STUDY ON THE DETECTION OF TORQUE TENO SUS VIRUS BY NESTED POLYMERASE CHAIN REACTION FROM PIGS REARED IN CONVENTIONAL FARMS

コンベンショナル農場で飼養されている豚からのトルクテノススウィルスの nPCR による検出

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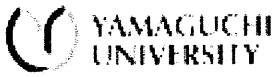

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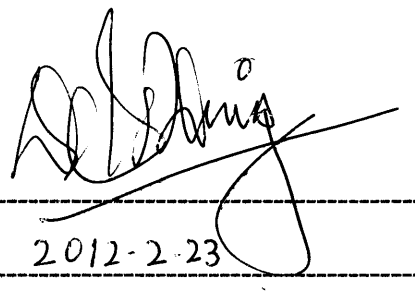
Signature of the author: 

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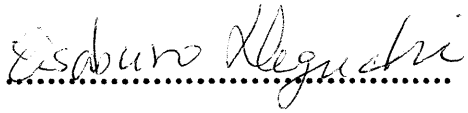

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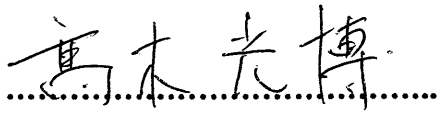

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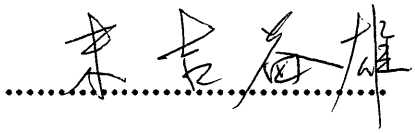

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DEDICATION

This thesis is dedicated to my newly born daughter Kelzang Hana Tshogyel. Welcome Baby! There's so much to see and do, so much adventure waiting for you, time for fun and time to run, everything's new under the sun. May you grow strong and happy under the love and care of your dad, mum, brother and sister.

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ABSTRACT

Torque teno virus (TTV) is a small (30-50nm in diameter), non-enveloped virus that contains a circular single stranded DNA genome of negative polarity. TTV was first isolated in 1997 from a patient with posttransfusion hepatitis of unknown etiology. Subsequently, viruses related to human TTV have been found in a number of other vertebrate animals including pigs. TTV of pigs is currently designated as *Torque teno sus virus* (TTSuV) and consist of 2 distinct species/types, TTSuV *1* (TTSuV1) and *2* (TTSuV2). They are grouped under the *iotatorquevirus* genius of the *Anelloviridae* family. TTSuVs have been detected from serum, faeces, nasal secretion, semen, colostrum and various tissues of pigs exclusively by polymerase chain reaction (PCR) with a prevalence rate ranging from 24% to 100% in different parts of the world. However, many aspects of the biology of TTSuVs are still unknown. This thesis describes the existence of a sow-to-foetus transmission route, seroprevalence in postweaning multisystemic wasting syndrome (PMWS)-suspected pigs and *Porcine circovirus* type *2* (PCV2)-vaccinated normal pigs, detection in tissues of stillborn piglets, and infection dynamics in serum and peripheral blood mononuclear cells (PBMCs) of TTSuVs.

In the first study (Chapter 3), the presence of a sow-to-foetus transmission route during normal pregnancy and a possibility of a sow-to-piglet transmission via colostrum of TTSuVs were examined. For this purpose, a total of 6 sows that were bred to TTSuVs-negative semen of boars were allowed to complete their normal gestation period. Serum samples obtained from sows at farrowing, and their newborn piglets immediately after birth before suckling colostrum and at 24 hrs after suckling colostrum were investigated for the presence of TTSuV1 and TTSuV2 by a nested PCR (nPCR) method. TTSuVs DNA were detected in the sera of newborn piglets delivered by both TTSuV-positive and -negative sows, confirming the existence of sow-to-foetus transmission of TTSuVs. Moreover, the sera of newborn piglets were examined 24 h following their colostrum intake, which indicated that TTSuVs nucleic acids were present in the

sera of those piglets that were TTSuV-negative prior to suckling colostrum, suggesting the possibility of the occurrence of a sow-to-piglet transmission of TTSuVs.

In the second study (Chapter 4), the seroprevalence of TTSuVs in PMWS-suspected pigs and PCV2-vaccinated normal pigs across 3 commercial pig farms (farms A, B, C) in Kagoshima prefecture, Japan was determined. For this purpose, a total of 38 blood samples of PMWS-suspected pigs (from 7, 18, and 13 pigs on farms A, B, and C, respectively) and 43 blood samples of PCV2-vaccinated normal pigs from the same farm (from 8, 22, and 13 pigs on farms A, B, and C, respectively) were investigated for the presence of TTSuVs by a nPCR method. It was observed that the detection rate of TTSuV2 was significantly higher in PMWS-suspected pigs (94.4%) compared to that of the PCV2-vaccinated normal pigs (81.4%). Furthermore, the detection rate of TTSuV2 (94.4% and 81.4%) was significantly higher than that of TTSuV1 (71.1% and 58.1%) in both PMWS-suspected pigs and PCV2-vaccinated pigs, respectively. These results indicate that TTSuV1 and TTSuV2 infections are highly prevalent in both PMWS-suspected pigs and PCV2-vaccinated normal pigs, with TTSuV2 seroprevalence more likely to be higher in PMWS-suspected pigs than in the PCV2-vaccinated normal pigs.

In the third study (Chapter 5), the liver, heart, spleen, and tonsils of Berkshire stillborn piglets derived from sows with increased stillbirths at the time of sampling were examined for the presence of TTSuVs. The results showed that the detection rates of TTSuV1 and TTSuV2 were not uniformly distributed among the tissues. Specifically, TTSuV1 was detected at its highest level in the liver, followed by the heart, spleen, and tonsils, whereas TTSuV2 was detected at its highest level in the tonsils, followed by the liver, spleen, and heart. These results may be suggestive of a presence of different tissue tropism of TTSuV1 and TTSuV2, particularly in the tissues of stillborn piglets and suggest that TTSuVs infection may be associated with stillbirths in sows.

In the fourth study (Chapter 6), the detection of TTSuVs in PBMCs and the infection dynamics of TTSuVs in paired PBMCs and serum samples were investigated. For this purpose, a total of 24 littermates (3 littermates per sow) were seletecd. The PBMCs and serum samples obtained from these litermates at 14, 30, 60, 90, 120 and 150 days of age were examined for the presence of TTSuV1 and TTSuV2 by a nPCR method. In sera, the detection rate of TTSuV1 and TTSuV2 increased with age reaching the peak level at 60 days of age for both TTSuV1 and TTSuV2 (TTSuV1: 75%, TTSuV2: 100%); however, a gradual decrease in the detection rate was seen for TTSuV1 after 90 days of age whereas the peak level was maintained for TTSuV2 until 150 days of age. In PBMCs, the detection rate of TTSuV1 and TTSuV2 increase with age reaching the peak level at 60 days of age (TTSuV1: 75%, TTSuV2: 100%), and thereafter maintaining these peak levels until 150 days of age. In both sera and PBMCs, the detection rate of TTSuV2 was higher than TTSuV1. These results indicate that TTSuVs are present in the body of the conventional pigs as long as 150 days of age, and suggest that TTSuV1 and TTSuV2 may have different infection dynamics under same or different epidemiological conditions.

In conclusion, by using nPCR the current study confirmed the presence of sow-to-foetus transmission of TTSuVs, and showed high prevalence of TTSuVs in both PMWS-suspected pigs and PCV2-vaccinated normal pigs, and also in the tissues of the stillborn piglets derived from sows with increased stillbirths. Furthermore, for the first time, the current study detected TTSuV from PBMCs, and describes the infection dynamic of TTSuVs in the paired PBMC and serum samples which indicated a lifelong presence of the 2 virus types in both PBMCs and serum of pigs raised on conventional farms.

ORIGINAL PUBLICATIONS

This thesis is based on the original publications mentioned below. The original publications are reproduced with the permission of copyright holders.

1. Tshering, C., Takagi, M. and Deguchi, E. 2012. Seroprevalence of *Torque teno sus virus* types *1* and *2* in postweaning multisystemic wasting syndrome-suspected pigs and porcine circovirus type 2 vaccinated pigs in southern Japan. *J. Vet. Med. Sci.* **74**: 107–110.
2. Tshering, C., Takagi, M. and Deguchi, E. 2012. Detection of *Torque teno sus virus* types *1* and *2* by nested polymerase chain reaction in sera of sows at farrowing and of their newborn piglets immediately after birth prior to suckling colostrum and at 24 hours following suckling colostrum. *J. Vet. Med. Sci.* **74**: 315–319.
3. Tshering, C., Takagi, M. and Deguchi, E. 2011. Distribution of *Torque teno sus viruses* in tissues of stillborn piglets delivered by sows at natural farrowing. *J. Vet. Sci.* **13** (3): xxx–xxx (In press).
4. Tshering, C., Takagi, M. and Deguchi, E. 2011. Infection dynamics of *Torque teno sus virus* types *1* and *2* in sera and peripheral blood mononuclear cells. *J. Vet. Med. Sci.* **74** (4): xxx–xxx (In press).

ABBREVIATIONS

ADV	Aujeszky's disease virus
ALT	Alanine amino transferase
BBTV	Banana bunchy top virus
Bp	Base pair
CAV	Chicken anaemia virus
CSFV	Classical swine fever virus
EMV	Encephalomyocarditis virus
GV	Getah virus
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HEV	Hepatitis E virus
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HVR	Hypervariable region
IMM	Idiopathic inflammatory myopathy
JEV	Japanese encephalitis virus
LW	Large white
nPCR	Nested polymerase chain reaction
NSP	Non-structural protein
ORF	Open reading frame
PBFDV	Psittacine beak and feather disease virus

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV2	Porcine circovirus type 2
PCVAD	Porcine circovirus-associated disease
PEV	Porcine enterovirus
PMWS	Postweaning multisystemic wasting syndrome
pPBMC	Pooled peripheral blood mononuclear cell
PRRSV	Porcine reproductive and respiratory syndrome virus
pSE	Pooled serum
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAV	Small anellovirus
ssDNA	Single-stranded deoxyribonucleic acid
TLMV	Torque teno virus-like mini virus
TP	Total serum protein
TTMDV	Torque teno midi virus
TTMV	Torque teno mini virus
TTSuV	Torque teno sus virus
TTV	Torque teno virus
UTR	Untranslated region
VP	Viral protein

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CHAPTER 1

GENERAL INTRODUCTION

The Torque teno virus (TTV) is a nonenveloped virus with a circular, icosahedral, and single-stranded DNA (ssDNA) genome belonging to the family *Anelloviridae*. TTV was initially discovered in humans (Nishizawa, 1997) and later identified in other species including pigs (Okamoto et al., 2002). Two different TTV species, *Torque teno sus virus 1* (TTSuV1) and *Torque teno sus virus 2* (TTSuV2), both members of the *Iotatorquevirus* genus, have been described so far in domestic pigs and wild boars.

Although TTSuV was first detected in 1999 by Leary and his co-researchers (Leary et al., 1999), the presence of this virus was retrospectively detected as early as 1985 by Segales and his coworkers (Segales et al., 2009). Based exclusively on polymerase chain reaction (PCR) results, TTSuV has been considered to be widespread in domestic swine population, with prevalence varying between 24% and 100% in different parts of the world (Bigarre et al., 2005; Brassard et al., 2008; Gallei et al., 2010; Jarosova et al., 2011; Kekarainen et al., 2006; Martelli et al., 2006; McKeown et al., 2004; Takacs et al., 2008). The viruses have been detected in plasma/sera, faeces, colostrum, semen, and various tissues of pigs, including those of foetuses, suggesting that the virus can be transmitted by both horizontal and vertical routes (Brassard et al., 2008; Kekarainen et al., 2007; Martinez-Guino et al., 2009; Martinez-Guino et al., 2010; Pozzuto et al., 2009). However, a definitive study confirming these routes of transmission was still lacking when this study was initiated. Therefore, the experiments described in Chapter 3 of this thesis were performed to clarify the existence of sow-to-foetus transmission during normal pregnancy, and sow-piglet transmission via colostrums of TTSuVs.

Considering its ubiquitous nature in pigs of conventional farms worldwide, TTSuVs have traditionally been considered non-pathogenic. However, some studies have suggested that TTSuV might potentiate certain diseases in pigs, particularly postweaning multisystemic wasting syndrome (PMWS), which is a porcine circovirus-associated disease (PCVAD).With regard to this effect, TTSuV1-containing homogenates were shown to partially contribute to the

experimental induction of PMWS (Ellis et al., 2008; Krakowka et al., 2008) when co-infected with *Porcine circovirus* type *2* (PCV2) in a gnotobiotic pig model, and a higher prevalence of TTSuV2 in PMWS-affected pigs than in non-PMWS-affected pigs was observed in a field study (Kekarainen et al., 2006; Nieto et al., 2011). However, contradictory results were reported by a Korean group of researchers (Lee et al., 2010), who found no significant differences in the viral loads of either TTSuV species between PCV2-negative pigs and PMWS-affected pigs. Recently, PMWS has been successfully controlled by the introduction of routine PCV2 vaccination in pig farms. To provide further insights into the potential role of TTSuV in the occurrence of PMWS, a comparative study on the seroprevalence of PMWS-suspected pigs and PCV2-vaccinated normal pigs was performed, which is described in Chapter 4 of this thesis.

The detection of TTSuVs in semen, colostrum, sera of stillborn piglets, and various tissues of foetuses suggests that these 2 virus types could be transmitted vertically (Kekarainen et al., 2007; Martinez-Guino *et al.*, 2009; Martinez-Guino *et al.*, 2010; Pozzuto *et al.*, 2009). However, it is unknown whether vertical transmission of the virus could be related to any reproductive disease or performance loss. Martinez-Guino *et al.* (2010) found no significant differences between the detection rate of TTSuVs in aborted and non-aborted foetuses, but another research group reported an association between TTSuV2 detection in the sera of sows and the number of stillbirths (Sibla *et al.*, 2009b). Moreover, TTSuVs was detected in the sera of stillborn piglets (Martínez-Guinó *et al.*, 2009) but there are no previous studies showing the presence of TTSuVs in the tissues of stillborn piglets. Chapter 5 of this thesis reports the detection of TTSuVs in selected individual tissues of stillborn piglets delivered by sows with increased stillbirths at natural farrowing.

One of the most important issues that remain unanswered about TTSuVs is its site or target of replication. In humans, peripheral blood mononuclear cells (PBMCs) and bone marrow haematopoietic cells have been suggested as the 2 possible sites of TTV replication (Maggi *et*

al., 2001a; Mariscal *et al.*, 2002; Zhong et al., 2002). However, little is known about the primary infection with TTSuV and the sites of viral persistence and reactivation after infection in pigs. Moreover, there have been no studies conducted to examine the presence of TTSuVs in PBMCs of pigs. In addition, the infectious dynamics of TTSuVs after it infects the host remain largely unknown, though limited longitudinal studies employing serum (Nieto *et al.*, 2011; Sibla *et al.*, 2009) and few cross-sectional studies employing serum and tissues (Aramouni *et al.*, 2010; Taira *et al.*, 2010) have shown that TTSuV infection increases with age. Chapter 6 of this thesis describes the infection dynamics of TTSuV in paired serum and PBMCs of growing-finishing pigs from 14 to 150 days of age.

Therefore, the overall objectives of this thesis were as follows: (1) To clarify the existence of sow-to-foetus transmission of TTSuVs during pregnancy and sow-to-piglet transmission via colostrum; (2) To determine the seroprevalence of TTSuV in PMWS-suspected pigs and PCV2-vaccinated pigs in order to provide insights into the pathogenic potentials of TTSuV in the occurrence of PMWS; (3) To determine the tissue distribution of TTSuVs in stillborn piglets delivered by sows with increased stillbirths in order to provide some insights into the possible association of TTSuVs with stillbirths and (4) To investigate the infection dynamics of TTSuVs in paired serum and PBMCs of pigs reared on conventional farms.

CHAPTER 2

LITERATURE REVIEW

2.1. History of discovery

Viruses that cause hepatitis include hepatitis A–E viruses, which are broadly classified into 2 categories: those with haematogenous spread [hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis D virus (HDV)] and those with enteric spread [hepatitis A virus (HAV) and hepatitis E virus (HEV)]. However, there are still numerous hepatitis cases of unknown aetiology. In 1997, a novel virus associated with post-transfusion hepatitis was identified by representational difference analysis of 3 patients from Japan who developed elevated serum aminotransferase concentrations following transfusion and tested negatively for all known hepatitis viruses (Nishizawa et al., 1997). This novel virus was referred to as 'TT virus' for the initials of the patient in whom it was isolated or as 'transfusion-transmitted virus' (TTV). The virus exhibited hepatotropism, and its titres correlated with elevation in serum aminotransferase concentrations, suggesting that it was a true hepatitis virus. Later studies showed that TTV was the first circular single-stranded DNA (ssDNA) virus found to infect humans, and that it is similar in structure to other animal viruses classified within the *Circoviridae* family (Miyata et al., 1999; Mushahwar et al., 1999). Recently, the International Committee on Taxonomy of Viruses proposed a new meaning for 'TT', namely *Torque teno virus*, deriving from the Latin terms 'torque' meaning 'necklace' and 'tenuis' meaning 'thin'. These terms were chosen to reflect the organisational arrangement of the TTV genome (Todd et al., 2005).

