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

AGENTES VIRAIS POTENCIALMENTE ASSOCIADOS À SÍNDROME MULTISSISTÊMICA DO DEFINHAMENTO DOS SUÍNOS

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Porto Alegre, fevereiro de 2012.

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Trabalho apresentado como requisito parcial para obtenção do grau de Mestre em Ciências Veterinárias na área de Microbiologia Veterinária - Virologia.

Orientador: Paulo Michel Roehe

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Aprovada em 29 de fevereiro de 2012

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Aos meus pais, pelo apoio e presença incondicionais.

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“*Apenas quando somos instruídos pela realidade é que podemos mudá-la”.*

Bertolt Brecht

RESUMO

Com os avanços recentes nos métodos de detecção de patógenos, a importância das doenças polimicrobianas tornou-se mais evidente e a identificação de interações de patógenos e seus mecanismos de potenciação de enfermidades se tornou um tema de grande interesse. O circovírus suíno tipo 2 (PCV2) está associado a várias síndromes, tais como a síndrome multissistêmica do definhamento dos suínos (SMDS), a síndrome da dermatite e nefropatia dos suínos (SDNS), o complexo das doenças respiratórias dos suínos (CDRS), falhas reprodutivas e tremores congênitos, coletivamente chamadas "doenças associadas ao circovírus suíno tipo 2" (PCVAD), e que, além do PCV2, podem apresentar diferentes graus de envolvimento de outros agentes. Dentre estas, a SMDS é a que apresenta maior impacto na cadeia produtiva de suínos. Sendo a SMDS uma doença multifatorial e polimicrobiana, estudos buscando identificar associações do PCV2 com outros patógenos são importantes. No presente trabalho foram desenvolvidos ensaios moleculares para a detecção de alguns agentes cuja potencial participação nas PCVAD não havia ainda sido estudada com maior profundidade. Dessa forma, o citomegalovírus suíno (PCMV) e os recém-identificados bocavírus suínos do tipo 1, 2, 3 e 4 (PBoV1-4) foram pesquisados em amostras de animais afetados ou não pela SMDS, em diferentes faixas etárias. O PCMV foi detectado em altas taxas em ambos os grupos de animais, afetados ou não pela SMDS, sendo que nenhum tipo de associação com a SMDS pode ser inferida quanto à detecção de genomas de PCMV e o desenvolvimento da síndrome. A detecção de genomas de PBoV2 e 3 foi associada com animais afetados pela SMDS, enquanto nenhum tipo de associação foi inferida com a detecção de genomas de PBoV1 e do PBoV4. Animais afetados pela síndrome apresentam frequência de detecção e carga viral de PBoV2 significantemente superior à frequência encontrada em animais saudáveis com idade equivalente, revelando uma associação positiva entre PBoV2 e a SMDS. Por outro lado, animais adultos possuem uma carga viral significativamente superior a dos animais jovens sem sinais clínicos de SMDS. Os resultados obtidos possibilitaram detectar, pela primeira vez, os quatro tipos PBoV em amostras de suínos no Brasil. Paralelamente, esse estudo mostrou, pela primeira vez, a presença de genomas circulantes de PBoV1, PBoV2 e PBoV4 em animais adultos clinicamente saudáveis. Além disso, permitiu sugerir uma possível associação entre SMDS e PBoV2 e PBoV3, mostrando que mais estudos devem ser realizados para elucidar tal associação.

ABSTRACT

With recent advances in pathogen detection methods, the importance of polymicrobial diseases has become more evident, and identification of pathogen interactions and their disease potentiation mechanisms has become a topic of great interest. The porcine circovirus type 2 (PCV2) is associated with several syndromes, such as Postweaning multisystemic wasting syndrome (PMWS), Porcine dermatitis and nephropathy syndrome (PDNS), Porcine respiratory disease complex (PRDC), reproductive failures and congenital tremors, collectively called "Porcine circovirus type 2 associated diseases" (PCVAD), that beyond PCV2, may have different degrees of involvement of other agents. Among these, PMWS have the main impact on pig production chain. As PMWS is a multifactorial and polymicrobial disease, studies attempting to identify associations of PCV2 with other pathogens are important. In the present work, we developed molecular assays for detection of some agents whose potential role in PCVAD had not yet been studied in greater depth. Thus, porcine cytomegalovirus (PCMV) and newly identified porcine bocavirus type 1, 2, 3 and 4 (PBoV1-4) were investigated in samples of animals with or without PMWS, in different age groups. PCMV was detected at high rates in both groups of animals, and any type of association with PMWS could be inferred regarding detection of PCMV genomes and syndrome development. The PBoV2 and PBoV3 genomes detection was associated with animals affected by PMWS, while no association could be inferred from detection of genomes PBoV1 and PBoV4. Animals affected by the syndrome had significantly higher PBoV2 frequency of detection and viral load than the healthy animals with equivalent age, showing a positive association between PBoV2 and PMWS. Moreover, adult animals had significantly higher viral load than young animals without clinical signs of PMWS. Obtained results showed, for the first time, the four types of PBoV in Brazilian pig samples and the presence of circulating genomes of PBoV1, PBoV2 and PBoV4 in clinically healthy adult animals. It also allowed suggesting a possible association between PBoV2 and PBoV3 with PMWS, showing that more studies are needed to elucidate this association.

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1. INTRODUÇÃO

O Circovírus suíno (PCV) pertence à família *Circoviridae*, gênero *Circovirus* (ALLAN *et al.*, 1995). O PCV é um dos menores vírus animais, com um diâmetro aproximado de 17 nm, destituído de envelope e com um genoma de DNA circular de fita simples (HAMEL *et al.*, 1998). Duas espécies de PCV já foram descritas, PCV1 e PCV2 (ALLAN *et al.*, 1998). O PCV1 é apatogênico para suínos (ALLAN *et al.*, 1995) e foi originalmente identificado como um contaminante de células da linhagem de células renais de suíno PK15 (TISCHER *et al.*, 1982). O PCV2 está envolvido em várias síndromes, coletivamente chamadas de "doenças associadas ao circovírus suíno tipo 2" (PCVAD). A mais importante delas é a síndrome multissistêmica do definhamento dos suínos (SMDS) (ALLAN e ELLIS, 2000). Além da SMDS, o vírus está associado a outras manifestações clínicas, como a síndrome dermatite e nefropatia dos suínos (SDNS) (ALLAN, MCNEILLY, KENNEDY, *et al.*, 2000; ROSELL, SEGALES, RAMOS-VARA *et al.*, 2000; CHOI *et al.*, 2001; CHAE, 2005), e falhas reprodutivas (LADEKJAER-MIKKELSEN *et al.*, 2001; KIM *et al.*, 2004). O vírus também tem sido associado a doenças do complexo de doenças respiratórias dos suínos (DCRS) (ELLIS *et al.*, 1999) e à síndrome do tremor congênito (STEVENSON *et al.*, 2001; KENNEDY *et al.*, 2003).

Não obstante, a SMDS é a que apresenta maior impacto econômico para a cadeia produtiva de suínos. Esta condição foi identificada pela primeira vez no Canadá em 1991 (CLARK, 1996; HARDING, 1996). Desde então, vem sendo verificada em várias regiões do mundo, evidenciando que a infecção pelo PCV2 já se encontrava em caráter endêmico praticamente em todas as regiões onde a suinocultura industrial é praticada (ALLAN e ELLIS, 2000; CHAE, 2004) sendo relatada na Ásia, nas Américas, na Europa e na Oceania (SEGALES *et al.*, 1997; ALLAN *et al.*, 1998; SPILLANE *et al.*, 1998; ONUKI *et al.*, 1999; SAOULIDIS *et al.*, 2002; SARRADELL *et al.*, 2002; GRAU-ROMA *et al.*, 2011). O primeiro registro da SMDS no Brasil foi realizado no ano de 2000, no Estado de Santa Catarina, embora o PCV2 tenha sido identificado em tecidos de suínos estocados desde 1988 (CIACCI-ZANELLA *et al.*, 2000).

Embora o PCV2 seja o principal vírus associado à SMDS, nem todos os suínos que são infectados pelo PCV2 desenvolvem a síndrome. Isso indica que o curso de muitas infecções por PCV2 é subclínico, sendo necessários outros cofatores para o

desencadeamento dos sinais da SMDS (WELLENBERG, STOCKHOFE-ZURWIEDEN, DE JONG, *et al.*, 2004; TOMAS *et al.*, 2010).

Um desses cofatores pode ser representado por co-infecções com outros agentes virais (ELLIS *et al.*, 2004). A SMDS tem sido frequentemente reportada em animais infectados com outros patógenos, como o vírus da Síndrome respiratória e reprodutiva dos suínos (PRRSV), o Parvovírus suíno tipo 1 (PPV1), o vírus da Influenza suína (SIV), *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae*, *Streptococcus suis* e *Mycoplasma hyopneumoniae* (PALLARES *et al.*, 2002; ROVIRA *et al.*, 2002; KIM *et al.*, 2003). Entretanto, muitos outros agentes podem estar envolvidos, e cujo papel nesse quadro não tenha ainda sido estudado. Entre esses agentes, encontram-se o citomegalovírus suíno (PCMV) e os bocavírus suínos.

O PCMV é o agente causador da rinite por corpúsculos de inclusão e de infecções generalizadas em animais jovens (WATT *et al.*, 1973; EDINGTON *et al.*, 1976). Anticorpos contra o PCMV são descritos em altas prevalências em vários locais do globo (RONDHUIS *et al.*, 1980; ASSAF *et al.*, 1982; TAJIMA *et al.*, 1993; HAMEL *et al.*, 1999) sendo, como o citomegalovírus humano (HCMV), um vírus ubíquo em suínos, à semelhança do citomegalovírus humano (HCMV ou HHV-5) na espécie humana.

