UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

DETECÇÃO E ISOLAMENTO DE ANELOVÍRUS EM SUÍNOS E CULTIVOS CELULARES

THAIS FUMACO TEIXEIRA

Porto Alegre
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Tese submetida ao Programa de Pós-Graduação em Ciências Veterinárias da UFRGS, como requisito parcial para obtenção do grau de Doutor em Ciências Veterinárias, área de concentração Microbiologia Veterinária - Virologia.

Orientador: **Prof. Dr. Paulo Michel Roehe**

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DETECÇÃO E ISOLAMENTO DE ANELOVÍRUS EM SUÍNOS E CULTIVOS CELULARES

Aprovada em 30 de março de 2012

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Dedico esta tese aos meus pais Acacio e Elenir e ao meu irmão Anderson que de muitas formas me incentivaram e ajudaram para que fosse possível a concretização deste trabalho.

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“A melhor de todas as coisas é aprender. O dinheiro pode ser perdido ou roubado, a saúde e força podem falhar, mas o que você dedicou à sua mente é seu para sempre."

-- *Louis L'Amour*

DETECÇÃO E ISOLAMENTO DE ANELOVÍRUS EM SUÍNOS E CULTIVOS CELULARES

RESUMO

Estudos preliminares visando a identificação de possíveis agentes virais associados à síndrome multissistêmica do definhamento dos suínos (SMDS) revelaram uma possível associação inversa entre a presença de TTSuV1 e a ocorrência da SMDS. Com base neste achado, foi formulada a hipótese de que o TTSuV1 poderia ser capaz de inibir a multiplicação do PCV2, impedindo assim o desenvolvimento da SMDS. Buscando esclarecer esta questão, seria necessário desenvolver um sistema eficiente de replicação para este vírus, até o presente ainda não disponível. Em vista disso, foi desenvolvido um método de detecção de infecções por TTSuV em cultivos celulares para a avaliação de possíveis linhagens a serem potencialmente utilizadas para isolamento e multiplicação destes vírus. Genomas de TTSuVs foram detectados em células de linhagem de origem suína e não suína assim como em um dos lotes de tripsina. Os soros utilizados como suplemento para o meio de cultivo não apresentaram genomas de TTSuV. Desta forma, o lote de tripsina contaminado pode ser considerado uma importante fonte de contaminação, principalmente em células de origem não suína. Com o objetivo de avaliar uma possível associação entre os TTSuVs e a ocorrência da SMDS, a frequência de detecção e quantificação de genomas de TTSuV1 e TTSuV2 em tecidos e soros de suínos com e sem SMDS foram determinadas. A análise feita nos diferentes tecidos de suínos revelou uma aparente correlação inversa entre a presença do genoma de TTSuV1 e a ocorrência da SMDS. Quanto ao TTSuV2 em tecidos de suínos com e sem a SMDS, nenhuma diferença estatística foi observada. A distribuição do genoma de TTSuV1 e TTSuV2 nos diferentes tecidos examinados não revelou um órgão alvo específico. A frequência de detecção e a carga viral de TTSuV1 e 2 nas amostras de soro de suínos com e sem a SMDS não apresentaram diferença significativa. No entanto, a carga viral de TTSuV2 foi mais alta do que a carga viral de TTSuV1 nos soros de todos os grupos de animais estudados. Estes resultados indicam uma alta frequência de detecção de ambas as espécies de TTSuV em amostras de tecidos e soros de suínos com e sem a SMDS.

Palavras-chave: Torque teno sus vírus, TTSuV, anelovírus, suíno, co-infecção, tecidos, qPCR, cultivos celulares

DETECTION AND ISOLATION OF ANELLOVIRUSES IN PIGS AND IN CELL LINEAGES

ABSTRACT

Preliminary studies aiming the identification of possible viral agents associated with the postweaning multisystemic wasting syndrome (PMWS) revealed a possible negative association between TTSuV1 and occurrence of PMWS. Based on this finding was hypothesized that TTSuV1 might be able to inhibit the PCV2 multiplication, preventing the development of PMWS. To better clarify this, would be require an efficient system of replication for this virus, which has not been reported in the literature. In view of this, a method for detection of TTSuV infections in cell culture was developed to assess possible cell lineages to be potentially used for virus isolation and multiplication. TTSuV genomes were detected in cell lineages of porcine and non-porcine origin as well as a batch of trypsin. Sera used as media supplement was not found to contain TTSuV genomes. Thus, the contaminated batch of trypsin can be considered an important source of contamination, especially in cells of non-porcine origin. In order to evaluate a possible association between the TTSuVs and the occurrence of PMWS, the frequency of detection and quantification of TTSuV1 and TTSuV2 genomes in tissues and sera from pigs with and without PMWS were determined. The analysis in the different tissues of pigs reveal an apparent inverse correlation between the frequency of detection of TTSuV1 genomes and the occurrence of PMWS. Regarding TTSuV2 in tissues of PMWS and non-PMWS-affected animals no significant differences was observed. The distribution of TTSuV1 and TTSuV2 genomes in tissues did not reveal any particular target organ. The frequency of detection and viral load of TTSuV1 and TTSuV2 in sera samples were no significant statistically among animals PMWS-affected and healthy pig. The mean of TTSuV2 viral load was significantly highest than TTSuV1 in sera of all groups studied. These results indicate a high frequency of detection of both TTSuV species in tissues and sera samples from PMWS-affected and healthy pig.

Keywords: Torque teno sus virus, TTSuV, anellovirus, swine, co-infection, tissue, qPCR, cell cultures

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LISTA DE ABREVIATURAS E SÍMBOLOS

ACRMP	Amplificação por Círculo Rolante com Múltiplos Primers
AIDS	Síndrome da imunodeficiência adquirida
HIV	Vírus da imunodeficiência humana
ICTV	*International Committe on Taxonomy of Viruses*
IgG	Imunoglobulina G
kb	Mil pares de base
mRNA	RNA mensageiro
NK	Células natural killer
nm	Nanômetros
nt	Nucleotídeos
ORF	Fase aberta de leitura
pb	Pares de base
PCR	Reação em cadeia da polimerase
PCV1	Circovírus suíno tipo 1
PCV2	Circovírus suíno tipo 2
PDNS	Síndrome de Dermatite e Nefropatia Suína
PMWS	*Postweaning Multisystemic Wasting Syndrome*
PRRSV	Vírus da Síndrome Respiratória e Reprodutiva dos suínos
RDA	*Representational Differences Analysis*
SAV	*Small anellovirus*
SAV1	*Small anellovirus 1*
SAV2	*Small anellovirus 2*
Sd-TTV1p	Primeiro genoma de TTSuV1 caracterizado no Brasil
Sd-TTV2p	Primeira denominação do TTSuV2
Sd-TTV31	Primeira denominação do TTSuV1
SISPA	*Sequence-Independent Single Primer Amplification*
SMDS	Síndrome Multissistêmica do Definhamento dos Suínos
TLMV	TTV-like mini vírus
TTMDV	Torque teno midi-vírus
TTMV	Torque teno mini-vírus
TTSuV	Torque teno sus vírus
TTSuV1	Torque teno sus vírus 1

TTSuV2	Torque teno sus vírus 2
TTV	Torque teno vírus
UTR	U*ntranslated region*

SUMÁRIO

1 INTRODUÇÃO

Os Torque teno vírus (TTVs) são vírus pequenos, não-envelopados e com genoma de DNA circular de fita simples, classificados na família *Anelloviridae* (ICTV-2009). O primeiro TTV foi identificado em um paciente humano com hepatite pós-transfusional de etiologia desconhecida em 1997 (NISHIZAWA et al., 1997). Desde então, TTVs tem sido descrito em suínos, cães, gatos, bovinos, frangos, ovinos, tupaias e primatas (CONG et al. 2000; LEARY et al. 1999; OKAMOTO et al. 2001; VERSCHOOR et al. 1999). Em suínos, duas espécies de TTV foram descritas até o momento: *Torque teno sus virus 1* (TTSuV1) e *2* (TTSuV2) (NIEL et al. 2005; OKAMOTO et al. 2002). A primeira descrição deste vírus em suínos foi publicada em 1999 (LEARY et al. 1999), embora sua presença tenha sido demonstrada em suínos (SEGALÉS et al., 2009) e em células de cultivo celular (TEIXEIRA et al., 2011) desde 1985.

Genomas de ambas as espécies de TTSuVs tem sido detectadas em soros de suínos em todo o mundo, com taxas de detecção que variam de 24% a 100% (BIGARRÉ et al., 2005; KEKARAINEN et al., 2006; MARTELLI et al., 2006; TAIRA et al., 2009; GALLEI et al., 2010). É muito provável que ambas as espécies sejam ubíquas em suínos domésticos e em javalis (KEKARAINEN e SEGALÉS, 2009). Os TTSuVs também têm sido encontrados em fluidos biológicos, como sêmen, colostro, cavidade nasal e fezes (KEKARAINEN et al., 2007; MARTÍNEZ-GUINÓ et al., 2009; SIBILA et al., 2009a), sugerindo a ocorrência de transmissão vertical e horizontal (MARTÍNEZ-GUINÓ et al., 2009; POZZUTO et al., 2009; SIBILA et al., 2009a,b; ARAMOUNI et al., 2010).

Até o presente, não existe nenhuma associação clara entre esses agentes e qualquer patologia. Em função disso foi proposto que os TTSuVs seriam vírus "órfãos", ou seja, não associados a enfermidades (BENDINELLI et al., 2001). No entanto, a infecção por TTSuV tem sido sugerida como um fator de agravamento em co-infecções com outros patógenos, como seria o caso em infecções pelo circovirus suíno tipo 2 (PCV2). Este último é um vírus ubíquo que tem sido associado a diferentes síndromes em suínos, denominadas enfermidades associadas ao circovírus suíno, ou “porcine circovirus associated diseases” (PCVAD). Entre elas, a síndrome multissistêmica do definhamento dos suínos (SMDS) tornou-se uma das principais causas de perdas econômicas na suinocultura nacional.

Parece haver uma diferença marcante entre a frequência de detecção das diferentes espécies de TTSuV em diferentes quadros. A prevalência de TTSuV2, mas não do TTSuV1, foi significativamente maior em animais com a SMDS do que em animais saudáveis (KEKARAINEN et al., 2006). O TTSuV1 facilitaria a indução de SMDS por PCV2 em suínos gnotobióticos infectados experimentalmente (ELLIS et al., 2008). Por outro lado, em um estudo recente, embora com limitado número de animais (n = 22), não foi detectada associação entre a frequência de detecção de genomas de TTSuV1 e TTSuV2 e o desenvolvimento de SMDS (LEE et al., 2010).

Estudos realizados por nosso grupo visando a identificação de possíveis agentes virais associados à SMDS, mostrou uma possível associação negativa entre a presença entre TTSuV1 e a ocorrência da SMDS foi detectada (TEIXEIRA, 2008). Tal achado sugere que este agente poderia estar exercendo algum papel inibidor sobre a multiplicação do PCV2 nos hospedeiros ou sofrendo algum tipo de inibição por este último.

Em função das incertezas com relação aos co-fatores potencialmente envolvidos na SMDS, é de grande importância o estabelecimento dos agentes potencialmente envolvidos no quadro além do estudo de outros agentes que possam estar de alguma forma associados ao PCV2 e à ocorrência dessa síndrome.

Para avaliar com maior profundidade possíveis associações entre TTSuVs e SMDS seria importante contarmos com um sistema eficaz para a replicação dos TTSuVs. Neste trabalho foram avaliadas diferentes linhagens celulares visando um sistema de isolamento para TTSuVs. Infelizmente, até o momento, tal sistema ainda não foi obtido. Paralelamente, foram desenvolvidos ensaios moleculares para a detecção e quantificação das duas espécies de TTSuVs em soros e tecidos de suínos com e sem a SMDS uma vez que estes vírus possam ter papéis diferentes na co-infecção com o PCV2.

2 REVISÃO BIBLIOGRÁFICA

2.1 Histórico

O vírus Torque teno (TTV) foi descoberto em 1997 no Japão, em um paciente humano com uma hepatite pós-transfusional de etiologia desconhecida e foi nomeado com as iniciais do nome do paciente (T.T.), contudo essas iniciais também foram utilizadas para designar "transfusion transmitted virus" ou vírus transmitido por transfusão (NISHIZAWA et al., 1997, BENDINELLI et al., 2001).

Em 2000, um vírus pequeno, relacionado ao TTV, foi identificado acidentalmente através de uma PCR em amostras de plasma humano. Usando iniciadores específicos para TTV, foi obtido um amplicon visivelmente menor que o esperado que foi nomeado provisoriamente como "TTV-like mini virus" (TLMV). Usando a estratégia de PCR invertida, vários genomas circulares de cerca de 2.900 nt foram caracterizados (TAKAHASHI, et al., 2000a;b).

Em 2005, o Comitê Internacional de Taxonomia de Vírus propôs um novo significado para TTV e TLMV, ou seja, Torque teno vírus (TTV) e Torque teno mini vírus (TTMV), onde "torque" significa colar e "teno" significa pequeno. Estes termos foram escolhidos para refletirem o arranjo organizacional do genoma do TTV (BIAGINI et al., 2005).

Posteriormente, através do método de amplificação "*Sequence-Independent Single Primer Amplification*" (SISPA), dois novos vírus, chamados de "*small anellovirus 1"* (SAV1) e "*small anellovirus 2"* (SAV2), foram isolados de soros de pacientes com infecção viral aguda (JONES et al., 2005). Em 2007, no intuito de amplificar SAV no soro humano, produtos maiores do que o esperado foram obtidos, e os clones completos apresentavam 3.242-3.253 nt. Estes isolados foram denominados de Torque teno midi-vírus (TTMDV) (NINOMIYA et al., 2007).

No entanto, a infecção por TTV não é restrita a humanos. O DNA de TTV também foi detectado com frequência em amostras de soro de chimpanzés, macacos japonês, saguis-de-barriga-vermelha, saguis-cabeça-de-algodão e douroucoulis. Sequências virais completas amplificadas por PCR com primers invertidos demonstraram que todos os TTVs de primatas não-humanos eram circulares e com genoma de 3,5–3,8 kb (OKAMOTO et al., 2000b). Além disso, verificou-se que os

chimpanzés apresentam TTMVs de 2,8 kb, semelhante aos seres humanos (OKAMOTO et al., 2000b).

Vírus semelhantes ao TTV também tem sido detectados em espécies de animais não-primatas, incluindo animais domésticos (gatos e cães), de produção (gado, galinhas, porcos e ovelhas), animais selvagens (javalis) e tupaias (BRASSARD et al., 2008; LEARY et al., 1999; MARTINEZ et al., 2006; OKAMOTO et al. 2001a; 2002).

A primeira descrição de TTV em suíno foi publicada em 1999 (LEARY et al., 1999), embora sua presença tenha sido comprovada desde 1985 em um estudo retrospectivo (SEGALÉS et al., 2009; TEIXEIRA et al., 2011). Até o momento, dois genogrupos foram identificados em suínos domésticos (NIEL et al., 2005) e recentemente, foram definidos como espécies: o *Torque teno sus vírus 1* (TTSuV1) e o *Torque teno sus vírus 2* (TTSuV2) (HUANG et al., 2010).