TTV has been identified in humans and in nonhuman primates and vertebrate animals, including swine, poultry, cattle, sheep, cats, dogs, gibbons, and camels (Abe et al., 2000; Al-Moslih et al., 2007; Cong et al., 2000; Inami et al., 2000b; Leary et al., 1999; Nopporpanth et al., 2001; Okamoto et al., 2000a; Okamoto et al., 2000b; Okamoto et al., 2001b; Okamoto et al., 2002; Romeo et al., 2000; Tawara et al., 2000, Thom et al., 2003; Verschoor et al., 1999). Furthermore, the complete genomic sequences of species-specific TTVs infecting humans, non-human primates, tree shrews (Tupaia spp.), domestic pigs, cats, and dogs have been determined.

2.2. Taxonomy and molecular and biological properties of torque teno viruses

Virus families with circular ssDNA genomes include *Microviridae* (bacterial viruses), *Geminiviridae* (plant viruses), and *Circoviridae* (plant and animal viruses). The genus *Circovirus* within the family *Circoviridae* contains animal and plant viruses, such as PCV, psittacine beak and feather disease virus (PBFDV), and banana bunchy top virus (BBTV). Their genomes are ambisense and have a common 9-nt stem-loop structure at the replication origin. Chicken anaemia virus (CAV), which has a negative-strand genome without the 9-nt structure common to circoviruses, is classified into the genus *Gyrovirus*. TTV has some similarity to CAV in the family *Circoviridae* and genus *Gyrovirus*, but possesses a significantly larger genome size. Thus, TTV was initially classified into a new genus *Anellovirus*, which is named for the Latin meaning 'ring' (Biagini et al., 2005; Hino, 2002). However, recently, anelloviruses were assigned a new separate family '*Anelloviridae*' and deemed to consist of 9 distinct genera (Biagini et al., 2007; Brassard et al., 2008; Niel et al., 2005).

TTV is estimated to be 30–50 nm in diameter (Itoh et al., 2000; Mushahwar et al., 1999) (Fig. 1) and possesses a circular ssDNA genome, with an 89% to 90.6% GC-rich region of 117 nucleotides (Fig. 2). The transcription regulation sites are similar to those of CAV, and possess a negative-sense DNA (Miyata et al., 1999; Mushahwar et al., 1999) with an icosahedral and nonenveloped genome (Okamoto et al., 1998b). The genomes of TTVs that infect humans and chimpanzees range from 3.7 to 3.9 kb in size, those infecting pigs and dogs are 2.8 and 2.9 kb in length, respectively, and the TTV with the smallest genome identified to date, 2.1 kb, was detected in cats (Erker et al., 1999; Hallett et al., 2000; Heller et al., 2001; Hijikata et al., 1999b; Miyata et al., 1999; Okamoto et al., 2002; Peng et al., 2002). The genome contains a coding region of approximately 2.6 kb and an untranslated region (UTR) of 1.2 kb.

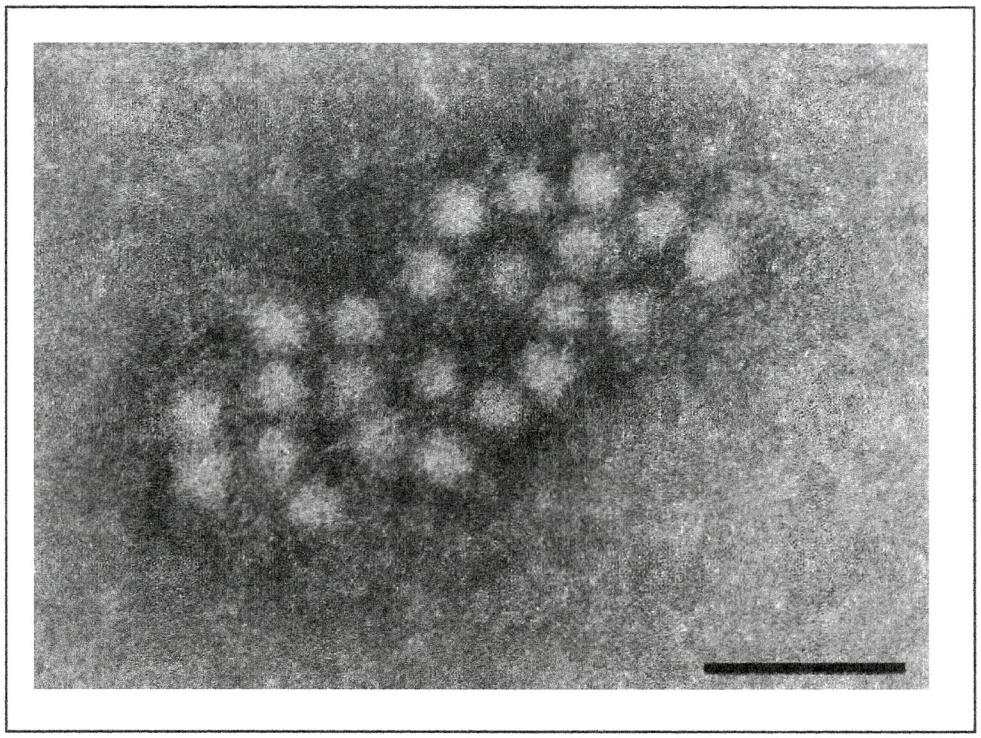


Fig. 1. Electron microscopy of TTV aggregates in a serum sample with a TTV level of 10^8 copies/mL. The scale bar is equal to 100 nm (Itoh et al., 2000).

Reports of the TTV genome sequence have described it as extremely variable across TTV isolates; however, certain areas are conserved (Erker et al., 1999; Hallett et al., 2000; Heller et al., 2001; Hijikata et al., 1999b; Mushahwar et al., 1999; Tanaka et al., 1998b; Viazov et al., 1998a). At the nucleotide level, the UTR is more conserved than the coding area, and contains elements for transcription regulation (Kamada et al., 2004; Miyata et al., 1999; Suzuki et al., 2004). Divergences of 47% to 70% have been reported at the amino acid level (Biagini et al., 1999; Luo et al., 2002). However, the high degree of divergence is not distributed uniformly across the genome. In all isolates, a GC-rich region of 108–160 nucleotides is present in the UTR (Hallett et al., 2000b; Heller et al., 2001; Peng et al., 2002). The poly-A sequence downstream and the TATA box upstream of the coding regions are also conserved (Erker et al., 1999; Hallett et al., 2000; Heller et al., 2001; Hijikata et al., 1999). Interestingly, the coding regions of TTV are less conserved than the UTR. For instance, the coding region of open reading frame (ORF) 1

contains 3 hypervariable regions (HVRs) in tandem (Mushahwar et al., 1999; Nishizawa et al., 1999). The variability within ORF1, which is believed to code for the TTV capsid protein, may be crucial for evasion from the host immune system (Takahashi et al., 1998b). The UTR contains conserved stem-loop structures (Hijikata et al., 1999b; Okamoto et al., 2002), which are the sites of transcription factor binding, promoters, and enhancer elements that may be crucial for efficient replication and transcription (Kamada et al., 2004; Miyata et al., 1999; Suzuki et al., 2004).

Initially, analysis of the full-length sequences identified 2–3 ORFs (Erker et al., 1999; Miyata et al., 1999; Mushahwar et al., 1999; Okamoto et al., 1998b). However, recent studies indicated expression of at least 6 proteins, designated as ORF1, ORF2, ORF1/1, ORF2/2, ORF1/2, and ORF2/3, from 3 or more spliced mRNAs (Kakkola et al., 2008; Mueller et al., 2008; Qiu et al., 2005).

ORF1 is the longest ORF and encodes a capsid protein (Takahashi et al., 1998b). The predicted length of the ORF1-encoded protein varies, ranging from 719–770 amino acids (Erker et al., 1999; Heller et al., 2001; Tanaka et al., 2000b; Ukita et al., 2000). In certain isolates, stop codons are located in the middle of ORF1, shortening or interrupting the putative protein (Erker et al., 1999; Jelcic et al., 2004; Luo et al., 2002). It has been suggested that the interrupted ORF1 could form defective viruses (Pollicino et al., 2003). The ORF1 sequence varies at the amino acid level among TTV isolates, showing amino acid differences in the order of 50–70% (Luo et al., 2002; Ukita et al., 2000). However, these differences are not uniformly distributed since ORF1 contains HVRs in which mutations/nucleotide changes occur, leading to amino acid changes occurring more frequently than in the remaining part of the protein (Jelcic et al., 2004; Luo et al., 2002; Mushahwar et al., 1999; Nishizawa et al., 1999; Takahashi et al., 1998b). It has been suggested that TTV could escape immunological responses by mutating these HVRs.

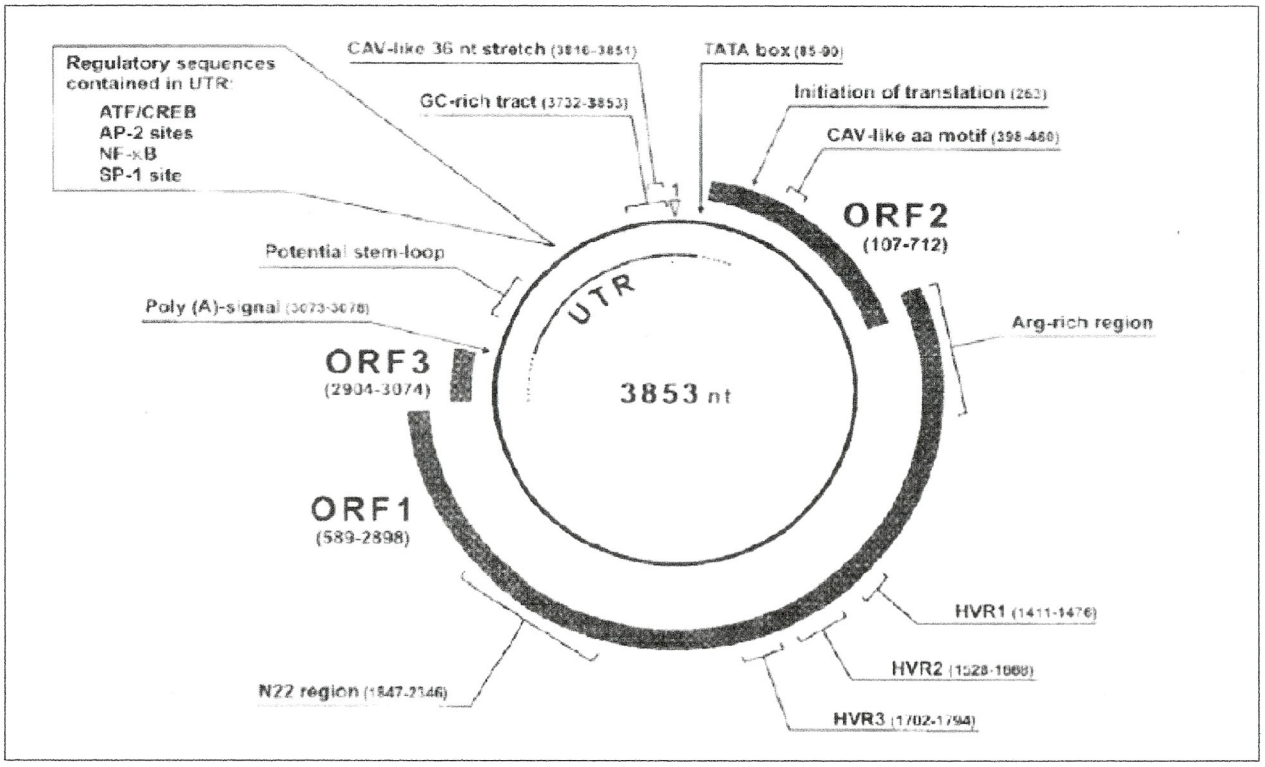


Fig. 2. Detailed structure of human TTV genome (Bendinelli et al., 2001).

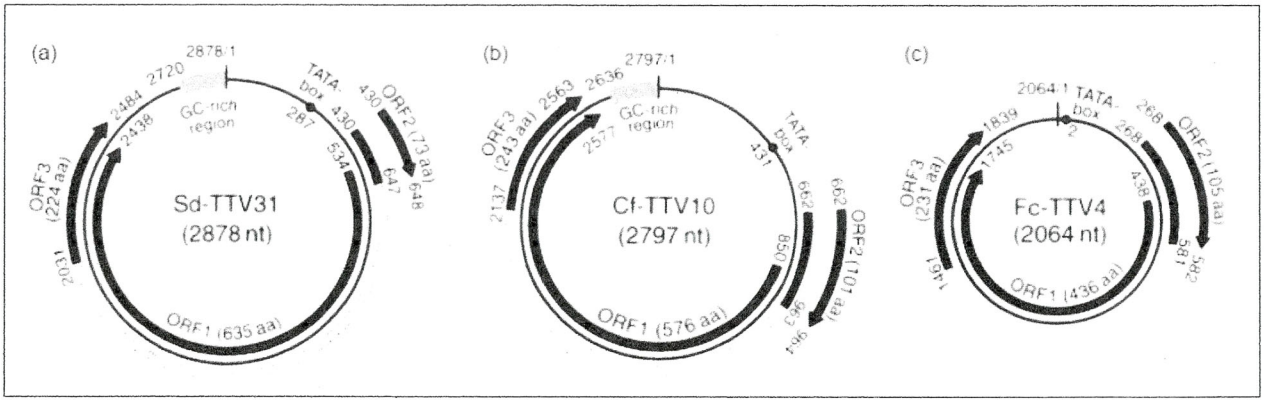


Fig. 3. Comparison of predicted genomic organisation of swine (a), canine (b), and feline (c) TTV isolates. The circumference of each circle represents the relative size of the genome. The closed arrows represent the ORFs. The open boxes located between an upstream closed box and downstream closed arrow in ORF3, which encodes a putative joint protein, represents an area corresponding to an intron in the shorter mRNA. The shaded box indicates the GC-rich stretch and the smaller closed circle represents the position of the TATA box (Okamoto et al., 2002).

The ORF1-encoded protein is also assumed to be involved in replication, since ORF1 has 2 rolling circle replication motifs, so-called Rep-motifs, of which at least one seems to be conserved among the majority of TTV isolates (Erker et al., 1999; Luo et al., 2002; Mushahwar et al., 1999; Tanaka et al., 2001). In addition, the ORF1-encoded protein has an arginine-rich N-terminus (Hijikata et al., 1999b; Mushahwar et al., 1999; Okamoto et al., 1998b; Takahashi et al., 1998b; Tanaka et al., 2001) that might have DNA-binding activity and function in packaging of viral DNA into capsids (Erker et al., 1999). The capsid protein, viral protein (VP) 1, of CAV has Rep-motifs and an arginine-rich N-terminus similar to the ORF1-encoded protein of TTV, and is assumed to function as a replication-associated protein (Niagro et al., 1998). The TTV ORF1-encoded protein also contains potential glycosylation sites (Tanaka et al., 2000b) that vary between isolates, and could have some effect on protein function and/or antigenicity (Bendinelli et al., 2001; Hijikata et al., 1999b).

ORF2 encodes a putative protein of approximately 200 amino acids. However, in several genotypes, a stop codon divides ORF2 into 2 smaller protein-coding areas, ORF2a and ORF2b. ORF2b is less conserved in amino acid sequence than ORF2a (Tanaka et al., 2000b; Ukita et al., 2000); however, ORF2 (or ORF2b) has a conserved amino acid motif, WX7HX3CX1CX5H, which is also found in the CAV protein (Hijikata et al., 1999b; Peng et al., 2002; Tanaka et al., 2001). This conserved motif corresponds to the protein-tyrosine phosphatase (PTPase) signature motif, and the protein might be involved in the regulation of cellular and/or viral proteins during infection (Peters et al., 2002).

The nomenclature of the remaining TTV proteins/ORFs varies to some extent among publications. The ORF3-encoded protein, i.e. ORF2/2 and ORF2–4 on the work of Kakkola et al., (2008) and Kamahora et al., (2000), respectively, of approximately 280 amino acids has a serine-rich domain preceded by basic amino acids at the C-terminus (Peng et al., 2002; Tanaka et al., 2001). Although, the exact function of this protein during the viral life cycle is not yet

known, it has been suggested that like in other viruses where the phosphoprotein usually take part in virus replication or in the regulation of cellular proteins, the phosphorylated TTV-ORF3 protein at the serine residues in the C-terminal region may be involved in perfoming similar functions observed in other viruses (Asabe et al., 2001). The protein encoded by ORF2–5 (i.e. ORF2/3) (Kakkola et al., 2008), which is approximately 280 amino acids, has been shown to have similarities with proteins possessing transcription-factor activity and could thus have some role in the regulation of TTV transcription (Kamahora et al., 2000). A conserved amino acid motif, E-X8-R-X2-R-X6-P-X12-19-F-X1-L, has been found in the C-terminus of the protein (Okamoto et al., 2000b; Peng et al., 2002), but has an unknown function. One putative protein of 105 amino acids, named TTV-derived apoptosis-inducing protein (TAIP), has been shown to induce apoptosis in hepatocellular carcinoma cells, and thus resembles the apoptin protein of CAV that causes apoptosis in cancer cells (Kooistra et al., 2004). However, whether this TAIP is expressed by all TTV isolates, or has some cell/tissue-type specificity, remains to be elucidated. In addition to the proteins already listed, other ORFs have been suggested to arise by intragenomic rearrangements and by alternative splicing (Leppik et al., 2007), thus potentially further expanding the coding capacity and the variability of TTV isolates.