Nos últimos anos, com os avanços das técnicas de metagenômica, vários agentes virais potencialmente associados a doenças suínas foram descritos. Utilizando-se dessa metodologia, vários bocavírus suínos foram descritos, sendo posteriormente agrupados em 4 grandes grupos (ZHANG *et al.*, 2011). Os *Bocavírus* são vírus pertencentes à família *Parvoviridae,* sendo potencialmente relacionados ao desenvolvimento de infecções respiratórias e entéricas, principalmente em hospedeiros jovens (ALLANDER *et al.*, 2005; MANTEUFEL *et al.*, 2008; SCHILDGEN *et al.*, 2008). Entretanto, poucos estudos de associação com doenças desses vírus recém-descobertos foram realizados (BLOMSTROM *et al.*, 2010; ZHAI *et al.*, 2010; ZHANG *et al.*, 2011). 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. No presente estudo, por meio de técnicas de amplificação de ácidos nucléicos, buscou-se associar a presença de genomas virais de PCMV e dos recém-descobertos bocavírus suínos em animais acometidos ou não pela SMDS.

2. REVISÃO BIBLIOGRÁFICA

2.1. Os circovírus suínos

O circovírus suíno (PCV) foi descoberto como contaminante de culturas celulares de rim de suíno (PK15; American Type Culture colection, ATCC, código CCL33) nos anos 70 (TISCHER *et al.*, 1974). Mais tarde, foi verificado que se tratava de um vírus com material genético constituído por DNA de fita simples (ssDNA) e circular, sendo alocado na família *Circoviridae* (TISCHER *et al.*, 1982). Uma vez verificado que nenhum efeito citopático foi associado ao vírus e que estudos sorológicos mostravam que anticorpos contra o vírus eram prevalentes na população suína (TISCHER *et al.*, 1986; ALLAN *et al.*, 1995), o agente foi considerado apatogênico.

Em 1996, o PCV foi associado a surtos de SMDS no Canadá (ou *postweaning multisystemic wasting syndrome – PMWS*) (ALLAN, G. *et al.*, 1998). O vírus isolado era similar, mas não idêntico ao PCV descrito previamente. A sequência de nucleotídeos tinha uma identidade menor que 80% quando comparado com o PCV previamente reportado (MEEHAN *et al.*, 1998). Em função disso, os dois distintos PCVs foram dividido em dois tipos; PCV1 (o vírus apatogênico anteriormente descrito) e o PCV2, associado à doença clínica então identificada.

2.2. Classificação dos circovírus

Os circovírus suínos estão agrupados dentro da família *Circoviridae,* gênero *Circovirus* (*International Commitee on Viral Taxonomy,* ICTV, revisão de 2009). Este gênero é o taxon que contém o maior número de agentes virais conhecidos para esta família (Tabela 1). O gênero *Gyrovirus* contém três espécies conhecidas, o vírus da anemia infecciosa das galinhas (CAV) (NOTEBORN *et al.*, 1991) e os recém-descritos girovírus aviário tipo 2 (DOS SANTOS *et al.*, 2011; RIJSEWIJK *et al.*, 2011) e o girovírus humano tipo 1 (SAUVAGE *et al.*, 2011). Recentemente, vários genomas similares à *Circovirus* foram descrito, e um novo gênero (*Cyclovirus*) foi proposto (LI *et al.*, 2010).

Tabela 1. Vírus pertencentes à família *Circoviridae* (*NCBI Taxonomy*, fevereiro de 2012).

Circovirus	**Nome**	**Sigla**	**Referência**
Beak and feather disease virus	Vírus da doença do bico e das penas	PBFD	(NIAGRO *et al.*, 1998)
Canary circovirus	Circovírus dos canaries	CaCV	(TODD, WESTON, BALL, *et al.*, 2001)
Goose circovirus	Circovírus dos gansos	GoCV	(TODD, WESTON, SOIKE, *et al.*, 2001)
Porcine circovirus 1	Circovírus suíno tipo 1	PCV1	(NIAGRO *et al.*, 1998)
Porcine circovirus 2	Circovírus suíno tipo 2	PCV2	(HAMEL *et al.*, 1998)
Bovine circovirus	Circovírus bovino	BoCV	AF109397
Swan circovirus	Circovírus dos cisnes	SwCV	(HALAMI *et al.*, 2008)
***Circovirus* não classificados**			
Barbel circovirus	Circovírus dos barbos	BaCV	(LORINCZ *et al.*, 2011)
Bat circovirus ZS/China/2011			JF938130
Bat circovirus ZS/Yunnan-China/2009			JN377581
Chimpanzee stool avian-like circovirus Chimp17			(LI, KAPOOR, *et al.*, 2010)
Circovirus NG1, NG3, NG_chicken17, NG_chicken20, NG_chicken21, NG_chicken25, NG_chicken38, NG_chicken33, NG_chicken39 and NG_chicken5			
Columbid circovirus	Circovírus dos columbídeos	CoCV	(MANKERTZ *et al.*, 2000)
Duck circovirus	Circovírus dos patos	DuCV	(BANDA *et al.*, 2007)

Finch circovirus	Circovírus dos tentilhões	FiCV	(TODD *et al.*, 2007)
Gull circovirus	Circovírus das gaivotas	GuCV	
Mulard duck circovirus			(HATTERMANN *et al.*, 2003)
Muscovy duck circovirus			(CHEN *et al.*, 2006)
Raven circovirus	Circovírus dos corvos	RvCV	(STEWART *et al.*, 2006)
Sewage associated circovirus			(BLINKOVA *et al.*, 2009)
Starling circovirus	Circovírus dos estorninhos	StCV	(JOHNE *et al.*, 2006)
Gyrovirus			
Chicken anemia virus	Vírus da anemia infecciosa das galinhas	CAV	(NOTEBORN *et al.*, 1991)
Avian gyrovirus 2	Girovírus aviário tipo 2	AGV2	(RIJSEWIJK *et al.*, 2011)
Human gyrovirus type 1	Girovírus humano tipo 1	HGV1	(SAUVAGE *et al.*, 2011)
Genomas não classificados da família *Circoviridae*			
Circoviridae NG24			(LI, KAPOOR, *et al.*, 2010)
Circoviridae PKbeef21			
Circoviridae PorkNW2			
Circoviridae PorkNW2/USA/2009, SFbeef/USA/2010 and SFpork/USA/2010			(LI, L., SHAN, T., SOJI, O. B., *et al.*, 2011)
Circoviridae TM-6c			(LI, VICTORIA, *et al.*, 2010)

Circoviridae TN4	(LI, KAPOOR, *et al.*, 2010)
Meles meles circovirus-like virus	(VAN DEN BRAND *et al.*, 2012)
Po-Circo-like virus 21, 22, 41 and 51	(SHAN, LI, *et al.*, 2011)
Novo gênero (sugerido) *Cyclovirus*	
Bat cyclovirus GF-4c	(LI, VICTORIA, *et al.*, 2010)
Cyclovirus bat/USA/2009	
Cyclovirus Chimp11, 12, 13, 32, 53 and 73	(LI, L., SHAN, T., SOJI, O. B., *et al.*, 2011)
Cyclovirus NG12, NG14, NG15, NG22, NG23, NG6 AND NG8 and NG14	
Cyclovirus NG_camel27, 37 and 40	(LI, KAPOOR, *et al.*, 2010)
Cyclovirus NG_chicken clade 3	
Cyclovirus NG_cow12, 2 and 23	
Cyclovirus NG_sheep50	
Cyclovirus NGchicken15/NGA/2009	
Cyclovirus PK10, 14, 15, 16, 2, 2096, 2178, 2291, 3, 4, 5006, 5016, 5034	
Cyclovirus PK5222, 5510, 5727, 6197, 6527 and 5192	
Cyclovirus PKbeef23, 22, 24 and 25	
Cyclovirus PKgoat11, PAK/2009, 12, 21, 21/PAK2009, 22, 23, 24 and 25	
Cyclovirus TN10, 11, 12, 15-18, 2, 22, 25, 26, 6, 8, 9	
Dragonfly cyclovirus	(ROSARIO *et al.*, 2011)
Human stool-associated circular virus NG13	(LI, KAPOOR, *et al.*, 2010)

2.3. Características dos PCVs

Os circovírus suínos estão entre os menores vírus conhecidos que replicam de forma autônoma. O genoma dos circovírus consiste em uma fita simples de DNA circular, não envelopado, de simetria icosaédrica, com aproximadamente 17 nm (TISCHER *et al.*, 1982), como representado na Figura 1. A densidade das partículas virais é de 1,36-1,37 g/mL em gradiente de cloreto de césio. O vírus não possui atividade hemaglutinante e é resistente ao clorofórmio, temperaturas altas e pH igual ou maior que 3 (ALLAN *et al.*, 1994).

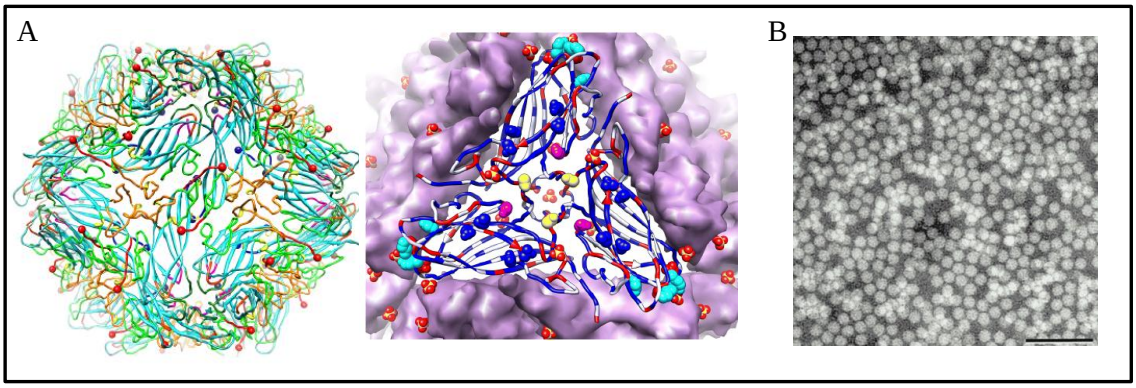

FIGURA 1. Estrutura do capsídeo do PCV2. (A) Modelos gerados a partir de imagenas de criomicroscopia eletrônica do capsídeo do PCV2 (KHAYAT *et al.*, 2011). (B) Microscopia eletrônica, aumento de 250.000 vezes. A barra indica 100 nm.

