. The horizontal dotted line shows the cut-off value at 0.6 for ORF1 and 0.2 for ORF3. The antibody dynamics within each group is shown with the average of value (AVG).

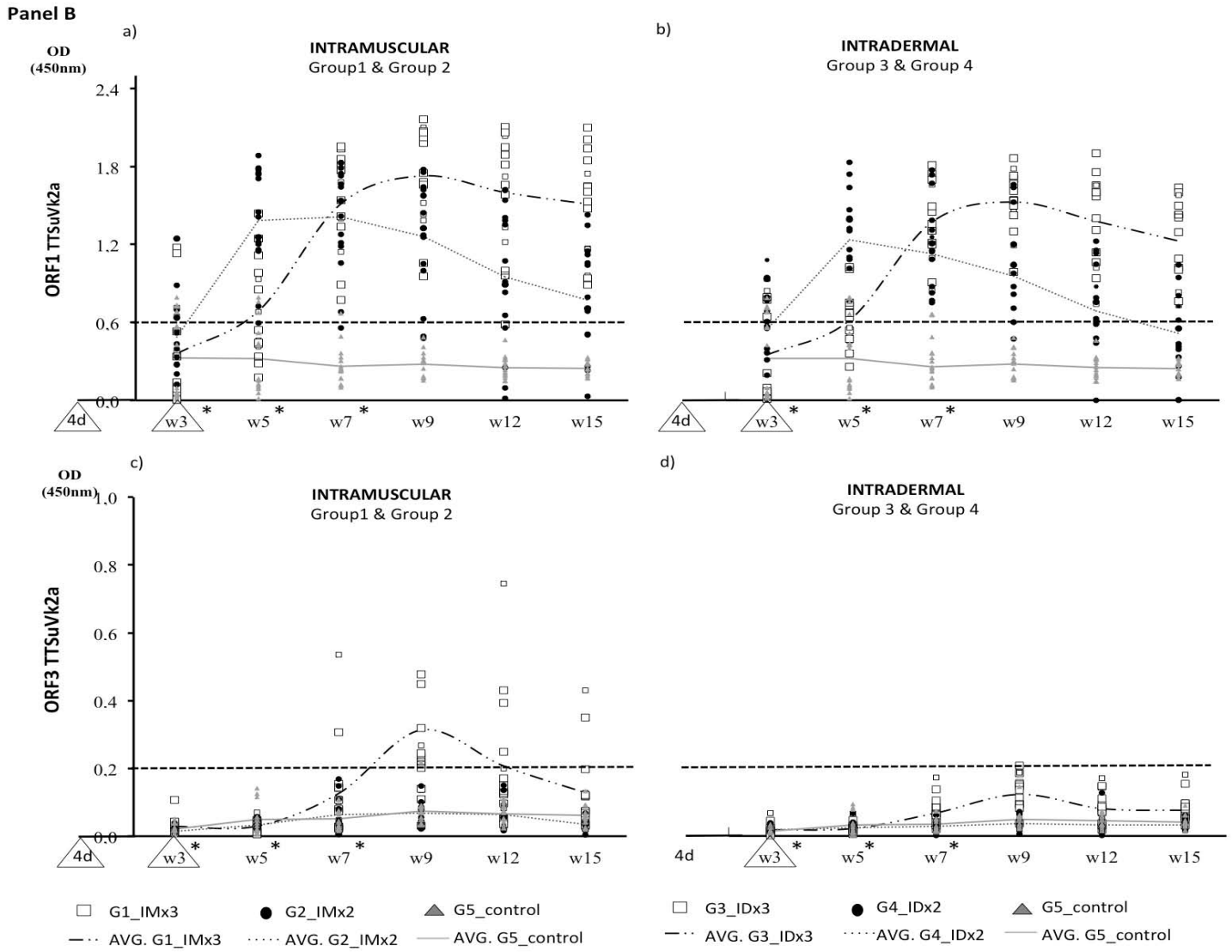

Figure 4.8 Specific antibodies against multivalent vaccine detected by ELISA based on baculovirus expressed proteins. Antibody dynamics against ORF1 (a and b) and ORF3 (c and d) proteins at different ages is displayed in both panels for TTSuVk2a. Panels display intramuscular (groups 1 and 2) (a and c) and intradermal groups (group 3 and 4) (b, d). The triangle 4 days (4d) and 3 weeks (w) indicate vaccination of animals of group 2 and 4. Asterisk drawn at 3w, 5w and 7w indicate vaccination of animals of group 1 and 3. Values were obtained by reading the ELISA plates at OD 450 nm. The horizontal dotted line shows the cut-off value at 0.6 for ORF1 and 0.2 for ORF3. The antibody dynamics within each group is shown with the average of value (AVG).

Western blot analyses were performed to confirm that antibodies detected by ELISA were specifically recognizing vaccine antigens. Indeed, it was confirmed that serum from immunized animals contained antibodies, which specifically detected a ~70 kDa and ~30kDa sized band corresponding to the ORF1 and ORF3 proteins, respectively (Figure 4.9).

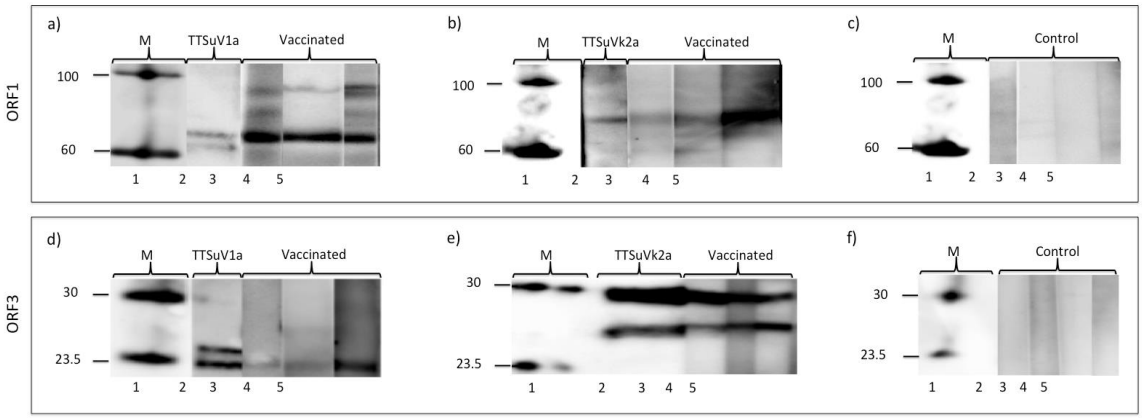

Figure 4.9 Specific antibodies against ORF1 and ORF3 in pigs by Western blot assay. As a positive control, ORF1 TTSuV1a (a), ORF1 TTSuVk2a (b), ORF3 TTSuV1a (d) and ORF3 TTSuVk2a (e) purified proteins were detected with an anti-Strep monoclonal antibody (line 2). Sera from vaccinated group (lanes 3 to 5), with value higher than 0.2 for ORF3 and 0.6 for ORF1 and four control sera from group 5 (lanes 2 to 5) were analysed.

Furthermore, to determine the TTSuV specificity of serum antibodies, all serum samples were tested by an ELISA based on *E. coli* expressed ORF1-A (this protein shares its N-terminal parts with that of the ORF1 protein) and ORF2 (this protein is identical to the N-terminus of the ORF3 protein) as antigens (Figure 4.10 and 4.11). Using this method anti-TTSuVs antibodies were detected against ORF1-A in 60% (9/15) of animals of group 1 (IMx3) at 7 weeks of age (Figure 4.10). In group 4 (IDx2), 3 animals had antibodies against TTSuV1 ORF1-A already at 3 weeks of age, being at higher level against TTSuVk2a (Figure 4.11b). In contrast, just a few animals in the control group had low levels of antibodies at 3, 7, 9, and 12 weeks of age.

Taken together, vaccinated animals developed an antibody response against the ORF1 and ORF3 proteins upon vaccination. In general, pigs that received early vaccination, group 2 (IMx2) and group 4 (IDx2), showed the maximum seroconversion rate by 5 weeks

of age, while groups that received the first vaccination at 3 weeks old, group 1 (IMx3) and 3 (IDx3), reached the peak of antibodies at 9 weeks of age.