2.2 Taxonomia

Inicialmente, devido a suas características morfológicas, o TTV foi caracterizado como um parvovírus (MIYATA et al., 1999). No entanto, observações posteriores sobre a estrutura biofísica e molecular do TTV o enquadraram junto aos circovírus em função do seu genoma circular não-segmentado e dos capsídeos isométricos (HIJIKATA et al.,1999, BENDINELLI et al., 2001). Recentemente, todos os TTVs detectados em humanos e os vírus relacionados ao TTV detectados em animais foram oficialmente designados dentro de uma nova família, *Anelloviridae*. Esta família contem nove gêneros: *Alphatorquevirus*; *Betatorquevirus*; *Gammatorquevirus*; *Deltatorquevirus*; *Epsilontorquevirus*; *Zetatorquevirus*; *Etatorquevirus*; *Thetatorquevirus* e *Iotatorquevirus*. Neste último estão classificadas as duas espécies de TTVs em suínos: *Torque teno sus virus 1* (TTSuV1) e *Torque teno sus virus 2* (TTSuV2) (ICTV-2009).

2.3 Propriedades do vírion

O TTV é um vírus pequeno e icosaédrico (BENDINELLI et al., 2001; CROWTHER et al., 2003). A partícula viral possui densidade de 1,31-1,33 g/cm3 para TTV em soro e 1,33-1,35 g/cm3 para TTV em fezes (OKAMOTO et al., 1998). Na circulação sanguínea, foram observadas partículas de TTV ligadas a imunoglobulina G

(IgG), formando imuno-complexos (ITOH et al. 2000). Por conseguinte, partículas de TTV com um diâmetro de 30-32 nm recuperado dos soros de seres humanos foram observadas como agregados de vários tamanhos em microscopia eletrônica. Por outro lado, partículas de TTV nas fezes apresentam-se como vírions livres. Partículas de TTV do genótipo 1a com um diâmetro de 30-32 nm e bandas em 1.33–1.35 g/cm3 têm sido visualizados no sobrenadante fecal através de microscopia eletrônica usando γ-globulinas do plasma humano contendo anticorpos específicos para o genótipo 1a de TTV (ITOH et al. 2000; TSUDA et al. 1999).

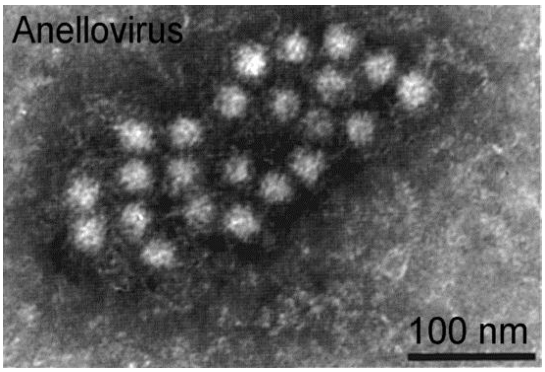

Figura 1. Microscopia eletrônica de contraste negativo de partículas de um isolado de Torque teno vírus, coradas com acetato de uranilo (ITOH et al., 2000).

2.4 Características moleculares

Todos os TTVs identificados, até o presente, possuem um genoma de DNA circular de fita simples e polaridade negativa, apresentando grande variabilidade no tamanho de seus genomas (NISHIZAWA et al., 1997; ITOH et al., 2000; OKAMOTO et al., 2002).

Os genomas dos *Anellovirus* suínos apresentam 2,9 kb, ao contrário do genoma encontrado nos TTVs humanos (3,4 – 3,9 kb) (OKAMOTO et al., 2002). O TTV com o menor genoma identificado até o momento (2,1 kb) foi detectado em felinos domésticos (OKAMOTO et al., 2002).

2.5 Organização genômica

O genoma dos TTSuVs é dividido em uma região não codificadora (UTR) que ocupa 24-31 % do genoma viral (OKAMOTO et al., 2002) e uma região codificadora

que contém três fases abertas de leitura (ORFs). A UTR contém sequências altamente conservadas entre os TTVs que infectam humanos, primatas não-humanos e outros TTVs encontrados em animais. Tipicamente a UTR contém uma região rica em G-C que forma estruturas do tipo “stem-loop” (OKAMOTO et al., 2002) que são importantes sequências reguladoras da replicação e da transcrição (MANKERTZ et al., 2004).

A organização genômica dos TTSuVs assemelha-se aos TTVs que infectam seres humanos, embora a similaridade entre as sequências seja menor que 50% (OKAMOTO et al., 2002; NIEL et al., 2005). Três mRNAs de diferentes tamanhos foram descritos a partir do genoma do TTSuV (OKAMOTO et al., 2002). Duas fases abertas de leitura (ORF1 e ORF2) podem ser deduzidas diretamente da sequência de nucleotídeos do TTSuV, enquanto que a ORF3 é produzida através de “splicing” (Fig. 1). A ORF1 (nucleotídeos 534 a 2438) codifica a proteína do capsídeo de 635 aminoácidos. Esta sequência inclui segmentos (motivos) característicos de proteínas envolvidas na replicação de vírus de DNA circular de dupla fita. A ORF2, localizada entre os nucleotídeos 430 a 648, codifica uma proteína de 73 aminoácidos que contém sequências característica de tirosina fosfatase, enquanto a função da ORF3, que codifica uma proteína de 224 aminoácidos, ainda tem de ser determinada (OKAMOTO et al., 2000c; BIAGINI et al., 2001).

Até o momento, apenas três sequências completas de TTSuVs foram publicadas (OKAMOTO et al., 2002; NIEL et al., 2005). Duas sequências, denominadas pelos autores como, Sd-TTV31 e Sd-TTV1p, partilham um grau moderado de identidade (69,6%), enquanto a terceira (Sd-TTV2p) apresenta apenas 44% de identidade com as outras 2 linhagens (NIEL et al. 2005). Devido à diversidade genética entre estes genomas, foi sugerido que as cepas Sd-TTV 31 e Sd-TTV2p representariam os protótipos das espécies de TTSuV1 e TTSuV2, respectivamente (OKAMOTO et al., 2002; NIEL et al., 2005). Entretanto, a variação da sequência nucleotídica entre TTSuV depende muito da região do genoma viral analisada. Enquanto que uma região de aproximadamente 100 pb na UTR apresenta homologia de 78-99% entre genomas (BIGARRÉ et al., 2005), a análise de uma região de 260 pb na UTR entre TTSuV1 e 2 revelou homologia de 91-97% e de 93-99%, respectivamente (KEKARAINEN et al., 2006).

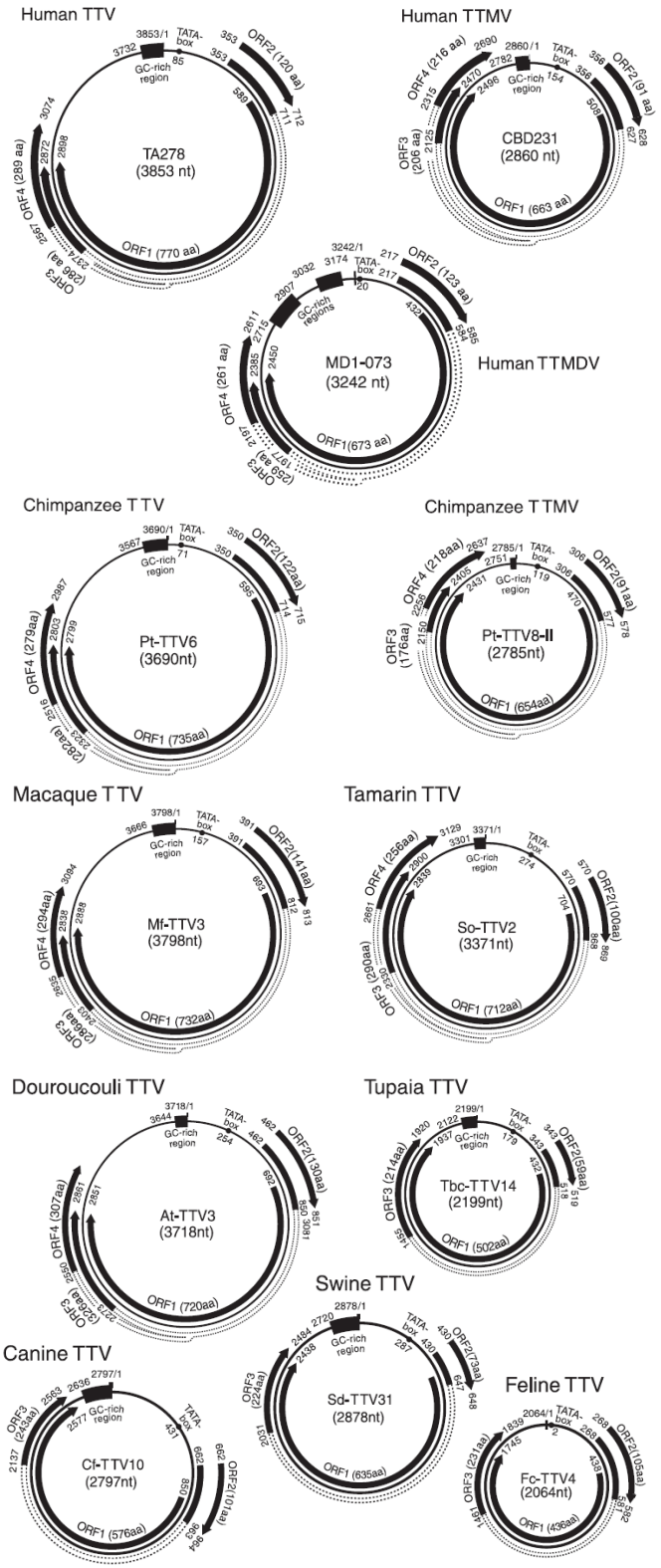

Figura 2. Organização genômica de 12 Torque teno vírus (TTV) de diferentes espécies. A circunferência de cada círculo representa o tamanho relativo do genoma. As setas fechadas representam as ORFs (ORF1-3). A caixa aberta localizada no centro da ORF3 representa uma área correspondente a um intron no mRNA mais curto. As caixas fechadas indicam a região rica em GC e o ponto representa a posição do TATA Box. (OKAMOTO et al., 2002).

2.6 Epidemiologia

2.6.1 Epidemiologia em humanos

O TTV, assim como o TTMV e o TTMDV, tem distribuição mundial (BENDINELLI et al., 2001; HINO & MIYATA, 2007). Elevadas frequências de detecção de genomas tem sido demonstradas em indivíduos saudáveis em diversos lugares do mundo (PRESCOTT et al., 1999). A transmissão fecal–oral é considerada uma das principais rotas de propagação de TTV (BIAGINI, 2004). A identificação de DNA de TTV na bile indica que esta pode ser uma das principais fontes de TTV encontrado nas fezes (ITOH et al., 2001). Foi levantada a hipótese da existência de uma via aérea de transmissão em função da carga viral elevada de TTV em suabes de secreção nasal de crianças (MAGGI et al., 2003). A via de disseminação sexual foi considerada viável baseada em trabalhos que encontram DNA de TTV no sêmen (INAMI et al., 2000) e no colo uterino de indivíduos infectados (CALCATERRA et al., 2001). Ainda tem-se considerado a transmissão vertical, uma vez que o TTV foi encontrado no sangue de cordão umbilical (MORRICA et al., 2000). A detecção de vírus no leite materno também sugere que esta seria outra possível via de transmissão pós-natal (GERNER et al., 2000).

2.6.2 Epidemiologia em suínos

Tanto o TTSuV1 como o TTSuV2 parecem estar amplamente difundidos nos criatórios em várias regiões do mundo (LEARY et al., 1999; OKAMOTO et al., 2002; McKEOWN et al., 2004; BIGARRÉ et al., 2005; NIEL et al., 2005; KEKARAINEN et al., 2006), incluindo o Brasil (NIEL et al., 2005). A detecção do TTSuV1 em amostras de soro de suínos domésticos de diferentes regiões geográficas (Canadá, China, Coréia, Espanha, Itália, França, Tailândia e E.U.A.) revelou uma frequência variando de 24% a 100% (McKEOWN et al., 2004; BIGARRÉ et al., 2005; KEKARAINEN et al., 2006; MARTELLI et al., 2006). A frequência do TTSuV2 em suínos, na Espanha, foi de até 77% dos animais infectados (KEKARAINEN et al., 2006; SEGALES et al., 2009). Da mesma forma, na Espanha, 58 % e 66 % dos javalis testados estavam infectados com o TTSuV1 e 2, respectivamente e a frequência da infecção variou de acordo com a região geográfica (MARTINEZ et al., 2006).

O TTSuV tem sido frequentemente detectado em fezes e secreção nasal de suínos, sugerindo a via fecal-oral como a forma mais importante de transmissão deste vírus (BRASSARD et al., 2008). No entanto, evidências de transmissão vertical tem sido sugeridas desde que TTSuV1 e TTSuV2 foram detectados em amostras de colostro e no soro de porcas e seus natimortos (MARTINEZ-GUINO et al., 2009), assim como no útero (POZZUTO et al., 2009). Uma elevada frequência de detecção de genomas de TTSuV também foi detectada em sêmen de suínos, sugerindo que a via sexual talvez possa contribuir para a disseminação viral (KEKARAINEN et al., 2007).

2.7 Patogenia

Apesar de o DNA de TTV ter sido detectado em células mononucleares do sangue periférico (OKAMOTO et al., 1999; OKAMURA et al., 1999). Até o momento, não são conhecidas quais células os TTVs utilizam para sua replicação. Tem sido observado que tais células quando mitógeno-estimuladas e infectadas com o vírus liberam vírions, sugerindo que a replicação pode ocorrer nessa população de célula (MAGGI et al., 2001; MARISCAL et al. 2002). Igualmente, formas replicativas do genoma viral tem sido detectadas no fígado e na medula óssea, sugerindo que os TTVs podem replicar em células dentro destes órgãos (OKAMOTO et al., 2000a; ZHONG et al., 2002). Entretanto, a replicação do TTV não parece estar restrita a estes órgãos. Uma alta carga viral de formas replicativas e mRNA também foram detectadas nos tecidos do pulmão, pâncreas, baço (OKAMOTO et al., 2001b; JELCIC et al., 2004), e outros tecidos linfóides (KAKKOLA et al. 2004).

2.8 Resposta imune

A maioria dos estudos realizados, até o momento, referem-se à resposta imune do TTV em humanos. Mesmo assim, pouco se sabe sobre as relações de imunidade e sobre a produção de anticorpos anti-TTV na circulação sanguínea. Anticorpos contra o virion do TTV (TSUDA et al. 1999) e de proteínas recombinantes da ORF1 (HANDA et al. 2000; OTT et al. 2000) são detectados em indivíduos virêmicos e não virêmicos. Como já citado, partículas de TTV na circulação tem sido muitas vezes ligadas a IgG, formando imunocomplexos (ITOH et al. 2000). No entanto, até o presente não há

evidência para indicar uma associação com doenças evocadas pela deposição de complexos imunes, como a glomerulonefrite.