O genoma do PCV2 contém 1767 ou 1768 nucleotídeos (MEEHAN *et al.*, 1998). Este codifica dois genes principais, denominados *rep* (codificando a proteína replicase) e *cap* (que codifica a proteína do capsídeo viral, CAP). O gene *rep* tem uma fase de leitura aberta (ORF1), que se estende do nucleotídeo 51 ao 995 e está localizado na fita viral positiva. O *cap* localizado na ORF2 da fita viral negativa, se estende do nucleotídeo 1735 ao 1034. Na região intergênica está a origem de replicação do DNA (ORI), onde duas proteínas REP ligam-se a uma estrutura palindrômica e iniciam o processo de replicação do tipo "círculo rolante" (MANKERTZ *et al.*, 2004). A proteína CAP tem função estrutural (NAWAGITGUL *et al.*, 2000). Outra proteína viral, que está associada à indução da apoptose, localizada na ORF3 da fita viral negativa, se estende no nucleotídeo 671 ao 375 (LIU *et al.*, 2005). O mapa genômico do PCV2 está ilustrado na Figura 2.

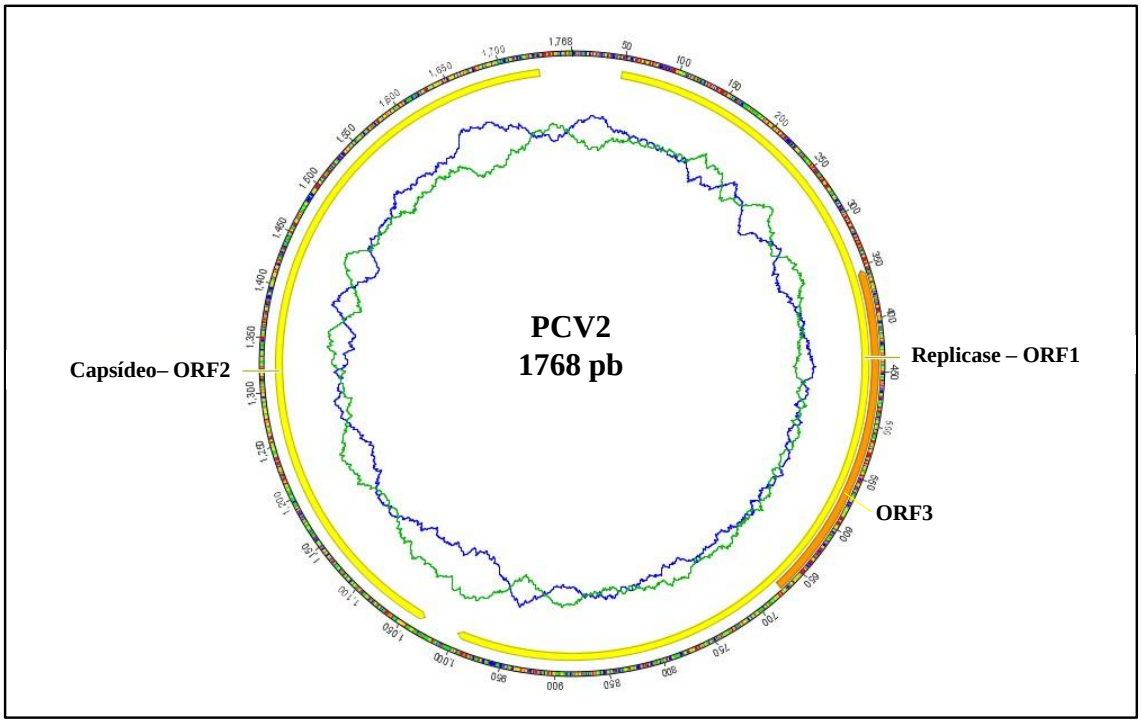

FIGURA 2. Organização genômica do PCV2 (NC_005148). A ORF3 está sobreposta à ORF1. A ORF2 e ORF3 são transcritas a partir da fita viral negativa, enquanto a ORF1 da fita viral positiva. O mapa genômico do PCV2 foi realizado no software *Geneious* (DRUMMOND *et al.*, 2011).

2.4. Classificação do PCV2 em genogrupos

As primeiras análises filogenéticas mostraram que sequências genômicas do PCV2 variavam de acordo com a origem geográfica do vírus isolado (HAMEL *et al.*, 1998; FENAUX *et al.*, 2000; MEEHAN *et al.*, 2001). Posteriormente, baseando-se nas sequências de aminoácidos e nucleotídeos, três genogrupos puderam ser identificados, sem relação nenhuma com a localização geográfica do isolado (DE BOISSESON *et al.*, 2004; OLVERA *et al.*, 2007; GAGNON *et al.*, 2008; GRAU-ROMA *et al.*, 2008). Em alguns estudos, certos genogrupos de PCV2 foram fortemente associados a surtos de doenças relacionadas ao PCV2 (OPRIESSNIG *et al.*, 2006; GRAU-ROMA *et al.*, 2008; TIMMUSK *et al.*, 2008). Entretanto, muitos autores não encontraram esse tipo de associação (DE BOISSESON *et al.*, 2004; ALLAN *et al.*, 2007; OLVERA *et al.*, 2007).

Atualmente, a nomenclatura PCV2a, PCV2b e PCV2c tem sido adotada (SEGALES *et al.*, 2008). Estudos retrospectivos mostraram que os genótipos de PCV2 circulam mundialmente e que existiu uma predominância de determinado genótipo em décadas distintas. O genótipo "c" circulou predominantemente na década de oitenta. Na década de noventa, estudos mostraram que o genótipo que mais circulou foi o PCV2a, enquanto o PCV2b predominou de 2001 em diante, sugerindo uma potencial mudança na predominância de genótipos ao longo do tempo (DUPONT *et al.*, 2008). Entretanto, recentemente, publicações de diferentes partes do mundo, relataram a emergência de outros possíveis genogrupos de PCV2, sendo que sua distribuição e associação com a gravidade das doenças associadas ao PCV2 carecem de estudos (CHEUNG *et al.*, 2007; DUPONT *et al.*, 2008; GRAU-ROMA *et al.*, 2008; TIMMUSK *et al.*, 2008; WANG *et al.*, 2009; GUO *et al.*, 2010).

2.5. Ciclo de replicação viral

Embora não seja conhecida qual é a célula preferencial de replicação para o PCV2 *in vivo*, estudos *in vitro* com diferentes tipos de células geraram valiosas informações a respeito dos mecanismos de infecção do PCV2. O ciclo de replicação viral se inicia com a adsorção do PCV2 a glicosaminoglicanos da célula hospedeira (MISINZO *et al.*, 2006), sendo após endocitado por intermédio de clatrinas (MISINZO *et al.*, 2005; BOETTNER *et al.*, 2012).

Após a internalização, a replicação do PCV2 ocorre no núcleo das células infectadas por um mecanismo de replicação via círculo rolante, como ocorre nos geminivírus (GUTIERREZ, 1999; FAUREZ *et al.*, 2009; FINSTERBUSCH *et al.*, 2009). Após a infecção, o genoma viral (DNA fita simples) é convertido por fatores do hospedeiro em uma forma replicativa (DNA dupla fita). A origem de replicação é caracterizada por uma estrutura em alça ou "*stem-loop*" com uma sequência característica de nove nucleotídeos (5'-AAGTATTAC-3'), que é conservada em todos os circovírus. Próximo a essa estrutura, repetições do hexâmero 5'-CGGCAG-3' e de pentâmeros também são encontradas. A ligação da proteína REP e REP' na origem desestabiliza relaxa a fita dupla de DNA e induz a exposição da sequência de nove nucleotídeos como DNA de fita simples (DNA viral), o qual é reconhecido e clivado pelo complexo REP/REP'. Enquanto a proteína REP/REP' está covalentemente ligada ao fosfato-5'do DNA clivado, a síntese unidirecional da fita "*leading*" é iniciada a partir do grupo hidroxila-3' que funciona como primer para a DNA polimerase do hospedeiro,

uma vez que o PCV2 não sintetiza sua própria DNA polimerase para a produção de intermediários replicativos, ele depende de DNA polimerases do hospedeiro para completar seu ciclo de replicação (TISCHER *et al.*, 1987; STEINFELDT *et al.*, 2007; VEGA-ROCHA *et al.*, 2007). Após um ciclo de replicação, a nova fita de DNA sintetizada é clivada novamente dentro da origem regenerada e o fosfato-5' é ligado no novo grupo hidroxila-3'. Eventos com relação à montagem e a liberação do PCV da célula ainda não foram esclarecidos (STEINFELDT *et al.*, 2007; FAUREZ *et al.*, 2009; FINSTERBUSCH *et al.*, 2009).