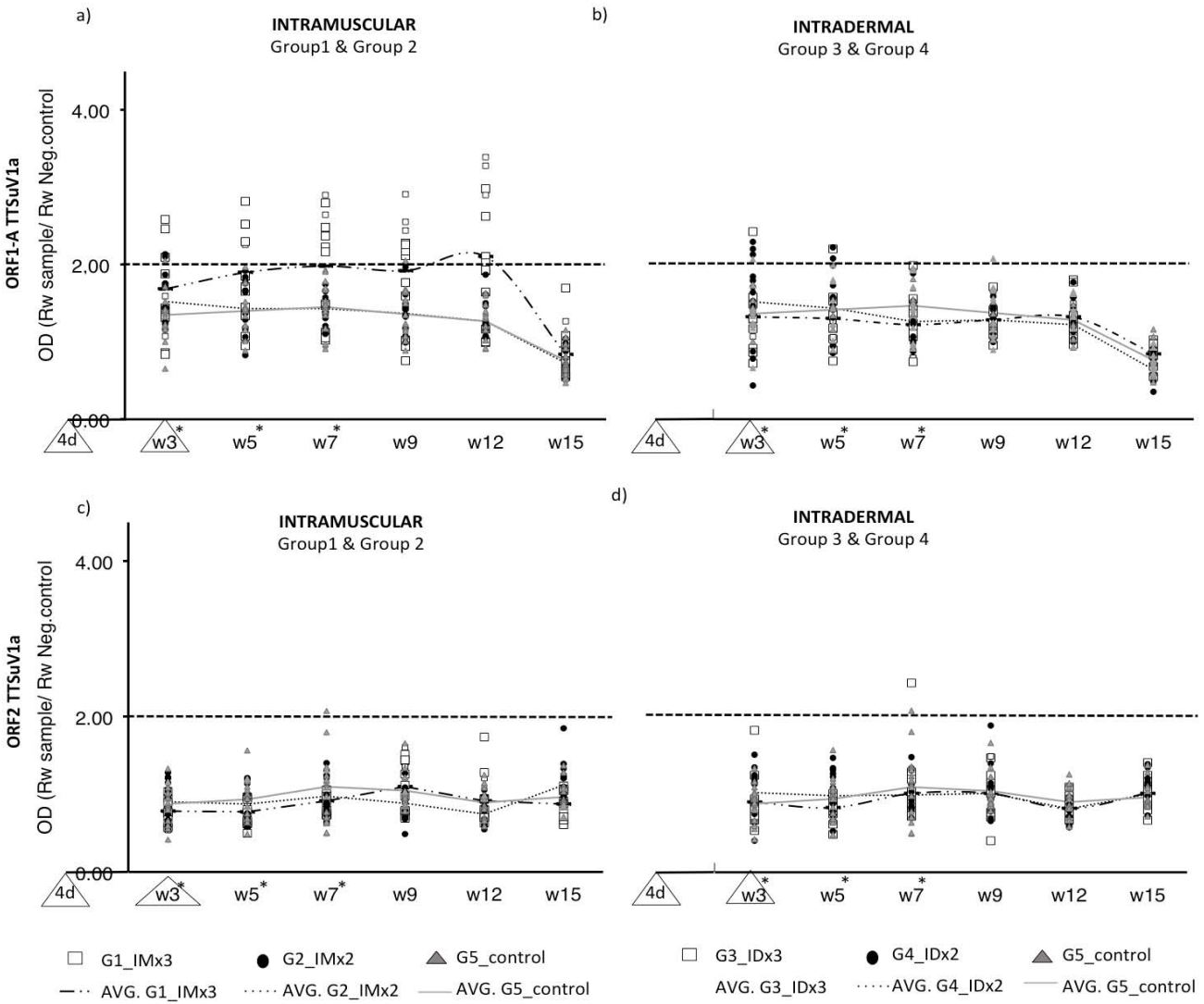

Figure 4.10. TTSuV antibodies in vaccinated and control pigs were analysed by ELISA based on *E.coli* expressed ORF1-A and ORF2 TTSuV1a proteins. Serum samples taken from intramuscular groups (group1 and 2) (a and c) and intradermal groups (group3 and 4) (b, d) at different age were tested. The triangle drawn at 4 days (4d) and 3 weeks (w) indicate vaccination of animals of group 2 and 4. Asterisk drawn at 3w, 5w and 7w indicate vaccination of animals of group 1 and 3. The values are presented as the ratio of the mean sample OD over the mean negative control OD. Horizontal dotted line shows the cut-off value at 2.

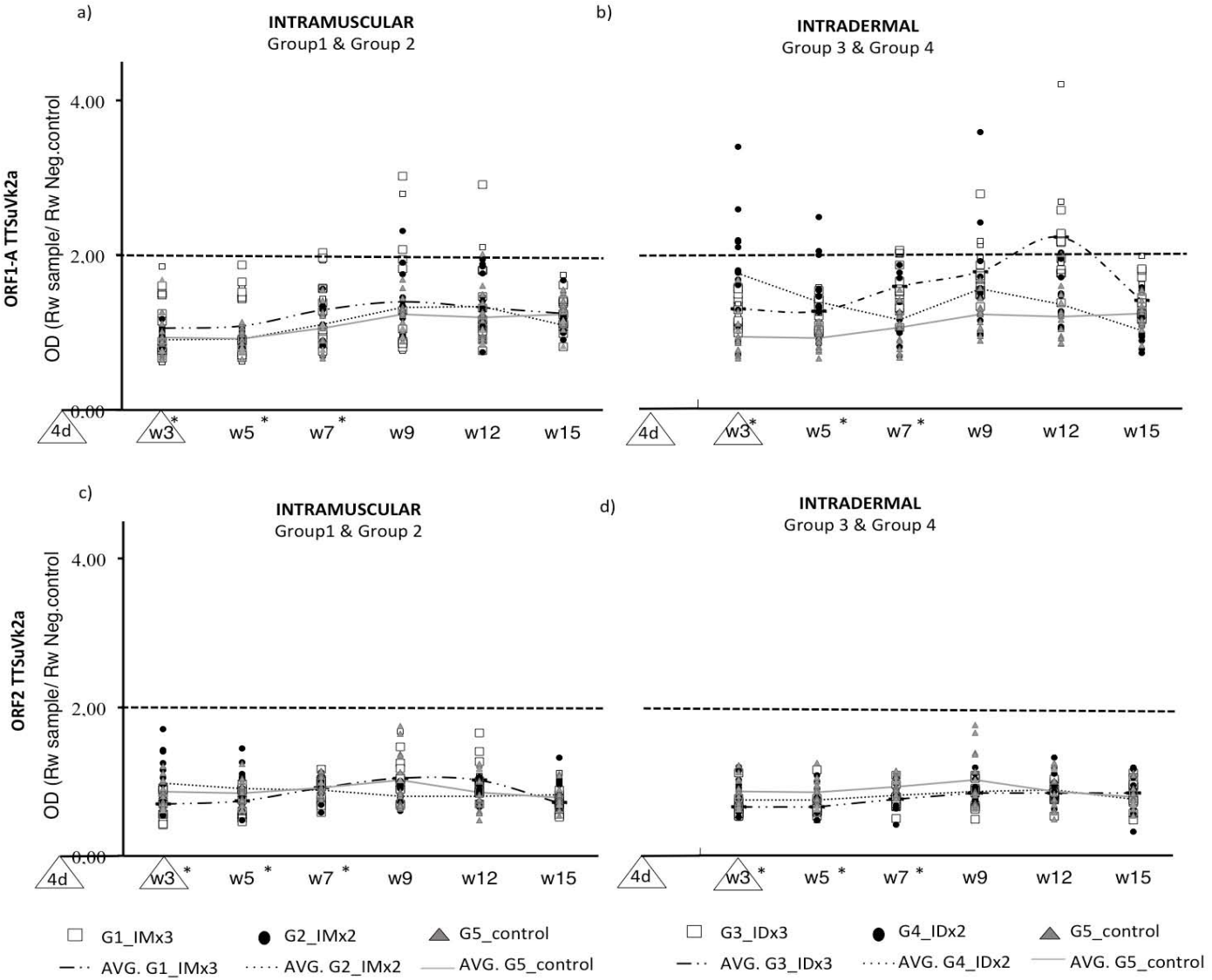

Figure 4.11. TTSuV antibodies in vaccinated and control pigs were analysed by ELISA based on *E.coli* expressed ORF1-A and ORF2 TTSuVk2a proteins. Serum samples taken from intramuscular groups (group1 and 2) (a and c) and intradermal groups (group3 and 4) (b, d) at different age were tested. The triangle drawn at 4 days (4d) and 3 weeks (w) indicate vaccination of animals of group 2 and 4. Asterisk drawn at 3w, 5w and 7w indicate vaccination of animals of group 1 and 3. The values are presented as the ratio of the mean sample OD over the mean negative control OD. Horizontal dotted line shows the cut-off value at 2

4.4. Discussion

This study describes, firstly, the production of four proteins (i.e. the ORF1, ORF1-A, ORF2, and ORF3 proteins) for the currently known TTSuV species (i.e. TTSuV1a, TTSuV1b, TTSuVk2a, and TTSuVk2b) using the baculovirus expression system. Secondly, a multivalent vaccine prototype containing recombinant ORF1 and ORF3 proteins of the four known TTSuV species was tested during natural TTSuV infection by monitoring viral genome load in blood and seroconversion by ELISA.

To our knowledge, putative capsid protein (ORF1 protein) has never been successfully expressed as a full-length protein using either bacterial, insect cell or mammalian expression systems (Kakkola *et al.*, 2008; Kakkola *et al.*, 2002), although at transcriptional level ORF1 mRNA has been detected (Kakkola *et al.*, 2008; Kakkola *et al.*, 2002; Martínez-Guinó *et al.*, 2011). However, the ORF1-A, ORF2, and ORF3 proteins of both human TTV and TTSuVs have already been expressed in mammalian cells, insect cells or bacterial cells (Huang *et al.*, 2011; Huang *et al.*, 2012b; Kakkola *et al.*, 2008; Martínez-Guinó *et al.*, 2011). In this study, the four open reading frames (ORF1, ORF1-A, ORF2 and ORF3) of TTSuV1a, 1b, TTSuVk2a, and k2b were expressed successfully using Sf9 insect cells. To improve protein expression in the present study, all genes included in the baculovirus expression vectors were codon optimized to the polyhedrin gene of baculoviruses. In addition, perhaps the short affinity-tags (Strep- or His-tag) used here may have facilitated the expression of the full-length proteins. The GST- and GST-6XHis-tags used in other studies have a size of ~26 kDa while Strep- (eight aa sequence) or His-tags (polyhistidine residues) are approximately ~1 kDa.

ORF1-A and ORF3 glycosylation or excessive binding to DNA or RNA were discarded as reasons for higher MW observed in SDS-PAGE analysis. Such differences in the observed and expected protein sizes have already been reported for the ORF3 (also known as ORF2/2) protein expressed in mammalian and bacterial cells (Lu *et al.*, 2013). In general, ORF1, ORF1-A, ORF2 and ORF3 proteins for the four TTSuVs had optimal expression.

Subsequently, different purifying methods were carried out to obtain the TTSuV proteins. Unfortunately, the ORF1-A isoform could not be efficiently purified and one reason could be the folding of the protein making the Strep-tag inaccessible for the anti-Strep microbeads used in the purification protocol. Affinity purification could also not be applied for the ORF1 protein and the putative viral capsid protein could be successfully purified using an ultracentrifugation method. The usefulness of this method may indicate that the expressed ORF1 protein formed virus like particles (VLPs). In the future, electron microscopy should be used to proof that the protocol used in this study would result in TTSuV VLPs.

ORF1 protein was considered a good protein to be included in the vaccine prototype since ORF1 protein is the putative capsid protein containing known antigenic domains (Huang *et al.*, 2011; Huang *et al.*, 2012a; Jarosova & Celer, 2013). ORF3 protein was also considered ideal to be included in the vaccine prototype, since it covered also the aa sequence of ORF2 protein (the N-terminal region of ORF3 is identical to almost the entire ORF2 protein). Therefore, the multivalent vaccine prototype containing ORF1 and ORF3 proteins of currently circulating TTSuV species was considered for testing by means of two administration routes (intramuscular and intradermal) and two vaccination schedules (two or three administrations).