Tem sido demonstrado um aumento da carga viral de TTV em pacientes infectados com o vírus da imunodeficiência humana (HIV) e uma elevada carga viral de TTV foi associada com uma baixa contagem de células CD4, indicando um potencial papel do sistema imunológico no controle da replicação do TTV (CHRISTENSEN et al. 2000; SHIBAYAMA et al. 2001; THOM & PETRIK 2007; TOUINSSI et al. 2001; ZHONG et al. 2002).

É muito provável que a infecção por TTV seja adquirida no início da infância, o que pode levar a algum tipo de tolerância imunológica, em analogia com o descrito para outros vírus, como o vírus da hepatite B. Embora ainda não esteja claro qual o papel que o sistema imunológico desempenha no curso natural da infecção viral, o TTV pode atuar como um patógeno oportunista em hospedeiros imunocomprometidos, análogo ao citomegalovírus humano em pacientes infectados pelo HIV-1 (OKAMOTO, et al., 2009).

O único trabalho que descreve a detecção de anticorpos em suínos mostrou que animais com a SMDS apresentam um nível de anticorpos anti-TTSuV2 menor que os animais saudáveis (HUANG, et al., 2011). O baixo nível de anticorpos anti-TTSuV2 em suínos afetados pela SMDS pode ser um resultado da imunossupressão resultante desta síndrome (OPRIESSNIG et al., 2007).

2.9 Potencial patológico

Em humanos tem sido sugerido que o TTV estaria associado a diferentes patologias auto-imunes, incluindo doenças reumáticas (GERGELY et al., 2006), patologias hepáticas (NISHIZAWA et al. 1997; MUSHAHWAR et al., 1999; KASIRGA et al., 2005) e condições respiratórias (BIAGINI et al., 2003; MAGGI et al., 2003). No entanto, não existem dados definitivos indicando que o TTV é responsável por qualquer doença em humanos.

No caso dos suínos, os TTSuVs infectam uma elevada proporção de animais aparentemente saudáveis (KEKARAINEN & SEGALE, 2009). No entanto, o seu papel durante a co-infecção com outros patógenos não tem sido investigado em detalhes. A taxa de detecção de genomas de TTSuV foi significativamente maior em soros de animais com SMDS quando comparados a animais não afetados pela síndrome 97% e

78% respectivamente. Além disso, suínos com SMDS (91%) foram mais propensos à infecção por TTV2 do que os suínos não afetados (72%) (KEKARAINEN et al., 2006). Outros autores propuseram que a presença do TTSuV1 facilita a indução da SMDS pelo PCV2 (ELLIS et al., 2008).

2.10 Métodos de detecção de TTV

Os TTVs estão entre os vírus que começaram a ser identificados a partir do momento em que se tornou possível buscar genomas heterólogos em órgãos ou tecidos, independente da presença de uma enfermidade sugestiva de infecção. Até o momento, nenhum sistema de cultivo celular que permita a propagação eficiente de TTV foi identificado. Dois sistemas de cultivo celular que suportam a replicação do vírus e a formação de vírions apartir de clones de TTV humano transfectados foram relatados, mas em nenhum caso a propagação do vírus foi eficiente (KAKKOLA et al., 2007; LEPPIK et al., 2007).

2.10.1 Métodos dependentes da sequência de nucleotídeos

A PCR é o método mais usado para a detecção do TTV. Métodos de PCR utilizando oligonucleotídeos específicos para a região não codificante (UTR) do genoma do TTV são capazes de reconhecer praticamente todos os genótipos conhecidos, enquanto PCRs com primers desenhados para as regiões codificantes tendem a ser espécie-específicas (KEKARAINEN et al., 2006; NINOMIYA et al., 2007).

Até recentemente, todos os estudos sobre TTSuV detectavam a fraquência deste vírus baseada em PCR qualitativa (positivo x negativo) (BIGARRÉ et al., 2005; KEKARAINEN et al., 2006; MARTELLI et al., 2006; MARTÍNEZ-GUINÓ et al., 2009; MARTÍNEZ-GUINÓ et al., 2010; POZZUTO et al., 2009; SEGALÉS et al., 2009; SIBILA et al., 2009a,b). Estudos recentes descreveram a técnica de PCR em tempo real (qPCR) para quantificação de TTSuV1 e TTSuV2 (BRASSARD et al., 2010; GALLEI et al., 2010; LEE et al., 2010; NIETO et al., 2011).

2.10.2 Métodos independentes da sequência de nucleotídeos

Se a PCR tem sido amplamente utilizada no diagnóstico, o desenvolvimento de metodologias independentes da sequência tem proporcionado a investigação de novas variantes virais. Estes superam tanto o problema das variações na suposta sequência como, por exemplo, mutações pontuais quanto à obtenção de fragmentos de sequências localizadas em todos os alvos do genoma viral. Assim, a combinação dos métodos de Amplificação por Círculo Rolante com Múltiplos Primers (ACRMP) e SISPA "Sequence-Independent Single Primer Amplification" tem demonstrado seu potencial na identificação de novos anelovírus no homem e nos animais (NIEL et al., 2005; BIAGINI et al., 2007; TEIXEIRA, 2008).

2.10.3 Outras abordagens

Em complementação à detecção do genoma viral, um número muito baixo de publicações abordaram o diagnóstico sorológico, baseados na detecção de IgG e IgM específicos utilizando proteínas recombinantes como antígeno (TSUDA et al., 1999; TSUDA et al., 2001; HUANG et al., 2011). Abordagens para a detecção *in situ*, por hibridização ou por PCR também foram descritas (CHENG et al., 2000; COMAR et al., 2006).

3 OBJETIVOS

3.1 Objetivo geral

- Contribuir para um melhor entendimento da biologia dos TTSuVs e verificar se estes agentes apresentam algum tipo de associação com a ocorrência de SMDS.

3.2 Objetivos específicos

- Desenvolver uma duplex PCR para detectar a presença de genomas de TTSuV1 e TTSuV2 em células de linhagem;

- Desenvolver uma PCR espécie-específica para detecção de TTSuV1 e TTSuV2 em diferentes órgãos de suínos com e sem Síndrome Multissistêmica do Definhamento dos Suínos (SMDS);

- Avaliar a carga viral de TTSuV1 e TTSuV2 em soro de suínos com e sem SMDS.

4 CAPÍTULO 1

4.1 Torque teno sus virus (TTSuV) in cell cultures and trypsin.

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Torque teno sus virus (TTSuV) in cell cultures and trypsin

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Abstract

Torque teno sus virus (TTSuV), a member of the family *Anelloviridae*, is a single-stranded, circular DNA virus, widely distributed in swine populations. Presently, two TTSuV genogroups are recognized: *Torque teno sus virus 1* (TTSuV1) and *Torque teno sus virus 2* (TTSuV2). TTSuV genomes have been found in commercial vaccines for swine, enzyme preparations and other drugs containing components of porcine origin. However, no studies have been made looking for TTSuV in cell cultures. In the present study, a search for TTSuV genomes was carried out in cell culture lineages, in sera used as supplement for cell culture media as well as in trypsin used for cell disaggregation. DNA obtained from twenty-five cell lineages (ten from cultures in routine multiplication and fifteen from frozen ampoules), nine samples of sera used in cell culture media and five batches of trypsin were examined for the presence of TTSuV DNA. Fifteen cell lineages, originated from thirteen different species contained amplifiable TTSuV genomes, including an ampoule with a cell lineage frozen in 1985. Three cell lineages of swine origin were co-infected with both TTSuV1 and TTSuV2. One batch of trypsin contained two distinct TTSuV1 plus one TTSuV2 genome, suggesting that this might have been the source of contamination, as supported by phylogenetic analyses of sequenced amplicons. Samples of fetal bovine and calf sera used in cell culture media did not contain amplifiable TTSuV DNA. This is the first report on the presence of TTSuV as contaminants in cell lineages. In addition, detection of the viral genome in an ampoule frozen in 1985 provides evidence that TTSuV contamination is not a recent event. These findings highlight the risks of TTSuV contamination in cell cultures, what may be source for contamination of biological products or compromise results of studies involving *in vitro* multiplied cells.

Introduction

Torque teno viruses (TTVs) are small, non-enveloped viruses that contain a circular single-stranded DNA genome of negative polarity [1], presently classified in the family *Anelloviridae* [2]. TTVs were first detected in 1997 in a Japanese patient with post-transfusion hepatitis of unknown etiology [3]. Since then, other human TTVs have been described with distinct genome sizes; Torque teno "midi viruses" (TTMDV) comprises viruses with genomes sizes with about 3.2 kb [4], whereas Torque teno "mini viruses" (TTMV) have genome sizes between 2.8 kb and 2.9 kb [5]. TTVs are not restricted to human hosts and have also been identified in a number of other species, including non-human primates, tupayas, cats, dogs, pigs, chickens, cows and sheep [1,6-11].

In swine, two distinct genogroups, *Torque teno sus virus 1* (TTSuV1) and *Torque teno sus virus 2* (TTSuV2), have been identified [1,7,12]. Torque teno sus viruses (TTSuVs) are widely distributed in swine populations, though reported prevalences are quite variable [13-16]. The association of TTSuVs with disease is currently subject of studies; data suggest that TTSuVs may participate as coadjuvants in other pathological conditions of swine, such as post-weaning multisystemic syndrome (PMWS) and porcine dermatitis and nefropathy syndrome (PDNS), diseases primarily associated to porcine circovirus type 2 infections [13,17-18].

TTSuVs have also been detected in colostrum and in stillborns, suggesting vertical transmission of the virus [19]. The finding of TTSuV genomes in semen of boars indicates that the virus may possibly be transmitted by natural or artificial reproduction [20]. Others have raised the possibility of TTSuV transmission by contaminated biological products, since TTSuVs genomes have been identified in commercial vaccines for swine and in enzyme preparations and other drugs formulated

with components of porcine origin [21]. This possibility, however, awaits further investigation.

We have been attempting to propagate TTSuV *in vitro* cultured cells. However, this would require previous testing of cells and media to ensure that no preexisting contamination would undermine virus isolation. To date, no previous data on the presence of TTSuV in cell cultures is available. In view of that, a search was made for TTSuV genomes in a number of available established cell lineages. In addition, other frequent sources of cell culture contaminants, such as fetal calf sera and trypsin used routinely in cell culture manipulation were also tested for the presence of TTSuV.

Results

A duplex PCR was designed to amplify genome fragments of both TTSuV1 and TTSuV2 in a same reaction. The sensitivity of the duplex PCR was determined by performing the reaction with different concentrations of DNA extracted from pCR2.1 plasmid containing TTSuV1 or TTSuV2 PCR products. The minimum number of TTSuV copies that could be identified with this method was determined by testing tenfold dilutions of plasmid DNA in the duplex PCR. With this approach, it was determined that the lowest number of genome molecules detectable by the assay was 100 molecules of TTSuV1 and 1000 molecules of TTSuV2 per reaction.

Once the sensitivity of the tests was determined, the search for the presence of TTSuV contamination in cell cultures and related products was carried out. The results of these findings are summarized on Table 1. Fifteen cell culture lineages tested contained amplifiable TTSuV1 and/or TTSuV2 genomes, including cells that were tested as soon as thawed out of the liquid nitrogen. Some cell culture lineages of swine origin (PK15 PCV1 free, ST and PK15) were co-infected with both TTSuV1 and

TTSuV2. All samples from sera that had been used as cell culture media supplement resulted negative for the presence of amplifiable TTSuV DNA. One batch of trypsin contained genomes of two distinct TTSuV1 as well as TTSuV2. This batch of trypsin was in use on the ten cell lineages that were currently being multiplied in the laboratory. These were found to be contaminated with either TTSuV1 or TTSuV2. The three cell lineages of porcine origin, on which the same trypsin batch was also been used, was found contaminated with both types of TTSuV. The other four batches of trypsin tested did not contain amplifiable TTSuV DNA (Table 1).

Amplicons with the expected size (107 bp for TTSuV1 and 103 bp for TTSuV2) were excised from 1% agarose gels, cloned and sequenced. Twenty one nucleotide sequences corresponding to such amplicons were submitted to GenBank (accession numbers GU574709 to GU574729).

The phylogenetic tree (Figure 1) inferred by the neighbor-joining method allowed the grouping of virus genomes in TTSuV1 and TTSuV2 genogroups. Eleven sequences were clustered within the TTSuV1 genogroup, displaying between 88.71 % to 100 % sequence similarity to the reference strains in genogroup 1 (AB076001, AY823990). Ten other sequences clustered within the TTSuV2 genogroup, with 83.79 % to 100 % sequence similarity to the reference strain (AY823991). TTSuV1 genomes identified in trypsin-b were nearly identical to those found in eight of the contaminated cells and TTSuV2 genome detected in trypsin-c was nearly identical to those found in seven cells showing that maybe these cells can be contaminated by residual trypsin. It can also be seen that sequences from PK15-b and SK6 lineages were the most filogenetically distant sequences within the TTSuV2 genogroup, suggesting either a different source of contamination, or that the original virus sequence had been mutated during replication.

Discussion

Koch´s postulates are being once more challenged by molecular methods of genome detection. Diagnostic methods have evolved in such a way that in many instances the genome of an agent can be identified without the need for its previous isolation. While searching for DNA- containing agents that may be infecting swine - regardless of any association with disease - using methods that allow genome amplification without previous knowledge of nucleotide sequences [6,7] our group identified TTSuV contamination in farming pigs [22]. In order to proceed on the study of such agents, a natural development was to try to multiply such viruses in cell cultures. However, this would require cultured cells free of TTSuV contamination. Thus, the present study was set up to examine whether the cells available in our laboratory would be contaminated. As result of this search, TTSuV genomes were detected in cell lineages of porcine and non-porcine origin. Indeed, fifteen out of the 25 cells tested revealed TTSuV contamination. Three of the cell lineages of porcine origin were infected with both TTSuV1 and TTSuV2.

Once contamination was detected in cultured cells, the identification of the source of contamination was imperative. One batch of trypsin was contaminated with two distinct variants of TTSuV1 as well as with TTSuV2. The other trypsin batches tested were negative for the presence of TTSuV. The sera used as media supplement was not found to contain TTSuV, a result which might be expected, since all sera were of non-porcine origin. Therefore, these findings were pointing towards the contaminated batch of trypsin as source of TTSuV contamination. Phylogenetic analyses suggest that the TTSuV genomes detected in most cell lineages were closely related - but not identical - to those detected in the contaminated batch of trypsin. Therefore, this seems in fact the most likely source for contamination of cultures. This batch of trypsin was

being used on all cells (BHK-21, CER, CrFK, H407, PK15, PK-2 nd, PK15 PCV1 free, SK6, ST, and Vero) being multiplied in the laboratory at the time this study was being carried out. All these cells were found to contain at least one TTSuV variant. Clearly, the finding of TTSuV genomes in cells treated with contaminated trypsin does not ensure that virus multiplication took place in such cells. Indeed, it may be argued that virus carried over by residual trypsin might have been the source of TTSuV contamination for at least some of the cells. However, if this was the case, all infected cells should reveal contamination with both types of TTSuV detected in the contaminated trypsin. Moreover, the nucleotide sequences of the recovered fragments should be very similar, which was not the case. In fact, phylogenetic analysis shows that, although some sequences are indeed very similar, others are quite phylogenetically apart, indicating that either contamination originated from distinct sources, or the original virus had undergone distinct evolutionary pathways during replication. As an example, the phylogenetic distance between the TTSuV2 fragments from SK6 and PK15-b cells (Figure 1) suggests these viruses probably originated from distinct sources - perhaps another batch of contaminated trypsin used in the past, or yet the tissues from which cells were originally prepared. In such cases, however, the precise source of contamination can only be guessed with the data here available.