Estudos revelam que a dinâmica do ciclo replicativo varia com o tipo de célula infectada. Em cardiomiócitos fetais e macrófagos alveolares suínos, antígenos virais no núcleo podem ser identificados após 48 horas em menor proporção do que em células PK15 (CHEUNG *et al.*, 2002; MEERTS *et al.*, 2005). Nestas últimas, a proteína do capsídeo e expressa entre 6-12 horas pós-inoculação (hpi) e é deslocada para o núcleo 12 a 24 hpi, quando a proteína Rep e detectada no núcleo antígenos virais são detectados no citoplasma e núcleo em torno de 18 hpi, sendo as partículas virais liberadas 32 hpi (CHEUNG *et al.*, 2002). Este ciclo de replicação difere do observado em monócitos e macrófagos, nos quais a proteína do capsídeo do PCV2 é detectada no citoplasma, mas nenhuma evidência de replicação foi observada. Isto provavelmente ocorre porque estas células estão em estágio de diferenciação terminal, onde a maquinaria de replicação celular não é, na maioria das vezes, ativa (GILPIN *et al.*, 2003).

O PCV2 é frequentemente encontrado no citoplasma de monócitos, macrófagos e células dendríticas. Entretanto, a ausência de formas replicativas de dsDNA e progênie de vírus infectiva indica que a replicação não ocorre dentro dessas células (GILPIN *et al.*, 2003; VINCENT *et al.*, 2003). Nenhum receptor específico para o PCV2 foi encontrado até o momento, mas glicosaminoglicanas heparan sulfatadas e condroitina B foram identificadas como receptores de adsorção para o PCV2 em monócitos (MISINZO *et al.*, 2005; MISINZO *et al.*, 2006). Além disso, essas estruturas servem como receptores para vários vírus, e é sugerido que o PCV2 seja internalizado nos monócitos e nas DC via endocitose mediada por clatrinas (VINCENT *et al.*, 2003; MISINZO *et al.*, 2006). Além do mais, a infecção de células monocíticas pelo PCV2, demonstrou ser dependente do ambiente ácido provido através do sistema endossomal (MEERTS *et al.*, 2005).

2.6. Doenças associadas ao PCV2

O PCV2 está associado a várias síndromes, tais como a Síndrome multissistêmica do definhamento dos suínos (SMDS), Síndrome da dermatite e nefropatia dos suínos (SDNS) (SMITH *et al.*, 1993; THIBAULT *et al.*, 1998), ao Complexo das doenças respiratórias dos suínos (CDRS) (THACKER, 2001; OPRIESSNIG, GIMENEZ-LIROLA, *et al.*, 2011), a falhas reprodutivas (WEST *et al.*, 1999; LADEKJAER-MIKKELSEN *et al.*, 2001; O'CONNOR *et al.*, 2001; KIM *et al.*, 2004), além do tremor congênito dos suínos (STEVENSON *et al.*, 2001; KENNEDY *et al.*, 2003; HA *et al.*, 2005). Dentre estas, a SMDS é a que apresenta maior impacto na cadeia produtiva de suínos, como comentado a seguir.

2.6.1. Síndrome multissistêmica do definhamento do suíno – SMDS (*Postweaning multisystemic wasting syndrome – PMWS*) ou circovirose

Existem muitos debates sobre a associação entre PCV2 e SMDS devido às dificuldades do cumprimento dos postulados de Koch para a reprodução do quadro. Entretanto é aceito que o PCV2 é o agente necessário para o desenvolvimento da SMDS, embora outros co-fatores sejam importantes para a manifestação da síndrome.

Os primeiros casos de SMDS foram identificados em granjas com alto padrão sanitário no Canadá em 1991. Mais tarde, a denominação "síndrome multissistêmica do definhamento dos suínos" (SMDS) foi proposto para descrever o quadro, com base na apresentação clínica e características das lesões histopatológicas observadas na enfermidade, então emergente (CLARK, 1996; HARDING, 1996).

Pesquisas subsequentes mostraram que animais com SMDS apresentavam abundância de genomas de PCV2 e antígenos virais associados às lesões em diferentes tecidos (ELLIS *et al.*, 1998). Durante a primeira década dos anos 2000, a SMDS espalhou-se para todos os países produtores de suínos e atualmente constitui-se no maior problema sanitário para criadores de suínos.

Clinicamente, a SMDS é uma doença caracterizada pela perda de peso, aumento do volume dos linfonodos, dispnéia e, menos frequentemente, palidez, icterícia e diarréia, a qual acomete principalmente leitões logo após a fase de desmame (ALLAN *et al.*, 1999; ALLAN e ELLIS, 2000; CHOI *et al.*, 2000; CHAE, 2004). Atualmente, a SMDS é endêmica em muitos países, sendo a principal causa de refugarem em plantéis suínos (CHAE, 2004).

A manifestação clínica da doença ocorre em animais na 5ª a 16ª semana de vida (ALLAN e ELLIS, 2000; PALLARES *et al.*, 2002; SEGALES e DOMINGO, 2002), apresentando baixa taxa de morbidade, porém alta taxa de mortalidade. A morbidade da doença varia de 4-30% entre granjas, embora algumas propriedades reportem estatísticas próximas aos 60%. A mortalidade entre animais afetados pela síndrome varia de 70-80% (SEGALES e DOMINGO, 2002; DARWICH *et al.*, 2004).

Até o presente momento, a SMDS tem sido diagnosticada em países dos cinco continentes e na maioria dos países produtores de carne suína (GRAU-ROMA *et al.*, 2011). O vírus está amplamente disseminado nas populações suínas. A soroprevalência de anticorpos anti-PCV2 chega a 100% em animais em fase de terminação, sendo que em nenhuma granja todos os animais do plantel foram soronegativos (LAROCHELLE *et al.*, 2003). Em média, a percentagem de animais soropositivos nos rebanhos norte-americanos é de 50% (NAWAGITGUL *et al.*, 2002).

A presença de co-infecções dentro de granjas com animais com SMDS vem sendo estudada. A correlação positiva entre PRRSV e o desenvolvimento da SMDS já foi verificado em vários trabalhos (ROSE *et al.*, 2003; WELLENBERG *et al.*, 2004; TEIXEIRA, 2008; WOODBINE *et al.*, 2010; STADEJEK *et al.*, 2011). Outros patógenos que possuem uma relação positiva com SMDS são o Parvovírus suíno tipo 1, influenza suína, *Mycoplasma hyopneumoniae*, hepatite suína do tipo E e o vírus da pseudoraiva (ELLIS *et al.*, 2004). A natureza e os mecanismos de interação desses agentes com o PCV2 para o desenvolvimento da SMDS e de todos os sinais clínicos ainda não foram elucidados.

Vários estudos epidemiológicos têm sido realizados a fim de identificar os fatores que podem estar influenciando no aumento do risco de surtos de SMDS. O risco para o desenvolvimento da SMDS é aumentado em propriedades com grandes rebanhos (WALLGREN *et al.*, 2007; WOODBINE *et al.*, 2007), quando há um alto grau de endocruzamentos e quando o vazio sanitário entre um lote e outro é de curta duração (ROSE *et al.*, 2003; WALLGREN *et al.*, 2007).

2.7. Patogenia

A maioria dos animais infectados pelo PCV2 não apresentam doença clínica, porém a infecção pode ser evidenciada pela soroconversão (QUINTANA *et al.*, 2001). Acredita-se que a imunossupressão, caracterizada pela depleção linfóide e linfopenia, seja o evento determinante do desencadeamento da síndrome (SEGALES, DOMINGO,

et al., 2004; OPRIESSNIG *et al.*, 2007). Alguns autores sugerem que para o completo desenvolvimento da síndrome, existiria a necessidade de co-infecções virais, como o parvovírus suíno tipo 1 e o vírus da síndrome respiratória e reprodutiva dos suínos (ALLAN *et al.*, 1999; CHOI *et al.*, 2000; HARMS *et al.*, 2001) ou cofatores imunoestimulatórios (KRAKOWKA *et al.*, 2001; KYRIAKIS *et al.*, 2002).

Antígenos e material genético do PCV2 têm sido detectados em vários tipos de células de suínos infectados. O material viral tem sido encontrado principalmente no citoplasma de histiócitos e células da linhagem monocítica/macrofágica, tais como macrófagos alveolares, células de Kupffer e células dendríticas (ROSELL *et al.*, 1999; GILPIN *et al.*, 2003).

Devido ao fato do vírus ser frequentemente detectado em células epiteliais (ROSELL, SEGALES e DOMINGO, 2000), foi sugerido que este tipo de célula possa ser o tipo celular primário mais permissível à replicação do PCV2 (DARWICH *et al.*, 2004). Após a infecção e replicação primária nestas, acredita-se que o PCV2 seja transportado para os principais alvos (linfonodos e/ou corrente sanguínea), contribuindo para a disseminação viral (ROSELL *et al.*, 1999). Com a evolução da enfermidade, o vírus distribui-se em órgãos como intestino, rins e fígado, podendo causar alterações que colaboram para ocorrência da SMDS (SEGALES, DOMINGO, *et al.*, 2004).

Infecções causadas por PCV2 tem uma evolução crônica. Em animais inoculados com PCV2 que não desenvolveram a doença, observou-se viremia a partir do 7° dia, com pico virêmico no 21° dia pós-infecção (pi), seguido da redução gradual da carga viral ate o 69° dia pi (RESENDES *et al.*, 2004).

2.8. Lesões em suínos afetados pela SMDS

Suínos com SMDS mostram diferentes graus de definhamento, linfoadenopatia generalizada, falta de colabamento pulmonar e atrofia tímica. Com menor frequência, podem apresentar atrofia hepática e lesões renais (ALLAN e ELLIS, 2000; LADEKJAER-MIKKELSEN *et al.*, 2002; KIM *et al.*, 2003).