The effect of immunization was evaluated by monitoring viral genome load in blood. Prevalence and load of TTSuVs are known to increase with the age of the animals and maximum prevalence in healthy animals is reached at about 3 months of age (Martínez-Guinó *et al.*, 2009; Nieto *et al.*, 2011; Sibila *et al.*, 2009a). Indeed, such increments on prevalence and TTSuV loads were also observed in this study and non-significant differences in viral loads were observed between control and vaccinated animals. Notably, TTSuV1 loads in serum of animals from all groups were unexpectedly low. This may be due to low infection pressure in the farm used in this study

Since no differences were observed in viremia between vaccinated and control groups, the vaccine ability to induce antibodies was evaluated. For that, the ORF1 and ORF3 proteins from TTSuV1a and TTSuVk2a were selected as representing species of each

genera of TTSuVs, as antibody cross-reactivity has been demonstrated to exist even between different TTSuV types (aa identity about 50%), but not between TTSuV from different species (aa identity about 25%) (Huang *et al.*, 2012a). Indeed, vaccinated animals generated antibodies against antigens included in the vaccine. Since baculovirus ELISA assays used the same antigens included in the vaccine, we further wanted to assay the specificity of antibodies using antigens produced in the *E. coli* expression system. Indeed, specific antibodies against TTSuVs were detected in vaccinated animals. Noteworthy, this study was based on natural TTSuV infection and therefore antibodies could develop also in control group animals. However, only 4 pigs in the control group generated detectable levels of TTSuV antibodies at 3, 7, 9 and 12 weeks of age. It is known that TTSuV-specific antibodies appear around 10 weeks of age and peak when animals reach around 20 weeks of age (Jarosova & Celer, 2013). Differences between the present and published results may be related with the immunological methods used in antibody detection or different infection dynamics of the analysed farms as already mentioned above. However, the present study showed that vaccination resulted in the development of humoral immunity against antigens used in the vaccine and, therefore, validated the success of seroconversion elicited by vaccination. It has to be noted that further optimization of the used ELISA techniques should be done in the case of their utilization in studies aimed to study humoral immune responses in non-vaccination scenarios.

In some cases, the vaccinated animals had higher, but non-significant, TTSuV loads than non-vaccinated control animals. It could be that vaccination activated humoral immune responses resulting in non-neutralizing antibodies; those might enhance the viral infection through interactions of complement system and antibody receptors, creating antibody-virus complexes, up-regulating TTSuV replication (Boonnak *et al.*, 2008; Willey *et al.*, 2011). Indeed, exacerbation has been described with other experimental vaccines, like in the case of an as *African swine fever virus* (ASFV) vaccine prototype. Specifically, one experimental vaccine study observed a good response after immunization of pigs; however, it did not confer protection against ASFV lethal challenge and a viremia exacerbation was observed in vaccinated pigs (Argilaguet *et al.*, 2011). However, little is

known about the interaction that TTVs establish with the host immune system and more work is needed to study this phenomenon.

Although vaccinated animals did mount humoral responses against TTSuVs, they did not have any effect on the virus loads in blood. Although the used vaccine prototype did include predicted antigenic epitopes, the lack of a virus neutralization assay makes impossible to conclude if these epitopes evoked neutralizing antibodies (Huang *et al.*, 2011; Jarosova & Celer, 2013). Furthermore, both cellular and humoral immune responses are believed to be necessary in controlling viral infections. In the present study, it could be that the vaccine protein only stimulated the humoral response, which may not be sufficient to control TTSuV viremia. Thus, it may be needed to improve the overall immune responses by using alternative prime-boost strategies, which are effective in generating high levels of T-cell memory (Ramshaw & Ramsay, 2000). Several studies have demonstrated the efficient use of priming with DNA, followed by a different agent like recombinant virus or protein as a 'boost' dose to obtain enhanced protective efficacy (Lu *et al.*, 2009; Woodland, 2004). In further studies, the multivalent vaccine should be used in combination with a DNA vaccine to see if such approach would improve the outcome.

It is known that anelloviruses are genetically variable and their infection is composed of different variants. For that reason, to control their infection, our goal was to design a multivalent vaccine to induce early immunity against all 4 known, currently circulating TTSuV species. The representative strains used to prepare the multivalent vaccine may not elicit a robust neutralising antibody response to TTSuVs and produce antibodies of the appropriate specificity. Another reason for the failure of our prototype vaccine is that TTSuVs may have developed multiple mechanisms to evade the neutralizing antibody response. This could indeed explain our observation, because during natural infection and in older pigs, antibodies and TTSuV virus co-exist.

In conclusion, the multivalent vaccine prototype developed in this work, based on baculovirus expressed TTSuV ORF1 and ORF3 proteins, was not able to control viremia during TTSuV natural infection. Since the immunized animals seroconverted, it is suggested that generated antibodies were not sufficient to control the viremia.

5. Study III

Vaccination of pigs reduces Torque teno sus virus viremia during natural infection

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5.1. Introduction

Currently, four species have been found to infect swine, namely *Torque teno sus virus* (TTSuV) 1a and 1b (TTSuV1a, TTSuV1b; genus *Iotatorquevirus*), TTSuVk2a and TTSuVk2b (genus *Kappatorquevirus*). TTSuV infection in pigs is distributed worldwide and characterized by a persistent viremia with high prevalence of TTSuV1 and TTSuVk2a species (Sibila *et al.*, 2009a), while prevalence of TTSuVk2b is lower (according the study I) For the most prevalent TTSuVs, circulating levels of up to 10^6 DNA copies per ml of sera for TTSuV1 and up to 10^7 for TTSuVk2a have been detected (Nieto *et al.*, 2011).

The TTSuV genome contains four open reading frames (ORF), ORF1, ORF1/1 (also known as ORF1-A), ORF2, and ORF3 (also known as ORF2/2). ORF3 can only be translated after splicing of the mRNA precursor, and splicing of the ORF1 mRNA transcript results in different protein isoforms (Huang *et al.*, 2012b; Martínez-Guinó *et al.*, 2011), as is also indicated in human TTV (Müeller *et al.*, 2008; Qiu *et al.*, 2005). The predominant transcripts detected *in vitro* and *in vivo* of ORF1 are spliced and full-length ORF1 transcripts have not been detected (Huang *et al.*, 2012b; Martínez-Guinó *et al.*, 2011). Splicing of TTSuVk2a ORF1results in three protein isoforms (ORF1-A, -B and -C) and the aa composition between them varies, depending on the splicing site used (Huang *et al.*, 2012b; Martínez-Guinó *et al.*, 2011).

ORF1 encodes the largest TTSuV protein with a predicted viral capsid domain in the N-terminal half of the protein and a putative replication-associated domain in its C-terminal half (Maggi & Bendinelli, 2009). It has been suggested that the ORF2 protein is involved in viral replication (Hijikata *et al.*, 1999; Huang *et al.*, 2010b). At present, the role of the ORF3 protein is unknown.

The pathogenic potential of anelloviruses is still controversial. TTSuVs persistently infect a high proportion of animals that are apparently healthy (Sibila *et al.*, 2009b). Thus, infection by itself does not cause immediate disease and therefore TTSuV by itself is not considered pathogenic. However, it is believed that TTSuVs can influence the

development of some diseases or even affect their outcome (Kekarainen & Segales, 2012).

In the present study, the impact of pig immunization against TTSuVk2a was evaluated by following the specific seroconversion and the viral load dynamics during natural infection. Our strategy was to use a combination of DNA and protein immunizations. Quantitative PCR techniques were used to detect and quantify the viremia levels of each TTSuV species separately and together while the induction of specific antibodies where followed by indirect ELISA. This showed that vaccination against TTSuVk2a resulted in a specific antibody induction and in a significant reduction of the TTSuVk2a loads, while DNA levels of the other TTSuV species were not affected. Further studies are needed to elucidate the intrinsic mechanisms involved in protection.

5.2. Materials and methods

5.2.1. Experimental design of the animal trial

Thirty six (36) male piglets (Landrace x Piétrain genetic origin) that were tested TTSuVk2a negative by PCR (Segalés *et al.*, 2009) were selected for this study. The 3-week old animals were randomly distributed over group A, immunized animals (n=18), and group B, control animals (n=18), but taking into account the piglets' weight, sow, and PCR positivity to TTSuV1 (3 animals tested PCR positive for TTSuV1 at the starting time point) (Figure 5.1). Throughout the study animals from both groups were housed together. Pigs in Group A were DNA immunized twice (when 3 and 5 weeks old) followed by a protein boost (at 7 weeks of age). Pig weight was recorded at 3, 7 and 15 weeks of age and serum samples were collected every two weeks until pigs reached 15 weeks of age. Treatments, housing and husbandry conditions conformed to the European Union Guide lines for animal welfare and the study was performed according to *Good Experimental Practices*. At the end of the experiment (when the animals were 15-weeks old), after final blood

sampling and weighing, all animals were euthanized using an intravenous (jugular vein) lethal dose of sodium pentobarbital.

The assays to detect antibodies against Porcine circovirus 2, Porcine reproductive and respiratory syndrome virus (PRRSV), *Mycoplasma hyopneumoniae* (MH) and *Actinobacillus pleuropneumoniae* (APP) were performed by the R&D Service Laboratory at MSD Animal Health, Boxmeer (The Netherlands), according to standard procedures.

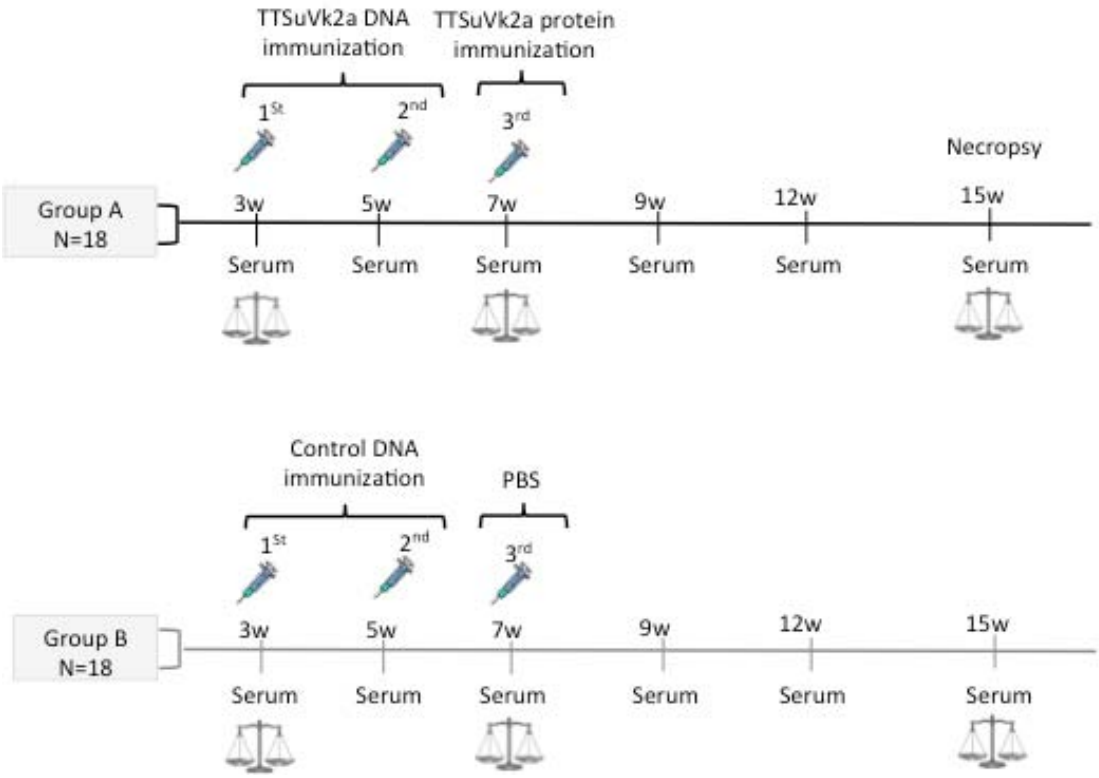

Figure 5.1. Overview of experiment design. The animal groups, age of animals at each point immunizations and samples taken is shown