Despite that, there still remains the possibility that some of the cells were in fact carrying virus from residual trypsin. The sensitivity threshold of the PCR employed in this study was 100-1000 molecules of viral DNA, indicating that relatively high viral loads were needed to be detected in the cell lineages. In addition, in attempting to minimize chances of amplifying viral genomes that could be present in residual trypsin, the supernatant medium was carefully washed out with PBS three times before DNA extractions. However, the possibility of residual trypsin contamination carry over cannot

be fully discarded and must remain as an additional risk to be considered when searching for anelloviruses in cell cultures.

It must also be reminded that other TTSuV variants could be present in cells and trypsin and might have remained undetected by the method employed here; this possibility also cannot be completely ruled out. In view of the specificity of the primers designed for this study, these would not be detected. Likewise, it is also possible - and quite probable, in our belief, based on the apparently wide dispersion of anelloviruses in nature - that sera may act as a potential source for anelloviruses derived from other animal species. This might eventually lead to infection of other cultured cell lineages. However, this must also be taken into account when dealing with cultured cells.

Interestingly, from the results obtained here, it became apparent that TTSuV contamination of cultured cells is not a recent event. A cell lineage that had been ampouled in 1985 and was tested as soon as thawed out of the liquid nitrogen was also found to contain TTSuV1. Therefore, such viruses have been circulating for at least more than 25 years. This adds to the evidence for the circulation of such viruses, as indeed detected in a retrospective study on swine sera collected in the same year, revealing that TTSuV1 and TTSuV2 were already detected in the original source species [23].

Knowledge on TTSuV- as well as on anelloviruses in general - is still in its early days; clear association between these viruses and disease has not yet been fully established. It is possible that TTSuV might act as incidental pathogens, where disease would become evident only under exceptional circumstances. In some infections, the viral load is a critical for the development of disease. It has been suggested that anelloviruses might be comensal agents under normal circumstances, incapable of exceeding the threshold of a disease-causing load [24]. Also interesting is the

198 observation that anelloviruses may be able to impair replication of other viruses. An
199 association was detected between a higher prevalence of TTSuV1 in healthy, non-
200 PMWS-affected pigs, than in PMWS-affected animals [22]. In this sense, anelloviruses
201 might somehow bring some benefit the host, an aspect hitherto unexplored [25].

202 In addition, the possibility of xenotransplantation of swine tissues to humans
203 would require that no adventitious agents are present in tissues of potential donors to
204 ensure no contamination of transplant recipients [26]. Therefore, TTSuV contamination
205 must be examined in light of such possibility.

206 Whichever is the case, appropriate measures should be taken to ensure that no
207 TTSuV contamination occurs through the usage of contaminated cell culture or the
208 reagents used for *in vitro* cell multiplication and maintenance. Further studies should be
209 conducted to confirm whether TTSuV might give rise to productive infections in non-
210 porcine cell lineages.

211

212 **Material and Methods**

213 **Cells, sera and trypsin**

214 Twenty-five cell lines (ten from cultured cells and fifteen from ampoules
215 stocked in liquid nitrogen) obtained from the laboratory cell bank were used in the
216 experiments (Table 1). Cell culture multiplication was performed following standard
217 methods [27]. Cell lineages were multiplied in Eagle's minimal essential medium
218 (MEM) supplemented with 10 % fetal bovine serum and antibiotics (penicillin 100
219 IU/mL; streptomycin 100 μg/mL). In addition, nine different batches of sera from
220 different species [bovine (05), equine(3), ovine(1)] used as supplements to cell culture
221 media in different moments in the cell culture laboratory, as well as and five batches of
222 trypsin from different manufacturers were included in this study (Table 1).

DNA extraction

DNA extraction from cultured cells was performed as follows: the culture medium was removed and the confluent monolayer washed with PBS (0.15 M NaCl, 0.07 M Na_2HPO_4, 8 mM NaH_2PO_4, pH 7.4). The PBS was discarded and 4 ml of lysis buffer [20 mM Tris–HCl (pH 8.0); 2 mM EDTA (pH 8.0), 300 mM NaCl; 100 µg proteinase K, 1% sodium duodecyl sulphate (SDS)] were added to flasks and incubated for 90 minutes at 37 ºC. Subsequently, 500 µl of the digested material were transferred to new tubes. The DNA was extracted with phenol and after with chloroform/isoamyl alcohol (24:1) [28]. The DNA was precipitated with ethanol, the pellet dried and resuspended in 50 µl TE (10 mM Tris, 1 mM EDTA, pH 7.4) containing 20 µg/ml RNase A. DNA extraction from cells thawed from liquid nitrogen was carried out as follows: ampoules were thawed and centrifuged for 1 min at 9,000 × *g*. The supernatant was removed and the cell pellet resuspended in 500 µl of PBS, 3 % SDS, 200 µg/ml proteinase K and incubated for 90 minutes at 37 ºC. The DNA was extracted as mentioned above. The sera DNA extraction was performed with 500 µl of serum and the trypsin DNA extraction was performed with 50 mg of trypsin diluted in 500 µl Milli-Q water. The DNA was extracted with phenol/chloroform/isoamyl alcohol as mentioned above. DNA from samples was quantified with known amounts of lambda/Hind III DNA as standard in 1 % agarose gels, stained with ethidium bromide and visualized on a UV source. To avoid cross-contamination, DNA extraction was performed in different days, with each cell line being processed separately and with filter tips; after each extraction, laminar flow cabinets were cleaned with ethanol and UV-sterilized for at least 30 minutes before working with another cell lineage. No more than three cell lineages were processed on a same working day.

248

249 **Detection of TTSuV**

250 To detect simultaneously TTSuV1 and TTSuV2, a duplex PCR was designed.
251 PCR primers were based on sequences available at GenBank (AB076001– AY823991)
252 and were designed to amplify the non-coding regions of TTSuV1 and TTSuV2. Two
253 forward primers and one common reverse primer were designed: primer "forward-1" (5'
254 GGG AGC TCA AGT CCT CAT TTG 3') and a common reverse primer (5' GCG
255 GCA TAA ACT CAG CCA TTC 3') targeted a 107 bp DNA fragment (nucleotide
256 positions 221-328 on TTSuV1 genome), whereas primer "forward-2" (5' GGG CCW
257 GAA GTC CTC ATT AG 3') plus the common reverse primer were expected to
258 amplify a 103 bp fragment (nucleotide positions 170-273 on TTSuV2 genome). The
259 PCR was carried out in 25 µl volumes with contained 2 µl of DNA (100 ng), 5 pmol
260 primer forward-1, 5 pmol primer forward-2, 5 pmol primer reverse, 0.8 mM dNTP, 1.5
261 mM $MgCl_2$ and 1 U Taq DNA polymerase (Invitrogen). The PCR program consisted of
262 an initial reaction at 94°C for 3 min, followed by 35 cycles at 94 °C (30s), 65 °C (30s)
263 and 72 °C (30s), with a final extension period of 10 min at 72 °C. Amplicons were
264 electrophoresed in 1 % agarose gel and purified using a commercial kit (GFX™
265 Purification Kit; Amersham Biosciences).

266 All PCR products were cloned into plasmids using a TA cloning strategy (pCR
267 2.1 TOPO Cloning, Invitrogen). At least three recombinant plasmids of each reaction
268 were sequenced on both strands using M13-forward and M13-reverse oligonucleotides
269 as primers in a MegaBACE 500 apparatus with the Dyenamic ET terminator cycle
270 sequencing kit (Amersham Biosciences) following the manufacturer's protocol.
271 Sequence identification was performed using NCBI nucleotide BLAST searches
272 (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

To avoid contamination, filter tips were used to prepare the PCR reactions and separate rooms were used to prepare reaction buffers, to extract DNA, and to examine PCR products. A negative control (with ultra pure water instead of sample DNA) was included in every ten PCR tubes as additional contamination controls. Positive controls consisted of reactions with cloned TTSuV1 and TTSuV2 DNA (see below).

Sensitivity assay

In order to determine the PCR sensitivity, amplicons from TTSuV1 (107 bp) and TTSuV2 (103 bp) were cloned into plasmids as described above. The sensitivity of the PCR was determined by amplification of tenfold dilutions of known amounts of each plasmid DNA in the duplex PCR. These experiments were repeated three times. The same plasmids were also used as positive controls in the duplex PCR assays.

Phylogenetic analyses

The obtained sequences were aligned with two sequences proposed as TTSuV1 prototypes (accession n°AY823990 and AB076001) and one sequence proposed as TTSuV2 prototype (accession n° AY823991) available at GenBank [1,7]. A human TTV sequence was included in the alignment as outgroup (accession n° AB041007). Sequences were aligned using the ClustalW program within the MEGA 4 package. The construction of phylogenetic tree was carried out using the neighbor-joining (NJ) method in the MEGA 4 software package, based on Kimura two-parameter distance estimation method. Bootstrap resampling was performed for each analysis (1000 replications).

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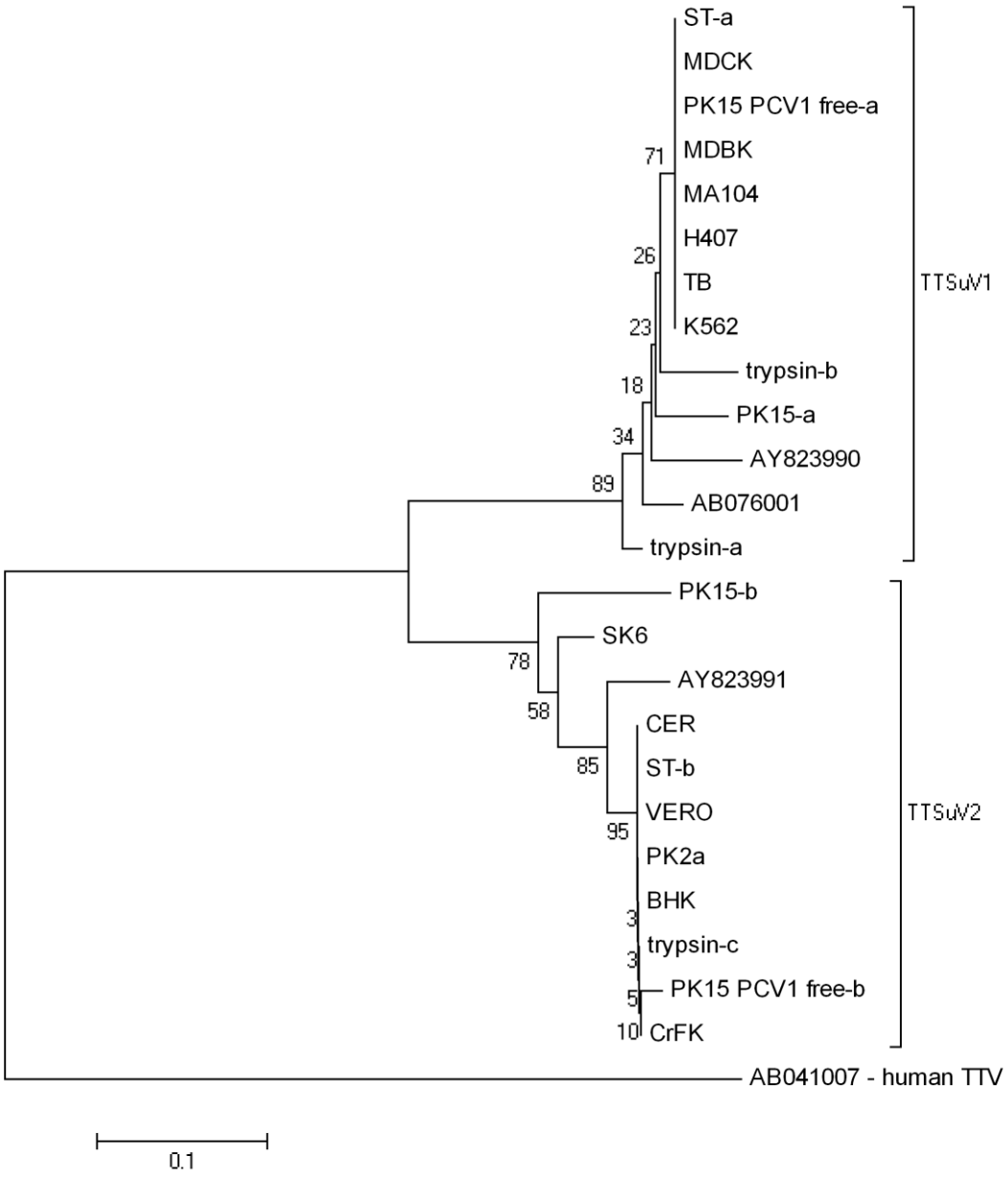

Figure 1 – Neighbour-joining phylogenetic tree constructed based on the nucleotide sequences of the noncoding region of TTSuV genomes.
Bootstrap values are indicated above major branches. AB076001 and AY823990 are reference sequences for TTSuV1 and AY823991 is the reference strains for TTSuV2. Small letters (-a,-b,-c) after names refer to different sequences identified in a particular cell lineage (or trypsin batch).

Table 1. Detection of genomes of *Torque teno sus virus 1* (TTSuV1) and *2* (TTSuV2) in cell lineages, sera and trypsin batches.