Em animais com a síndrome, as lesões microscópicas observadas com maior frequência são: depleção linfóide e infiltração histiocítica. Estas lesões, nos animais gravemente acometidos, são observadas em praticamente todos os tecidos, incluindo os linfonodos, tonsilas, placas de Peyer, baço e timo (ROSELL *et al.*, 1999). Outras lesões observadas são a inflamação granulomatosa e presença de corpúsculos de inclusões intracitoplasmáticos. A inflamação granulomatosa é observada nos linfonodos, fígado,

baço, timo, placas de Peyer e linfonodos inguinais superficiais, consistindo de um infiltrado de células epitelióides e células gigantes multinucleadas (CHAE, 2004).

Os corpúsculos de inclusão intracitoplasmáticos são grandes, numerosos e basofílicos, sendo observados no citoplasma de histiócitos e células gigantes multinucleadas (ROSELL *et al.*, 1999; ALLAN e ELLIS, 2000; GILPIN *et al.*, 2003; CHAE, 2004). Esporadicamente, é possível detectar o vírus no citoplasma de células renais, epitélio respiratório, endotélio vascular e no núcleo de monócitos, macrófagos, células da musculatura lisa, hepatócitos e enterócitos (ROSELL *et al.*, 1999; ROSELL, SEGALES, RAMOS-VARA, *et al.*, 2000).

2.9. Diagnóstico

O PCV2 pode ser detectado em animais sadios, portanto a simples detecção da presença viral não é confirmatória para a síndrome. Para diagnóstico de SMDS deve ser observada a presença de PCV2 nas lesões, sinais clínicos compatíveis e lesões microscópicas características (SEGALES, ALLAN, *et al.*, 2005; SEGALES, 2011).

Embora o definhamento e as alterações respiratórias observadas na fase de maternidade e creche contribuam para o diagnóstico da SMDS, os sinais clínicos e as lesões desta doença são inespecíficos e, portanto, não são suficientes para diagnosticar a doença. Além disso, o fato do PCV2 ser cosmopolita na população suína dificulta o diagnóstico da síndrome. Atualmente, existem três critérios utilizados para diagnosticar a SMDS (SORDEN, 2000; SEGALES, ALLAN, *et al.*, 2005): *i.* presença de sinais clínicos compatíveis, incluindo o definhamento ou retardo no crescimento; *ii.* presença de lesões histopatológicas moderadas a graves, incluindo depleção linfocitária e infiltrado histiocitário; *iii.* detecção de quantidades moderadas a altas de PCV2 nas lesões de tecidos linfóides e outros tecidos de suínos afetados.

Vários métodos têm sido desenvolvidos para detectar PCV2 nos tecidos e correlacionar a sua detecção com a presença de lesões. Entre eles, a hibridização *in situ* e a imunohistoquímica são os testes mais utilizados rotineiramente (MCNEILLY *et al.*, 1999; ROSELL *et al.*, 1999). Esses métodos mostraram uma forte correlação entre a quantidade de acido nucleico ou do antígeno de PCV2 e da gravidade de lesão microscópicas do tecido linfóide (ROSELL *et al.*, 1999; QUINTANA *et al.*, 2001). Consequentemente, devido ao fato do diagnóstico de SMDS exigir a detecção de quantidades moderadas a altas de PCV2 nos tecidos linfóides, isso acarreta na presença de lesões microscópicas moderadas a graves, nos tecidos dos suínos afetados. Os suínos

que estão na primeira semana da infecção clínica são os que mais facilmente preenchem os critérios de diagnóstico (SEGALES, ROSELL, *et al.*, 2004), enquanto que suínos convalescentes ou recentemente infectados podem apresentar lesões microscópicas leves e uma pequena quantidade de antígeno/acido nucléico viral presente nas lesões (QUINTANA *et al.*, 2001).

A imunohistoquímica e hibridização *in situ* são as técnicas mais comumente utilizadas para estabelecer o diagnóstico da SMDS, produzindo resultados semelhantes (MCNEILLY *et al.*, 1999; ROSELL *et al.*, 1999). No entanto, outras técnicas para detectar o antígeno de PCV2 ou ácidos nucleicos e seus anticorpos são relatados na literatura. Entre eles, a PCR tem se mostrado um teste muito sensível (LAROCHELLE *et al.*, 1999; HAMEL *et al.*, 2000; KIM *et al.*, 2001; CALSAMIGLIA *et al.*, 2002), sendo utilizada em várias espécimes tais como soro, excreções, secreções e tecidos (QUINTANA *et al.*, 2001; SHIBATA *et al.*, 2003). No entanto, a PCR não é capaz de diferenciar entre infecções clínicas e subclínicas (MCNEILLY *et al.*, 2002). Levando em consideração que infecções subclínicas por PCV2 são frequentes e que a infecção por PCV2 nem sempre resulta em doença clínica, a PCR não-quantitativa não deve ser usada para diagnosticar SMDS (MCNEILLY *et al.*, 2002; SEGALES, ALLAN, *et al.*, 2005). Da mesma forma, um grande número de técnicas sorológicas relatadas na literatura, incluindo ensaio de imunoperoxidase em monocamada (IPMA) (RODRIGUEZ-ARRIOJA *et al.*, 2000), imunofluorescência indireta (ALLAN e ELLIS, 2000) e ELISA (WALKER *et al.*, 2000; TRUONG *et al.*, 2001; WU *et al.*, 2008) são úteis para monitorar a infecção pelo PCV2, porem não devem ser usados para fins de diagnóstico (RODRIGUEZ-ARRIOJA *et al.*, 2000; SIBILA *et al.*, 2004).

Técnicas como PCR quantitativo (qPCR), ELISA de captura de antígeno e analise imunocitoquímica de criocortes permitem a quantificação do vírus em tecidos (MCNEILLY *et al.*, 2002; ZHAO *et al.*, 2010; LIU *et al.*, 2011; VLASAKOVA *et al.*, 2012). Levando em consideração que existe uma correlação entre a quantidade de acido nucleico de PCV2 ou do antígeno viral com a gravidade das lesões microscópicas características de SMDS (ROSELL *et al.*, 1999; QUINTANA *et al.*, 2001), estas técnicas poderiam ser usadas para diagnosticar a SMDS. Assim, alguns autores sugeriram valores-limite, 10^7 cópias do genoma viral por mL de sangue, para diagnosticar a SMDS nos animais, usando técnicas de qPCR (BRUNBORG *et al.*, 2004; OLVERA *et al.*, 2004; SEGALES, CALSAMIGLIA, *et al.*, 2005).

2.10. Interação do PCV2 com o sistema imune do hospedeiro

Animais com SMDS apresentam monocitose, neutrofilia, linfopenia e diminuição de células $CD8^+$, $CD4^+$ e IgM^+ (DARWICH *et al.*, 2002). A linfopenia induzida por PCV2 em animais sintomáticos levou a hipótese que eventos imunossupressores seguidos de infecção por PCV2 teriam um importante papel no desenvolvimento de doenças associadas ao vírus (NIELSEN *et al.*, 2003). Sabe-se também que o vírus se associa a células da linhagem monocítica *in vivo* (ROSELL *et al.*, 1999; KRAKOWKA *et al.*, 2002) e *in vit*ro, sem mostrar qualquer sinal de replicação (GILPIN *et al.*, 2003; VINCENT *et al.*, 2003). A habilidade do vírus de se replicar e persistir por um longo tempo no organismo indica uma falha na resposta imunológica do hospedeiro em reconhecer ou remover as partículas virais e as células infectadas (MEERTS *et al.*, 2006).

O comprometimento imunológico em animais com SMDS é evidente, uma vez que ocorre depleção linfóide (ROSELL *et al.*, 1999; LADEKJAER-MIKKELSEN *et al.*, 2002) e infecções por patógenos oportunistas, como *Pneumocystis carinii, Chlamydia spp e Aspergillus spp*. nos pulmões de animais acometidos (SEGALES e DOMINGO, 2002). A imunossupressão induzida com ciclosporina, um agente imunossupressor forte, produz um quadro clínico mais grave da doença do que animais tratados com corticóides, agentes imunossupressores fracos (KRAKOWKA *et al.*, 2002).

Suínos com SMDS tem padrões alterados de expressão gênica de citocinas. Ocorre um aumento da expressão de IL-10 no timo e IFN-γ nas tonsilas. O aumento de IL-10 tem sido associado à depleção e atrofia tímica em animais doentes. A diminuição da expressão gênica foi observada para IL-2 no baco, IL-4 nas tonsilas e linfonodos, IL-12p40 em linfonodos inguinais e esplênicos e IFN-γ e IL-10 em linfonodos inguinais. Estes achados, associados ao fato da redução de células CD4+ e CD8+ no sangue e depleção e atrofia tímica são indicativos de uma imunossupressão de células T, o que prejudicaria o sistema imune de suínos afetados pela síndrome (DARWICH, BALASCH, *et al.*, 2003).

Células mononucleares do sangue periférico derivadas de animais com SMDS não produziram níveis normais de IFN-γ ou IL-1β, IL-2, IL-4 ou IL-8 quando desafiadas com fitohemaglutinina ou superantígenos, mas produziram IFN-γ ou IL-10 quando desafiadas com antígenos de PCV2, evidenciando que animais com SMDS tem

uma redução na habilidade de realizar suas funções imunológicas normais quando expostos a vírus ou moléculas imunoestimulatórias (DARWICH *et al.*, 2003).

2.11. O Citomegalovírus suíno

O citomegalovírus suíno (PCMV) é um *Betaherpesvirus* que, assim como outros membros da família *Herpesviridae*, causam infecções latentes (TUCKER *et al.*, 1999; GARKAVENKO *et al.*, 2004; DAVISON *et al.*, 2009). Estudos filogenéticos, baseados em sequências da glicoproteína B, da DNA polimerase e da principal proteína do capsídeo ("*Major capsid protein",* MCP), mostraram que o PCMV está mais relacionado com os *Roseolovirus* (herpesvírus humano tipo 6, HHV6 e herpesvírus humano tipo 7, HHV7) do que com os *Cytomegalovirus* (citomegalovírus humano, HHV5 ou HCMV, por exemplo) (RUPASINGHE *et al.*, 1999; GOLTZ *et al.*, 2000; RUPASINGHE *et al.*, 2001; WIDEN *et al.*, 2001). A Figura 3 ilustra essa evidência.