2.3. Human TTV

The genome of human TTV exhibits an astonishingly high genetic diversity and is classified into nearly 39 genotypes that incorporate 3 hypervariable regions within ORF1 (Peng et al., 2002; Prescott et al., 1999). In addition to these genotypes, other small anelloviruses (SAV), such as TTV-like minivirus, Torque teno virus-like mini virus (TLMV; later termed Torque teno mini virus (TTMV)), and Torque teno medi viruses (TTMDV), which resemble TTV in their genomic structures, have been identified (Biagini et al., 2001; Biagini et al., 2006a;

2006b; Biagini et al., 2007; Chung et al., 2007; Jones et al., 2005; Niel et al., 2001; Takahashi et al., 2000).

2.3.1. Prevalences and geographic distributions

After the discovery of TTV from the patient with post-transfusion hepatitis of unknown aetiology, the need to investigate its prevalence among healthy individuals and various patient groups became important for establishing possible disease associations. Although a low level of prevalence was detected in initial studies, later studies revealed high and global distribution of TTV that increases with age (Abe et al., 1999; Hsieh et al., 1999; Okamoto et al., 1999b; Prescott et al., 1999; Saback et al., 1999; Umemura et al., 2001a; Viazov et al., 1998a; Zhong et al., 2001a). For example, TTV DNA positivity in the sera of blood donors was found to be 46–62% in Brazil (Devalle et al., 2004; Niel et al., 1999) 51.6–82.7% in Turkey (Erensoy et al., 2002; Yazici et al., 2002), 90% in Norway (Huang et al., 2001), and 53.3% in China (Zhong et al., 2001a). The occurrence of variation in geographical prevalence of certain genotypes or evolution of genotypes within isolated ethnic groups has been reported (Prescott et al., 1999; Umemura et al., 2003; Vasconcelos et al., 2003). However, results showing no geographical differences in TTV DNA prevalence or genotype/genogroup prevalence have also been presented (Gallian et al., 2000; Huang et al., 2001; Manni et al., 2002; Mushahwar et al., 1999; Pisani et al., 1999). It seems that the knowledge of TTV prevalence is continuously changing. New variants are discovered and new methods are developed (Biagini et al., 2007; Niel et al., 2005). Nevertheless, it is obvious that this virus (or these viruses) is frequently (or constantly) present in the blood of the majority of healthy individuals.

2.3.2. Detection in tissues and body fluids

TTV DNA has been detected in several organs, cells, and body fluids, including peripheral blood mononuclear cells (PBMCs), saliva, urine, faeces, throat swabs, liver, bile, cervical swabs, semen, hair, skin, bone marrow, haemopoietic cells, lymph node, muscle, thyroid gland, lung, spleen, pancreas, kidney, cerebrospinal fluid, gastrointestinal tract, and brain, exclusively by PCR methods (Chan et al., 2001a; Deng et al., 2000; Desai et al., 2005; Fornai et al., 2001; Gallian et al., 2000; Inami et al., 2000a; Ishikawa et al., 1999; Itoh et al., 2000; Jiang et al., 2000; Kanda et al., 1999; Lopez-Alcorocho et al., 2000; Maggi et al., 2001b; Maggi et al., 2003a; Martinez et al., 2000; Matsubara et al., 2000; Okamoto et al., 1998b; Okamoto et al., 1999a; Okamoto et al., 2000c; Okamoto et al., 2000d; Okamoto et al., 2001a; Okamura et al., 1999; Osiowy et al., 2000; Pollicino et al., 2003; Rodriguez-Inigo et al., 2000; Rodriguez-Inigo et al., 2001; Ross et al., 1999; Sospedra et al., 2005; Suzuki et al., 2001; Tanaka et al., 2000a; Ukita et al., 2000; Yamamoto et al., 1998; Yu et al., 2002; Zhong et al., 2002). Furthermore, it seems that there is no preference for a given genotype to exist in certain tissues (Nakagawa et al., 2000; Pollicino et al., 2003). In addition to being present in these normal tissues, TTV DNA has also been detected in tissues of several cancer types (de Villiers et al., 2002), including brain tumours (Sospedra et al., 2005).

2.3.3. Modes of transmission

TTV has been detected in various body tissues and fluids, suggesting the possibility of several transmission routes. The finding of higher TTV DNA prevalence in patients who received blood transfusion relative to that in non-recipients suggested that the virus is transmitted through blood and blood products (Forns et al., 1999; Maeda et al., 2000; Prati et al., 1999; Simmonds et al., 1998; Viazov et al., 1998b). In fact, some researchers have termed TTV as a

'transfusion transmitted virus'. However, transmission via blood does not explain the global prevalence of TTV. Transmission via the faeco-oral route has also been suggested since the virus is found in faeces (Lin et al., 2002; Okamoto et al., 1998a; Ross et al., 1999; Ukita et al., 1999), even among 7–12-month-old healthy children (Lin et al., 2000). TTV detection in surface water from Japan (Haramoto et al., 2005a) and in wastewater (Haramoto et al., 2005b; Vaidya et al., 2002) indicates that water could possibly be one of the sources of TTV transmission for the faeco-oral route. In addition, saliva has been found to contain higher TTV loads than serum (Deng et al., 2000; Gallian et al., 2000), and SAV/TTMDV has been detected in throat swabs (Ishikawa et al., 1999) and nasopharyngeal aspirates (Chung et al., 2007), suggesting the possibility of transmission via the respiratory tract and droplets. Furthermore, TTV DNA has been detected in cervical swabs (Calcaterra et al., 2001; Chan et al., 2001c; Fornai et al., 2001) and semen (Inami et al., 2000a; Martinez et al., 2000), suggesting a sexual mode of transmission.

Human beings are believed to acquire TTV infection(s) very early in life, probably due to the occurrence of *in utero* transmission since TTV DNA has been detected in the blood of newborns, in cord blood, and in amniotic fluid (Bagaglio et al., 2002; Gerner et al., 2000; Matsubara et al., 2001; Morrica et al., 2000; Saback et al., 1999). In addition, it has been shown that newborns acquire TTV from the environment within a few months (Davidson et al., 1999; Kazi et al., 2000; Komatsu et al., 2004). This environmentally acquired infection could be transmitted from the mother to the child, since TTV has been found in breast milk (Gerner et al., 2000; Iso et al., 2001; Matsubara et al., 2001; Ohto et al., 2002; Schröter et al., 2000). However, results contradictory to *in utero* transmission have also been presented; showing TTV DNA-negative cord blood samples and TTV DNA appearance from a few months of age upwards (Davidson et al., 1999; Hsieh et al., 1999; Iso et al., 2001; Kazi et al., 2000; Ohto et al., 2002; Salakova et al., 2004; Simmonds et al., 1999; Sugiyama et al., 1999). The comparison of TTV sequences from children and from mothers has shown both similar and divergent sequences,

indicating both mother-child transmission and environmental transmission (Bagaglio et al., 2002; Davidson et al., 1999; Lin et al., 2002; Ohto et al., 2002; Sugiyama et al., 1999). Also of note, TTMV has been detected in cord blood, amniotic fluid, breast milk, and newborn sera, with sequence similarity to that of the mothers (Matsubara et al., 2001). The mother-to-child transmission route could partly explain the high prevalence and persistent infectious characteristics of TTV. It is apparent that such a prevalent virus group as anelloviruses may have several modes of transmission; faeco-oral and droplet spread currently being the most probable candidate routes.

2.3.4. Infections and pathogenic effects

TTV infections in humans were initially shown to be either transient or persistent (Nishizawa et al., 1997). TTV viraemia has been shown to persist for several years (Azzi et al., 2001; Ball et al., 1999; Biagini et al., 1999; Irving et al., 1999; Lefrere et al., 2000; Sugiyama et al., 1999), but clearance of the virus has also been detected (Ohto et al., 2002; Prati et al., 1999; Simmonds et al., 1998; Wilson et al., 2001). In persisting TTV infections, the DNA sequence of the virus has been shown to either remain unchanged or change with time (Ball et al., 1999; Biagini et al., 1999; Irving et al., 1999), indicating true persistence, superinfections, and/or evolution of quasispecies. TTV infection has been suggested to be a very dynamic process; it has been estimated that over 90% of the virions are cleared every day, with generation of 3.8×10^{10} virions to replace the cleared ones (Maggi et al., 2001c). In general, the levels of TTV viraemia in healthy subjects are assumed to be low; the viral levels in PBMCs of healthy individuals were shown to be 2 logs lower than in PBMCs of cancer patients (Zhong et al., 2001b). In addition, it has been shown that, among the study subjects, the levels of TTV DNA either remained steady or fluctuated, in the range of 10^2–10^8 copies/mL (Ball et al., 1999; Maggi et al., 2005b; Pistello

et al., 2001; Zhong et al., 2001a). Co-infections with multiple TTV genotypes are common, even in healthy individuals (Ball et al., 1999; Biagini et al., 1999; Chan et al., 2001a; Forns et al., 1999; Gallian et al., 1999; Jelcic et al., 2004; Mushahwar et al., 1999; Niel et al., 2000; Okamoto et al., 1999b; Okamoto 2009; Okamoto and Mayumi, 2001; Prescott et al., 1999; Takayama et al., 1999b). The presence of mixed infections could suggest 1) that TTV is not cleared from the system, 2) that re-infections and super-infections occur frequently, 3) that the virus is spread via several routes, 4) that the mutation rate is high, and/or 5) that a previous infection does not protect against a re-infection (White et al., 2000). Of these possibilities, at least infections with new genotypes have been documented to occur during follow-up (Gallian et al., 2000; Maggi et al., 2006). In addition to mixed infections with various TTV genotypes, mixed infections with several TTMV isolates (Vasconcelos et al., 2002), as well as co-infections with TTMV and TTV, have been reported to occur in healthy individuals (Biagini et al., 2006b; Moen et al., 2002a; Niel et al., 2001; Vasconcelos et al., 2003). Also co-infections with the 3 anelloviruses (TTV, TTMV, and SAV/TTMDV) have been reported (Biagini et al., 2006b; Ninomiya et al., 2007a). In any case, data on sewage waters suggest that there are no seasonal changes in TTV infections (Haramoto et al., 2005b; Vaidya et al., 2002). In other words, it appears that we can acquire TTV throughout the year and throughout our lives.

Although a definitive association of TTV with disease(s) has not been found, it has been suggested that some isolates/genotypes, either alone or co-infected with other TTV strains or pathogens, could cause certain diseases. It is likewise possible that TTVs *per se* do not cause any disease, but that they somehow have an effect (either good or bad) on the outcome or the progression of some disease(s). Also of note, the levels of TTV in tissue and/or blood could affect any of the conditions mentioned above. Finally, it is possible that TTV does not have any effect on our well-being. Taking into consideration the wide array of different isolates/genotypes, and bearing in mind that the only methods in current use for detection of TTV infections (only

acute and persisting, not past infections) are PCR-based, it is obvious that the results on disease associations of TTVs are inconsistent. Two excellent review articles exist on this topic (Bendinelli et al., 2001; Hino et al., 2007), in addition to 2 noteworthy editorials (Naoumov, 2000; Simmonds, 2002). The following section simplifies and briefly summarises what has been studied and found previously.

Liver diseases: Because of the discovery of TTV from a patient with hepatitis (Nishizawa et al., 1997), TTV was initially suspected to be the cause of hepatitis. The initial studies did show an association between TTV prevalence and/or loads and various hepatic disorders (Charlton et al., 1998; Huang et al., 2001; Ikeda et al., 1999; Ikeuchi et al., 2001; Iwaki et al., 2003; Kanda et al., 1999; Naoumov et al., 1998; Nishiguchi et al., 2000; Nishizawa et al., 1997; Okamura et al., 2000; Takayama et al., 1999a; Tanaka et al., 1999a; Tokita et al., 2002; Watanabe et al., 2000). However, contradictory results indicating that TTV is not associated with alanine aminotransferase (ALT) levels or with hepatic disorders have also been found (Hijikata et al., 1999a; Hsieh et al., 1999; Huang et al., 2000; Nakano et al., 1999; Naoumov et al., 1998; Niel et al., 1999; Prati et al., 1999; Viazov et al., 1998b; Yamamoto et al., 1998). Some researchers have hypothesised that certain genotypes/genogroups could be associated with hepatic disorders (Okamura et al., 2000; Tokita et al., 2001; Tuveri et al., 2000), but it seems that infection is not related to hepatitis or other liver diseases (Akiba et al., 2005; Kao et al., 2002; Momosaki et al., 2005; Schröter et al., 2003; Shibata et al., 2001; Umemura et al., 2001a; Yoshida et al., 2002). In summary, several studies conducted over the past decade have indicated that TTV does not fulfil the criteria for being a hepatitis virus: it does not appear to cause any damage to the liver cells (Akiba et al., 2005; Campo et al., 2000; He et al., 2003; Hsu et al., 2003; Kato et al., 2000; Kao et al., 2002; Momosaki et al., 2005; Schröter et al., 2003; Shibata et al., 2001; Shimizu et al., 2000; Tuveri et al., 2000; Umemura et al., 2001a; Umemura et al., 2002a; Yoshida et al., 2002).

Respiratory tract disorders: TTV infection has been suggested to play a role (either active or opportunistic) in children with acute respiratory disease. TTV has been found in high loads in nasal swabs of such children, particularly in patients with a more severe respiratory tract disease, such as bronchopneumonia (Maggi et al., 2003b). The prevalence of SAV was also found to be higher in children with acute respiratory syndrome (Chung et al., 2007). In addition, children with high TTV loads in their nasal specimens were shown to have worse spirometric values, and TTV was suggested to contribute to the pathogenesis of asthma. It was suggested that TTV replication could twist the immunobalance towards the Th2 response, which is known to play a role in the pathogenesis of asthma (Pifferi et al., 2005). In idiopathic pulmonary fibrosis patients, a poorer survival rate was shown to correlate with TTV infection. Interestingly, TTV replication has been shown to occur in lung tissue (Bando et al., 2001). Furthermore, high TTV loads have been associated with the severity of bronchiectasis (Pifferi et al., 2006). However, as with all the other disorders mentioned, it is not known whether TTV is the cause or the result of (or a bystander in) the disease. In one study, a newborn and the child's parents were followed to assess the TTV loads in their saliva, which showed that the newborn acquired a TTV strain from the mother within a few days after delivery, and showed high viral loads in saliva associated with benign rhinitis (Biagini et al., 2003).

Co-infections with other pathogens: Co-infection of cervical cells with TTV and human papillomavirus (HPV) has been shown to occur. Even though women with multiple HPV infections had a higher prevalence of TTV, there was no correlation to high-risk or low-risk HPV strains (Calcaterra et al., 2001). Higher TTV loads have been detected in gastric tissue of patients with gastritis and with *Helicobacter pylori*, possibly suggesting some role for TTV infection (Maggi et al., 2003a).

Immunocompromised patients: It has been suggested that certain genotypes could be more common in human immunodeficiency virus (HIV)-infected patients than in healthy

individuals (Shibayama et al., 2001). In addition, TTV viral loads have been shown to increase in HIV patients who are progressing towards AIDS, and to correlate with a low CD4+ cell count (Christensen et al., 2000; Sagir et al., 2005a; Sagir et al., 2005b; Shibayama et al., 2001; Thom et al., 2007; Touinssi et al., 2001). However, this most likely reflects the overall immune status of the patient and has no consequence on the underlying disease.

Cancer: TTV DNA has been found in various lymphoma types as often as in healthy individuals, and the virus has been shown to localise in non-neoplastic cells. However, it was postulated that TTV could somehow modulate the T cells it infects and thus have some role in the pathogenesis of lymphomas (Garbuglia et al., 2003). zur Hausen and coworkers presented an interesting hypothesis in which they suggest that persistent TTV infections increase the risk for specific translocations, thus having an effect on the development of childhood leukaemia and lymphomas (zur Hausen et al., 2005). Co-infection with HPV and TTV genotype-1 has been related to poor outcome of laryngeal carcinoma, but it is not known if TTV has any effect on the cancer progression (Szladek et al., 2005). TTV DNA has been detected in a wide variety of neoplastic tissues (de Villiers et al., 2002); however, this could be due to tissue inflammation, or to the rapidly dividing cancer cells supporting TTV replication. The presence of TTV in colorectal cancer specimens has been suggested to have some pathogenetic role; however, the same TTV isolates have also been found in non-cancerous tissue (de Villiers et al., 2007). TTV DNA has also been detected more often in the sera of patients with classical Kaposi's sarcoma than in the sera of healthy subjects. In addition, TTV DNA has been detected in both lesional skin and healthy skin of patients, but not in the skin of healthy individuals. It has been suggested that TTV infection could, for example by immunosuppression, have some effect on the replication of human herpes virus 8 and thus have a role in the disease pathogenesis (Girard et al., 2007). TTV has been shown to occur in 100-times higher loads in the PBMCs of any cancer patient tested than in the PBMCs of healthy controls. However, it is not clear if this finding is

due to cancer or represents something in common with the seriously ill patients (Zhong et al., 2001b).