Cell lineages, serum and trypsin	**Origin**	**N° of Passages[#]**	**Presence of viral DNA of TTSuV1**	**Presence of viral DNA of TTSuV2**
Baby hamster kidney (BHK-21)	A. Lutz[a]	75	-	+
Chicken embryo related (CER)	VLA[b]	130	-	+
Crandell feline kidney (CrFK)	Unk[c]	241	-	+
Human embryonic intestine (H407)	UFRGS[d]	19	+	-
Human leukemic cell (K562) (28/05/04)*	UFRGS	7	+	-
African green monkey kidney embryonic (MA-104) (28/04/93)*	UFSM[e]	34	+	-
Madin-Darby bovine kidney (MDBK) (17/08/01)*	Panaftosa[f]	129	+	-
Madin-Darby canine kidney (MDCK) (25/10/05)*	Unicamp[g]	60	+	-
Porcine kidney PK15	UFSM	26	+	+
Porcine kidney (PK-2a)	VLA	38	-	+
Porcine kidney PK15 PCV1 free (PKsC3)	Cloned from PK15 at IPVDF	35	+	+
Swine kidney (SK6)	VLA	120	-	+
Swine testicle (ST)	Embrapa[h]	56	+	+
Bovine thyroid cell (TB) (27/02/85)*	Flow[i]	13	+	-
African green monkey kidney (Vero)	Fiocruz[j]	118	-	+
Canine Carcinoma (A-72) (2/07/08)*	VLA	45	-	-
Mutant MDBK Resistant to BVDV Infection (CRIB) (16/01/06)*	UFSM	120	-	-
Embryonic Bovine Trachea (EBTr) (29/12/04)*	IPVDF[k]	30	-	-
Equine Dermis (ED) (9/07/08)*	UFPEL[l]	17	-	-
Foetal Lamb Kidney (FLK) (6/12/90)*	UFPEL	137	-	-
Murine Fibrosarcoma (L929) (3/06/08)*	UFRJ[m]	10	-	-
Monkey Kidney (LLC-MK_2) (15/07/86)*	UFRJ	61	-	-
Murine Neuroblastoma (N2A) (18/10/04)*	VLA	202	-	-
Rabbit Kidney (RK13) (13/01/93)*	UFPEL	54	-	-
Murine myeloma (SP2/O-Ag14)	VLA	34	-	-
Fetal Bovine Serum	Manufacturer A	na[§]	-	-

Fetal Bovine Serum	Manufacturer B	na	-	-
Fetal Bovine Serum	Manufacturer C	na	-	-
Calf Serum (treated in house with polyethylene glycol)	IPVDF	na	-	-
Calf serum	IPVDF	na	-	-
Horse serum (inactivated)	IPVDF	na	-	-
Horse serum (1 donor)	IPVDF	na	-	-
Horse serum (pool)	IPVDF	na	-	-
Ovine serum	IPVDF	na	-	-
Trypsin	Manufacturer A	na	+	+
Trypsin	Manufacturer B	na	-	-
Trypsin	Manufacturer C	na	-	-
Trypsin	Manufacturer D	na	-	-
Trypsin	Manufacturer E	na	-	-

*Date of ampouling/freezing in liquid nitrogen; [a]Adolfo Lutz Institute, Brazil; [b]Veterinary Laboratories Agency, Weybridge, UK; [c]Unknown origin; [d]Federal University of Rio Grande do Sul, Brazil; [e]Federal University of Santa Maria, Brazil; [f]Panaftosa; [g]Campinas University, Brazil; [h]Empresa Brasileira de Pesquisa Agropecuária, Brazil; [i]Flow Laboratories, USA; [j]Fundação Oswaldo Cruz, Brazil.
[k]Prepared at Fepagro Saúde Animal – IPVDF; [l]Federal University of Pelotas, RS, Brazil; [m]Federal University of Rio de Janeiro, RJ, Brazil; [#]refers to the number of passages continuing the sequencial passage number as received from the source; [§]not applicable.

5 CAPÍTULO 2

5.1 *Torque teno sus virus* (TTSuV) in tissues of pigs and its relation with the occurrence of post weaning multisystemic wasting syndrome.

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Torque teno sus virus (TTSuV) in tissues of pigs and its relation with the occurrence of post weaning multisystemic wasting syndrome.

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ABSTRACT

Torque teno sus virus (TTSuV) is a member of the recently created family *Anelloviridae.* Two distinct species of TTSuVs, 1 (TTSuV1) and 2 (TTSuV2) have been reported so far in domestic pigs and wild boars. Although TTSuVs have not been clearly linked to any specific pathology of pigs, a relation between TTSuV infections and postweaning multisystemic wasting syndrome (PMWS) has been suggested. To examine further this possibility, the present study was conducted in search for TTSuV1 and TTSuV2 genomes in tissues of PMWS and non-PMWS-affected animals. PMWS diagnosis was established by clinical signs, characteristic macroscopic and histopathologic lesions and the presence of PCV2 DNA. Samples of five different tissues (lungs, kidneys, livers, spleens and lymph nodes) from 76 PMWS-affected and 11 non-PMWS-affected pigs were examined. Two specific PCR assays were developed to amplify TTSuV1 and TTSuV2 genome segments. TTSuV1 DNA was detected in tissues of non-diseased animals to significantly higher levels than in tissues of PMWS-affected pigs ($p \leq 0.001$). Regarding TTSuV2, viral genomes were detected in nearly all samples from both PMWS-affected (94.7 %) and non-affected pigs (100 %), with no significant differences in the frequencies of detection of TTSuV2 genomes in both groups. No significant differences were detected on the distribution of TTSuV1 and TTSuV2 in the different tissues examined ($p=0.970$). These findings reveal an apparent inverse correlation between the frequency of detection of TTSuV1 DNA (but not TTSuV2 DNA) in pig tissues and the occurrence of PMWS.

Key words: *Anelloviridae*, TTSuV1, TTSuV2, PMWS, distribution, tissues.

1. Introduction

Torque teno sus virus (TTSuV) is currently classified in the genus *Iotatorquevirus* within the *Anelloviridae* family (ICTV-2009). To date, two distinct TTSuV species (TTSuV1 and TTSuV2) have been identified in domestic pigs (Niel et al., 2005; Okamoto et al., 2002).

TTSuVs have been found worldwide in swine sera, with prevalence rates ranging from 24 % to 100 % (Bigarré et al., 2005; Kekarainen et al., 2006; Martelli et al., 2006; Taira et al., 2009; Gallei et al., 2010). Although TTSuV was first described in 1999 (Leary et al., 1999), TTSuVs were already detected in pigs back in 1985 (Segalés et al., 2009) as well as in cell cultures frozen since 1985 (Teixeira et al., 2011), suggesting that the virus was circulating even before such findings. TTSuVs have been also found in other biological fluids, such as semen and colostrum, as well as in nasal cavities and faeces (Kekarainen et al., 2007; Martínez-Guinó et al., 2009; Sibila et al., 2009a), suggesting that transmission probably occurs either vertically or horizontally (Martínez- Guinó et al., 2009; Pozzuto et al., 2009; Sibila et al., 2009a, b; Aramouni et al., 2010).

Although TTSuVs have not been clearly linked to any specific porcine disease, the possibility of a role for such agents as coadjuvants in pathological conditions still remains to be investigated. The prevalence of TTSuV2 was reported significantly higher in postweaning multisystemic wasting syndrome (PMWS)-affected pigs than in healthy animals (Kekarainen et al., 2006). Furthermore, experimental infection of gnotobiotic pigs with TTSuV1 and porcine circovirus type 2 (PCV2) was shown to trigger PMWS (Ellis et al., 2008). More recently, TTSuV1 and TTSuV2 were reported as not related to the occurrence of PCV2- associated disease (PCVAD) (Lee et al., 2010).

To investigate more deeply possible associations between TTSuVs and PCV2, this study was conducted in search for TTSuV1 and TTSuV2 genomes in different tissue samples (lungs, kidneys, livers, spleens and lymph nodes) of PMWS and non-PMWS-affected pigs.

2. Materials and methods

2.1 Samples

Tissue samples of five organs (lung, kidney, liver, spleen and lymph nodes) were collected from 76 PMWS-affected pigs (1-4 months old). The PMWS diagnosis was established with basis on the clinical signs, characteristic macroscopic and histopathologic lesions and the detection of PCV2 DNA (Segalés et al., 2005). Tissues were collected at necropsy. Negative controls samples of the same tissues were collected from eleven healthy pigs in an abattoir. All samples were stored at –70 °C until DNA extraction.

2.2 DNA extraction

DNA extraction was performed essentially as described by Dezen et al. (2011). Briefly, tissue fragments (about 25 mg) were placed in 885 µL lysis buffer [20 mM Tris–HCl (Invitrogen), pH 8.0; 2 mM EDTA (Invitrogen), pH 8.0, 300 mM NaCl (Nuclear); 100 µg proteinase K (USB Corporation), 1% SDS (Promega) and incubated for 4 h at 37 ºC. After this, the mixture was centrifuged at 12,000 g for 1 min and 300 µL of supernatant transferred to a new tube. The DNA was extracted with 6 M NaI and chloroform/isoamyl alcohol. After precipitation with ethanol, the pellet was dried and resuspended in 50 µL TE (10 mM Tris pH 7.4; 1 mM EDTA pH 8.0) containing RNase A. The quantity and quality of the DNA was checked by spectrophotometry.

2.3 Plasmids

Positive controls were prepared by cloning TTSuV1 and TTSuV2 amplicons into a pCR2.1 vector (Invitrogen) according to the manufacturer methods. Such recombinant plasmids were named “positive control 1” (C+1) and “positive control 2” (C+2). The concentration of plasmid DNA was evaluated by spectrophotometry and diluted in a tenfold series to determine the analytic sensitivity of the assay.

To detect false negative reactions, an internal control was constructed as follows: A PCR with primers designed to amplify TTSuV1 (see below) was performed on DNA extracted from the continuous swine kidney cell lineage SK6 under low stringency conditions (annealing temperature: 40 °C). Another PCR designed to amplify TTSuV2 was performed at low stringency conditions on DNA extracted from cells of the porcine kidney cell lineage (PK15; previously cloned to eliminate endogenous PCV1). Non-specific bands from different sizes were produced in both reactions. A 293 bp amplicon originated from the PCR with TTSuV1 primers was selected and cloned into plasmid vector pCR2.1. This plasmid was named “internal control 1” (IC1). Another 441 bp-long amplicon generated in the TTSuV2-primed reaction was cloned as above and the resulting plasmid named “internal control 2” (IC2). All PCRs performed in search for TTSuV genomes included IC controls.

2.4 PCR

Primer pairs were designed to amplify the 5’ non-coding regions of TTSuV1 and TTSuV2 genomes. For TTSuV1 PCR, the forward primer was: 5’-GGG TTC AGG AGG CTC AAT TTG G-3’, and the reverse primer: 5’-CCC AGT CGC TAG ACA GTT CTG T-3’. These were aimed to give rise to an amplicon of 349 bp. The TTSuV1 PCR was performed as follows: in 25 μL volumes, reactions contained 2 μL of DNA

(100 ng), 5 pmol of forward TTSuV1primer, 5 pmol of the reverse TTSuV1 primer, 0.8 mM of each dNTP, 1.5 mM $MgCl_2$, 5 % dimethyl sulfoxide (DMSO), 1U Taq DNA polymerase (Invitrogen) and 10% PCR buffer. To each reaction, 100 molecules of IC1 were added. The amplification was performed in an Eppendorf Mastercycler PCR reactor. Cycling was initiated by heating for 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 62 °C, 30 s at 72 °C and a final extension period of 5 min at 72 °C.

For TTSuV2 PCR, primers were: forward: 5'-CCA CAA AGT ATT ACA GGA AAC TGC -3', and reverse: 5'- AAC CAT TGT ATG ACC GGA GCT TT -3'. These were aimed to amplify 572 bp along the TTSuV2 genome. The TTSuV2 PCR was performed as follows: in 25 μL, the reactions were set up with 2 μL of DNA (100 ng), 5 pmol of forward primer, 5 pmol of reverse primer, 0.8 mM of each dNTP, 1,5 mM $MgCl_2$, 2,5 % dimethyl sulfoxide, 1U Phusion Hot Start DNA polymerase (Finnzymes) and 10 % HF PCR buffer. To each reaction, 50 molecules of IC2 were added. The amplification was performed in an Eppendorf Mastercycler starting with 3 min at 98°C, followed by 35 cycles of 30 s at 98°C, 30 s at 59°C, 30 s at 72°C and a final extension step of 5 min at 72 °C. The negative controls used in both TTSuV PCRs consisted of sterile water instead of extracted DNA and were included after every ten samples. The amplified products were run in a 1.5 % agarose gel stained with ethidium bromide and photographed on a UV transilluminator.

2.5 Sequencing and Phylogenetic analysis

Eighteen selected amplification products with the expected size (8 from TTSuV1 and 10 from TTSuV2) were excised from the agarose gels and purified with GFX PCR DNA gel band purification kit (GE Healthcare). Sequencing was performed on both strands of the obtained products with the PCR primers described above, employing the

Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems), in the ABI Prism 3100 genetic analyzer (Applied Biosystems). Sequences were aligned with the program Clustal W enclosed in the MEGA4 software package. Sequences with accession numbers NC_014070, AY_823990, HM_633251 and HM_633245 were used as references for TTSuV1; and the sequences GU_456385, HM633239, HM_633214 and HM_633238 was used as reference for TTSuV2. The nucleotide sequences obtained in this study were deposited at GenBank. Phylogenetic analyses of nucleotide sequences were carried out using the Neighbour-Joining (NJ) method within the MEGA4 software package, based on Kimura two-parameter distance estimation method. Bootstrap resampling was performed for each analysis (1000 replications).

2.6 Statistical analysis

Tissues samples were grouped in two different groups: tissues from PMWS-affected pigs and non-PMWS-affected pigs. The frequency of detection of TTSuV1 and TTSuV2 genomes between both groups and the distribution of both TTSuV genogroups among tissues were evaluated statistically using Chi-Square and Fisher's Exact Test. Statistical analysis was performed with the SPSS software v.16. The level of significance was set to $p \leq 0.05$.

3. Results

3.1. Detection of TTSuV DNA

The sensitivity of the TTSuV1 and TTSuV2 PCRs were determined by titrating tenfold dilutions of known quantities of plasmids C+1 or C+2. Both PCRs were able to detect a minimum of 100 molecules of TTSuV1 and TTSuV2 per reaction (Figs. 1A and 1B).

The results of the frequencies of detection of TTSuV1 DNA in PMWS-affected pigs was significantly lower than non-PMWS-affected pigs; while 37/76 (48.7 %) of the PMWS-affected pigs carry TTSuV1 DNA, all (11/11) of the non-diseased pigs were found to bear TTSuV1 genomes (Table 1). Such difference was highly statistically significant ($p \leq 0.001$).

The search for TTSuV2 DNA revealed that most pigs (72/76; 94.7 %) of the PMWS-affected pigs bore viral genomes of this type. However, TTSuV2 genomes were also detected in all healthy, non-PMWS-affected pigs (11/11). Statistically, such difference was not significant ($p=1.000$). Co-infections (TTSuV1 and TTSuV2) were identified in 36/76 (47.3 %) of the PMWS-affected pigs, as well as in all eleven non-PMWS-affected pigs.

The frequency of detection of viral DNA in different tissues of pigs bearing TTSuV1 and TTSuV2 genomes was also examined. No significant differences ($p=0.970$) were found in the distribution of the two virus genomes in any of the tissues, regardless of whether these were from PMWS-affected pigs or from the animals on the healthy control group (Table 2).

3.2. Phylogenetic analysis

The sequences of the amplified 5' UTR region for eight TTSuV1 samples and ten TTSuV2 samples were determined. The parcial lengths of the TTSuV1 and TTSuV2 genome alignments revealed levels of genomic identity ranging from 90.5 % to 99.7 % among TTSuV1 genomes, from 85.4% to 98.2% among TTSuV2 genomes and from 48.8 % to 54.6 % between TTSuV1/TTSuV2 genomes (data not shown). The phylogenetic tree (Fig. 2) inferred by Neighboring Joining method showed the grouping of viruses according to their different species (TTSuV1 and TTSuV2).