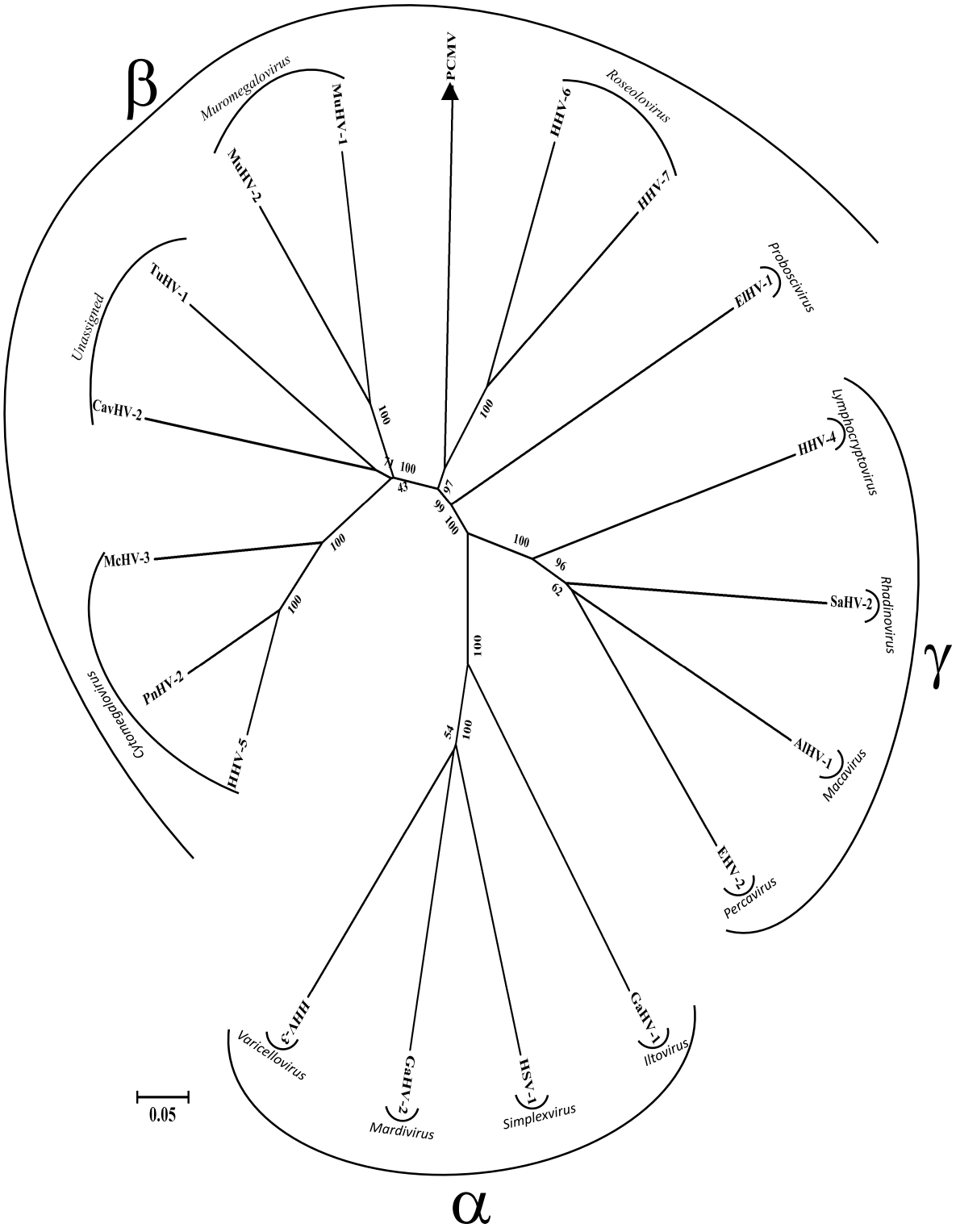

FIGURA 3. Árvore filogenética da família *Herpesviridae*. Sequências de aminoácidos da glicoproteína B de vários membros da família *Herpesviridae* foram alinhados com o *software* MUSCLE. Após, as análises filogenéticas foram realizadas no *software* MEGA5, utilizando-se Neighbor-Joining como método de inferência (somente valores de *bootstraps* superiores a 50% foram mostrados).

O PCMV é associado ao desenvolvimento de rinite por corpúsculos de inclusão nos suínos, além de desordens reprodutivas em fêmeas prenhas (EDINGTON *et al.*, 1976). O vírus infecta especialmente células da mucosa do trato respiratório superior, causando inclusões intranucleares que caracterizam a enfermidade. Em fêmeas gestantes, o vírus pode cruzar a placenta e causar mortalidade embrionária ou fetal (EDINGTON *et al.*, 1976).

O vírus está presente em forma ubíqua nos suínos, geralmente sem induzir sinais clínicos (RONDHUIS *et al.*, 1980; ASSAF *et al.*, 1982; TAJIMA *et al.*, 1993; HAMEL *et al.*, 1999; GOLTZ *et al.*, 2000). Quando ocorre doença, geralmente são afetados leitões de duas a três semanas, mas pode ocorrer em suínos com mais de 10 semanas de vida. Os leitões se infectam diretamente da mãe ou por via transplacentária. O período de incubação é de 7 a 10 dias; o vírus infecta a mucosa da cavidade nasal e o trato respiratório superior. Após a viremia primária, o vírus pode ser isolado das secreções nasal e ocular, de macrófagos pulmonares, da urina, do fluido cervical, dos testículos e do epidídimo. A morbidade é alta, mas a mortalidade é baixa (EDINGTON *et al.*, 1976).

As lesões macroscópicas são de rinite catarral ou purulenta. Lesões generalizadas podem ser observadas em leitões muito jovens ou recém-nascidos, sendo que, as principais são: hemorragias petequiais e edema difuso envolvendo, principalmente, a cavidade torácica e tecido subcutâneo. Nos casos não complicados, a infecção pelo citomegalovírus, em leitões com mais de três semanas de idade, pode se apresentar de maneira assintomática (EDINGTON *et al.*, 1976).

O isolamento do vírus pode ser feito em cultivo de macrófagos pulmonares, células de mucosa nasal, glândulas salivares, pulmões ou células renais (L'ECUYER *et al.*, 1966; WATT *et al.*, 1973; KAWAMURA *et al.*, 1996). A presença da infecção no rebanho pode ser confirmada pela pesquisa de anticorpos usando a imunofluorescência indireta ou ELISA (ASSAF *et al.*, 1982; TAJIMA *et al.*, 1993; TAJIMA *et al.*, 1994). A doença pode ser reconhecida pelos sinais clínicos e pela demonstração em cortes histológicos das inclusões intranucleares, típicas de citomegalovírus (L'ECUYER *et al.*, 1966; WATT *et al.*, 1973; EDINGTON *et al.*, 1976).

A infecção do PCMV pode ser reativada e a replicação viral aumentada sobre condições de imunodepressão (FISHMAN *et al.*, 1998; MUELLER *et al.*, 2002; GOLLACKNER *et al.*, 2003; MUELLER *et al.*, 2003). Muitos estudos a respeito da reativação do PCMV foram realizados no início dos anos 2000, em vista do suíno ser

um potencial doador de órgãos para xenotransplantes (TUCKER *et al.*, 1999; MUELLER *et al.*, 2002; GOLLACKNER *et al.*, 2003; MUELLER *et al.*, 2003; GARKAVENKO *et al.*, 2004; MUELLER, KUWAKI, *et al.*, 2004; MUELLER, LIVINGSTON, *et al.*, 2004; GARKAVENKO *et al.*, 2008; WHITTEKER *et al.*, 2008).

Apesar da alta prevalência do PCMV em suínos de todo o mundo (RONDHUIS *et al.*, 1980; ASSAF *et al.*, 1982; TAJIMA *et al.*, 1993; HAMEL *et al.*, 1999), as associações entre PCMV e SMDS não foram investigadas. Como o PCV2 é um vírus com atividade imunodepressora, tornou-se de interesse examinar se o PCMV iria desempenhar qualquer papel no desenvolvimento de SMDS.

2.12. Bocavírus suínos

Os membros da família *Parvoviridae* são vírus pequenos, com tamanho aproximado de 20 nm, esféricos, com capsídeo icosaédrico e desprovidos de envelope (Figura 4). Seu peso molecular é de 5,5 a 6,2 x 10^6 Daltons, com aproximadamente metade do peso dividido em sua massa protéica e outra metade composta por seu genoma (MUZYCZKA *et al.*, 2001), o qual é constituído por uma molécula de DNA linear fita simples (CRAWFORD *et al.*, 1969). Uma característica marcante dos parvovírus é que para sua replicação existe uma dependência de células na fase S (*synthesis*) do ciclo celular. Nesta fase, as enzimas de replicação celular estão em maior concentração, propiciando a replicação do vírus (MUZYCZKA *et al.*, 2001).