5.2.2. Preparation and application of DNA vaccine

Constructs, based on the pcDNA3.1 plasmid, containing the three ORF1 isoform proteins to cover as much of the antigenic regions variability as possible, being TTSuVk2a ORF1 splicing variants ORF1-A, ORF1-B, and ORF1-C, as well as ORF2 genes under the control of the cytomegalovirus (CMV) promoter were used for the first two immunizations. Plasmid DNA purification was carried out using the Endofree Plasmid Purification Maxi Kit

(QIAGEN) following the manufacturer's instructions. Each animal from group A received 400 µg of pooled plasmids (i.e. 100 µg per plasmid) in both the first and second immunization. Control animals in group B received the empty pcDNA3.1 plasmid. The DNA in a total volume of 1.5 ml of saline solution (0.9% w/v of NaCl) was administered intramuscularly in the neck, alternating right and left side in subsequent immunizations.

5.2.3. Preparation and application of protein vaccine

TTSuVk2a ORF1-A and ORF2 genes were cloned into *E. coli* expression plasmid pET24a in frame with a 6xHistidine tag (Novagen). For protein production, *E. coli* Rosetta 2 (DE3) cells were used. The pET24a-transformed bacteria were cultured in 5 ml of Luria broth (LB) medium with kanamycin (30 mg/ml) and chloramphenicol (34 mg/ml) for 8 h at 37ºC. This starter culture was subsequently inoculated into 1 l of LB containing kanamycin (30 mg/ml) and chloramphenicol (34 mg/ml) and grown until the optical density reading at 600 nm (OD_{600}) reached 0.6. At this point, protein expression was induced by adding 1 mM isopropyl ß-D-1-thiogalactopyranoside (IPTG) for 4 h. Cell pellets were collected by centrifugation at 4,000x g at 4ºC for 30 min and stored at -80ºC until use. Protein purification was done using His SpinTrap columns (GE Healthcare Life Science) according to the recommended protocol. The ORF1-A protein was purified under denaturing conditions using 8 M urea and the ORF2 protein under native conditions. Purified proteins were further dialysed 4 times with PBS using Dialysis cassettes with 20 kDA molecular weight cut-off (Pierce). Protein preparations were analysed for purity by gel electrophoresis followed by coomassie staining and for identity by Western blot analysis using an anti-His antibody. Protein concentrations were determined using a BCA^{tm} Protein Assay Kit (Pierce) according to the manufacturer's instructions. Purified ORF1-A and ORF2 proteins were pooled and 27 µg/animal mixed 1:1 with an oil-in-water adjuvant (XSolve; MSD Animal Health) was used in the 3rd immunization. The eighteen pigs in control group B were each injected with saline solution mixed with adjuvant (1:1).

5.2.4. Detection of antibodies against TTSuVk2a ORF1-A and ORF2 by ELISA

Seroconversion of animals was tested by ELISA using either purified ORF1-A or ORF2 proteins as antigen. Low binding 96-well microtitre plates were coated with 50 µl per well containing 680 ng/ml protein in carbonate-bicarbonate buffer pH 9.6. After overnight incubation at 4ºC, plates were washed 3 times with TBS-T (Tris-buffered saline, 0.1% Tween20) and then blocked with blocking buffer (1x phosphate-buffered saline, 1% casein, 0.05% Tween20). After incubation at 37°C for 1 h, each well was washed 3 times with TBS-T. Serum samples were diluted 1:200 in blocking buffer and 50 µl was transferred to the corresponding well. After incubation at 37ºC for 2h, plates were washed 3 times with TBS-T. Then 50µl per well the diluted HRP-conjugated anti-swine whole IgG (1:40,000 in blocking buffer; Sigma) was added and plates were incubated at 37ºC for 1h. Finally, 50 µl of TMB substrate (3,3,5,5-Tetramethylbenzidine liquid, supersensitive, Sigma) was added and the plates were incubated in the dark at room temperature until the OD_{650} of the negative controls reached about 0.05. At this time-point the reaction was stopped by adding 50 µl/well of 2 M H_2SO_4 and the OD_{450} of each sample was determined. All serum samples were run in duplicate. Each plate contained one positive and one negative control serum. The ELISA cut-off value was calculated as the mean OD at 450nm of the negative control sample from each ELISA plate performed plus three times the standard deviation. The relative optical density was calculated by dividing the mean OD of a sample by the mean OD of the negative control, and the positive cut-off value was set at 2. To confirm the ELISA results, twelve samples were analysed by western blotting.

5.2.5. Detection of antibodies against TTSuVk2a ORF1-A and ORF2 by Western blot analysis

Western blot assays were used to confirm the seroconversion results obtained by ELISA. For this, purified TTSuVk2a ORF1-A and ORF2 proteins were run under denaturing conditions in a NuPageNovex 4-12% Bis-Tris polyacrylamide Gel (Invitrogen) and subsequently transferred to a Hybond ECL nitrocellulose membranes (GE Healthcare). The

membranes were blocked with blocking buffer (1x phosphate-buffered saline, 2% casein, 0.05% Tween20) and incubated with porcine sera diluted 1:100 in blocking buffer for 1h at room temperature, followed by addition of anti-swine whole IgG (Sigma) at a 1:150,000 dilution in blocking buffer for 1h at room temperature. Captured antibodies were detected by ECL Advance Western Blotting Detection Kit (Amersham Biosciences) and visualized by using a FluoroChem HD2 chemiluminescent workstation (Alpha Innotech). An anti-His (C-term) monoclonal antibody (Invitrogen) was used as positive control to detect TTSuVk2a ORF1-A and ORF2.

5.2.6. Detection and quantification of TTSuV in serum by PCR

DNA was extracted from 200 µl of pig serum using a Nucleospin® Blood DNA extraction kit (Macherey-Nagel GmbH & Co KG Düren, Germany) according to the manufacturer's instructions. DNA was eluted in 100 µl of 5 mM Tris/HCl, pH 8.5.

A standard PCR based on the untranslated region (UTR) was used for screening and selection of animals for the trial. The standard PCR was also used to pre-screen all serum samples and only positive ones were further analysed by quantitative PCR. Presence or absence of TTSuV1 (both genotypes TTSuV1a and 1b) and TTSuVk2a in serum samples was assessed as described (Segalés *et al.*, 2009). Amplification products were run on a 1.8% TAE-agarose gel followed by ethidium bromide staining. The limit of detection (LOD) of the standard PCRs is 400 molecules per ml of serum.

Quantitative PCR for detection of TTSuV1 (types a and b) and TTSuVk2a was performed according to a published method based on primers that bind to the UTR of the genome (Nieto *et al.*, 2011). The inter-assay variation was below 1.21% for TTSuV1 and 3.12% for TTSuVk2a. The amplification efficiency was 87.35% for TTSuV1 and 94.15% for TTSuVk2a. Total TTSuV (detection of all known TTSuVs) and TTSuVk2b quantifications were done according to the study I methods based on primers that bind to the UTR of the genome The LOD of the qPCR assays is 2500 viral genomes per 1 ml of serum (3.4 LOG_{10} molecules/ml).

5.2.7. Statistical analyses

Statistical analyses were done using IBM SPSS Statistics for Windows Version 20.0. Normal distribution was assessed using the Shapiro-Wilk test. In order to assess differences in viral loads between group A and B student's t-test was used for normally distributed samples and Mann-Whitney U test was used for non-parametric data. The Chi-square test was applied to evaluate the proportion of positive and negative results in PCR positive and negative samples. P-values <0.05 were considered statistically significant.

5.3. Results

5.3.1. Immunization resulted in seroconversion against TTSuVk2a

In this study 18 animals in group A were vaccinated against TTSuVk2a, while group B animals received a placebo. An ELISA was used to test if animals produced specific antibodies for TTSuVk2a ORF1-A or ORF2. Until 9 weeks of age, none of the animals had detectable amounts of antibodies. The number of animals in group A having antibodies against ORF1-A increased from 9 weeks onwards being 17% (3/18) at 9 weeks, 44% (8/18) at 12 weeks and 56% (10/18) at 15 weeks (Figure. 5.2a). A similar tendency was seen for antibodies against ORF2 (Figure. 5.2c); 56% (10/18) seroconverted at 9 weeks of age and 94% (17/18) from week 12 onwards. None of the animals in group B seroconverted to ORF2 (Figure 5.2d), while 3 pigs at week 15 had low levels of antibodies against ORF1-A (Figure 5.2b).

Detection of ORF1-A and ORF2 antibodies in sera by Western blot analysis was performed to confirm the ELISA results and to check the specificity of the serum antibodies. For this, serum samples from immunized animals (OD ratio in ELISA greater than 2) were used to detect TTSuVk2a ORF1-A and ORF2 proteins in comparison with serum sample from control animals. Serum obtained from group A pigs at 12 and 15 weeks of age specifically detected a ~30 kDa size protein, which corresponds to the size of the ORF1-A protein

(Figure 5.3a), and a ~12 kDa size protein that corresponds to the size of the ORF2 protein (Figure. 5.3b). In contrast, serum from control animals had no detectable antibodies against TTSuVk2a ORF1-A and ORF2 proteins (Figure. 5.3).

Taken together, the TTSuVk2a-immunized animals developed an antibody response against the ORF1-A and ORF2 proteins used for vaccination.