4. Discussion

Torque teno sus virus species are highly prevalent worldwide. Possible associations between TTSuVs and the occurrence of PMWS in pigs are still subject of investigation. In the present study, the results obtained reveal that the frequency of detection of TTSuV1 genomes was significantly higher in healthy animals (100 %) than in PMWS-affected pigs (48.7 %). These findings show an apparent inverse correlation between the detection of TTSuV1 genomes and the occurrence of PMWS in pigs. In a previous study, a significantly higher prevalence of TTSuV2 DNA in PMWS-affected pigs than in healthy ones, whereas no association (either positive or negative) between the detection of TTSuV1 and PMWS was detected (Kekarainen et al. 2006). Other authors reported that TTSuV1 would facilitate PCV2-induced PMWS in gnotobiotic swine (Ellis et al. 2008). The same group also reported successful transmission of TTSuV1 to gnotobiotic pigs via pooled leukocyte-enriched plasma (Krakowka and Ellis, 2008). Our findings, however, suggest an inverse correlation between the presence of TTSuV1 genomes in tissues and the occurrence of PMWS in pigs. As in the study here reported the pigs were conventionally reared, these findings must be compared to those of others with caution, since it is likely that more drastic responses could be expected from gnotobiotic animals subjected to experimental infections.

Previous exposure to the virus might also generate a pre-existing immune response to the virus and may also play some role in the virus/host relationship. Unfortunately, we have not been able to evaluate the serological responses to TTSuV1 and TTSUV2 infections, since at the time of writing of this report, there are no serologic tests available to detect TTSuV antibodies. It is quite possible that the apparent paradoxical observations reported on the previous studies (Ellis et al, 2008; Krakowka and Ellis, 2008) and the results obtained here might be related to the immune response

of animals to these agents; these will certainly become more transparent when such tests are made available. In relation to the detection of TTSuV2 DNA, in the present study, genomes of this type were found to frequencies that did not differ significantly in PMWS-affected and non-PMWS-affected pigs.

When examining TTSuV1 and TTSuV2 tissue distribution, the distribution of genomes of both species in tissues did not reveal any particular target organ. The viruses were evenly distributed in all organs examined here (lungs, kidneys, livers, spleens and lymph nodes). Similar findings regarding tissue distribution had also been reported previously in healthy, older animals (Aramouni et al. 2010) and then more recently detected in fetuses (Martínez-Guinó et al, 2010). Thus, it seems that TTSuV1 and TTSuV2 do not possess a particular target organ, at least not among the organs evaluated in the present study, where viral genomes could be detected throughout.

When compared to the findings reported here, these apparently conflicting findings are suggestive that the status of TTSuV infection in pigs may be vary irrespective of the PMWS status of the infected animal. In present days, detection of genomes regardless of its relationship with disease has become a common practice. This might just be the case with TTSuVs. Therefore, the significance of the identification of such genomes in the pathogenesis of disease - if any - will remain unclear until other approaches are used to further examine the question. In addition, as at stated above, the identification of immune responses to such agents might shed some light on the apparently incongruent results so far obtained in pathological and epidemiological studies.

In summary, in the present study, TTSuV1 genomes were detected significantly more often in healthy pigs than in PMWS-affected animals, suggesting an apparently inverse relationship between the presence of TTSuV1 genomes and the occurrence of

PMWS. Regarding TTSuV2, viral genomes were found equally distributed – and to high levels – in the PMWS- affected as well as in the control pig population, with no statistically significant differences between those. Future studies shall be conducted to evaluate more deeply the interaction between TTSuVs and its host species.

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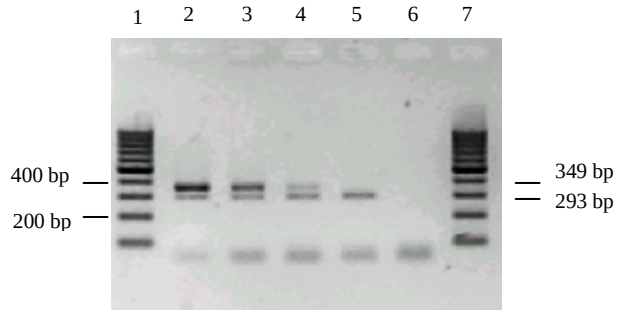
Table 1. Frequency of detection of TTSuV1 and TTSuV2 in tissue from PMWS-affected and non-affected pigs**.** Animals were considered positive when at least one of tissues was found positive.

	TTSuV1	**TTSuV2**	**TTSuV1/ TTSuV2**
Non-PMWS-affected pigs (n=11)	100 (11/11)	100 (11/11)	100 (11/11)
PMWS-affected pigs (n=76)	48.7 (37 /76)	94.7 (72 /76)	47.3 (36/76)
Total (n=87)	55.17 (48/87)	93.1 (81/87)	54.0 (47/87)

Table 2. Prevalence of TTSuV1 and TTSuV2 in five different tissue samples from PMWS-affected and non-affected pigs**.**

Groups		**Organs**				
		Lungs	**Livers**	**Kidneys**	**Spleens**	**Lymph nodes**
Healthy pigs	TTSuV1	100% (11/11)	100% (11/11)	90.9% (10/11)	100% (11/11)	100% (11/11)
	TTSuV2	81.8% (9/11)	81.8% (9/11)	81.8% (9/11)	100% (11/11)	90.9% (10/11)
PMWS-affected pigs	TTSuV1	37.7% (26/69)	41.9% (31/74)	37.8% (28/74)	38.5% (27/70)	30.4% (21/69)
	TTSuV2	76.8% (53/69)	81.1% (60/74)	59.4% (44/74)	77.1% (54/70)	68.1% (47/69)

A)



B)

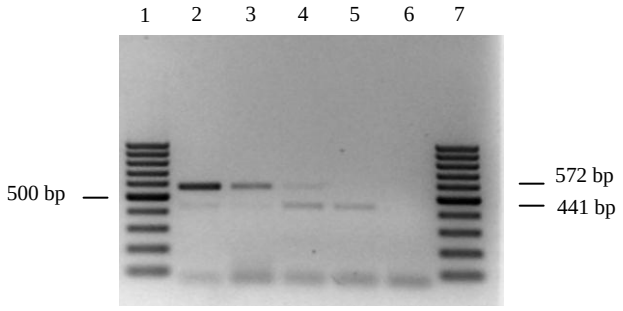


Fig. 1. PCR assay sensitivity. (A) Sensitivity of TTSuV1 PCR. 100 molecules of IC1 were added to each reaction. The total number of positive molecules added to the reaction was 10000 (lane 2), 1000 (lane 3), 100 (lane 4) and 10 (lane 5). Lane 6: negative control; lane 1 and 7: 100 bp DNA Ladder (Fermentas). (B) Sensitivity of TTSuV2 PCR. 50 molecules of IC2 were added to each reaction. The total number of positive molecules added to the reaction was 10000 (lane 2), 1000 (lane 3), 100 (lane 4) and 10 (lane 5). Lane 6: negative control; lane 1 and 7: 100 bp DNA Ladder (Fermentas).

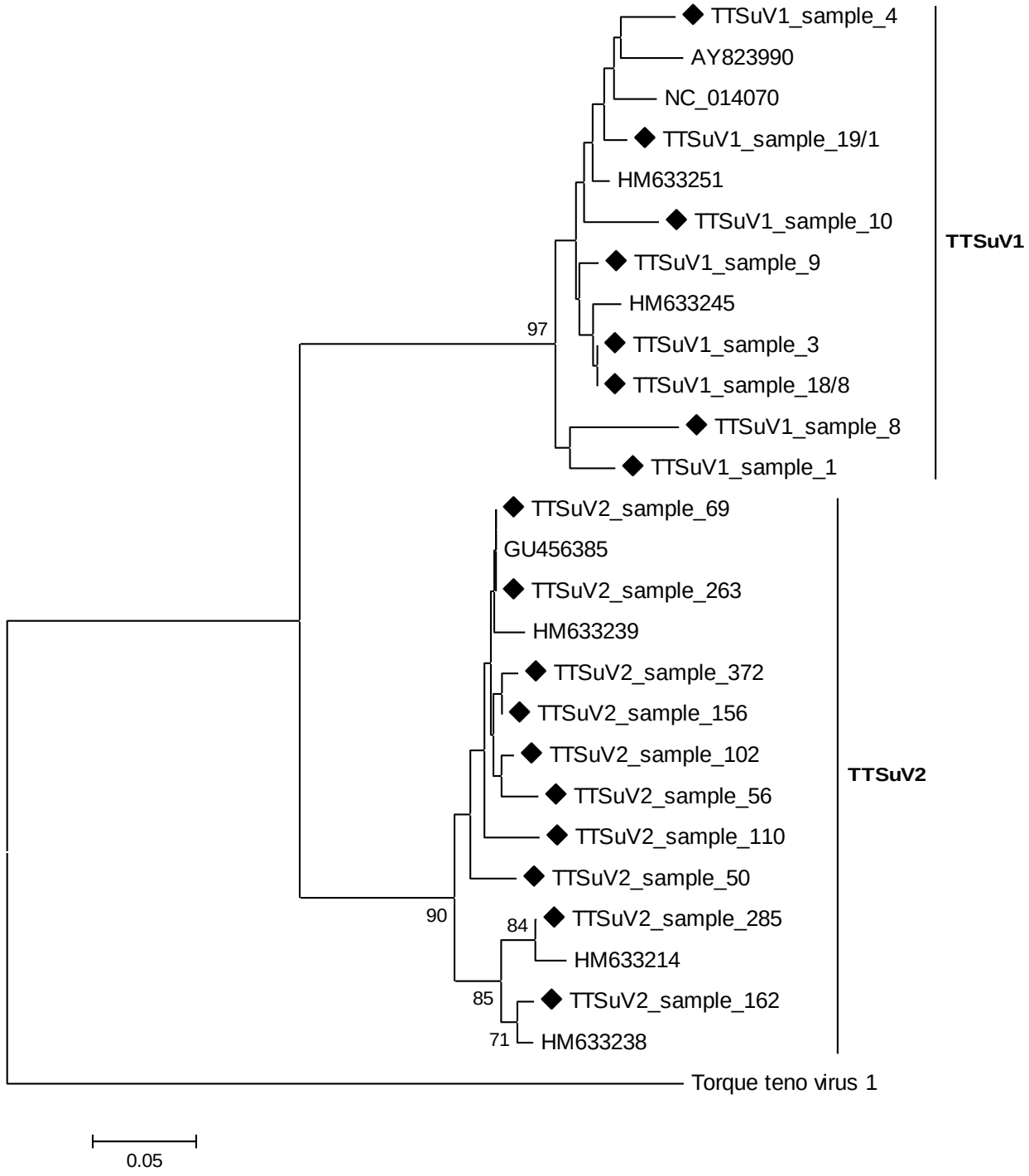


Fig. 2. Neighbour-joining phylogenetic tree based on the nucleotide sequences of untranslated region of TTSuV genomes. Sequences with accession numbers NC_014070, AY_823990, HM_633251 and HM_633245 were used as references for TTSuV1; and the sequences GU_456385, HM633239, HM_633214 and HM_633238 was used as reference for TTSuV2. Bootstrap values are indicated above major branches.

6 CAPÍTULO 3

6.1 *Torque teno sus virus 1* (TTSuV1) and *2* (TTSuV2) viral loads and its association with postweaning multisystemic wasting syndrome (PMWS).

Trabalho a ser submetido na forma de artigo científico.

Torque teno sus virus 1 **(TTSuV1) and** ***2*** **(TTSuV2) viral loads and its association with postweaning multisystemic wasting syndrome (PMWS).**

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Abstract

Torque teno viruses (TTVs), are small, non-enveloped agents with a circular single-stranded DNA genome. In domestic pigs, two distinct species have been reported: Torque teno sus virus 1 (TTSuV1) and 2 (TTSuV2). Associations of such viruses with the occurrence of postweaning multisystemic wasting syndrome (PMWS) have been reported with controversial results. To further examine the role for TTSuVs in PMWS, a SYBR Green-based quantitative PCR (qPCR) was designed to detect and quantify TTSuV1 and TTSuV2 genomes in sera of 49 PMWS-affected and 132 healthy pigs (including 50 piglets, 50 adults and 32 SPF animals). The frequency of detection of TTSuV1 and TTSuV2 was high (97.8%) and not significant different in all groups (PMWS-affected and unaffected pigs). Likewise, no significant differences were detected between the prevalence of TTSuV1 and TTSuV2 DNA and the ages of the sampled animals. The mean of TTSuV1 viral load was significantly lower than TTSuV2 ($p \leq 0.05$) in swine serum samples in all age groups. Most of animals with TTSuV1 DNA had genome loads between 10^3 and 10^5 DNA copies/mL, whereas TTSuV2 DNA-bearing animals had genome loads higher than 10^5 DNA copies/mL. However, although TTSuV2 could be detected in higher copy numbers than TTSuV1 in the different groups examined, neither TTSuV1 nor TTSuV2 DNA had any relation to the occurrence of PMWS. These findings indicate that TTSuV infections are highly prevalent in the sampled herds and are probably widespread among swine; moreover, these bear no association with the occurrence of PMWS.

Keywords: *Torque teno sus virus* (TTSuV); Anellovirus; postweaning multisystemic wasting syndrome (PMWS); quantitative PCR.

1. Introduction

Torque teno viruses (TTVs) are small, non-enveloped agents with circular single-stranded DNA genomes. TTVs are currently classified in the family Anelloviridae (ICTV-2009). TTV was discovered in a human Japanese patient with post-transfusion hepatitis of unknown aetiology in 1997 (Nishizawa et al., 1997). Since then, a large number of TTVs infecting humans, swine, poultry, cattle, sheep, cats and dogs have been identified (Kekarainen and Segalés et al., 2009; Okamoto et al., 2002; Okamoto, 2009).

In domestic pigs and wild boars, two genetically distinct TTV species have been identified, named Torque teno sus virus 1 (TTSuV1) and 2 (TTSuV2) (Niel et al., 2005; Martínez et al., 2006). Taxonomically, such viruses are currently grouped in the genus Iotatorquevirus (ICTV-2009). The first description of TTSuV was published by Leary et al. (1999), although evidence for TTSuV infections in Spanish pig farms (Segalés et al., 2009) and in cell cultures date as early as 1985, (Teixeira et al., 2011) though so far no TTSuV have been deliberately multiplied or visualized in vitro.

The role of TTSuV in swine disease is under investigation. It has been suggested that TTSuV infections could enhance the pathogenic effects of other microorganisms, such as porcine circovirus type 2 (PCV2). The latter is considered essential, though not sufficient, to induce postweaning multisystemic wasting syndrome (PMWS), presently the most economically important disease amongst a number of different conditions referred to as “porcine circovirus associated diseases” (PCVAD). One of such studies reported that combined infections with TTSuV1 and porcine reproductive and respiratory syndrome virus (PRRSV) were associated to porcine dermatitis and nephropathy syndrome (PDNS), another PCVAD (Krakowka et al., 2008). In addition, experimental infections of gnotobiotic pigs with TTSuV1 and PCV2 were shown to

trigger PMWS (Ellis et al., 2008). Others reported that the prevalence of TTSuV2 was higher in PMWS-affected pigs than in healthy ones (Kekarainen et al., 2006). Nontheless, others found no significant differences in TTSuV1 and TTSuV2 genome loads in PCVAD-affected pigs and PCV2-negative pigs (Lee et al., 2010).