Autoimmune disorders: Several autoimmune disorders still lack a definitive causative agent, and TTV has been studied in this regard as well. Gergely and coworkers noticed that peptides from ORF1 and ORF2 areas resemble human endogenous retrovirus-encoded nuclear protein, a common autoantibody-generating epitope of systemic lupus erythematosus (SLE). They showed that patients, in addition to having high TTV DNA prevalence, had antibodies against the peptides. However, it is not clear whether TTV has a role in autoantibody generation, or whether, due to immunological-dysfunction, the patients could be more prone to TTV infections (Gergely et al., 2005b). Sospedra and coworkers showed that T cells obtained from multiple sclerosis patients reacted against arginine-rich peptides similar to the TTV and TTMV ORF1 N-terminus. It was suggested that these T cells could be expanded due to repeated TTV infections, and in conjunction with some other predisposing (genetic/microbial) factors, could have some effect on the development of the disease (Sospedra et al., 2005). In addition, TTV has been found in patients with rheumatoid arthritis (Gergely et al., 2005a; Hirata et al., 1998) or with idiopathic inflammatory myopathy (IMM) (Gergely et al., 2005a). However, the TTV prevalence of these patients did not differ from that of blood donors (Gergely et al., 2005a). Maggi and coworkers showed that rheumatoid arthritis patients, when compared to healthy subjects, did not have elevated TTV loads, whereas patients with arthritis related to other autoimmune diseases and SLE did. The authors suggested that there could be a connection between TTV replication and arthritis, but as with the other disorders mentioned, causality is not known (Maggi et al., 2007). Interestingly, the expression of genotype-1 ORF1 in transgenic mice, leading to production of a spliced protein, has been shown to cause pathological alterations in the kidneys of the mice. Expression of the TTV protein seemed to interfere with

differentiation of the renal epithelial cells, the importance of which in TTV pathology remains to be ascertained (Yokoyama et al., 2002).

2.4. Porcine TTV

Soon after the discovery of human TTV, genetically related but distinct TTV forms from those discovered in humans were described in different species of farm vertebrate animals, such as primates, chickens, pigs, cows, sheep, cats, dogs, and tupaias (Bigarre et al., 2005; Kekarainen et al., 2006; Leary et al., 1999; Mckeown et al., 2004; Moen et al., 2003; Neil et al., 2005; Okamoto et al., 2002). Among these, porcine TTV has drawn a considerable amount of interest. Studies conducted over the last few years have explored the biological nature of the porcine TTV, including its impact on the pig's health. The following sections review the studies on porcine TTV conducted over the last decade.

2.4.1. Taxonomy, molecular and biological properties

Structurally, porcine TTV is similar to human TTV, but it possesses a much smaller genome size of 2.8 kb. It has been assigned into the genus *Iotatorquevirus*, under the same family of human TTV, *Anelloviridae*. Full-length sequence analyses of porcine TTV from various countries (Japan, Brazil, Canada, China, Germany, Spain, and USA) indicated low levels of nucleotide identity among them; these samples were initially categorised into 2 genogroups (Brassard et al., 2010; Cortey et al., 2010; Gallei et al., 2010; Huang et al., 2010; Niel et al., 2005; Okamoto et al., 2002). However, considering the amount of nucleotide sequence identity among 4 American strains, both genogroups have been recently updated to the species status by the International Committee of Taxonomy of Viruses (ICTV) (http://www.ictvonline.org/virusTaxonomy.asp?bhcp=1), *Torque teno sus virus 1* (TTSuV1) and

2 (TTSuV2), with 2 internal types for TTSuV1 and 3 subtypes for TTSuV2 (Huang et al., 2010). These genogroups share a sequence similarity of <50% with that of human TTV (Niel et al., 2005; Okamoto et al., 2002). However, the extent of nucleotide sequence variation between TTSuV strains appears to greatly depend on the region of the viral genome analysed. For example, a sequence homology of 78–99% was reported when analysing a region of approximately 100 bp within the UTR (Biggarre et al., 2005), whereas another study assessing a 260-bp region within the UTR reported 91–97% and 93–99% similarities for TTSuV1 and TTSuV2, respectively. TTSuVs possess 4 ORFs (ORF1, ORF2, ORF1/1, and ORF2/2) and have a short stretch with high GC content in the UTR (Huang et al., 2010b; Mueller et al., 2008). Similar to the encoding pattern of human TTV, ORF1 and ORF2 are suggested to be encoded by a ~2.8-kb viral mRNA and ORF1/1 and ORF2/2 by a spliced -1.2-kb viral mRNA (Huang et al., 2010).

2.4.2. Epidemiological features

TTSuV was first detected in pigs in 1999 (Leary et al., 1999), but a retrospective study by Segales and co-researchers (2009) showed the presence of the TTSuV in pigs as early as 1985. To date, TTSuV has been detected in several countries (Austria, Canada, China, Czech Republic, France Germany, Hungary, Italy, Japan, Korea, Spain, Thailand, and USA), with a varying prevalence ranging from 33% to 100% (Bigarre et al., 2005; Brassard et al., 2007; Gallei et al., 2010; Jarosova et al., 2011; Kekarainen et al., 2006; Martelli et al., 2006, McKeown et al., 2004; Takacs et al., 2008). Apart from domestic pigs, 58% of tested wild boars were found to be infected with TTSuV1 and 66% with TTSuV2, though the prevalence of infection varied with geographical region (Martinez et al., 2006). Surveys of pigs in Italy have indicated that the prevalence of TTSuV infection varies greatly, with certain farms having no evidence of TTSuV1

infection, whereas others had prevalence rates as high as 53% (Martelli et al., 2006). Although the prevalence of infection was found to be higher in 'finishing' herds (40% infection prevalence) than in 'farrow-to-finishing' herds (11% infection prevalence), no relationship was found between the prevalence of infection within herds and their size, general health, or biosecurity status (Martelli et al., 2006). These previous studies also reported that among domestic swine, nursery pigs (57% infection prevalence) were more likely to be infected with TTSuV1 than were fatteners (22% infection prevalence). Similar patterns of infection were reported for TTSuV2 infection in wild boar, whereby juveniles of 7–12 months of age (80% infection prevalence) were found to be more frequently infected than adults (58% infection prevalence). Furthermore, the prevalence of TTSuV infection in female wild boars (74% infection prevalence) was found to be higher than that in males (57% infection prevalence) (Martinezet al., 2006). Of note, TTSuV1 prevalence has been shown to be higher than TTSuV2 in some studies (Kekarainen et al., 2007; Martinez-Guino et al., 2009; Pozzuto et al., 2009; Sibla et al., 2009a; Sibla et al., 2009b), whereas the opposite result has been obtained in other studies (Kekarainen et al., 2006; Segale et al., 2009) and no difference was shown in another (Tara et al., 2009). Therefore, it is not currently possible to assess the relative importance of one or the other TTSuVs; this situation might vary depending on the animal population studied and the PCR analysis methods used for detection of TTSuVs.

TTSuVs, like its human counterpart, has been detected in the sera of various categories of pigs (Cortey et al., 2010; Gallei et al., 2009; Huang et al., 2010; Huang et al., 2011; Jarosova et al., 2011; Kekarainen et al., 2006; Kekarainen et al., 2007; Lee et al., 2010; Leary et al., 1999; Macera et al., 2011; Marteli et al., 2006; Martinez et al., 2006; Martinez et al., 2007; Martinez-Guino et al., 2009; Mckeown et al., 2004; Niel et al., 2005; Nieto et al., 2011; Okamoto, et al., 2002; Segales et al., 2009; Taira et al., 2009; Takacs et al., 2008), semen and reproductive apparatus of boars (Huang et al., 2010; Kekarainen et al., 2007; Ritterbusch et al., 2011),

colostrum (Martinez-Guino et al., 2009), faeces (Brassard et al., 2008; Sibla et al., 2009), nasal secretions (Sibla et al., 2009), and various tissues of pigs including those of foetuses (Aramouni et al., 2010; Bigarre et al., 2005; Blomstrom et al., 2009; Martinez-Guino et al., 2010; Pozzuto et al., 2009; Perez et al., 2011; Savic et al., 2010). These results suggest the possibility of the existence of TTSuVs transmission by both vertical and horizontal routes. In addition, TTSuVs DNA has been detected in commercial vaccines for swines, enzymes and drugs formulated with components of porcine origin, cell cultures, and trypsin (Kekarainen et al., 2009; Teixeira et al., 2011), highlighting the risks of TTSuVs transmission by mechanical routes and TTSuVs contamination in cell cultures.

2.4.3. Infections and pathogenic effects

The detection of TTSuVs in various tissues following natural infection suggests multi-systemic infection. However, virus was not detected in all organs of a pig and distinct virus subtypes were detected in different tissues (Bigarre et al., 2005; Okamoto et al., 2001a), suggesting that certain virus genotypes or subtypes might be better adapted to particular cell or tissue types. To date, no cell culture system that facilitates effective propagation of TTSuVs has been identified. Similar to PCV and CAV, TTSuVs might only replicate in actively dividing cells since these viruses require use of host cell DNA polymerase. It also remains unclear whether TTSuVs detection in different tissues reflects true infection of the constituent cells or represents virus circulating within the tissue vasculature. In humans, mitogen-stimulated PBMC cultures infected with TTV release infectious virus, suggesting viral replication is possible in that cell population (Maggi et al., 2001a; Mariscal et al., 2002). Furthermore, the detection of circular double-stranded forms of the viral genome in liver and bone marrow provides evidence that TTVs can replicate within cells in these organs (Okamoto et al., 2000a; Zhong et al., 2002).

TTSuVs, like their human counterparts, exhibit marked degree of genetic diversity (Bigarre et al., 2005; Kekarainen et al., 2006; Martinez et al., 2006; Niel et al., 2005). Co-infection of animals with both TTSuV types is common (Kekarainen et al., 2006; Martinez et al., 2006).

Currently, the disease-causing potential of TTSuVs is not well understood and is thus under investigation. The first indication of the pathogenic association of TTSuVs came from Spain where Kekarainen et al. (2006) reported a higher prevalence of TTSuV2 in PMWS-affected pigs than in PMWS-nonaffected pigs. PCV2 is essential but not a sufficient cause of PMWS, one of the economically most important diseases of PCVAD. In gnotobiotic piglets, TTSuV1 was found to induce the subclinical changes of interstitial pneumonia, transient thymic atrophy, glomerulopathy, and modest lymphocyte to histocytic infiltration in the liver (Krakowka and Ellis, 2008), and was also capable of inducing porcine dermatitis nephropathy syndrome (PDNS) in the presence of porcine reproductive and respiratory syndrome virus (PRRSV) (Krakowka et al., 2008), and PMWS in the presence of PCV2 (Ellis et al., 2008). Furthermore, an association between TTSuVs and infectious hepatitis of pigs in concomitant infections with PCV2 and/or HEV was observed. On the contrary, a study from Japan (Taira et al., 2009) reported no significant difference in the prevalence of TTSuVs between PMWS-suspected and normal pigs. Attempts to associate TTSuV infection with PMWS/PCVAD have revealed contradictory results, with a Korean group of researchers (Lee et al., 2010) reporting no significant difference in viral load between PCV2-negative and PCVAD-affected pigs, whereas others reported an increase of TTSuV2 viral load over time in PMWS-affected pigs but not in the case of healthy animals or for TTSuV1 (Nieto et al., 2011). Therefore, the disease-causing potential of TTSuVs remains debatable and warrants further investigation.

CHAPTER 3

DETECTION OF TORQUE TENO SUS VIRUS TYPES 1 AND 2 BY NESTED POLYMERASE CHAIN REACTION IN SERA OF SOWS AT FARROWING AND OF THEIR NEWBORN PIGLETS IMMEDIATELY AFTER BIRTH WITHOUT SUCKLING COLOSTRUM AND AT 24 HOURS AFTER SUCKLING COLOSTRUM

3.1. ABSTRACT

This study aimed to clarify the sow-to-foetus transmission pathway of *Torque teno sus virus* (TTSuV) types *1* (TTSuV1) and *2* (TTSuV2). For this purpose, detection of TTSuV1 and TTSuV2 (TTSuVs) in the sera of 6 sows (Sows 1–6) at parturition and in the sera of their newborn piglets immediately after birth without suckling colostrum was performed by nested polymerase chain reaction (nPCR). These sows were bred using semen that had tested negative for TTSuVs. For a TTSuV1- and TTSuV2-positive sow (Sow 1), TTSuV1 was detected in 4 of 12 newborn littermates, and TTSuV2 was detected in 5 littermates. For a TTSuV1-positive sow (Sow 2), TTSuV1 was detected in 1 of 8 newborn littermates. For 4 TTSuV1- and TTSuV2-negative sows (Sows 3–6), TTSuV1 was detected in 6 out of the 25 newborn piglets of 3 sows (Sows 3–5), whereas no TTSuVs were detected in 13 piglets of 1 sow (Sow 6). In addition, to investigate the possibility of a sow-to-piglet transmission pathway of TTSuV via colostrum, TTSuV1 and TTSuV2 in sera of 12 newborn piglets from Sows 1–3 were examined by nPCR. Immediately after birth before suckling colostrum, TTSuV1 and TTsuV2 were not detected in 10 and 8 of 12 piglets, respectively; however, at 24 hr after suckling colostrum, 6 piglets became positive for TTSuV1 and none for TTSuV2. These results confirmed the existence of a sow-to-foetus transmission pathway of TTSuVs during normal pregnancy and suggested a possibility of sow-to-piglet transmission of TTSuV via colostrum.

3.2. INTRODUCTION

Torque teno virus (TTV) is a small, nonenveloped virus with a single-stranded, circular DNA genome and belongs to the newly created family *Anelloviridae* (www.ncbi.nlm.nih.gov/ICTVdb/). TTV was first detected in a human patient with posttransfusion hepatitis of unknown aetiology (Nishizawa et al., 1997) and was later detected in other animals including swine (Biagini et al., 2007; Inami et al., 2000; Leary et al., 1999; Ng et al., 2009; Okamoto and Mayumi, 2001; Okamoto et al., 2002). In swine, 2 genetically distinct species of TTV have been identified: *Torque teno sus virus* (TTSuV) types *1* (TTSuV1) and *2* (TTSuV2), belonging to the genus *Iotatorquevirus* (Niel et al., 2005; Okamoto et al., 2002).

It has been reported that the prevalence rate of TTSuV1 and TTSuV2 (TTSuVs) ranged from 24% to 100% in different countries (Bigarre et al., 2005; Kekarainen et al., 2006; Martelli et al., 2006; Mckeown et al., 2005; Ng et al., 2009; Segales et al., 2009), and the prevalence rate was 51% in Japan (Taira et al., 2009). TTSuV appears to be a pathogenic trigger or cofactor of porcine circovirus associated disease (PCVAD) (Kekarainen et al., 2006; Krakowka and Ellis, 2008; Krakowka et al., 2008) and hepatitis of pigs infected with porcine circovirus type 2 (PCV2) and/or hepatitis E virus (Savic et al., 2010). However, the direct pathogenic effect of TTSuV remains unclear.

In addition, the TTSuV transmission pathway has not been fully elucidated in pigs. An intrauterine route of TTSuV infection has been assumed because TTSuVs were detected in the semen and sera of boars (Kekarainen et al., 2007; Martinez et al., 2006). Furthermore, sow-to-foetus transmission has been suggested because TTSuVs were observed in the sera or tissues of stillborn, gnotobiotic piglets and aborted and nonaborted foetuses (Aramouni et al., 2010; Kekarainen and Segales, 2009; Martinez-Guino et al., 2009; Martinez-Guino et al., 2010; Pozzuto et al., 2009). However, in these studies, the possibility of intrauterine infection by semen

was excluded since the semen was not examined for TTSuVs. Furthermore, it has been assumed that TTSuV can be transmitted from sow to piglet via colostrum, which has been shown to carry TTSuVs (Martinez-Guino et al., 2009). However, this particular transmission route has not been fully examined.

Therefore, this study aimed to clarify the existence of a sow-to-foetus transmission pathway of TTSuV by excluding the possibility of an intrauterine route of infection by semen. Therefore, sows were bred using TTSuVs-negative semen of boars and allowed to undergo normal pregnancy and parturition. Nested polymerase chain reaction (nPCR) was used to detect TTSuVs in the sera of sows at parturition and of their newborn piglets immediately after birth without suckling colostrum. In addition, to investigate the possibility of a sow-to-piglet transmission pathway of TTSuVs via colostrum, sera of piglets immediately after birth without suckling colostrum and at 24 h after suckling colostrum were analysed for the detection of TTSuVs by nPCR.

3.3. MATERIALS AND METHODS

3.3.1. Experiment 1

***Sow-to-foetus TTSuV transmission*:** This experiment was carried out on a commercial pig farm with 1500 sows in Kagoshima Prefecture, Japan. Six healthy sows (Sows 1–6) were used. All sows were bred by artificial insemination using the pooled semen of 8–10 boars obtained from a total of 13 boars. A portion of each individual semen sample was used to detect the presence of TTSuVs. Blood was collected from each sow via the *anterior vena cava* (approximately 8 mL) at parturition and from the 58 (7–13 piglets/sow) newborn piglets (approximately 4 mL) immediately after birth without suckling colostrum. The blood was

centrifuged at 1800 × *g* for 20 min, and the collected sera were stored at -28°C until nPCR was performed.