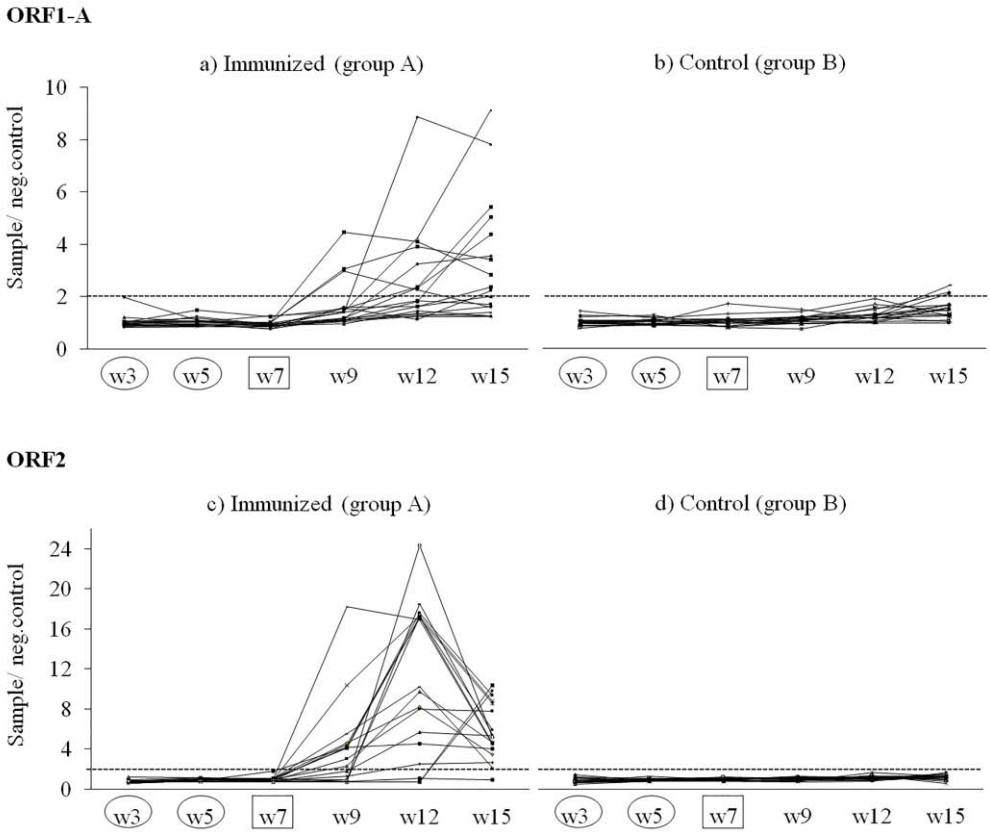

Figure 5.2 Seroconversion of animals against ORF1-A (a, b) and ORF2 (c, d) proteins measured by ELISA. Serum samples taken from group A animals (a, c) and group B animals (b, d) at different ages were tested. Values are presented as the ratio of the mean sample OD over the mean negative control OD. Horizontal dotted line shows the cut-off value at 2. Circles drawn at 3w and 5w indicate DNA immunization and the square at 7w indicates protein or PBS immunization

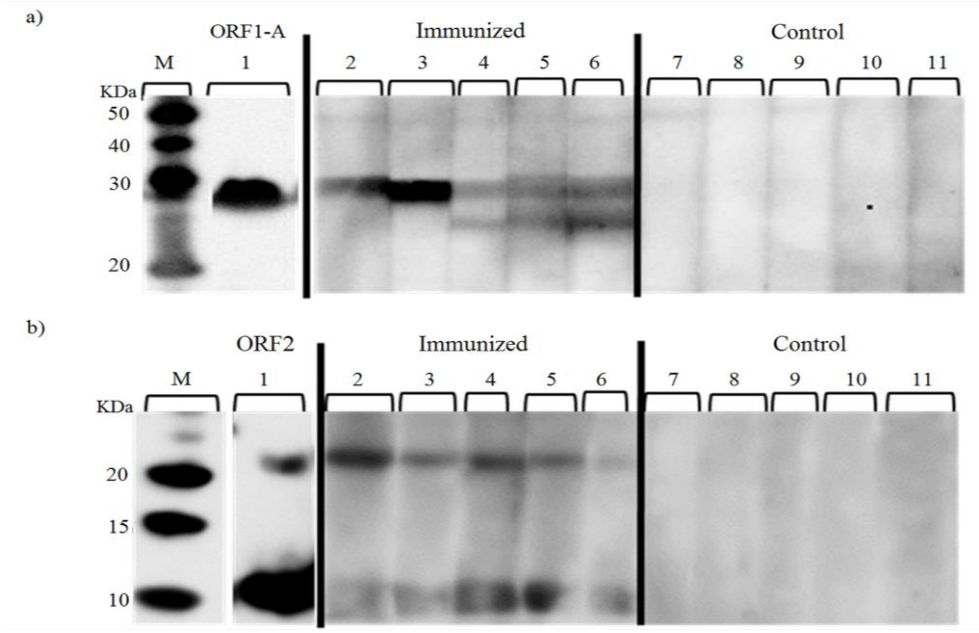

Figure 5.3. The presence of antibodies against ORF1-A and ORF2 in pigs determined by Western blot. As a positive control, ORF1-A (a) and ORF2 (b) purified proteins were detected with an anti-His (C-term) monoclonal antibody (lane 1). Sera from group A animals taken at 12 weeks of age (lanes 2 to 4) and 15 weeks of age (lanes 5 and 6), and five control sera from group B animals taken at 12 (lanes 7 to 9) and 15 weeks of age (lanes 10 and 11) were analysed.

5.3.2. Immunization led to delayed onset of TTSuVk2a infection

TTSuVk2a-positive animals were first detected by PCR at 3 weeks of age and reaching 100% (36/36) at week 12 in both groups (Figure 5.4a). This prevalence was observed in group B until the end of the study, while in group A the prevalence decreased to 83% (15/18). Furthermore, onset of TTSuVk2a infection was delayed in immunized animals (group A) in comparison to control group B. A few animals were already infected by TTSuV1 at 2 weeks of age and the prevalence reached 100% (18/18) in group A and 83% (15/18) in group B by the end of the study. There was no statistically significant difference in prevalence between the groups at any time.

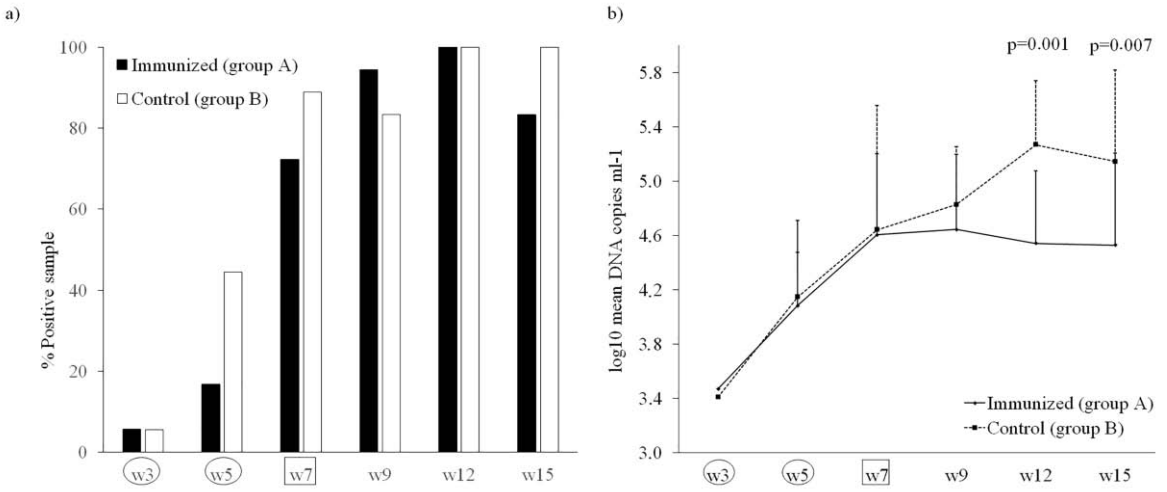

Figure 5.1. TTSuVk2a viral load dynamics and prevalence. (a) Prevalence of TTSuVk2a in immunized (group A, black bars) and control animals (group B, white bars) throughout the experiment. (b) Average viral loads and standard deviations at different sampling times (week 3 to 15) determined by qPCR in immunized group A (black line) and control group B (dotted line) are represented in $\log_{10}$ scale. P-values are shown where statistical differences between groups were observed. Circles drawn at 3w and 5w indicate time of DNA immunization and the square at 7w indicates time of protein or PBS immunization.

5.3.3. Vaccination against TTSuVk2a resulted in a specific decrease of TTSuVk2a loads and prevalence

The number of viral DNA copies in all PCR-positive samples was determined by quantitative PCR. TTSuVk2a loads did not differ between groups until week 7 (Figure 5.4b). Thereafter TTSuVk2a loads in group B increased until 12 weeks of age and decreased somewhat at 15 weeks. On the contrary, average TTSuVk2a loads in group A animals maintained at the same level from 7 weeks of age until the end of the study, being statistically lower than the average viral loads of group B animals.

During the study period only four animals in group A reached TTSuVk2a loads higher than $5\log_{10}$, and at 15 weeks of age all but three animals had viral titres below $5\log_{10}$ and 3 animals had cleared the viremia to below the detection limit of the qPCR (Figure 5.5). In comparison, all animals in group B were viremic at 15 weeks of age, with 9 animals having TTSuVk2a viral titres well above $5\log_{10}$. The delayed increase of TTSuVk2a viremia from 3 to 7 weeks in group A as compared to group B is again clearly observed if TTSuVk2a titres in individual animals are presented (Figure 5.4).