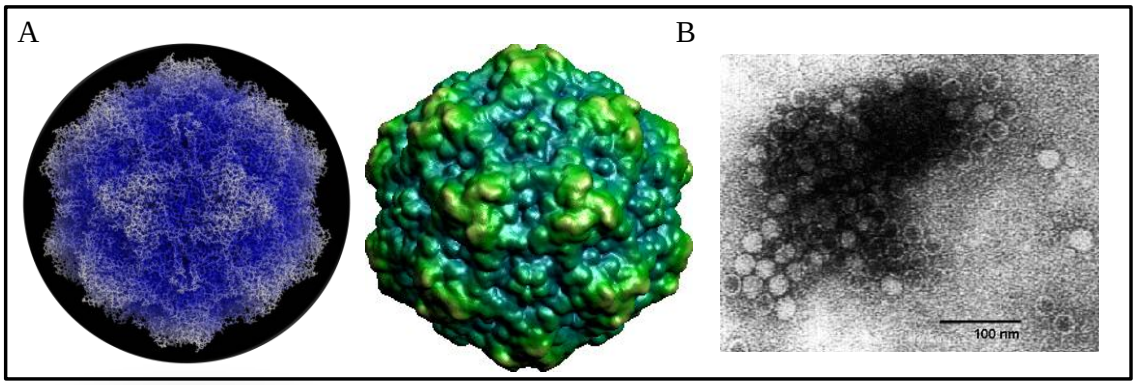

FIGURA 4. Estrutura do capsídeo do parvovírus canino tipo 1 (CPV1, também conhecido como *Canine minute virus, CMV*), um *Bocavirus.* (A) Imagem de crioscopia eletrônica do capsídeo do CPV1 (*Protein data bank,* pdb ID: 4dpv). As imagens foram tratadas no programa *Chimera* (PETTERSEN *et al.*, 2004). (B) Imagem de microscopia eletrônica do CPV1. A barra indica 100 nm (XIE *et al.*, 1996).

A família *Parvoviridae* é dividididda em duas grandes subfamílias: *Parvovirinae,* que contém agentes que infectam mamíferos e *Densovirinae,* que contém agentes que infectam invertebrados (MAYO *et al.*, 2000; FAUQUET *et al.*, 2005). Os principais membros da família *Parvoviridae* estão relacionados na Tabela 2.

Tabela 2. Membros da família *Parvoviridae* (de acordo com o ICTV, revisão de 2009).

Subfamília: *Densovirinae*	
	Gênero: ***Brevidensovirus***
	Espécie: *Aedes aegypti densovirus*
	Espécie: *Aedes albopictus densovirus*
	Gênero: ***Densovirus***
	Espécie: *Galleria mellonella densovirus*
	Espécie: *Junonia coenia densovirus*
	Gênero: ***Iteravirus***
	Espécie: *Bombyx mori densovirus*
	Gênero: ***Pefudensovirus***
	Espécie: *Periplaneta fuliginosa densovirus*
Subfamília: *Parvovirinae*	
	Gênero: ***Amdovirus***
	Espécie: *Aleutian mink disease virus*
	Gênero: ***Bocavirus***
	Espécie: *Bovine parvovirus*
	Espécie: *Canine minute virus*
	Gênero: ***Dependovirus***
	Espécie: *Adeno-associated virus-1*
	Espécie: *Adeno-associated virus-2*
	Espécie: *Adeno-associated virus-3*
	Espécie: *Adeno-associated virus-4*
	Espécie: *Adeno-associated virus-5*
	Espécie: *Avian adeno-associated virus*
	Espécie: *Bovine adeno-associated virus*
	Espécie: *Canine adeno-associated virus*
	Espécie: *Duck parvovirus*
	Espécie: *Equine adeno-associated virus*
	Espécie: *Goose parvovirus*

Espécie: *Ovine adeno-associated virus*
Gênero: ***Erythrovirus***
Espécie: *Human parvovirus B19*
Espécie: *Pig-tailed macaque parvovirus*
Espécie: *Rhesus macaque parvovirus*
Espécie: *Simian parvovirus*
Gênero: ***Parvovirus***
Espécie: *Chicken parvovirus*
Espécie: *Feline panleukopenia virus*
Espécie: *H-1 parvovirus*
Espécie: *HB parvovirus*
Espécie: *Kilham rat virus*
Espécie: *Lapine parvovirus*
Espécie: *LuIII virus*
Espécie: *Minute virus of mice*
Espécie: *Mouse parvovirus 1*
Espécie: *Porcine parvovirus*
Espécie: *RT parvovirus*
Espécie: *Tumor virus X*

O ICTV reconhece cinco gêneros dentro da subfamília *Parvovirinae*: *Amdovirus*, *Dependovirus*, *Erythrovirus*, *Parvovirus* e *Bocavirus* (Comitê Internacional de Taxonomia de Vírus, 2009) (FAUQUET *et al.*, 2005).

O gênero *Bocavirus* recebeu esse nome pela proximidade filogenética de seus membros com o ***Bo****vine parvovirus* e o ***Ca****nine minute virus* (*Bocavirus*) (ALLANDER *et al.*, 2005). Os bocavírus têm sido associados a infecções do trato respiratório e gastrointestinal, particularmente em hospedeiros jovens (MANTEUFEL *et al.*, 2008; SCHILDGEN *et al.*, 2008).

Nos últimos anos, com os avanços das técnicas de metagenômica, muitos genomas de *Parvovirus* foram caracterizados. Em suínos, vários genomas, que posteriormente foram agrupados no gênero *Bocavirus*, foram descobertos em animais apresentando doenças e também em animais clinicamente saudáveis (BLOMSTROM *et*

al., 2009; CHENG *et al.*, 2010; CHEUNG *et al.*, 2010; MCKILLEN *et al.*, 2011; SHAN, LAN, *et al.*, 2011; LI, MA, XIAO, FANG, *et al.*, 2012).

A organização genômica dos bocavírus é similar aos outros membros da subfamília *Parvoviridae,* possuindo duas ORFs principais, codificando uma proteína não estrutural (NS1, sendo associada com a replicação viral) e pelo menos duas proteínas do capsídeo viral (VP1 e VP2). Os membros do gênero *Bocavirus* são diferenciados de vírus de outros gêneros pela presença de uma terceira região promotora (ORF), como pode ser visualizado na Figura 5. Essa terceira região promotora gera uma proteína hiperfosforilada (NP1) cuja função ainda é desconhecida (VICENTE *et al.*, 2007).

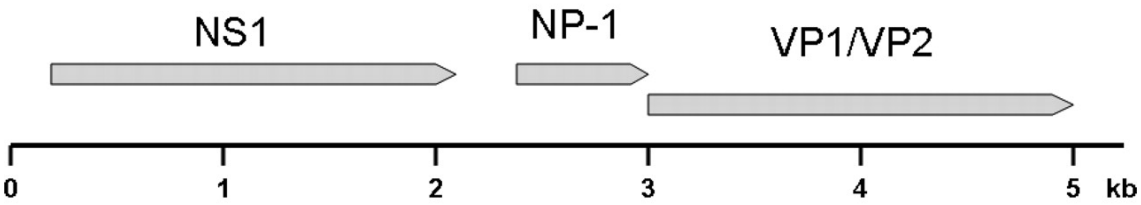

FIGURA 5. Organização genômica do bocavírus humano tipo 1 (HBoV1) (ALLANDER *et al.*, 2005).

Os bocavírus suínos ou porcinos (PBoV) são, entre os membros do gênero *Bocavirus,* os que apresentam uma maior diversidade genética (ZHANG *et al.*, 2011). Vários são os estudos de descrição de novos genomas de PBoV (BLOMSTROM *et al.*, 2009; CHENG *et al.*, 2010; CHEUNG *et al.*, 2010; MCKILLEN *et al.*, 2011; SHAN, LAN, *et al.*, 2011), entretanto, não existe um consenso a respeito da nomenclatura desses novos agentes. A Tabela 3 mostra os genomas de bocavírus identificados até fevereiro de 2012, depositados no *GenBank Taxonomy.* Em um estudo que avaliou a diversidade genética desses novos agentes, quatro grandes grupos foram observados e, baseados nesses grupos, uma nomenclatura provisória, foi proposta (ZHANG *et al.*, 2011) (Figura 6).