In view of such controversial results, this study was conducted to examine potential relations between the detection of TTSuVs and the occurrence of PMWS. In addition associations between viral loads and the occurrence of disease or age-related associations were also examined.

2. Materials and methods

2.1. Samples

Serum samples were collected in pig farms from the state of Rio Grande do Sul, Brazil. Fourty-nine sera from PMWS-affected pigs (1-4 months old), plus 50 serum samples from healthy, young pigs (1-2 months old) as well as from 50 healthy, adult pigs (6-19 months old). Additionally, 32 serum samples from SPF pigs (obtained from a research-directed, high biosecurity pig farm unit) were used in the present study. PMWS was diagnosed with basis on clinical signs, histopathological lymphoid lesions and identification of PCV2 in tissues by PCR as recommended (Segalés et al., 2005).

2.2. DNA extraction

DNA was extracted from 500 μL of serum with phenol-chloroform (Sambrook and Russel, 2001). The DNA was precipitated with ethanol; the pellet dried and resuspended in 50 μL of TE buffer containing 20 mg/mL RNase A. The quantity and quality of the extracts was analyzed with the aid of a spectrophotometer (Nanodrop® 1000).

2.3. Primers

The primers used for the SYBR Green real-time qPCR were designed previously by our group (Teixeira et al., 2011). The expected amplicon sizes were 107 bp for TSuV1 and 103 bp for TTSuV2.

2.4. Standards curves to TTSuV1 and TTSuV2 qPCR

TTSuV1 and TTSuV2 were amplified in a conventional PCR using the same primers used for the qPCR. The products were purified with a commercial kit (GFXTM Purification Kit; Amersham Biosciences) and cloned into the pCR2.1 vector (Invitrogen). Two recombinant plasmids (pCR2.1+TTSuV1 and pCR2.1+TTSuV2) were quantified with the aid of a fluorimeter (Qubit, Invitrogen) and used as standards. Standards dilutions of TTSuV were prepared in tenfold serial dilutions (109 to 10-1 molecules/μL) and tested by qPCR to plot the standard curves for TTSuV1 and TTSuV2, respectively.

2.5. Quantitative PCR (qPCR)

Reactions were carried out in 48-well plates. Each sample and standards were run in triplicate and a negative control was added between each eight wells, using sterile bi-distilled water instead of sample DNA. After optimization, the SYBR Green based real-time qPCR was performed in a 12.5 μL reaction mixture containing 3 μL of extracted DNA (diluted 1:4) or standard plasmid (to generate the standard curve), 6.25 μL of 2X Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 250 nM each of forward and reverse primers. Amplification and quantification were performed using StepOne™ Real-Time PCR System (Life Technologies) under the following conditions: 2 min at 50 °C, 2 min at 95 °C and 40 cycles of 15 s at 95 °C, 30 s at 60 °C. A dissociation curve was performed after amplification by a gradual rise in temperature

(0.3 °C) from 60 to 95 °C. The number of copies of viral DNA was determined by comparison with a standard curve. In order to simplify the results, viral loads were grouped in three categories based on viral load: less than 103, between 103 and 105 and higher than 105 DNA genomes copies/mL.

2.6. Statistical analysis

Descriptive statistics, a nonparametric Kruskal-Wallis test (with Dunn's multiple comparison as posttest) and $\chi 2$ were performed using the GraphPad Prism 5 software. Differences were considered significant when $P \leq 0.05$.

3. Results

The standard curve obtained with the TTSuV1 control (pCR2.1+TTSuV1) showed an efficiency of ~99.4%. The detection limit of the assay was 10 copies of plasmid DNA per reaction (Fig. 1). For TTSuV2 qPCR , the standard curve with the TTSuV2 control (pCR2.1+TTSuV2) showed an efficiency of ~99.8%, with a detection limit of 100 copies of plasmid DNA per reaction (Fig. 2). The regression coefficients (R2) were ≥0.999 on both curves. The observed temperature of dissociation for TTSuV1 and TTSuV2 qPCR were 78.75 °C and 77.41 °C, respectively. Negative controls did not give rise to any amplification product, as revealed by the absence of spurious peaks on the dissociation curve on both reactions.

The frequencies of detection of TTSuV1 and TTSuV2 in the four groups of pigs under study are summarized in Figure 3. Both TTSuV species were detected to high frequencies in sampled animals in all groups. Only 4 serum samples were negative for TTSuV genomes. TTSuV1 and TTSuV2 DNA was detected with no significant differences in PMWS-affected pigs and in healthy young, healthy old and SPF pigs,

indicating no association between TTSuV1 and TTSuV2 DNA and the occurrence of PMWS (Fig. 3).

The mean TTSuV1 and TTSuV2 genomes loads were quantified and compared in all of the groups of pigs under study. No significant differences were observed between TTSuV1 and TTSuV2genome loads in any of the different groups examined (Fig. 4). The mean TTSuV1 genome load in PMWS-affected pigs was 2.33x106 copies/mL, while in young, healthy animals (1-2 months old) the mean genome load was 1.88x105 copies/mL. In adult, healthy animals (6-19 mouths old) the mean genome load was 7.73x105 copies/mL. The mean TTSuV2 loads in PMWS-affected pigs was 2.84x106 copies/mL while in young, healthy animals (1-2 months old) was 7.64x107 copies/mL and in adult, healthy animals (6-19 mouths old) was 5.42x107 copies/mL. TTSuV1 and TTSuV2 were also detected in specific pathogen free animals. These animals had high levels of both, TTSuV1 and TTSuV2 (97.8 %), with high viral loads (6.52x104 for TTSuV1 and 1.00x106 for TTSuV2), not differing significantly from other animals groups (Fig. 4).

TTSuV2 mean genome loads were significantly higher than the TTSuV1 loads in all of the groups of animals under study (PMWS-affected; healthy young; healthy adult and SPF pigs). TTSuV1 and TTSuV2 viral loads did not differ when animals were infected with only one species of TTSuV (8.56x105 and 2.99x107, respectively) or co-infected (7.24 x 105 and 2.85 x 107, respectively) (Fig. 5).

Animals were grouped in one of viral load based categories: less than 103, between 103 and 105 and higher than 105 DNA genomes copies/mL of serum (Table 1). Most of the animals with TTSuV1 DNA had viral loads between 103 to 105 DNA copies/mL. In contrast, in most TTSuV2-bearing animals, viral loads were usually higher than 105 DNA copies/mL.

4. Discussion

TTSuV infections in pigs are widespread. Currently, there is debate on its disease association, especially with porcine circovirus associated disease (PCVAD). The increased prevalence of TTSuV in PMWS-affected pigs has raised several intriguing questions on the contribution of TTSuV to PCV2 pathogenesis (Kekarainen et al., 2006; Taira et al., 2009). In this study, a highly sensitive, specific and efficient qPCR to TTSuV1 and TTSuv2 detection and quantification were developed. The assays were applied to investigate possible associations between the presence of TTSuV DNA and the occurrence of PMWS. It was observed that the frequency of detection of TTSuV1 and TTSuV2 was high (97.8%) in all groups (PMWS-affected and unaffected pigs), regardless of their PMWS status, with no statistically significant differences between detection of TTSuV1 and/or TTSuV2 and the occurrence of PMWS. Others reported the reproduction of typical PMWS signs and mortality by infecting pigs with TTSuV1multiplied in vivo followed by a superinfection with PCV2 (Ellis et al., 2008). In addition, TTSuV2 was found more frequent in pigs displaying clinical signs of PMWS (Kekarainen et al., 2006).

As the frequency of detection, the mean viral load of TTSuv1 and TTSuV2 were no significant statistically among animals PMWS-affected and healthy pig at equivalent age and adult healthy pigs. However, the mean of TTSuV1 viral load was significantly lower than TTSuV2 ($p \leq 0.05$) in swine serum samples. These results do not support any role for TTSuV1 and TTSuV2 in the occurrence of PMWS in this conventional pigs population. Similar results were found by other authors, which found no association between TTSuVs and PMWS (Lee et al., 2010; Blomström et al., 2010). A possible reason for these results can be related with the sensitivity of the technique used in both studies and the different source and number of animals used (Aramouni et

al., 2011). Recently, all studies of TTSuV1 and TTSuv2 assessing the frequency of detection of infections based on the use of a qualitative PCR (Bigarré et al., 2005; Kekarainen et al., 2006; Martelli et al., 2006; Martínez-Guinó et al., 2009; Martínez-Guinó et al., 2010; Pozzuto et al., 2009; Segalés et al., 2009; Sibila et al., 2009). However, since recent studies have been described different qPCR techniques for the quantification of TTSuV1 and TTSuV2 (Brassard et al., 2010; Gallei et al., 2010; Lee et al., 2010; Nieto et al., 2011), obtained results may indicate a more accurate estimation of viral prevalence.

Interestingly, even in the SPF pig population sampled, TTSuV1 and TTSuV2 genomes were detected to high levels and at high prevalence (96.9 % were infected at least with one species of TTSuV). It may be necessary to test SPF pigs for the presence of TTSuV1 and TTSuV2 DNA to raises a concern regarding the biosecurity and biosafety of TTSuV. Future studies are required to address this aspect.

In summary, no differences in the frequency of detection and viral load were observed in pigs suffering of PMWS and healthy at equivalent age and adults pigs. These findings suggest no association between the presence of TTSuVs and the occurrence of PMWS.

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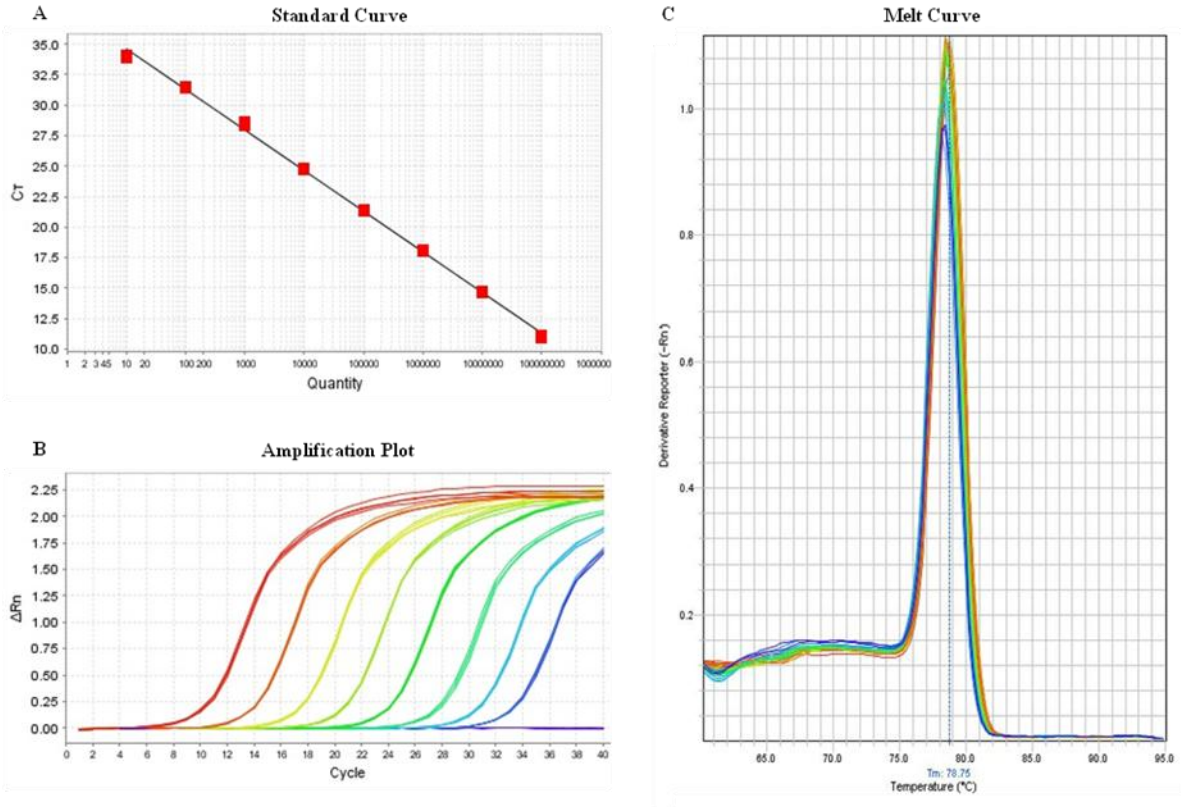

Figure 1. Standard curve to assess reaction optimization using a 10-fold dilution of a quantified TTSuV1 template and amplified by StepOne™ Real-Time PCR System (Applied Biosystems™). Each dilution was assayed in triplicate. (A) Standard curve with the threshold cycle (Ct) plotted against the log of the starting quantity of template for each dilution. (B) Dissociation curves of the SYBR Green real-time PCR products of TTSuV1 plasmids samples. (C) Amplification curves of the dilution series.

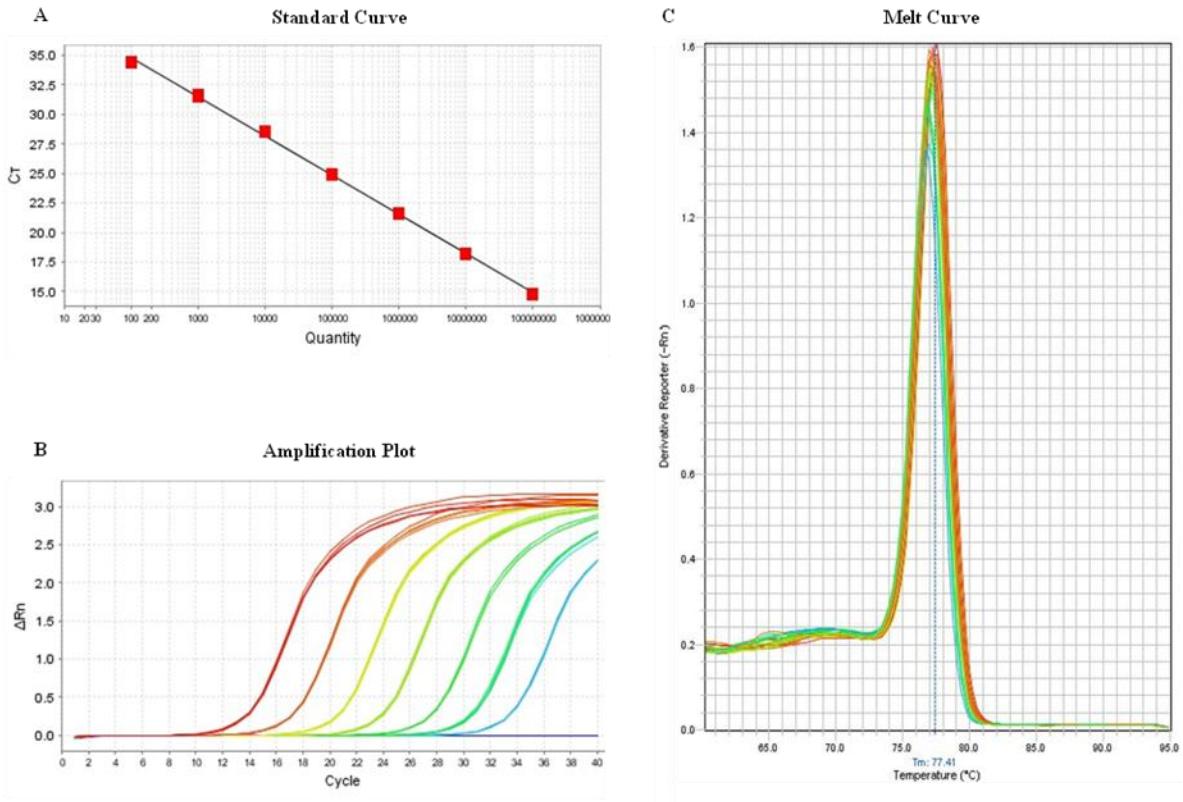

Figure 2. Standard curve to assess reaction optimization using a 10-fold dilution of a quantified TTSuV2 template and amplified by StepOne™ Real-Time PCR System (Applied Biosystems™). Each dilution was assayed in triplicate. (A) Standard curve with the threshold cycle (Ct) plotted against the log of the starting quantity of template for each dilution. (B) Dissociation curves of the SYBR Green real-time PCR products of TTSuV1 plasmids samples. (C) Amplification curves of the dilution series.