3.3.2. Experiment 2

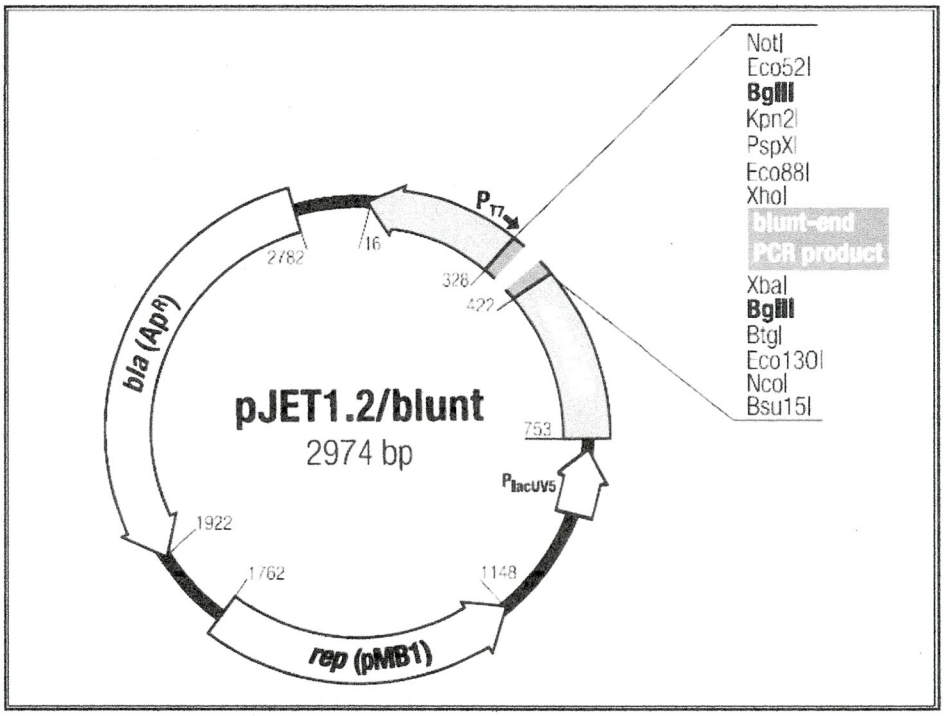
Sow-to-piglet transmission of TTSuV via colostrum: Immediately after birth, 12 newborn piglets from 3 sows (4 littermates/sow; Sows 1–3) in Experiment 1 were used, and each piglet was numbered for identification. These piglets were allowed to suckle the sow's colostrum *ad libitum* after collection of blood. Subsequently, blood was collected from all piglets via the *anterior vena cava* at 24 h after suckling colostrum, and the collected sera were stored at -28°C until nPCR was performed. The concentration of serum total protein (TP) for all the piglets immediately after birth without suckling colostrum and at 24 h after suckling colostrum was determined to assess the quantity of colostrums uptake by the piglets.

DNA extraction and nPCR for the detection of TTSuV1 and TTSuV2: DNA was extracted from 250 μL of boar semen and sera of sows and newborn piglets by a sodium iodide method using a DNA Extractor WB Kit (Wako Pure Chemical Industries, Osaka, Japan). The nPCR method used for detection of TTSuV1 and TTSuV2 was performed as described previously (Kekarainen et al., 2006; Mckeown et al., 2004). The first-round 50-μL PCR for TTSuV1 contained 5 μL of DNA, 20 pmole of the primer pair (5′- TACACTTCCGGGTTCAGGAGGCT-3′, forward and 5′-ACTCAGCCATTCGGAACCTCAC-3′, reverse), 2.5 mM dNTPs, 2 mM $MgCl_2$, and 0.5 U Tag DNA polymerase (rTag). The amplification conditions were as follows: 94°C for 5 min, 35 cycles of 94°C for 30 sec, 52°C for 20 sec and 72°C for 30 sec, followed by 72°C for 5 min. Five microlitres of amplified product were used as template for nPCR. The primer set for nPCR of TTSuV1 was 5′-CAATTTGGCTCGCTTCGCTCGC-3′ (forward) and 5′- TACTTATATTCGCTTTCGTGGGAA

C-3′ (reverse; 50 pmole of each), and the PCR reagents were 2.5 mM dNTPs, 2 mM $MgCl_2$, and 0.5 U rTag. The nPCR of TTSuV1 was performed under the same conditions as those of the first-round PCR. Amplified nPCR of TTSuV1 product was run on a 2% agarose electrophoresis gel, and a band of 260 bp was observed using ethidium bromide under ultraviolet (UV) fluorescence. TTSuV2 amplification was carried out under the same procedure as described for TTSuV1.Briefly, the primer pair for the first-round PCR was 5′- AGTTACACATAACCACCA AACC-3′ (forward) and 5′-ATTACCGCCTGCCCGATAGGC-3′ (reverse), and for the nPCR, the primer set was 5′-CCAAACCACAGGAAACTGTGC-3′ (forward) and 5′-CTTGACTCCGCTCTCAGGAG-3′ (reverse). After electrophoresis, the amplified product of a band of 230 bp was observed under UV fluorescence.

PCR sensitivity assay: To determine the PCR sensitivity, amplicons of TTSuV1 (260 bp) and TTSuV2 (230 bp) from the 2% agarose gel after electrophoresis were purified using a commercially available DNA purification kit (QIAquick® Gel Extraction Kit; QIAGEN, Japan) and cloned into a plasmid using a cloning strategy (pJET1.2/blunt cloning vector; Fermentas, Japan) at Hokkaido System Science Co., Ltd., Japan (Fig. 4). The sensitivity of the PCR was determined by amplification of 10-fold dilutions of known amounts of each plasmid DNA (TTSuV1clone: 5.5×10^8 copies/μL; TTSuV2-clone: 1.7×10^8 copies/μL) in PCR. To confirm the sensitivity result, the experiment was repeated once. The same plasmids and pure distilled water were used as positive and negative controls, respectively, in every PCR reaction.

Statistical analysis: The significance of the difference in the concentration of TP between the piglet group immediately after birth and at 24 h after suckling colostrum within the same litter was analysed using the Student's *t*-test.



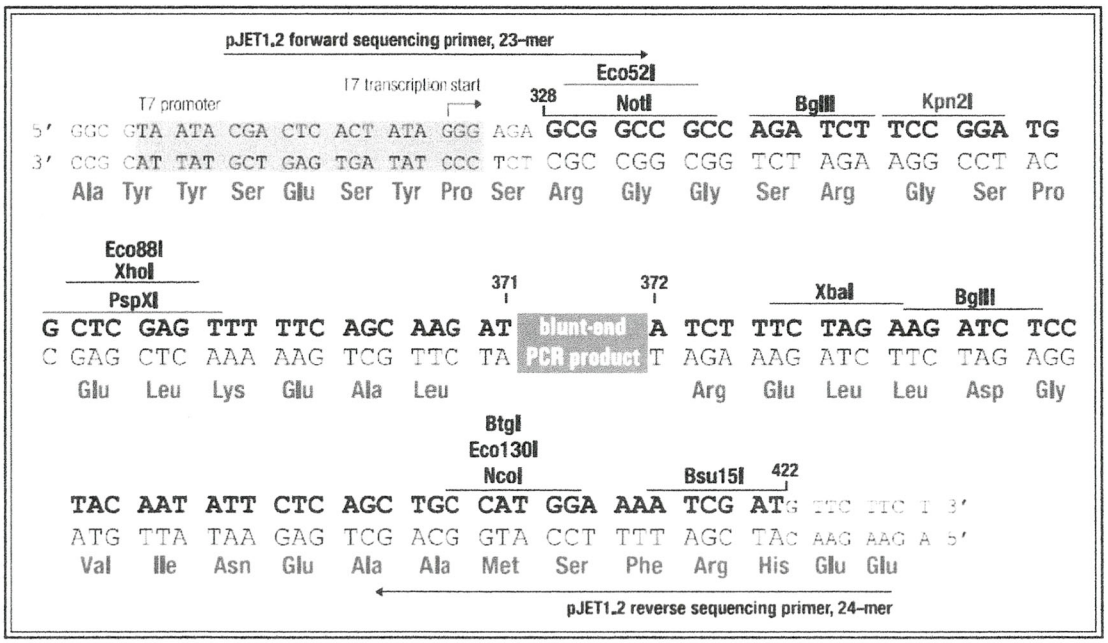


Fig. 4. pJET1.2/blunt cloning vector map and the DNA sequence of the MCS region (CloneJET^{TM} PCR cloning kit; Fermentas, Japan) used for cloning of TTSuVs.

3.4. RESULTS

Sensitivity of PCR: The minimum number of TTSuV1 and TTSuV2 copies that could be detected with the current PCR assay was 550 molecules of TTSuV1 and 14 molecules of TTSuV2 per reaction (Fig. 5).

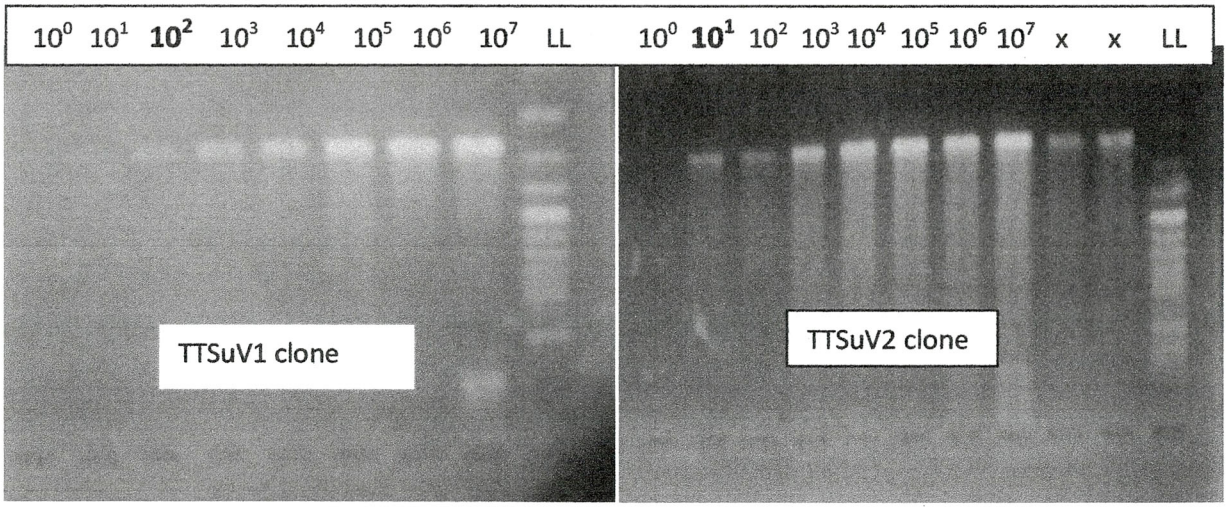


Fig. 5. PCR sensitivity of TTSuV1 and TTSuV2 determined with 10-fold dilutions of known amounts of TTSuV1 and TTSuV2 plasmid DNA. The lanes marked with x are TTSuV2 PCR samples with unknown concentration. The titres marked with bold values indicate the lowest concentration of TTSuV nucleic acid that could be detected using the current nPCR.

Detection of TTSuV1 and TTSuV2 in the semen and sera of pigs: Once the sensitivity of the PCR was determined, the detection of TTSuV1 and TTSuV2 in the semen of boars and sera of sows and their piglets was performed (Fig. 6).

The results of detection of TTSuVs in the semen of boars are shown in Table 1. In summary, neither TTSuV1 nor TTSuV2 were detected in any semen of the boars, therby ruling out any possibility of intrauterine transmission via semen.

The results of detection of TTSuVs in the sera of sows and their piglets are shown in Table 2. Both TTSuV1 and TTSuV2 were detected in Sow 1, TTSuV1 but not TTSuV2 in Sow 2, and neither TTSuV1 or TTSuV2 in Sows 3–6. For Sow 1, TTSuV1 was detected in 4 out of 12 newborn piglets, and TTSuV2 was detected in 5 piglets, whereas the 6 remaining piglets were negative for TTSuVs. For Sow 2, 1 out of 8 newborn piglets was positive for TTSuV1. For Sows 3–5, TTSuV1 was detected in 6 out of 25 newborn piglets, whereas neither TTSuV1 nor TTSuV2 was detected in any newborn piglet for Sow 6.

The results of detection of TTSuVs in the sera of 12 newborn piglets immediately after birth without suckling colostrum and at 24 h after suckling colostrum are shown in Table 3. In Sows 2 and 3, neither TTSuV1 nor TTSuV2 was detected in 8 newborn piglets before suckling colostrum, but TTSuV1 was detected in 5 of these piglets after suckling colostrum. In Sow 1, TTSuV1 was not detected in 2 piglets (Nos. 4 and 5) without suckling colostrum, but after suckling colostrum, TTSuV1 was detected in 1 of these piglets (No. 5). However, TTSuV2 was not detected in 8 piglets (Sows 2–3) before or after suckling colostrum.

The concentration of TP (g/dL) was 2.5–3 times higher in all individual piglets at 24 h after suckling colostrum (6.3 ± 0.7, 7.4 ± 0.4, and 6.5 ± 0.5) than the concentration immediately after birth without suckling colostrum (2.2 ± 0.1, 2.5 ± 0.3, and 2.2 ± 0.1); this difference was significant between the 2 groups of pigs ($P < 0.001$) from Sows 1–3 (results not shown), indicating that all piglets have adequate uptake of colostrum.

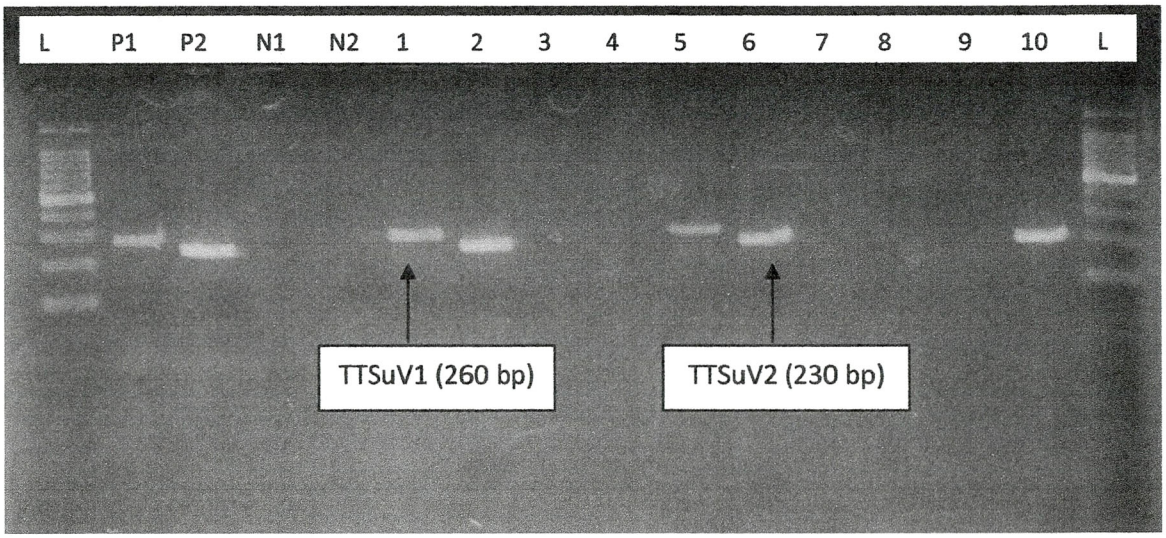


Fig. 6. Representative ethidium bromide-stained gel of PCR-amplified TTSuV1 (260 bp) and TTSuV2 (230 bp) DNA. L: 100-bp DNA ladder, P1 and P2: Positive control for TTSuV1 and TTSuV2, N1 and N2: Negative control for TTSuV1 and TTSuV2, Lanes 1–11: Serum samples collected from sows and their piglets.

Table 1. Results of detection of TTSuV1 and TTSuV2 by nested PCR in the semen of boars

Boar No.	Species	
	TTSuV1	TTSuV2
1	–	–
2	–	–
3	–	–
4	–	–
5	–	–
6	–	–
7	–	–
8	–	–
9	–	–
10	–	–
11	–	–
12	–	–
13	–	–

–: Not detected.

Table 2. Detection of TTSuV1 and TTSuV2 by nested PCR in the sera of sows at farrowing and of their newborn piglets immediately after birth without suckling colostrums

Sows			Newborn piglets					
No.	TTSuV1	TTSuV2	No.	TTSuV1	TTSuV2	No.	TTSuV1	TTSuV2
1	+	+	1	+	+	7	–	–
			2	+	–	8	+	+
			3	–	–	9	–	–
			4	–	+	10	–	–
			5	–	+	11	–	–
			6	+	+	12	–	–
2	+	–	1	–	–	5	–	–
			2	–	–	6	–	–
			3	–	–	7	–	–
			4	–	–	8	+	–
3	–	–	1	–	–	5	+	–
			2	–	–	6	+	–
			3	–	–	7	+	–
			4	–	–			
4	–	–	1	–	–	6	–	–
			2	–	–	7	+	–
			3	–	–	8	+	–
			4	–	–	9	–	–
			5	–	–	10	–	–
5	–	–	1	–	–	5	–	–
			2	–	–	6	+	–
			3	–	–	7	–	–
			4	–	–	8	–	–
6	–	–	1	–	–	8	–	–
			2	–	–	9	–	–
			3	–	–	10	–	–
			4	–	–	11	–	–
			5	–	–	12	–	–
			6	–	–	13	–	–
			7	–	–			

–: Not detected; +: Detected.

Table 3. Detection of TTSuV1 and TTSuV2 by nested PCR in the sera of sows and of their newborn piglets immediately after birth before suckling colostrum and at 24 h after suckling colostrum

Sows			Newborn piglets				
				Immediately after birth without suckling colostrum		24 hr after suckling colostrum	
No.	TTSuV1	TTSuV2	No.	TTVSu1	TTSuV2	TTSuV1	TTSuV2
1	+	+	4	–	+	–	–
			5	–	+	+	–
			6	+	+	+	+
			8	+	+	+	+
2	+	–	1	–	–	+	–
			2	–	–	+	–
			3	–	–	–	–
			4	–	–	+	–
3	–	–	1	–	–	–	–
			2	–	–	+	–
			3	–	–	–	–
			4	–	–	+	–

–: Not detected; +: Detected.