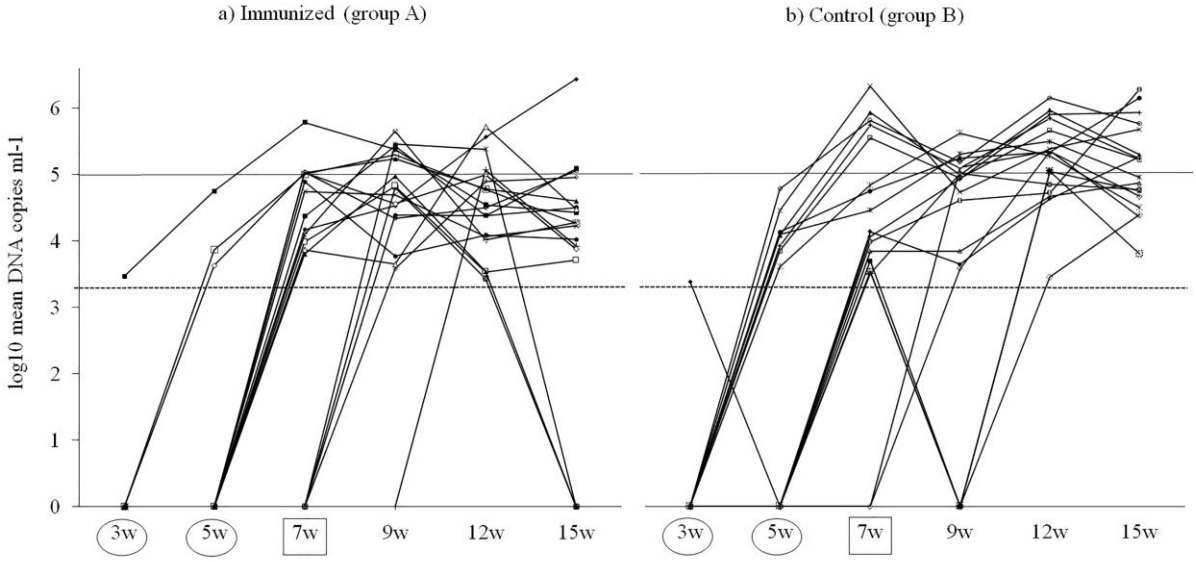

Figure 5.5. TTSuVk2a viral loads in individual animals in immunized (group A) (a) and control group (group B) (b) at different sampling times. PCR negative samples were given value 0. The dotted line represents the qPCR detection limit. The continuous line is drawn at $5\log_{10}$. Circles drawn at 3w and 5w indicate time of DNA immunization and the square at 7w indicates time of protein or PBS immunization.

To analyse if immunization was specifically affecting TTSuVk2a viremia, prevalence and viral loads of other TTSuV species were determined. Upon analysing the other TTSuV species (i.e. TTSuV1 and TTSuVk2b) no significant differences in the viral load or prevalence between groups were observed (Figure 5.5a and b). However, total TTSuV viral loads in group A reached its maximum at week 9, thereafter declining, while in group B a maximum was reached at week 12. At this age of the animals, the average total TTSuV loads in the immunized group was statistically lower than that in the unimmunized group

(Figure 5.6c). Therefore, reduction in TTSuVk2a viremia was also reflected in the total TTSuV qPCR results.

Average daily weight gain or the health status of animals was not different between the groups. None of the animals in the study developed antibodies against PCV2 and MH. Antibodies against PRRSV and APP were detected in immunized animals, being 22% (4/18) and 17% (3/18) respectively, and this was similar in control group animals, being 17% (3/18) for PRRSV and 11% (2/18) for APP.

Taken together, the onset of TTSuVk2a infection was delayed and lower TTSuVk2a titres were observed in animals immunized against TTSuVk2a and this immunization was specific, as no other TTSuV species were affected.

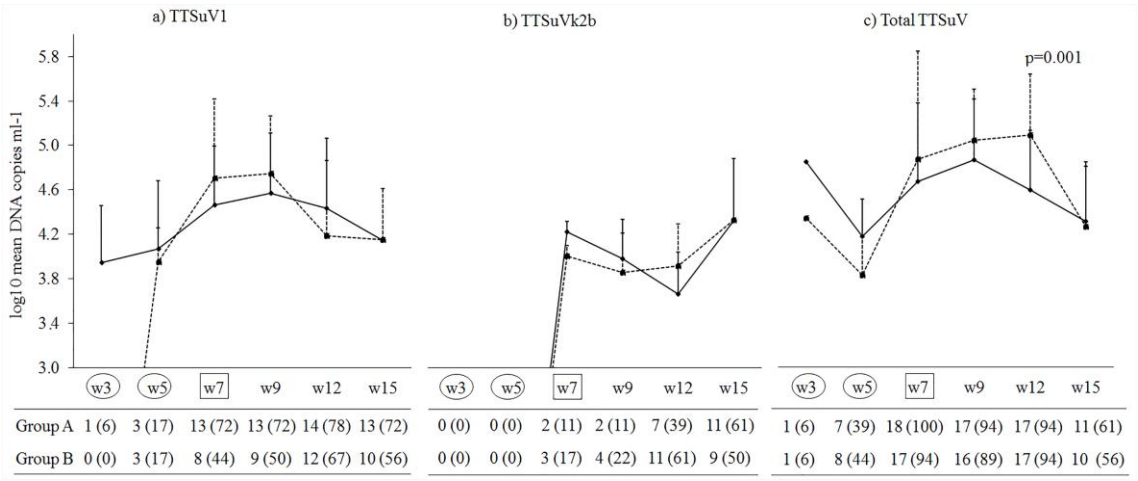

Figure 5.6. TTSuV1 (a), TTSuVk2b (b) and total TTSuV (c) viral load dynamics in immunized group (black line) and control group (dotted line) determined by qPCR. Average viral loads and standard deviations at different sampling times are represented in $\log_{10}$ scale. P-values are shown where statistical differences between groups were observed. Prevalence at different weeks of age is shown below the graphs and is expressed as the number of qPCR positive serum samples per group (on a total of 18) and the percentage positive is shown in parentheses. Circles drawn at 3w and 5w indicate time of DNA immunization and the square at 7w indicates time of protein or PBS immunization.

5.4. Discussion

This study describes for the first time the impact of successful pig immunization against TTSuVk2a during natural infection. Immunization was started at an early age and applied a total of three times at 14-day intervals. A combination of DNA and protein immunizations was chosen to increase the possibilities of activating both cellular and humoral immune responses. Pigs were housed in a local conventional farm with high hygiene levels until the age of 2.5 weeks, and thereafter kept under the same conditions as in conventional farms with the two study groups mixed. Thus, the results reflect a situation in which animals can infect each other as would occur in conventional farms.

It was found that TTSuVk2a viral loads were significantly lower in the immunized group than in the control group at the end of the study. Such differences in viremia were not observed for TTSuV1 or TTSuVk2b. It is known that the prevalence and titer of TTSuV increase with the age of the animals and maximum prevalence in healthy animals is normally reached at about 3 months of age (Sibila *et al.*, 2009b), which was also the case in this study. However, a delayed increase in TTSuVk2a prevalence was observed in immunized animals (group A), especially apparent in 5-weeks old animals, two weeks after the first DNA immunization. At that time, animals did not seroconvert and therefore it may be that the first DNA immunization activated a cellular immune response, which may have limited the infection. However, the role of cellular immune responses could not be proven in this study due to for example the lack of peripheral blood mononuclear cell fractions. However, DNA vaccines are known to stimulate immune responses in several animal infection models and in fact DNA vaccines participate in different molecular pathways involved in the immune system such as T cell responses (Bolhassani & Yazdi, 2009; Rodriguez & Whitton, 2000).

Not all immunized animals cleared TTSuVk2a infection and/or at the end of the study had relatively high viral titres. The rationale behind this reality could be explained by many different reasons including pig genetic differences or haplotypes. Also, it is known that anelloviruses infect the host as quasispecies, meaning that infection is composed of different, closely related genomic variants of the virus (Cortey *et al.*, 2012; Huang *et al.*,

2010a; Ng *et al.*, 2009a) . Therefore, one plausible explanation is that the strain used to prepare the DNA/protein used for immunization may not work against all TTSuVk2a strains potentially infecting the pigs in this study. Despite the fact that antibody cross-reactivity has been demonstrated to exist even between different TTSuV genotypes (aa identity about 50%), but not between TTSuV from different species (aa identity about 25%) (Huang *et al.*, 2012a), our results did not corroborate these observations. In our study, the generated antibodies were not cross-reacting with different genotypes (ORF1 protein is 58% identical between TTSuVk2a and TTSuVk2b (described in study I) or with different species (ORF1 protein is about 25% identical between TTSuVk2a and TTSuV1 (Huang *et al.*, 2010b)). Cross-protection would be a desirable feature of a vaccine that should reduce total TTSuV loads.

In our study, the antibodies against TTSuVk2a ORF1-A and ORF2 proteins were detected in serum in TTSuVk2a-vaccinated animals at 9 weeks of age until the end of the study (15 weeks of age). When antibodies are efficiently neutralizing viruses from circulation, it is expected that an increase in antibody titers is followed by a decrease in viral serum titers. However, in our study the antibody titers did not correlate with virus serum titers, indicating that they are not efficient at controlling the viral infection, perhaps explaining why during natural infection and in older pigs, antibodies and TTSuV co-exist.

In conventional healthy pigs the antibody levels during natural TTSuV infection increase with age. It has been observed in a recent study that TTSuV-specific antibodies started to appear when the animals were 10 weeks of age and peaked at week 20, using peptides based on antigenic regions in the C-terminal part of ORF1 (Jarosova & Celer, 2013). In fact, the highest TTSuVk2a seroprevalence has been detected in adult pigs, while being lower or absent in young animals (Huang *et al.*, 2011; Xiao *et al.*, 2012). In our study, only a few unimmunized animals generated detectable levels of ORF1-A antibodies at the end of the study. Since our experiment ended when pigs reached 15 weeks of age, it is impossible to say how the natural antibody response would have evolved in older animals. On the other hand, the difference in antibody detection between the vaccinated and non-vaccinated groups evidenced the success of the used immunization method to induce early humoral responses against TTSuVk2a.

Anelloviruses are considered non-pathogenic by themselves but it is believed that TTSuV play an important role during co-infection with other pig pathogens. TTSuV is often present in pigs with diseases like PCV2-SD (Aramouni *et al.*, 2011; Blomström *et al.*, 2010; Kekarainen *et al.*, 2006; Nieto *et al.*, 2011) and porcine respiratory disease complex (Rammohan *et al.*, 2012). In fact, TTSuV1 combined with porcine reproductive and respiratory syndrome virus (PRRSV) was considered to contribute to the experimental induction of a porcine dermatitis and nephropathy syndrome (PDNS)-like condition (Krakowka *et al.*, 2008). Furthermore, a previous study revealed that natural infection with TTSuV suppresses the host immune response and aggravates porcine reproductive and respiratory syndrome (PRRS) in pigs (Zhang *et al.*, 2012) . In our study a few animals were detected PRRSV positive by ELISA in both the immunized and control groups. Excluding the PRRSV positive samples from the analysis did not change the results with PRRSV positive samples included (data not shown).

Conversely, both the delay in TTSuVk2a infection and the TTSuVk2a virus load showed significant reductions directly after DNA immunization and in the absence of specific antibodies, indicating that specific T-cell responses might be involved in the observed partial protection. At this point, it is difficult to say how the infection would have evolved during the study if only DNA vaccines were used.