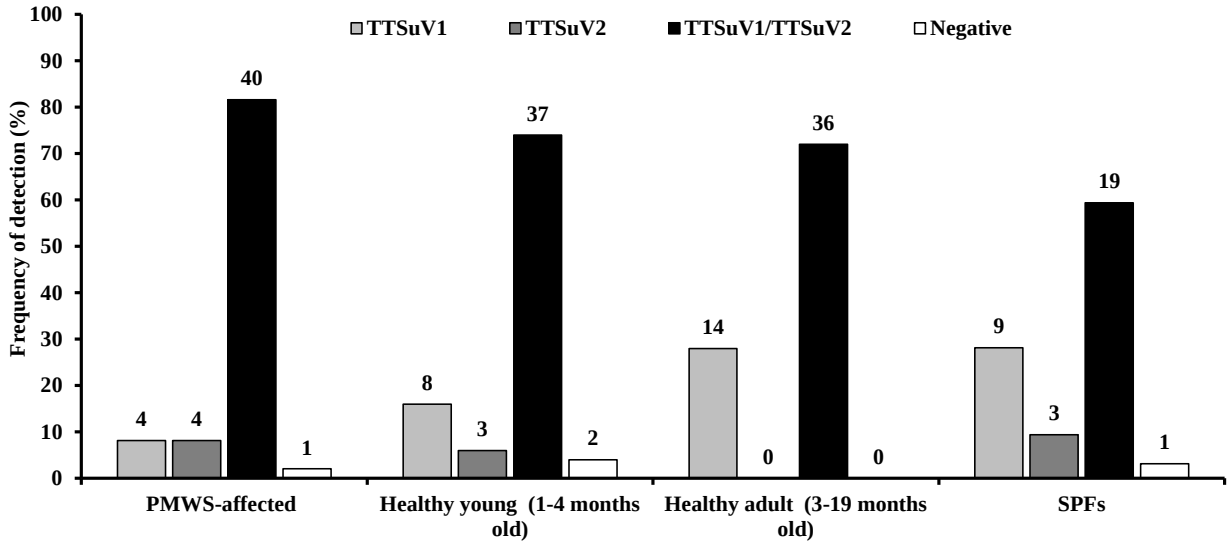

Figure 3. Frequency of detection of TTSuV1 and TTSuV2 in serum samples from PMWS-affected pigs, healthy young and adult pigs and in specific-pathogen-free (SPF) animals.

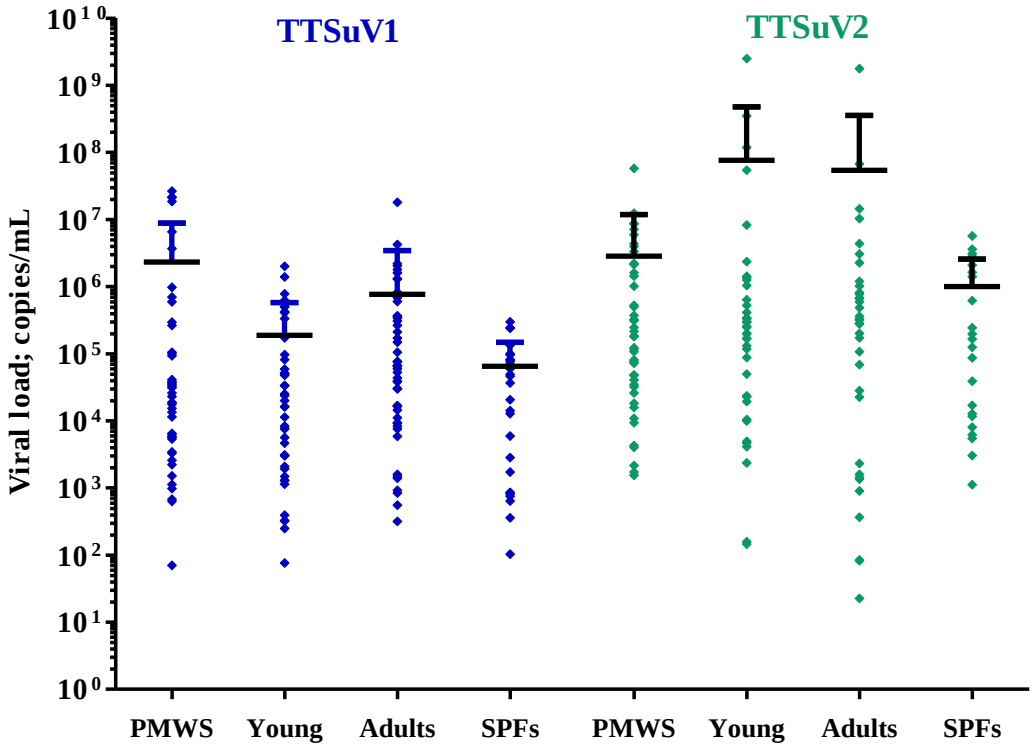

Figure 4. Dot plot and median serum viral load of TTSuV1 and TTSuV2 in PMWS-affected pigs, healthy young and adult pigs and in specific-pathogen-free (SPF) animals. Bars represent standard deviation.

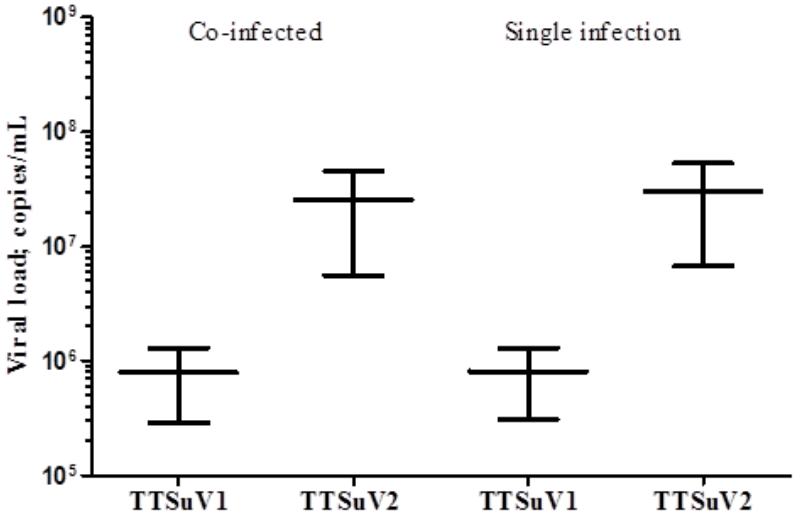

Figure 5. Median serum viral load of TTSuV1 and TTSuV2 in single infection and in co-infetion. Bars represent standard deviation.

Table 1. Distribution of viral loads for both TTSuV species among the different tested groups. Percentage of qPCR positive pigs is shown in parentheses.

Total (molecules/ml of serum)	PMWS		Healthy young		Healthy adult		SPF	
	TTSuV1	TTSuV2	TTSuV1	TTSuV2	TTSuV1	TTSuV2	TTSuV1	TTSuV2
	49	49	50	50	49	49	32	32
$\leq 10^3$	9 (18.4)	5 (10.2)	10 (20)	12 (24)	4 (8.2)	18 (36.7)	11 (34.4)	11 (34.4)
$>10^3$ -$<10^5$	27 (55.1)	17 (34.7)	28 (56)	13 (26)	25 (51.0)	7 (14.3)	15 (46.9)	10 (31.3)
$>10^5$	13 (26.5)	27 (55.1)	12 (24)	25 (50)	20 (40.8)	24 (49.0)	6 (18.8)	11 (34.4)

7 CONSIDERAÇÕES FINAIS

Neste estudo foram apresentados aspectos que visaram contribuir para um melhor entendimento da biologia dos TTSuVs e verificar se estes agentes apresentam algum tipo de associação com a síndrome multissistêmica do definhamento dos suínos (SMDS).

No primeiro estudo, uma duplex PCR foi desenvolvida para a detecção de TTSuV1 e TTSuV2 em diversas linhagens celulares de diferentes espécies, em soros utilizados como suplemento para o meio de cultivo dessas células e em tripsina utilizadas para individualizar as células. Este estudo foi realizado com o intuito de averiguar a existência de contaminação prévia de TTSuVs em linhagens celulares, uma vez que havia o intuito de isolar estes vírus. Inicialmente, como os TTVs são supostamente espécie-específicos, acreditava-se que os cultivos celulares pudessem ter TTVs específicos de cada tecido de origem. No entanto, não foi possível projetar primers capazes de detectar todos os TTVs descritos, ou pelo menos, a maioria deles, devido à grande diversidade genética existente entre estes vírus. Por esse motivo, os primers foram projetados para detectar apenas os TTSuVs de origem suína que eram os vírus de interesse para isolamento.

Genomas de TTSuV1 e TTSuV2 foram detectados em células de linhagem de origem suína e não suína. Interesantemente, uma linhagem celular que foi ampolada em 1985 e mantida desde então em nitrogênio líquido também estava contaminada com TTSuV1, mostrando a presença do vírus antes de ser descrito pela primeira vez em 1999. Uma vez que a contaminação foi detectada em células de cultivo, a identificação de uma suposta fonte de contaminação foi necessária. Foi verificado que um dos lotes de tripsina estava contaminado com duas variantes distintas do TTSuV1, bem como com o TTSuV2. Os outros lotes de tripsina testados foram negativos para a presença de TTSuV assim como os soros, o que era esperado, uma vez que todos os soros eram de origem não suína.

A identificação de genomas de TTSuV em células tratadas com tripsina contaminada não garante que ocorreu a multiplicação destes vírus em tais células, principalmente em células de origem não suína. Isso é reforçado pelo fato de, até o momento, não existirem cultivos celulares eficazes para a multiplicação destes anelovírus. Assim, pode-se argumentar que o genoma do vírus transportado pela tripsina residual poderia ter sido uma das fontes de contaminação dos TTSuVs em pelo menos

algumas das células. No entanto, as análises revelaram que as distâncias filogenéticas encontrada entre alguns fragmentos de TTSuV2 de células de suínos (SK6 e PK15-b) sugerem que outras fontes de contaminação devem existir. É possível que esta contaminação tenha ocorrido em algum momento no passado - talvez de um outro lote de tripsina contaminado anteriormente – ou ainda dos animais que serviram de fonte dos tecidos dos quais as células foram originalmente preparados. Em síntese, este estudo possibilitou relatar, pela primeira vez, a presença de TTSuVs como contaminante celular, assim como, questionar quais seriam as possíveis fontes da ocorrência de tal contaminação.

No segundo estudo, duas PCRs específicas para detectar genomas de TTSuV1 e TTSuV2 foram utilizadas em diferentes tecidos de suínos, buscando verificar a existência de algum tipo de associação entre estes e a ocorrência da SMDS. Para este trabalho foram projetados dois pares de primers para desenvolvimento de uma multiplex PCR. No entanto, isso não foi possível devido a baixa sensibilidade da técnica para a detecção de TTSuV2. Dessa forma, para aumentar a sensibilidade desta PCR foi necessário utilizar uma polimerase de alta fidelidade (Phusion Hot Start DNA polymerase) e por esse motivo foram padronizadas duas PCRs independentes.

Os resultados obtidos mostraram que a frequência de detecção de genomas de TTSuV1 foi significativamente maior em animais saudáveis (100%) do que em suínos com a SMDS (48,7%). Estes resultados revelam uma aparente correlação inversa entre a presença do genoma de TTSuV1 nos tecidos e a ocorrência da SMDS em suínos. Estes achados corroboram com um trabalho prévio de nosso grupo, onde esta mesma associação foi detectada (TEIXEIRA, 2008). Tal achado sugere que este agente pode estar exercendo algum papel inibidor sobre a multiplicação do PCV2 nos hospedeiros, o que poderia estar interferindo no desenvolvimento da SMDS. Em relação ao TTSuV2 nenhuma diferença estatisticamente significativa foi observada entre animais com e sem a SMDS. A distribuição do genoma de TTSuV1 e TTSuV2 nos diferentes tecidos examinados não revelou um órgão alvo específico.

No terceiro trabalho, duas PCRs quantitativas (qPCR) de alta sensibilidade e especificidade foram desenvolvidas buscando avaliar a carga viral de TTSuV1 e TTSuV2 em soro de suínos com e sem a SMDS. Apesar de alguns autores encontrarem diferença significativa entre a infecção por TTSuV e o desenvolvimento da SMDS. Uma elevada freqüência de detecção de TTSuV1 e TTSuV2 foi observada nos suínos com SMDS e em suínos saudáveis (jovens e adultos), não demonstrando diferenças

estatísticas entre os grupos de animais com SMDS e saudáveis de ambas as idades. O TTSuV1 inoculado em suínos antes do PCV2 foi capaz de reproduzir os sintomas típicos da SMDS (ELLIS et al., 2008). Outros estudos reportaram que o TTSuV2 foi encontrado mais frequentemente em suínos com a SMDS do que em suínos saudáveis (KEKARAINEN et al., 2006).

A carga viral de TTSuV1 e TTSuV2 em animais acometidos pela SMDS e saudáveis não apresentou diferença significativa. No entanto, verificou-se que as cargas virais de TTSuV2 foram significativamente maiores do que a de TTSuV1 para todos os animais avaliados. Além disso, suínos SPF também apresentaram uma elevada taxa de detecção e uma alta carga viral de TTSuVs. Estes resultados também apontam que não há associação entre TTSuV1 e TTSuV2 em suínos e o desenvolvimento da SMDS, corroborando com os resultados de outros autores (LEE et al., 2010; BLOMSTRÖM et al., 2010). Pode-se pensar que os TTSuVs, como exemplo de vírus altamente bem sucedidos e amplamente distribuídos, são capazes de estabelecerem uma relação comensal com seu hospedeiro.

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