3.5. DISCUSSION

In swine, it has been assumed that sow-to-foetus transmission of TTSuVs occurs during pregnancy because TTSuVs have been detected in the sera of stillborn and nonstillborn gnotobiotic piglets derived by caesarean section and in the tissues of aborted and nonaborted foetuses (Aramouni et al., 2010; Martinez-Guino et al., 2009; Martinez-Guino et al., 2010; Pozzuto et al., 2009). Further, intrauterine infection/transmission of TTSuVs in pregnant sows has also been reported as a possibility based on the detection of TTSuVs in the semen of boars by PCR (Huang et al., 2010; Kekarainen et al., 2007; Martinez-Guino et al., 2007). However, in these previous studies, detection of TTSuVs in the semen used for breeding the sows was not performed. Therefore, in the present study, to exclude the effect of TTSuVs infection/transmission to the foetus in the uterus by semen, sows were bred using semen in which TTSuV1 and TTSuV2 were not detected by nPCR.

Both TTSuV1 and TTSuV2 were detected in the sera of newborn piglets immediately after birth prior to them suckling colostrum. This result indicated the existence of a TTSuVs transmission pathway from sow to foetus during pregnancy. Moreover, TTSuV1 was detected in the piglets of sows without detectable levels of TTSuVs in sera at parturition, indicating that the sow-to-foetus TTSuV1 transmission pathway exists even in sows with undetectable levels of TTSuVs in the sera of sows at parturition. TTSuVs movement within and between tissues has previously been suggested because TTSuVs have been detected in different tissues and not simply in serum (Aramouni et al., 2010; Krakowka and Ellis, 2008; Pozzuto et al., 2009). Therefore, the detection of TTSuVs in the sera of newborn piglets from TTSuVs-negative sows in the present study may indicate the possible movement of the virus from serum to other tissues after the occurrence of sow-to-foetus transmission.

It has been assumed that suckling colostrum may be one of the routes of TTSuVs transmission in swine because TTSuVs have been detected in the colostrum of sows (Martinez-Guino et al., 2009). In this experiment, immediately after birth without suckling colostrum, TTSuV1 was not detected in 10, and TTSuV2 was not detected in 8, of 12 newborn piglets from 3 sows. However, at 24 h after suckling colostrum, TTSuV1 was detected in 6 of 10 piglets. The TTSuV1 detected in these piglets at 24 h after birth following suckling colostrum most likely originated from the colostrum since the concentrations of TP indicated that all the piglets had adequately consumed colostrum during this postnatal period. However, the possibility of piglet-to-piglet or sow-to-piglet transmission of TTSuVs via faeces, urine, or nasal secretions cannot be ignored, as the dissemination of TTSuVs via these routes has been reported by some authors (Brassard et al., 2008; Segales et al., 2009; Sibla et al., 2009a; 2009b). Further, TTSuV2 was not detected in any piglet at 24 h after suckling colostrum, indicating that TTSuV2 might be less transmissible via colostrum than TTSuV1.

In conclusion, it was confirmed that a sow-to-foetus transmission pathway of TTSuVs exists during normal pregnancy and that there might be a sow-to-piglet transmission pathway of TTSuVs via colostrum.

CHAPTER 4

SEROPREVALENCE OF TORQUE TENO SUS VIRUS TYPES 1 AND 2 IN PMWS-SUSPECTED PIGS AND PCV2-VACCINATED NORMAL PIGS IN SOUTHERN JAPAN

4.1. ABSTRACT

This study examines the seroprevalence rate of *Torque teno sus virus* types *1* (TTSuV1) and *2* (TTSuV2) in the sera of 38 post-weaning multisystemic wasting syndrome (PMWS)-suspected pigs and 43 porcine circovirus type 2 (PCV2)-vaccinated normal pigs on 3 commercial pig farms in southern Japan by nested polymerase chain reaction.The seroprevalence rate of TTSuV2 was significantly higher in the PMWS-suspected pigs than in the PCV2-vaccinated normal pigs (97.4% versus 81.4%, $P < 0.05$), whereas no such difference was observed for TTSuV1 between the 2 groups of pigs. In both pig groups, the seroprevalence rate of TTSuV2 was significantly higher than that of TTSuV1 ($P < 0.01$–0.05). A high seroprevalence rate of TTSuVs was observed in both PMWS-suspected pigs (100%) and PCV2-vaccinated normal pigs (90.7%). These results show that TTSuVs are highly seroprevalent in both PMWS-suspected pigs and PCV2-vaccinated normal pigs reared on conventional farms, with TTSuV2 being more seroprevalent in the former than in the latter.

4.2. INTRODUCTION

TTSuV1 and TTSuV2 (TTSuVs) have been detected in the semen, sera, plasma, faeces, saliva, nasal secretions, colostrum, and various tissues by polymerase chain reaction (PCR) (Aramouni et al., 2010; Brassard et al., 2008; Kekarainen et al., 2006; Martínez-Guinó et al., 2009; Pozzuto et al., 2009), and the seroprevalence rates in the general pig population have been reported to vary from 24% to 100% in different countries (Bigarre et al., 2005; Gallei et al., 2010; Lang et al., 2011; Martelli et al., 2006; Mckeown et al., 2008). In particular, the seroprevalence has reported to be 51% in postweaning multisystemic wasting syndrome (PMWS)-suspected pigs in Japan (Taira et al., 2009). However, the pathogenic association of TTSuV is still debatable. In gnotobiotic pigs, TTSuV1 partially contributed to PMWS and porcine dermatitis nephropathy syndrome in the presence of porcine circovirus type 2 (PCV2) and porcine reproductive respiratory syndrome virus infections, respectively (Ellis et al., 2008; Krakowka et al., 2008). Similarly, field studies have suggested TTSuV2 as a co-factor in PMWS since PMWS-affected pigs had a significantly higher seroprevalence rate and viral load than PMWS-nonaffected pigs (Aramouni et al., 2011; Kekarainen et al., 2006; Nieto et al., 2011). However, the difference in viral load between the TTSuVs in the sera of PMWS-affected pigs and PCV2-negative pigs was determined to be nonsignificant (Huang et al., 2011; Lee et al., 2010). Therefore, more studies are required to completely associate Torque teno virus (TTV) infection with PMWS or other specific diseases.

This study aimed to determine the seroprevalence rate of TTSuV1 and TTSuV2 in PMWS-suspected pigs and PCV2-vaccinated normal pigs to provide further insights into the potential role of TTSuVs in the occurrence of PMWS.

4.3. MATERIALS AND METHODS

4.3.1. Pig samples and blood collection

Three conventional pig farms (farms A, B, and C) in Kagoshima Prefecture, southern Japan were included in this study. The number of replacement and breeding sows on farms A, B, and C were 2400 Berkshire, 1500 hybrids (Hypor), and 400 (Large White) (LW), respectively. From April to August 2008, most of the growing pigs that had not been vaccinated for PCV2 showed typical clinical signs of PMWS, such as severe weight loss, respiratory disorders, diarrhoea, and palpable bilateral inguinal lymphadenopathy. From September 2008, regular PCV2 vaccination (Porcilis® PCV2, 2 mL intramuscularly; Intervet Schering-Plough Animal Health) of pigs at 21 days of age on all the farms was introduced. Thereafter, none of the PCV2-vaccinated pigs showed clinical signs of PMWS. Analysis of the mortality data on the 3 farms revealed mortality rates of 6–8% before the disease outbreak (historical) period, which increased to 12–14% during the disease outbreak period (PMWS-suspected period); however, a significant decrease in mortality rates on all farms, to 2–4%, was observed after the introduction of PCV2 vaccines (PCV2-vaccination period) (Fig. 7). Based on these observations, all clinically affected pigs were defined as PMWS-suspected pigs, and all PCV2-vaccinated pigs as PCV2-vaccinated normal pigs. A total of 38 blood samples (from 7, 18, and 13 pigs on farms A, B, and C, respectively) were collected from the anterior vena cava of 90–140-day-old clinically affected PMWS-suspected pigs in June–July 2008, and 43 blood samples (from 8, 22, and 13 pigs on farms A, B, and C, respectively) were collected in a similar manner from PCV2-vaccinated normal pigs in the same age range in June–July 2010. All blood samples were centrifuged at 1800 × *g* for 20 min, and the obtained sera were stored at -28°C until they were used for DNA extraction.

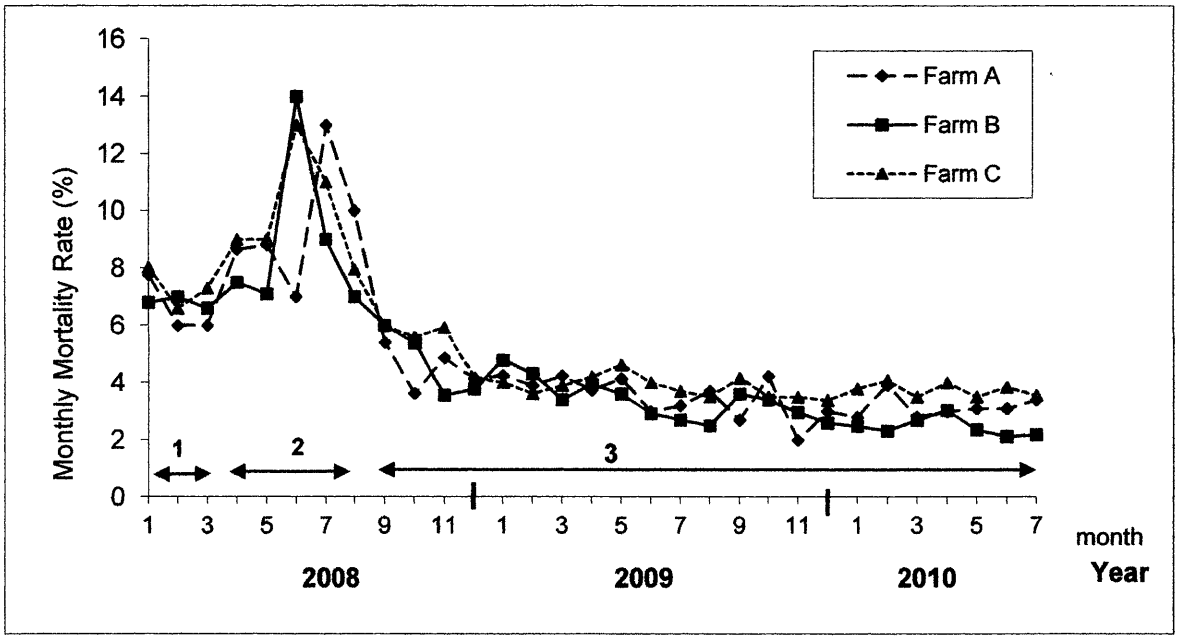


Fig. 7. Monthly mortality rates of growing pigs in the historical, PMWS-suspected, and PCV2-vaccinated periods. 1: Historical period (January–March 2008); 2: PMWS-suspected period (April–August 2008); 3: PCV2-vaccinated period (September 2008 to July 2010).

4.3.2. Detection of TTSuV1 and TTSuV2 DNAs by polymerase chain reaction

DNA was extracted from 250 μL of serum collected from the PMWS-suspected and PCV2-vaccinated normal pigs using a DNA Extractor WB Kit (Wako, Japan), and TTSuVs nucleic acid were detected by an nPCR method previously described (Kekarainen et al., 2006). A chi-square test or Fisher's exact test was used to analyse the differences in the detection rates of the viruses between the pig groups, and significance was set at $P < 0.05$.

4.4. RESULTS

As shown in Table 4, the seroprevalence rate of TTSuVs was high in both PMWS-suspected and PCV2-vaccinated normal pigs (100.0% and 90.7%, respectively). Globally, TTSuV2 was more frequently detected than TTSuV1 on all farms; the seroprevalence rate of TTSuV2 was significantly higher in PMWS-suspected pigs than that in PCV2-vaccinated normal pigs (97.4% versus 81.4%, $P < 0.05$). Furthermore, in both PMWS-suspected pigs and PCV2-vaccinated normal pigs, the seroprevalence rate of TTSuV2 was significantly higher than that of TTSuV1 (97.4% versus 71.1% and 81.4 versus 58.1, respectively; $P < 0.01$–0.05). However, no significant difference was observed in TTSuV1 between the 2 groups of pigs.

Table 4. Seroprevalence rate of TTSuV1 and TTSuV2 in PMWS-suspected pigs and PCV2-vaccinated normal pigs.

Farm	Group	TTSuV1	TTSuV2	TTSuV1 and TTSuV2	TTSuV1 or TTSuV2
A	I	71.4 (5/7)†	100.0 (7/7)	71.4 (5/7)	100.0 (7/7)
	II	37.5 (3/8)	75.0 (6/8)	37.5 (3/8)	75.0 (6/8)
B	I	77.8 (14/18)	94.4 (17/18)	72.2 (13/18)	100.0 (18/18)
	II	63.6 (14/22)	86.4 (19/22)	50.0 (11/22)	95.5 (21/22)
C	I	61.5 (8/13)	100.0 (13/13)	61.5 (8/13)	100.0 (13/13)
	II	61.5 (8/13)	76.9 (10/13)	46.2 (6/13)	92.3 (12/13)
Total	I	71.1 (27/38)^{c}	97.4 (37/38)^{a}	68.4 (26/38)	100.0 (38/38)
	II	58.1 (25/43)^{d}	81.4 (35/43)^{b}	46.5 (20/43)	90.7 (39/43)

a > b ($P < 0.05$), a < c ($P < 0.001$), b > d ($P < 0.05$)

†: % (Number of detected pigs/Total number of examined pigs)

I: PMWS-suspected pigs

II: PCV2-vaccinated normal pigs

4.5. DISCUSSION

TTSuV infection is highly prevalent globally, with seroprevalence rates varying from 24% to 100% (Bigarre et al., 2005; Gallei et al., 2010; Kekarainen et al., 2006; Lang et al., 2011; Martelli et al., 2006; Mckeown et al., 2004). In Japan, the seroprevalence rate of TTSuV in PMWS-suspected pigs, tested across 16 prefectures, varied from 0% to 100%, with an average prevalence rate of 51%. In particular, 58% (7/12) of the samples from Kagoshima Prefecture tested seropositive for TTSuVs (Taira et al., 2009). In the present study, high seroprevalence rates of TTSuV1 and TTSuV2 in both PMWS-suspected pigs and PCV2-vaccinated normal pigs (100% and 90.7%, respectively) across 3 conventional pig farms were observed, indicating that TTSuVs infection was widespread in these farms.

The pathogenic association of TTSuV, especially with PMWS, has been a subject of debate. Initially, TTSuV1 and TTSuV2 in gnotobiotic and conventional pigs, respectively, were implicated as possible co-factors of PCV2 in the occurrence of PMWS (Ellis et al., 2008; Kekarainen et al., 2006; Krakowka et al., 2008; Martin-Vallis et al., 2008). More recently, studies from Spain have reported an association between TTSuV2 viral load and PMWS, and have suggested that TTSuV2 may play an important role in the occurrence of PMWS (Aramouni et al., 2011; Nieto et al., 2011). However, a group of Korean researchers (Lee et al., 2010) found no difference with regard to TTSuV2 viral load between PMWS-affected pigs and PCV2-negative pigs. In the present study, the seroprevalence rate of TTSuV2 was significantly higher in PMWS-suspected pigs than in PCV2-vaccinated normal pigs. Furthermore, when the virus types were considered, it was observed that the seroprevalence rate of TTSuV2 was significantly higher than that of TTSuV1 in both PMWS-suspected and PCV2-vaccinated normal pigs. Additionally, it is tempting to speculate that a direct correlation exists between PCV2 and TTSuVs infection, since a decrease in the seroprevalence rate of TTSuVs was observed in

PCV2-vaccinated pigs. However, further studies are required to completely elucidate the synergistic mechanisms of PCV2 and TTSuVs infections that may result in clinical expression of PMWS.

In conclusion, the results of the present study indicated that TTSuV1 and TTSuV2 infections are highly prevalent in both PMWS-suspected pigs and PCV2-vaccinated normal pigs, with seroprevalence of TTSuV2 morelikely to be higher in PMWS-suspected pigs than in PCV2-vaccinated pigs.

CHAPTER 5

DETECTION OF TORQUE TENO SUS VIRUS TYPES 1 AND 2 IN TISSUES OF STILLBORN PIGLETS DELIVERED BY SOWS AT NATURAL FARROWING

5.1. ABSTRACT

In this study, the presence of *Torque teno sus virus* types *1* (TTSuV1) and *2* (TTSuV2) in tissues (liver, heart, spleen, and tonsil) obtained from 18 stillborn piglets was examined using nested polymerase chain reaction. TTSuV1 was detected at its highest levels in the liver (72%), followed by the heart (56%), spleen and tonsil (38%); whereas TTSuV2 was highest in the tonsil (38%), followed by liver (33%), spleen (25%), and heart (17%). Overall, 83% of the stillborn piglets were positive for TTSuV1 or TTSuV2 showing 78% and 50% positivity for TTSuV1 and TTSuV2, respectively. These results indicate that TTSuVs are commonly present but not uniformly distributed in the tissues of stillborn piglets.

5.2. INTRODUCTION

Torque teno sus virus types *1* (TTSuV1) and *2* (TTSuV2) have been detected by polymerase chain reaction (PCR) in various tissues of pigs, including those of foetuses (Aramouni et al., 2010; Martínez-Guinó et al., 2010), and in serum, plasma, semen, faeces, colostrum, and nasal secretions. In stillborn piglets, TTSuVs have been detected from the sera of caesarean-derived and occasionally delivered stillborn piglets (Martínez-Guinó et al., 2009). To our knowledge, there are no existing reports on the detection of TTSuVs in the tissues of stillborn piglets. Moreover, it remains unclear whether TTSuVs have any role in the reproductive failures of sows. Therefore, to provide further insights on the dynamics of TTSuVs in tissues of stillborn piglets, we report the detection rates of TTSuVs from selected tissues of stillborn piglets examined by nested PCR (nPCR).