While the average viral titer of TTSuVk2a in group A never reached $5\log_{10}$, group B averages were well above $5\log_{10}$ for the two last sampling points. The viral titers in group B are in accordance with earlier data showing that TTSuVk2a viral serum titers in healthy animals older than 2 months are higher than 5 $\log_{10}$ (Nieto *et al.*, 2011). Even though the TTSuVk2a viremia was lowered by immunization, this was not reflected in the average daily weight gain. It is to be seen if immunization against all TTSuV variants would lower the total TTSuV loads in such a way that a gain in average daily weight can be obtained.

In summary, it can be concluded that a combination of DNA and protein immunization can control TTSuVk2a viremia, but clearly further studies are needed to improve vaccine efficacy, to know the mechanism behind this, and to assess the impact for pig producers.

6. General discussion

Novel anelloviruses have been described in several vertebrate species since the first description in humans in 1997. The first anelloviruses were discovered by PCR methods and the improvements in molecular techniques developed in the last years have been used for investigating anellovirus variability and discovering diverse novel anelloviruses in several species as wild rodents (Nishiyama *et al.*, 2014) or horses (Li *et al.*, 2015). All anelloviruses share certain characteristics such as high genetic diversity, conserved UTR region, similar genome organization and similar gene expression strategy. This occurs in the case of TTSuVs, which infect pigs and wild boar. Since TTSuVs discovery in 1999, a significant research focus has been placed on them and their infections, variability, sequencing of full-length genomes and diagnostic methods.

Study I of this PhD thesis included the description of a new TTSuV species. After such discovery, up to four pig infecting viral species are known, namely TTSuV1a and TTSuV1b (genus *Iotatorquevirus*), and TTSuVk2a and TTSuVk2b (genus *Kappatorquevirus*). The phylogenetic analysis carried out in the study I is currently being used in the taxonomic proposal made by the ICTV to generate the genus *Kappatorquevirus* with its corresponding species. Furthermore, the study I also describes the presence of TTSuVk2b in commercial pig sera. Interestingly, TTSuVk2b was not previously detected although there had been attempts to discover novel pig anelloviruses (Macera *et al.*, 2011). Before the results of the present PhD thesis, the primers used in published TTSuV1- or TTSuVk2a-specific PCRs (Brassard *et al.*, 2010; Gallei *et al.*, 2010; Huang *et al.*, 2010a; Nieto *et al.*, 2011) were not able to amplify TTSuVk2b given the mismatches of these described primers with TTSuVk2b sequences. The phylogenetic analysis used in the study I grouped TTSuVk2b close to TTSuVk2a and clearly apart from TTSuV1, further supporting the existence of a novel Kappatorquevirus genus. The different qPCR techniques, broad spectrum and species-specific qPCR, developed in the study I were used to determine the epidemiology of this novel anellovirus as well as its potential association with pig diseases, mainly with PCV2-SD.

TTSuV species existing so far before the knowledge of the novel one are widespread and ubiquitous in domestic pig and wild boar (Kekarainen & Segalés, 2012a). Epidemiological studies carried out in study I by species-specific qPCR confirmed that the novel species

was detected in almost all 17 countries scrutinized, but showed lower viral load compared to other TTSuV species. However, in three countries where TTSuVk2b was apparently absent, the number of serum samples analysed was probably low; therefore, more samples and farms should be analysed to rule out the presence of TTSuVk2b in these countries. Also, more viral genomic sequences should be obtained to confirm that the annealing sites of the used primers are highly conserved, thus guaranteeing the detection of all species variants. A recent study detected and confirmed the lower viral load of TTSuVk2b compared to other TTSuVs in Italian pig herds (Blois *et al.*, 2014).

TTSuVs are currently considered non-pathogenic. TTSuV infection by itself does not cause immediate disease, but it is believed that TTSuVs influence the development of some economically important viral diseases or even affect their outcome. Several studies have associated TTSuVs to a number of pig diseases, especially with PCVDs. These conditions have been grouped because of the underlying aetiological agent, PCV2. PCVDs represent some of the most significantly economic diseases in pig production worldwide.

The epidemiological study carried out in the first study of this PhD Thesis showed that both TTSuVk2a and TTSuVk2b DNA loads were significantly higher in PCV2-SD-affected pigs, compared to healthy counterparts, while no significant differences were observed in the case of TTSuV1. This confirms previous findings about the association of TTSuVk2a but not TTSuV1 with PCV2-SD (Aramouni *et al.*, 2011; Kekarainen *et al.*, 2006; Nieto *et al.*, 2011). This result suggests that the species included in the *Kappatorquevirus* genus (TTSuVk2a and k2b) could be a factor of clinical aggravation in co-infection with other pathogens. Perhaps only certain types or subtypes of TTSuV may be particularly pathogenic or potentially associated with some pig diseases. Moreover, TTSuV1 combined with PRRSV has been considered to contribute to the experimental induction of a porcine dermatitis and nephropathy syndrome (PDNS)-like condition (Krakowka *et al.*, 2008). Indeed, in the complex association of TTSuVs with potential pig diseases, it should be taken into account not only the TTSuV infection, but also the specific health status of each animal, possible co-infecting pathogens and the immune status of the host. Thus, nowadays the disease association of TTSuVs is under debate. In fact, the idea that some species are assumed to be more virulent than others has been suggested for human TTVs

as well as possible implications of co-infections with other pathogens (Okamoto, 2009a). As an example, among other ssDNA viruses such as porcine circoviruses, PCV2 is described a pathogenic and causing PCVDs, while PCV1 is considered non-pathogenic. In fact, PCV2 and PCV1 share only about 76% of nucleotide sequence identity and have similar genomic organizations (Hamel *et al.*, 1998; Meehan *et al.*, 1998; Morozov *et al.*, 1998). Furthermore, PCV2 is ubiquitous in the swine population, like TTSuV, and it is necessary but not sufficient for the onset of PCV2-SD (Segalés *et al.*, 2005).

In addition, in study I, serum samples of PCV2-SD-affected and healthy pigs were used to study disease association with the novel TTSuVk2b. These samples were used in the past by means of different qPCR methods to detect the so far existing TTSuVs (Aramouni *et al.*, 2011; Nieto *et al.*, 2011). Indeed, the literature shows methods like PCR or qPCR that have been developed by several laboratories using different technologies, methods and/or primer pairs. This may lead to disagreements on the results and even misinterpretation of them. For that, it would be important to compare the currently used methods for TTSuV detection and quantification. Thus, in the study I, a ring-trial to estimate the reliability for TTSuV detection was done in two different laboratories. The applied qPCRs in study I were based on SYBR Green-based and Light Upon Extension (LUX) qPCR techniques. Both assays detected TTSuVk2a with good concordance, but the LUX assay quantified about 10-fold higher loads compared to the SYBR Green technique. These results should not be considered surprising since they use different quantification methods as well as primer pairs. In the LUX qPCR, one fluorogenic primer was used and labelled with a fluorophore close to the 3'-end that was quenched by the hairpin structure of the primer. On the formation of the PCR product, the fluorescence increased due to extension of the hairpin structure. On the contrary, the SYBR Green-based qPCR technology was based on a double-stranded DNA binding dye and unlabelled primers. The primers used in each technique hybridized at different regions of the viral genome and were likely to cause differences in detection rates between technologies, especially considering the high nucleotide variability of TTSuVs (Cortey *et al.*, 2011; Huang *et al.*, 2010b). It should be added, as well, that the samples were not tested contemporaneously

with both techniques (1-year difference), so, it could not be ruled out that the quality of them was in a better shape when the LUX assay was performed.

For studies II and III of this PhD Thesis, several proteins were produced in different platforms, such as *E. coli* and baculovirus expression systems. At the beginning of the studies, it was already known that the TTSuV genome contains four ORFs, ORF1, ORF1/1 (also known as ORF1-A), ORF2, and ORF3 (also known as ORF2/2) (Huang *et al.*, 2012b; Lu *et al.*, 2013; Martínez-Guinó *et al.*, 2011). ORF3 can only be translated after splicing of the mRNA precursor, and splicing of the ORF1 mRNA transcript results in different protein isoforms (Huang *et al.*, 2012b; Martínez-Guinó *et al.*, 2011), as also observed in human TTVs (Müeller *et al.*, 2008; Qiu *et al.*, 2005).

Even if TTSuVs are currently considered non-pathogenic, it is hard to assume that a viral infection would not have consequences to its host. As another example, farm performance is improved when PCV2 (also a single stranded circular DNA virus) infection is controlled by vaccination even in farms without disease, displaying only a subclinical infection (Beach & Meng, 2012). Currently, commercial vaccines against PCV2 are efficient in reducing the serum viral load, preventing the disease and improving the performance of the farm. Nevertheless, the prevention and control against TTSuV infections have not yet been established, but it was hypothesized the possibility that the subclinical infection by TTSuVs had relevant consequences for the pig production. In consequence, two experimental (study II and III) vaccines against TTSuVs were developed and tested in this PhD Thesis.

The second study evaluated the impact of a multivalent vaccine in naturally TTSuV infected pigs. It is known that anelloviruses are genetically variable and different variants may cause infection. In fact, multiple infections of TTSuV with different genotypes or subtypes in a single pig have been reported (Blois *et al.*, 2014; Gallei *et al.*, 2010; Huang *et al.*, 2010b), as observed in Study I. Also, it is known that anelloviruses infect the host as quasispecies, meaning that infection is composed of different, closely related genomic variants of the virus (Cortey *et al.*, 2012; Huang *et al.*, 2010b; Ng *et al.*, 2009a). For that reason, the multivalent vaccine was a *priori* a good tool to cover the maximum of the

TTSuV diversity. The development and use of multivalent vaccine strategies have been successfully used in *human papilloma virus* (HPV), also a genetically diverse virus (IARC, 2007). The development of a multivalent vaccine against HPV types 6, 11, 16 and 18 afforded protection against two diseases (cervical and genitals warts) and has demonstrated that this 4-valent vaccine was able to broaden the reach of immune response against four circulating strains of HPV (Garland *et al.*, 2007; Group., 2007; Muñoz *et al.*, 2009).

The multivalent vaccine prototype (including ORF1 and ORF3 proteins) against the four known TTSuV species was not able to control viremia during TTSuV natural infection despite of seroconversion of vaccinated animals. This result suggested that generated antibodies were not able to neutralize the virus sufficiently to control viremia. It could be that the protein vaccine only stimulated the humoral response, which may not be enough to control TTSuV viremia.