5.3. MATERIALS AND METHODS

5.3.1. Stillborn piglets and their tissue samples

The tissue samples used in this study were obtained from frozen stillborn piglets submitted to the Laboratory of Farm Animal Production Medicines, Faculty of Agriculture, Kagoshima University, Japan in October 2008. A total of 18 stillborn Berkshire piglets delivered by 16 sows were received from a conventional Berkshire farm of 2400 breeding sows. All stillborn piglets were delivered by sows at natural farrowing (1–2 stillborn piglets/sow) along with other liveborn piglets, and the average litter size of the sows ranged from 9 to 11 piglets. All sows in the study farm were regularly vaccinated for the major reproductive failure-causing pathogens, including Aujeszky's disease virus (ADV), encephalomyocarditis virus (EMV), getah virus (GV), Japanese encephalitis virus (JEV), porcine enterovirus (PEV), porcine parvovirus (PPV), and porcine reproductive and respiratory syndrome virus (PRRSV). At the time of

farrowing, all sows appeared apparently normal and healthy without any other clinical signs of reproductive problems. The body weights of these stillborn piglets at birth (1.2–1.4 kg) were also comparable to those of the liveborn piglets. Analysis of 1 year's reproductive data of the farm during the sampling month showed an increased number of stillborn piglets to 0.7 per litter compared to that of 6 months before (0.25 stillborn piglets·litter^{-1}·month^{-1}) and after (0.35 stillborn piglets·litter^{-1}·month^{-1}) the sampling month. At necropsy, all stillborn piglets showed similar macroscopic findings, including collapsed lung and enlarged liver, but the heart and spleen were normal.

5.3.2. Detection of TTSuV1 and TTSuV2 DNA, PCV2 DNA, and PRRSV RNA

Individual tissues, including heart, liver, spleen, and tonsil, were collected from the stillborn piglets, and were homogenised in 20% phosphate buffer saline (PBS; pH 7.4) solution. The mixture was centrifuged at 1800 × *g* for 20 min to obtain the supernatant. DNA was extracted from 250 μL of the tissue supernatant using a DNA Extractor WB Kit (Wako Chemicals, Japan). Porcine circovirus type 2 (PCV2) and TTSuVs nucleic acids were detected by PCR methods as previously described for PCV2 (Ellis et al., 1999) and TTSuVs (Kekarainen et al., 2006), respectively. For PRRSV detection, RNA was extracted from the same quantity of individual tissue supernatant as for DNA extraction using a commercially available kit (Isogen-LS; Nippon Gene, Japan), and the presence of PRRSV RNA was detected using a reverse transcriptase PCR (RT-PCR) method, as described previously (Stadejek et al., 2002).

5.4. RESULTS

As shown in Table 5, 83% (15/18) of the stillborn piglets were infected with either TTSuV1 or TTSuV2. TTSuV1 and TTSuV2 were detected in 78% (14/18) and 50% (9/15) of the stillborn piglets, respectively, whereas 44% (3/18) of the stillborn piglets were co-infected with the 2 virus types. In tissues, TTSuV1 was detected more frequently than TTSuV2, particularly in the liver (72%, 13/18 versus 33%, 6/18) heart (56%, 10/18 versus 17%, 3/18), and spleen (38%, 3/8 versus 25%, 2/8). The detection rate of TTSuV1 or TTSuV2 was the highest in the liver (78%, 14/18), followed by tonsils and spleen (63%, 5/8), and heart (61%, 11/18). PCV2 and PRRSV were not detected in the tissues of any of the stillborn piglets (results not shown).

Table 5. Detection rate of TTSuV1 and TTSuV2 in tissues of stillborn piglets delivered by sows at natural farrowing

Tissue	TTSuV1	TTSuV2	TTSuV1 and TTSuV2	TTSuV1 or TTSuV2
Liver	72 (13/18)†	33 (6/18)	28 (5/18)	78 (14/18)
Heart	56 (10/18)	17 (3/18)	11 (2/18)	61 (11/18)
Spleen	38 (3/8)	25 (2/8)	0 (0/8)	63 (5/8)
Tonsil	38 (3/8)	38 (3/8)	13 (1/8)	63 (5/8)
Stillborn piglets infected	78 (14/18)	50 (9/18)	44 (8/18)	83 (15/18)

†: % (positive samples/total number of samples examined)

5.5. DISCUSSION

TTSuVs have been detected by PCR in various tissues of healthy pigs, including those of foetuses (Aramouni et al., 2010; Martínez-Guinó et al., 2010). However, to date, there are no reports of TTSuVs detection in tissues of stillborn piglets, though the viruses have been detected in the sera of stillborn piglets (Martínez-Guinó et al., 2009). Therefore, in the present study, selected tissues of stillborn piglets delivered by sows at natural farrowing were examined by nPCR for the presence of TTSuV1 and TTSuV2. TTSuVs were detected in the tested tissues at a high percentage, indicating that, in addition to being detected in serum, TTSuVs can also be detected in tissues of stillborn piglets. Interestingly, the detection rate was different among the tissues for the 2 TTSuV types. TTSuV1 was more frequently detected in the liver, followed by the heart, spleen, and tonsils, whereas the detection rate of TTSuV2 was the highest in the tonsils, followed by the liver, spleen, and heart. Although the detection of TTSuV1 and TTSuV2 in tissues may be attributed to the passive distribution of the viruses by normal blood circulation, it seems more likely that this represents the tissue tropism or preferential sites for replication and/or accumulation of TTSuVs, since the 2 virus types were not uniformly distributed among the tested tissues. However, the exact mechanism and biological implication of such a finding requires further investigation.

Globally, 83% of the stillborn piglets examined in this study were infected with either TTSuV1 or TTSuV2, and 70% and 50% were positive for TTSuV1 and TTSuV2, respectively. Interestingly, this value is comparatively higher than what was previously reported for the sera of caesarean-derived and occasionally delivered stillborn piglets (TTSuV1, 50% and TTSuV2, 7%) (Martínez-Guinó et al., 2009). It is unknown whether the high detection rate observed in this study could be related to the testing of tissue instead of serum, or to pathological conditions in stillborn piglets. Our recent report of TTSuVs prevalence in the sera taken from newborn piglets

prior to suckling colostrum from a different farm showed 18.9% and 8.6% positivity for TTSuV1 and TTSuV2, respectively (Tshering et al., 2011b), which is comparatively lower than the values found in the tissues examined in our current study. Furthermore, stillborn piglets used for this study came from apparently healthy sows, though it should be noted that the sow population had an increased stillbirth rate during the sampling month. PRRSV and PCV2 seemed not to have played a role in causing stillbirths since they were not detected by PCR in the present study. Moreover, all sows in the present study farm were regularly vaccinated for ADV, EMV, GV, JEV, PCV2, PEV, PPV, and PRRSV, making these viruses less probable to be associated with stillbirth in the present farm. Of note, the sows were not vaccinated for classical swine fever virus (CSFV) (Japan is free of CSFV). In addition, the farm has had no previous record of reproductive failure related to other infectious or non-infectious origins. Based on this result and our other observations, we speculate TTSuV as the potential cause of increased stillbirths in the present farm. However, since the present study was not designed to test the causal relationship of TTSuVs infection and stillbirths, such potential relationship must be interpreted with caution and deserves further investigation.

In summary, the results of this study indicate that TTSuVs were widely prevalent in the tissues of stillborn piglets derived from sows with increased stillbirths at natural farrowing. Furthermore, it was observed that TTSuV1 and TTSuV2 were not uniformly distributed among the examined tissues of the stillborn piglets, which might reflect the presence of a different tissue tropism for the 2 virus types under the same or different epidemiological conditions.

CHAPTER 6

INFECTION DYNAMICS OF TORQUE TENO SUS VIRUS TYPES 1 AND 2 IN SERUM AND PERIPHERAL BLOOD MONONUCLEAR CELLS

6.1. ABSTRACT

This study aimed to investigate the presence of *Torque teno sus virus* types *1* (TTSuV1) and *2* (TTSuV2) in longitudinally (14–150 days of age) collected, paired, pooled sera (pSE) and peripheral blood mononuclear cells (pPBMCs) using nested polymerase chain reaction. The detection rate of TTSuV1 in pSE increased from 14 to 90 days of age, but a progressive decline was observed from 120 to 150 days of age, whereas in pPBMCs, a high value was maintained until the end of the growing-finishing period. On the contrary, high detection rates of TTSuV2 were found in both pSE and pPBMCs in all sampling ages, except in PBMCs at 30 days of age. The detection rate of TTSuVs between pSE and pPBMCs was positively correlated at all sampling ages except for TTSuV1 at 150 days of age. This is the first study showing the presence of TTSuVs in PBMCs from pigs and describing the *in vivo* infection dynamics of TTSuV in paired serum and PBMCs during the entire growing and finishing periods of pigs reared in conventional farms; the results indication the lifelong presence of TTSuVs in the pigs reared on conventional farms.

6.2. INTRODUCTION

TTSuV1 and TTSuV2 (TTSuVs) have been detected, exclusively by polymerase chain reaction (PCR), in serum, plasma, semen, faeces, colostrum, nasal secretions, and various tissues (Brassard et al., 2008; Kekarainen et al., 2006; Martínez-Guinó et al., 2009; Sibila et al., 2009a; Tshering et al., 2011a; Tshering et al., 2011b). Different pig-rearing countries have reported prevalence rates ranging from 24% to 100% (Bigarre et al., 2005; Kekarainen et al., 2006; Martelli et al., 2006; Mckeown et al., 2004; Ng et al., 2009; Niel et al., 2005; Pozzuto et al., 2009; Segales et al., 2005; Sibila et al., 2009a). Limited longitudinal studies employing serum (Niel et al., 2005; Sibila et al., 2009a) and cross-sectional studies employing serum and tissues (Aramouni et al., 2010; Kekarainen et al., 2006; Kekarainen et al., 2007; Taira et al., 2009) have shown that TTSuV infection increases with age. However, little is known about primary infection with TTSuV and sites of viral persistence and reactivation after infection in pigs, and to the best of our knowledge, there have been no reports of detection of TTSuVs in peripheral blood mononuclear cells (PBMCs) of pigs. Therefore, this study aimed to investigate the presence of TTSuVs in PBMCs and to investigate the infection dynamics in paired serum and PBMCs.

6.3. MATERIALS AND METHODS

6.3.1. Blood and peripheral blood mononuclear cell collection

This study was conducted in a commercial hybrid (Hypor) pig farm in Kagoshima Prefecture, southern Japan with a total of 1500 breeding sows. Eight sows and their 24 pigs (3 piglets/sow) were used for this study. All piglets appeared apparently healthy during the whole study period. Blood samples were collected from the sows at 7 days before farrowing and their piglets at 14, 30, 60, 90, 120, and 150 days of age, and were centrifuged at 1800 × *g* for 20 min

to obtained sera. The resultant sera were stored at -28 °C until nPCR was performed. To obtain PBMCs, 2 mL of freshly collected heparinised blood per piglet was transferred to 15-mL heparinised tubes, which were pooled for the same sow (3 littermates^{-1}sow). The pooled heparinised blood samples were diluted to 3-fold dilutions using sterile phosphate-buffered saline (PBS), and centrifuged on a Ficoll-Conray gradient solution at 1400 × *g* for 20 min as described previously (Deguchi et al., 1997) (Fig. 8). The PBMC fraction was washed thrice with sterile PBS and stored below -28°C until used for analysis.

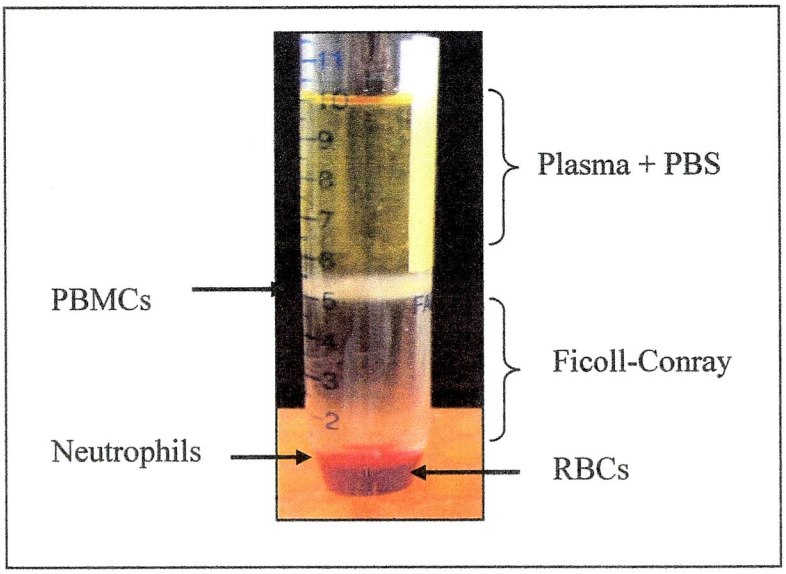


Fig. 1. Collection of PBMCs by using Ficoll-Conray gradient solution.

6.3.2. Detection of TTSuV1 and TTSuV2 in sera and peripheral blood mononuclear cells

DNA was extracted from 250 µL of the individual serum samples of sows, the pSE (pSE contain sera of 3 pigs from same sow), and pPBMCs of the pigs by a sodium iodide method using a kit (Wako Chemicals, Tokyo, Japan). The nPCR for the detection of TTSuV1 and TTSuV2 was performed using a method described previously (Kekarainen et al., 2006; Tshering et al., 2011). Specific amplicons of TTSuV1 (260 bp) and TTSuV2 (230 bp) were located after performing electrophoresis in a 2% agarose gel stained with ethidium bromide.

6.3.3. DNA sequencing

Eighteen paired amplified products of pSE and pPBMCs (10 from TTSuV1 and 8 from TTSuV2) of 2 sows and their piglets at 30 and 120 days of age were excised from the agarose gel and purified with a QIAquick gel extraction kit (Qiagen, Japan). The purified DNA products were sequenced from both strands according to the general sequencing protocol (Hokkaido System Science, Sapporo, Japan). The obtained sequences were aligned with the ClustalW program using the MEGA 4 package. The construction of the phylogenetic tree was performed using the neighbour-joining (NJ) method in the MEGA 4 software package (Tamura et al., 2007).

6.4. RESULTS

The results of detection of TTSuV1 and TTSuV2 in the serum and PBMC samples in sows and their pigs are shown in Table 6 and Fig. 10. In the individual serum samples of 8 sows analysed at 7 days before farrowing, TTSuV1 was detected in 1 sow (Sow 8) and TTSuV2 in all 8 sows.

In their growing pigs, the detection rate of TTSuV1 in pSE increased with age, with the lowest rate at 14 days of age (25%, 2/8) and the highest at 60 and 90 days of age (75%, 6/8), which then gradually declined at 120 (62.5%, 5/8) and 150 (25%, 2/8) days of age. On the other hand, TTSuV2 was detected in all pSE (100%, 8/8) at 14 days of age, but showed a reduced value of 62.5% (5/8) at 30 days of age; however, the virus was detected in all pSE (100%, 8/8) again at 60, 90, 120, and 150 days of age. The co-infection rates of the 2 virus types were 25.0% (2/8), 37.5% (3/8), 75.0% (7/8), 75.0% (7/8), 62.5%, and 12.5% (1/8) at 14, 30, 60, 90, 120, and 150 days of age, respectively. Of the 8 pSE samples analysed, 12.5% (1 out of 8) was TTSuV1 PCR-positive (No. 4), and 12.5% (1 out of 8) was negative (No. 2), at all 6 sampling ages.

Furthermore, 6 of 8 (75%) pSE samples analysed were TTSuV1 positive (No. 3–8) at more than one sampling time and showed consecutive PCR positivity in at least 2 sampling ages. On the contrary, 62.5% (5 out of 8) of pSE samples were TTSuV2 PCR positive (No. 3–6, 8) at all sampling ages, whereas none of the pSE samples were TTSuV2 PCR negative throughout the study period. All pSE (100%, 8/8) were TTSuV2 PCR positive in more than one sampling age and showed PCR positive in at least 2 consecutive sampling ages.

Regarding TTSuV1 and TTSuV2 in the pPBMCs of growing pigs, similar to the detection trend in pSE, the detection rates of TTSuV1 and TTSuV2 increased over time; the lowest values for TTSuV1 (12.5%, 1/8) and TTSuV2 (50%, 4/8) were detected at 30 days of age (TTSuVs detection was not analysed at 14 days of age), whereas the highest values for TTSuV1 (75%, 6/8) and TTSuV2 (100%, 8/8) were detected at 90 and 60 days of age, respectively. Thereafter, the highest value for TTSuV1 was maintained until the last sampling age, whereas for TTSuV2, a decreased detection rate from 100.0% at 60 days to 87.5% (7/8) at 100 days of age was observed. Co-infection of TTSuV1 and TTsuV2 increased progressively over time, with the lowest detection rate (12.5%, 1/8) observed at 30 days of age and the highest (87.5%, 7/8) observed at 120 days of age. One out of 8 pPBMC samples (12.5%) was TTSuV1 PCR positive (No. 4), and 1 out of 8 (12.5%) was negative (No. 5) at all 6 sampling ages. Seven of 8 (87.5%) pPBMC samples were TTSuV1 positive (No. 1–3, 5–8) in more than one sampling age, and 6 of 8 (75%) showed consecutive PCR positivity (No. 2–4, 6–8) in at least 2 sampling ages. On the contrary, 3 of 8 (37.5%) pPBMC samples were TTSuV2 PCR positive (No. 4, 6, 8), and none were PCR negative, at all sampling ages. All pPBMC samples (100%, 8/8) were TTSuV2 PCR positive in more than one sampling age and showed consecutive PCR positive in at least 2 sampling ages.

A comparison of the detection rates of the 2 virus types in pSE and pPBMCs showed no significant differences in the detection rates of either TTSuV1 or TTSuV2 between pSE and

pPBMCs at any sampling age. The detection rate of TTSuV1 between pSE and pPBMCs was positively correlated from 30 to 120 days of age and was negatively correlated at 150 days of age. On the contrary, the detection rate of TTSuV2 between pSE and pPBMCs showed a positive correlation at all sampling ages.

The phylogenetic tree (Fig. 9) shows the grouping of TTSuVs in 2 main types: TTSuV1 and TTSuV2. The sequence similarity among the respective TTSuV types (AB679090-AB679107) and to that of their respective references (TTSuV1:GU570208, TTSuV2:AB07600) ranged from 89% to 98%. It can also be observed that, except for 1 paired sample from piglets of Sow 8 at 30 days of age, the phylogenetic distances were different between the DNA sequences obtained from the paired pSE and pPBMCs.

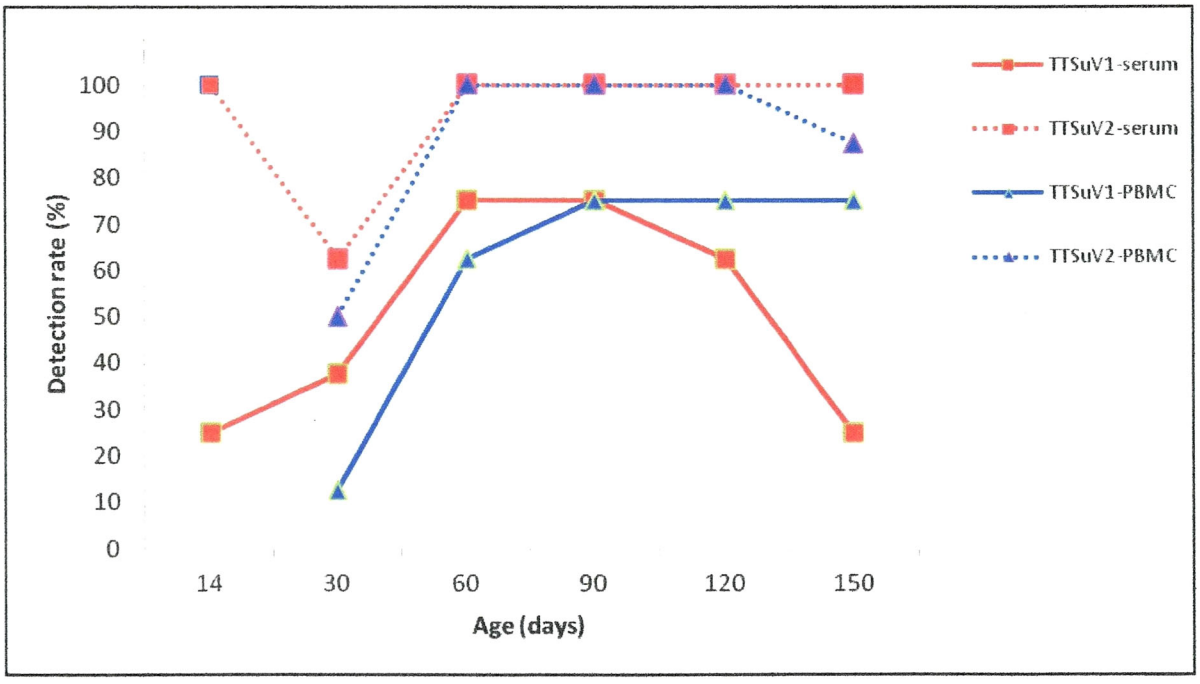


Fig. 9. Detection of TTSuV1 and TTSuV2 in individual serum samples of sows at 7 days before farrowing, and in paired pooled sera and peripheral blood mononuclear cells (PBMCs) of their growing and finishing pigs.

Table 2. Detection of TTSuV1 and TTSuV2 in individual serum samples of sows at 7 days before farrowing, and in paired pooled sera and peripheral blood mononuclear cells (PBMCs) of their growing and finishing pigs.

Type of sample	Sow			Growing and finishing pigs											
					14†		30		60		90		120		150	
	No.	TTSuV1	TTSuV2	No.	TTSuV1	TTSuV2	TTSuV1	TTSuV2	TTSuV1	TTSuV2	TTSuV1	TTSuV2	TTSuV1	TTSuV2	TTSuV1	TTSuV2
Serum																
	1	−	+	1	−	+	−	−	−	+	−	+	−	+	+	+
	2	−	+	2	−	+	−	−	−	+	−	+	−	+	−	+
	3	−	+	3	−	+	−	+	+	+	+	+	+	+	−	+
	4	−	+	4	+	+	+	+	+	+	+	+	+	+	−	+
	5	−	+	5	−	+	+	+	+	+	+	+	−	+	−	+
	6	−	+	6	−	+	−	+	+	+	+	+	+	+	−	+
	7	−	+	7	−	+	−	−	+	+	+	+	+	+	−	+
	8	+	+	8	+	+	+	+	+	+	+	+	+	+	+	+
Detection rate (%)		12.5	100.0		25.0	100.0	37.5	62.5	75.0	100.0	75.0	100.0	62.5	100.0	25.0	100.0
PBMCs																
	1			1			−	−	−	+	−	+	+	+	−	+
	2			2			−	−	−	+	+	+	+	+	+	+
	3			3			−	+	+	+	+	+	+	+	+	−
	4		NE	4		NE	+	+	+	+	+	+	+	+	+	+
	5			5			−	−	−	+	−	+	−	+	−	+
	6			6			−	+	+	+	+	+	+	+	+	+
	7			7			−	−	+	+	+	+	−	+	+	+
	8			8			−	+	+	+	+	+	+	+	+	+
Detection rate (%)							12.5	50.0	62.5	100.0	75.0	100.0	75.0	100.0	75.0	87.5

†: Age of piglets (days); -: Not detected; +: Detected; NE: Not examined.

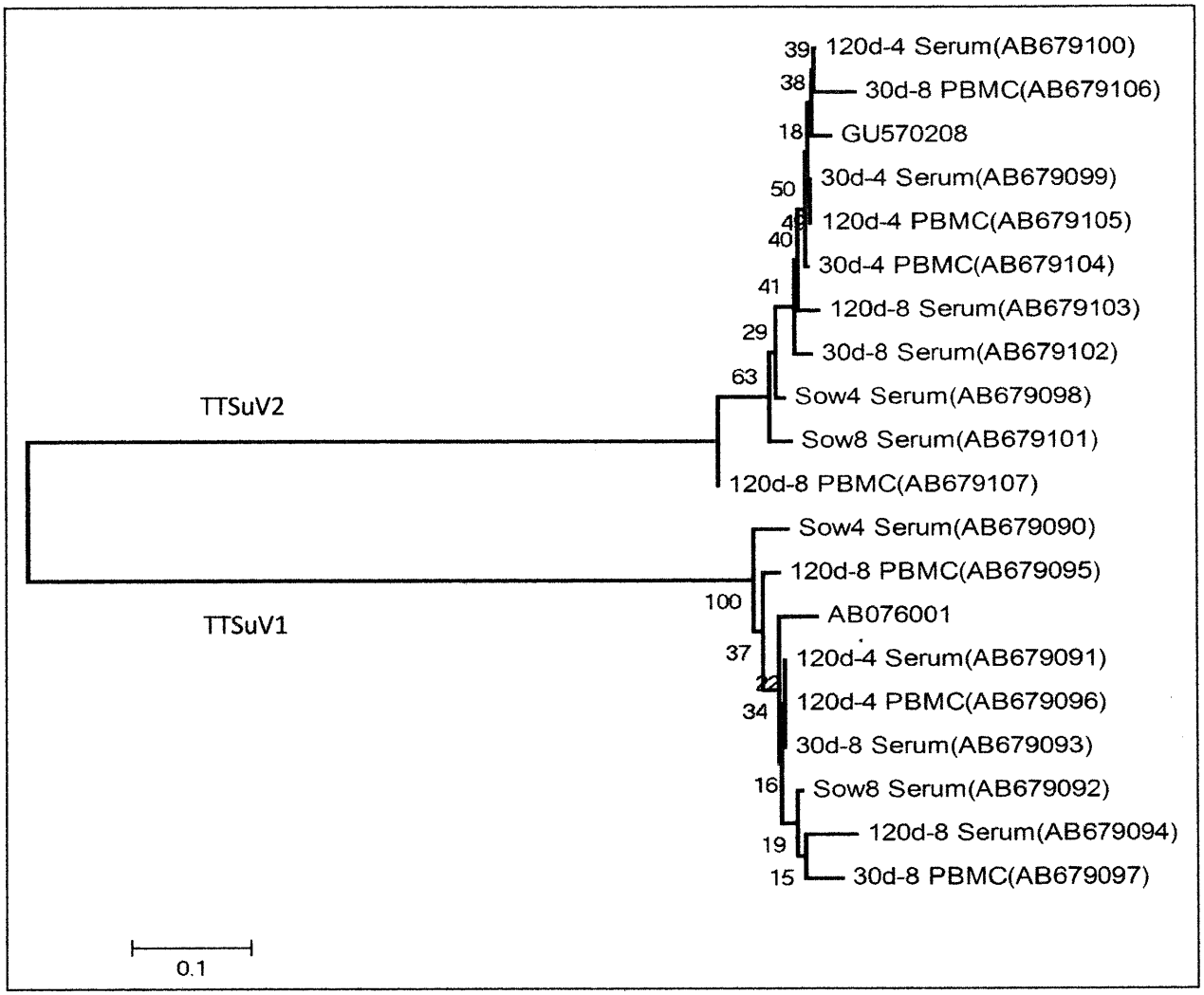


Fig. 10. Neighbour-joining phylogenetic tree constructed based on the nucleotide sequences of the noncoding region of TTSuV1 and TTSuV2. The bootstrap values are indicated above the major branches. GU570208 and AB076001 are reference sequences obtained from GenBank for TTSuV1 and TTSuV2, respectively. The reference number along with sample type of each sequence refers to the identification code of pigs. For example, Sow8 Serum indicates serum of Sow No. 8 and 30d-8 Serum indicates sera of piglets at 30 days of age of Sow No. 8. The GenBank accession numbers for TTSuV1 and TTSuV2 originating from different pigs in the phylogenetic analysis are indicated in parentheses.

6.5. DISCUSSION

In the present study, the presence of TTSuVs nucleic acids in paired serum and PBMCs during the entire growing-finishing period of pigs was investigated. To date, the prevalence of TTSuVs in PBMCs has not been studied, though the homologous counterpart of this virus has been detected in PBMCs from humans (Okamoto et al., 1999; Okamura et al., 1999). In the present study, the detection rate of TTSuV1 increased over time from 14 to 90 days of age and declined thereafter, whereas an increase in the detection rate of TTSuV2 was evident from 30 to 60 days (but a high value detected at 14 days of age) of age and a constant value was maintained until the last sampling age. Our finding is in partial agreement with the increasing prevalence of both TTSuVs in sera in a longitudinal study carried out with pigs aged from 7 to 105 days of age (Sibila et al., 2009a). However, the reason for the minor difference in prevalence rate, particularly for TTSuV2, is open to speculation. For example, it may be attributed to the high percentage of TTSuV2-positive sows observed in this study, since it has been suggested previously that TTSuVs can be transmitted by both vertical and horizontal routes (Brassard et al., 2008; Martínez-Guinó et al., 2009; Pozzuto et al., 2009; Tshering et al., 2011b), and as a result of these events, it is likely that TTSuV2 is more widespread than TTSuV1 in this farm. Furthermore, it is likely that the slightly higher PCR sensitivity of TTSuV2 compared to that of TTSuV1 (550 versus 14 molecules per reaction) reported in our previous study (Tshering et al., 2011b) might have contributed to the higher detection rate of TTSuV2 observed in this study. Another plausible explanation could be differential infection dynamics of the 2 virus types under same or different epidemiological settings. Further studies are required to elucidate these hypotheses.

The detection rate of TTSuVs in pPBMCs was similar to that in pSE. In fact, the rates were positively correlated at all sampling ages, except for the detection rate of TTSuV1 at 150

days of age, which showed significant negative correlation between the two sample types. In humans, PBMCs and bone marrow haematopoietic cells have been suggested as the 2 possible sites of Torque teno virus (TTV) replication (Maggi et al., 2001; Mariscal et al., 2002; Zhong et al., 2002). However, it is unknown whether the detection of TTSuVs in PBMCs in our study corresponds to the virus tropism to PBMCs. Apart from replication; TTSuVs could simply be engulfed without replication. Nevertheless, the detection of TTSuV in PBMCs opens room for further investigations, particularly to determine whether TTSuV can replicate in PBMCs, and if so, which cell subset may be involved in the viral replication.

The duration of TTSuV1 and TTSuV2 in pigs has been reported to last up to 56–77 and 105 days of age, respectively (Nieto et al., 2011; Sibila et al., 2009a), but the virus types have not been tested thereafter in the previous longitudinal studies. A few other cross-sectional studies (Aramouni et al., 2010; Taira et al., 2009), including our recent publication (Tshering et al., 2011a), have detected the 2 virus types in the sera of pigs from the last stage of their growing-finishing period. In the present study, TTSuV1 and TTSuV2 nucleic acids were detected in both sera and PBMCs samples from pigs 14 days to 150 days of age, further confirming the lifelong presence of the 2 virus types in pigs. Although TTSuVs were consecutively detected in most of the pSE, it was unclear whether it was due to virus persistence or re-infection. It is possible that TTSuVs in PBMCs being secluded from the circulating antibodies might be acting as a source of long-lasting viraemia, playing an important role in the transmission of the viruses in some clinical and epidemiological conditions.

In conclusion, to our knowledge, this is the first study showing the presence of TTSuVs in PBMCs from pigs and describing the *in vivo* infection dynamics of TTSuV in paired sera and PBMCs during the entire growing-finishing period. The obtained results indicate the lifelong presence of TTSuVs in both sera and PBMCs of pigs reared on conventional pig farms.

CHAPTER 7

GENERAL DISCUSSIONS AND CONCLUSIONS

Despite ongoing scientific interests, many important questions about TTSuVs remain unanswered. In this study, nPCR was used to investigate the existence of a sow-to-foetus transmission route of TTSuV and sow-to-piglet transmission via colostrum, to determine the seroprevalence of PMWS-suspected pigs and PCV2-vaccinated pigs, to examined the distribution of TTSuVs in individual tissues of stillborn piglets, and to investigate the infection dynamics of TTSuV in paired serum and PBMCs during the entire life of the growing-finishing pigs.

In the first study (Chapter 3), a significant number of piglets delivered by both TTSuV-seropositive and TTSuV-seronegative sows at farrowing were seropositive for the TTSuVs immediately after birth prior to suckling colostrum. In the same study, newborn piglets that were seronegative for TTSuVs prior to suckling colostrum became seropositive 24 h after suckling colostrums, suggesting the possible role of colostrum in TTSuV transmission. In the second study (Chapter 4), the investigation on the seroprevalence rate of TTSuVs showed a high values in both PMWS-suspected pigs and PCV2-vaccinated normal ones, with the seroprevalence rate of TTSuV2 being higher in the former group. In the third investigation (Chapter 5), it was observed that the detection rate of TTSuVs in individual tissues of stillborn piglets derived from sows with increased stillbirths were high, with a highest detection rates found in the liver, followed by spleen and tonsil and then by the heart. In study 4 (Chapter 6), TTSuVs were detected for the first time in pigs, and it was observed that the infection dynamics of TTSuVs in serum and PBMCs generally increased over time. Thus, from this thesis, following conclusions can be drawn:

1. TTSuVs can be transmitted from sow to fetus during the normal pregnancy period, and that there is possibility of a sow-to- piglet transmission via colostrum.

2. TTSuVs are highly prevalent in both PMWS-suspected and PCV2-vaccinated normal pigs, but TTSuV2 could be more frequently detected than TTSuV1 in PMWS-suspected pigs compared to that in PCV2-vaccinated group.
3. TTSuVs are highly prevalent but not uniformly distributed in the individual tissues stillborn piglets derived from sows with increased stillbirths suggesting the presence of a different tissue tropism for the 2 different TTSuV types.
4. TTSuVs are present in the PBMCs, and that the prevalence rates of TTSuVs in serum and PBMCs increase over time; the 2 virus types even being detected from these samples of pigs at 150 days of age, indicating a lifelong presence of TTSuVs in the growing-finishing pigs.

In general, the results of the present study to indicate that TTSuV infections may not be quite as new an entity as was previously thought, and that TTSuV infection alone may not sufficiently induce clinical diseases. However, it is tempting to speculate that it may be associated with diseases especially when these 2 virus types interact with other environmental (non-microbial) and microbial factors. Further studies should explore these interactive effects by employing more advanced and specific research tools such as cell culture system for virus propagation, immunohistochemical assay, in-situ hybridization, specific virus antibodies etc. In addition, it seems that the results of TTSuVs detections reported from different countries are influenced by the different methods of PCR, apart from the possible influences of epidemiological and environmental settings under which the studies are performed. Therefore, to address this issue, future research will also necessitate the standardization of the PCR methods used for detection of TTSuVs.

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