In study II, recombinant ORF1 and ORF3 proteins from TTSuV1 (1a and 1b) and TTSuVk2 (k2a and k2b) were considered good vaccine candidates *a priori*. On one hand, ORF1 is the putative capsid protein containing known antigenic domains (Huang *et al.*, 2011; Jarosova & Celer, 2013). On the other hand, ORF3 N-terminal region is identical with ORF2 and, therefore, ORF3 protein covers aa sequence of the two different viral proteins encoded by these genes. Although the vaccine prototype did include predicted antigenic epitopes, the lack of a reliable virus neutralization assay did not allow concluding if they evoked neutralizing antibodies (Huang *et al.*, 2011; Jarosova & Celer, 2013). It may happen that the specific strains used to prepare the multivalent vaccine did not elicit a robust neutralising antibody response to TTSuVs and, in consequence, did not produce antibodies of the appropriate specificity; it has to be reminded that there is a significant genetic variability even within the same species of TTSuV. Representative sequences from each of the 4 TTSuV groups were selected of each gene. Despite of that, maybe the TTSuV strains circulating in the pigs of this study were distinct from the vaccine strains. Therefore, it could be that viruses closest to the antigens in the vaccines would have been working better. On the other hand, it cannot be ruled out that the formulation of a multivalent prototype vaccine may cause interference between serotypes; maybe the

response against the dominant serotypes was low or even absent, thus not causing an efficient reduction of viremia.

Specifically, the ORF1 full-length capsid protein of four known TTSuVs was expressed using a baculovirus expression system for the first time. Other research groups have used bacterial, insect cell or mammalian expression systems. However, expression of entire full-length ORF1 protein had not been successful before (Kakkola *et al.*, 2008; Kakkola *et al.*, 2002), although ORF1 mRNA has been detected at transcriptional level (Huang *et al.*, 2012b; Kakkola *et al.*, 2008; Kakkola *et al.*, 2002; Martínez-Guinó *et al.*, 2011). Indeed, in the study II, TTSuV proteins were first codon optimized and expressed with a short affinity-tag (Strep or His) in their C-terminal end. This design may have improved the baculovirus expression and/or stability of the proteins, resulting in detectable levels of ORF1.

Although not significant, vaccinated animals with the multivalent vaccine prototype had higher TTSuV loads than non-vaccinated control animals. It could be that vaccination activated humoral immune responses resulting in non-neutralizing antibodies, which might enhance the viral infection through interactions with the complement system and its receptors, creating antibody-virus complexes that might up-regulate TTSuV replication (Boonnak *et al.*, 2008; Willey *et al.*, 2011). Indeed, viremia exacerbation has been described with other experimental vaccines and viral infections, such as *Dengue virus (DENV)*, *Feline Infectious Peritonitis virus* (FIPV) or *African swine fever virus* (ASFV) (Argilaguet *et al.*, 2011; Vennema *et al.*, 1990; Whitehead *et al.*, 2007). However, little is known about the interaction that TTVs establish with the host immune system and more work should be needed to study this phenomenon.

Since the seroconversion of vaccinated animals against the multivalent vaccine antigens could not control the TTSuV infection, a second animal trial (study III) was designed to apply a different strategy. In the new approach, a DNA prime-boost design was used to enhance the immune response combined with a protein third boost. Several studies have developed DNA vaccine strategies against different pathogens to improve the immune response. Thus, the overall immune responses using the prime-boost strategy might be

improved, and may be effective at generating high levels of T- memory cells (Ramshaw & Ramsay, 2000). Furthermore, the second animal trial included a vaccine against only one TTSuV species to compare the behaviour of a vaccine against a single species in front of a multivalent vaccine (study II). The TTSuVk2a was the species selected because of its documented relationship of co-infection with other viral pathogens. In the second vaccination approach (study III), the strategy was to get wider coverage of the antigenic regions of the ORF1 gene in order to increase the antigenic region. To cover as much of the antigenic region variability as possible, the 3 isoforms from ORF1 (A, B and C) were used in the new approach (study III) in front of the full length ORF1 protein in the multivalent vaccine prototype (study II). These isoforms shared the N-terminus region; however, the C-terminus varied depending on the splicing site, being the isoforms different in aa composition, thus increasing the variability. In fact, the C-terminus of ORF1 protein has been identified as the main antigenic region (Huang *et al.*, 2012a; Jarosova & Celer, 2013). On the other hand, ORF2 (DNA/protein vaccine) was used in study III in contrast with ORF3 (used in the multivalent vaccine prototype, study II).

The third study describes the immunization against TTSuVk2a during natural infection of pigs using a combination of viral DNA and protein. It was found that TTSuVk2a loads were significantly lower in the immunized group than in the control group at the end of the study. In fact, a number of animals of the immunized group cleared the TTSuVk2a viremia, which was not the case in the control group at the end of the study (15 weeks of age). Moreover, a delay in TTSuVk2a natural infection was observed after DNA immunization (used as prime boost to stimulate the cellular response). Taking into account that such situation occurred in absence of specific antibodies, it may be indicative of specific T-cell responses involved in the observed partial virological protection. In fact, the DNA vaccine was used as prime-boost in study III to improve the immune response and exert their effect in different molecular pathways involved in the immune system such as T cell responses (Bolhassani & Yazdi, 2009; Rodriguez & Whitton, 2000). The third immunization with a cocktail of proteins (ORF1-A isoform and ORF2 with adjuvant) was used to induce humoral responses against TTSuVk2a. Several studies have demonstrated that priming with DNA combined with a subsequent immunization with a

different product like recombinant viruses or proteins allows obtaining enhanced protective efficacy (Lu *et al.*, 2009; Woodland, 2004).

Overall, the results of study III, where TTSuVk2a infection was controlled by combination of DNA and protein vaccination, suggest that both cellular and humoral immune responses should be necessary to control TTSuV infection. It would have been very interesting to monitor experimentally vaccinated pigs by means of humoral and cellular immune responses, since both are believed to be necessary in controlling viral infections. Vaccine development studies have been shown the importance to enhance both humoral and cellular immune response, such as for *Hepatitis C virus* (HCV). Several reports have shown that an effective T-cell immune response is necessary for HCV clearance, while the neutralizing antibodies play an important role in eradicating HCV (Esumi *et al.*, 1999; Mancini *et al.*, 2009 ; Owsianka *et al.*, 2008; Puig *et al.*, 2004; Sun *et al.*, 2015).
It is known that TTSuV-specific antibodies increase with age in conventional healthy pigs during natural infection. Also, in a recent study, it has been observed that TTSuV-specific antibodies started to appear when the animals were 10 weeks of age and peaked at week 20. The same study detected antibodies at 4 weeks of age (probably residual maternal antibodies) and these decreased until 10 weeks of age (Jarosova & Celer, 2013). Another recent study corroborated these previous studies (Nieto *et al.*, 2015) .
Studies II and III were based on natural TTSuV infection and, therefore, antibodies developed also in control group animals. However, few animals of control group generated detectable levels in both studies (II and III). In study II, only 4 pigs in the control group generated detectable antibody levels at 3, 7, 9 and 12 weeks of age. In study III, only three non-immunized animals generated detectable levels of antibodies at the end of the study (15 week of age). Since studies II and III ended when pigs reached 15 weeks of age, it is impossible to know the natural antibody response would have evolved at older ages. Importantly, the difference in antibody detection between vaccinated and non-vaccinated groups evidenced the success of the used immunization methods in both studies to induce early humoral responses against TTSuVs.

In study III, the DNA/protein combination controlled viremia, but maybe not against all potentially existing TTSuVk2a variants. In fact, not all immunized animals cleared

TTSuVk2a infection and, at the end of the study, some of them had relatively high viral loads. Moreover, as observed in study III where the animals were vaccinated only against TTSuVk2a, difference in viral load of other TTSuVs (TTSuV1 or TTSuVk2b) was not observed. Moreover, antibody cross-reactivity has been demonstrated to exist even between TTSuV1a and 1b (aa identity about 50%), but not between TTSuV1 and TTSuVk2a (ORF1 protein is about 25% identical between TTSuVk2a and TTSuV1) (Huang *et al.*, 2012a). In study III, the generated antibodies were not cross-reacting between TTSuVk2a and k2b (ORF1 protein is 58% identical between TTSuVk2a and TTSuVk2b, as shown in study I). As discussed above, the high degree of genetic variability of TTSuV and the potential divergent TTSuV strains present during infection may explain why the vaccine prototype did not completely protect against TTSuVk2a. Regarding the genetic variability, cross-protection would be a desirable feature and, indeed, a multivalent vaccine seemed a good strategy *a priori* to reduce TTSuVs infection. Also, the use of isoforms from ORF1 genes to increase antigenic variability to cover antigenic regions was initially hypothesized as a good strategy. Anyway, practical results indicated that the multivalent vaccine was not efficient; maybe the multivalent vaccine did not activate the implicated mechanisms in protection against TTSuV infection, which is currently unknown.

As summary of the presented three studies, this Thesis contributes to increase the knowledge of TTSuVs by describing a novel species, which may be involved as a co-pathogen with other infectious agents. On the other hand, to our knowledge, two vaccine studies on TTSuVs were developed for the first time to provide novel information about viral infection control. In fact, TTSuVk2a infection was controlled by the administration of a DNA and protein combined vaccine strategy, while a multivalent protein-based vaccine was not efficient despite it induced specific antibodies. Further studies are needed to improve vaccine efficacy, to elucidate the protective mechanism behind, and to assess the impact of TTSuVs for pig producers. Maybe the multivalent vaccine should be used in combination with a DNA vaccine to see if such approach would improve the outcome.

7. Conclusions

1. A novel TTSuV species, named TTSuVk2b, was identified. According to the phylogenetic analysis, it belongs to the *Kappatorquevirus* genus together with the already known TTSuVk2a.

2. TTSuVk2a and TTSuVk2b are not only genetically related, but also their viral loads in serum were higher in PCV2-SD affected animals compared with healthy pen mates.

3. TTSuV proteins, namely ORF1, ORF1-A, ORF2 and ORF3, of the four known TTSuVs species were successfully expressed in a baculovirus system.

4. A multivalent vaccine prototype based on baculovirus expressed TTSuV proteins (ORF1 and ORF3 proteins) was not able to control viremia during TTSuV natural infection. This lack of efficacy occurred despite that immunized animals seroconverted against the virus. In consequence, the generated humoral immune response was not sufficient to control the viremia.

5. A combination of DNA and protein immunization was shown to control TTSuVk2a viremia during TTSuV natural infection. The onset of viremia was delayed and viral loads were lower in immunized animals. The vaccine prototype was specifically controlling TTSuVk2a infection and did not affect other TTSuV species.

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