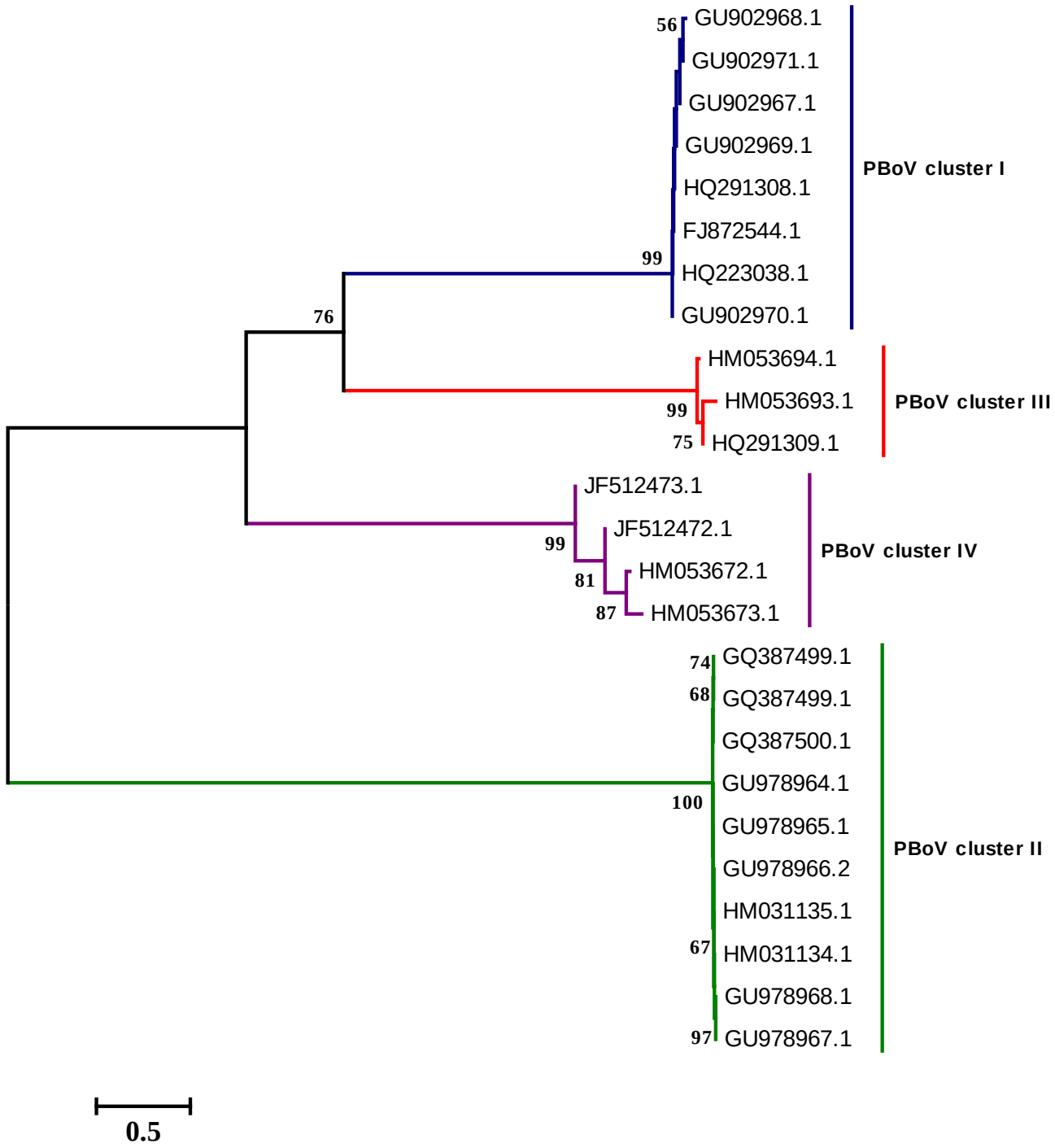

FIGURA 6. Árvore filogenética dos bocavírus suínos. Genomas completos dos bocavírus suínos foram alinhados no MUSCLE. Inferências filogenéticas foram realizadas no MEGA5, utilizando-se 2000 *bootstraps*. A árvore filogenética, originalmente não enraizada, é apresentada nessa forma para facilitar o entendimento.

Tabela 3. Bocavírus não classificados (*NCBI Taxonomy*, fevereiro de 2012). Os bocavírus suínos estão marcados com "▲".

***Bocavirus* não classificados**	**Referência**
Bocavirus chimpanzee/PT-LM1861/CMR	(SHARP *et al.*, 2010)
Bocavirus gorilla/GBoV1/2009	(KAPOOR *et al.*, 2010)
Bocavirus gorilla/GG-CP1426/CMR	(SHARP *et al.*, 2010)
▲*Bocavirus pig/6V/China/2006*	(CHENG *et al.*, 2010)
▲*Bocavirus pig/6V/China/2010*	HQ910439
▲*Bocavirus pig/7V/China/2006*	(CHENG *et al.*, 2010)
▲*Bocavirus pig/7V/China/2010*	HQ910441
▲*Bocavirus pig/China/2009*	(ZHAI *et al.*, 2010)
▲*Bocavirus pig/China/2011*	(LI *et al.*, 2012)
▲*Bocavirus pig/JSNJ1/China/2011*	(LI, 2011)
▲*Bocavirus pig/PBoV1/China/2010*	HQ910443
▲*Bocavirus pig/PBoV2/China/2010*	HQ910445
▲*Bocavirus pig/sw-107/SWE/2010*	(BLOMSTROM *et al.*, 2010)
▲*Bocavirus pig/sw-18/SWE/2010*	(BLOMSTROM *et al.*, 2010)
▲*Bocavirus pig/sw-90_1/SWE/2010*	(BLOMSTROM *et al.*, 2010)
▲*Bocavirus pig/sw-92_2/SWE/2010*	(BLOMSTROM *et al.*, 2010)
▲*Bocavirus pig/sw-A1/SWE/2010*	(BLOMSTROM *et al.*, 2010)
▲*Bocavirus pig/Sw-PBoV/China/2010*	HQ910447
▲*Bocavirus pig/Swebo_1/SWE*	(BLOMSTROM *et al.*, 2009)
▲*Bocavirus pig/SX/China/2010*	(ZENG *et al.*, 2011)
▲*Bocavirus pig/ZJD/China/2006*	(CHENG *et al.*, 2010)
California sea lion bocavirus 1	(LI *et al.*, 2011)
California sea lion bocavirus 2	(LI *et al.*, 2011)
California sea lion bocavirus 3	(LI *et al.*, 2011)
California sea lion bocavirus 4	(LI *et al.*, 2011)
Human bocavirus 1	(ALLANDER *et al.*, 2005)
Human bocavirus 2	(KAPOOR *et al.*, 2009)
Human bocavirus 3	(ARTHUR *et al.*, 2009)
Human bocavirus 4	(KAPOOR *et al.*, 2010)

Pine marten bocavirus	(VAN DEN BRAND *et al.*, 2012)
▲*Porcine boca-like virus*	JN400849
▲*Porcine bocavirus 1*	(CADAR *et al.*, 2011)
▲*Porcine bocavirus 1 pig/ZJD/China/2006*	(CHENG *et al.*, 2010)
▲*Porcine bocavirus 2*	(SHAN *et al.*, 2011)
▲*Porcine bocavirus 2 pig/ZJD/China/2006*	(CHENG *et al.*, 2010)
▲*Porcine bocavirus 3*	(LAU *et al.*, 2011)
▲*Porcine bocavirus 3/64-1/N.Ireland/2004*	(MCKILLEN *et al.*, 2011)
▲*Porcine bocavirus 4*	(LAU *et al.*, 2011)
▲*Porcine bocavirus 4-1*	(LAU *et al.*, 2011)
▲*Porcine bocavirus 4-2*	(LAU *et al.*, 2011)
▲*Porcine bocavirus 4/F41/N.Ireland/2004*	(MCKILLEN *et al.*, 2011)
▲*Porcine bocavirus 5*	(LI *et al.*, 2012)
▲*Porcine bocavirus 5/JS677*	(LI *et al.*, 2012)
▲*Porcine bocavirus 6V7V*	JN400879
▲*Porcine bocavirus type 1-2*	JN400875

Em 2009, foi realizado o primeiro relato de um bocavírus suíno (porcine boca-like virus) (BLOMSTROM *et al.*, 2009), que posteriormente, foi denominado bocavírus suíno tipo 1 (PBoV1) (ZENG *et al.*, 2011). Esse vírus foi identificado em linfonodos de animais acometidos pela SMDS. Em um trabalho realizado pelo grupo que identificou o agente, o PBoV1 foi associado com a SMDS, sendo a detecção de material genético viral duas vezes superior em animais acometidos pela síndrome do que em animais clinicamente saudáveis.

Em um trabalho realizado na China, o PBoV1 foi associado a infecções respiratórias, sendo encontrado em altas frequências nesses animais. Em soros de animais saudáveis o vírus foi encontrado em baixa frequência (ZHAI *et al.*, 2010). Outros autores não encontraram associação da presença do PBoV1 com doença (ZENG *et al.*, 2011; ZHANG *et al.*, 2011). Entretanto, não descartam a ideia de que o PBoV1 possa estar envolvido em patologias suínas.

O PBoV2, também denominado *Porcine parvovirus 4* (PPV4), foi identificado em 2010 por metagenômica em animais que morreram com sinais de SMDS (CHEUNG *et al.*, 2010). Esse vírus foi associado com animais doentes em um estudo realizado na China (sendo que no trabalho os autores não citam quais as doenças que acometiam os

animais) (HUANG *et al.*, 2010), entretanto, outro estudo não conseguiu associar o PBoV2 com nenhuma condição clínica (ZHANG *et al.*, 2011). O PBoV3, bem como o PBoV4, foram identificados primariamente em amostras de fezes de suínos (CHENG *et al.*, 2010; SHAN, LAN, *et al.*, 2011), não associados a enfermidades em suínos, sendo detectados em alta frequência tanto em animais saudáveis e doentes (CHENG *et al.*, 2010; SHAN, LAN, *et al.*, 2011; ZENG *et al.*, 2011).

3. OBJETIVOS

Geral

- Ampliar os conhecimentos sobre a participação de múltiplos agentes virais na patogenia da SMDS.

Específicos

- Pesquisar possíveis associações entre a ocorrência de SMDS e a presença de genomas de citomegalovírus suíno em soro e tecidos de animais saudáveis e com SMDS;
- Pesquisar possíveis associações entre a ocorrência dos recém-descobertos bocavírus suínos em animais saudáveis e com SMDS.

CAPÍTULO 1: Porcine cytomegalovirus infection is not associated to the occurrence of postweaning multisystemic wasting syndrome

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Porcine cytomegalovirus infection is not associated to the occurrence of postweaning multisystemic wasting syndrome

Running title: PCMV infection and PMWS

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Abstract

Porcine cytomegalovirus (PCMV) is a *Betaherpesvirus* that causes lifelong latent infections in swine; occasionally, it may be associated to inclusion body rhinitis in piglets and reproductive disorders in pregnant sows. Postweaning multisystemic wasting syndrome (PMWS) a condition where porcine circovirus type 2 (PCV2) infection is necessary – though not sufficient – to trigger disease, has become one of the major health problems to the porcine productive chain. Despite the high expected prevalence of both PCMV and PCV2 in swine-raising farms, no links between PCMV and PMWS have been investigated so far. In view of that, the present study was conducted to search for relations between PCMV infections and the occurrence of PMWS. Spleen and sera of PMWS-affected and non-PWMS-affected animals were examined in search for PCMV and PCV2 DNA. In PMWS-affected animals, PCMV DNA was detected in 88.4% of the spleen samples and 7.6% of the sera, whereas in non-PMWS affected pigs, PCMV DNA was detected in 72.7% of the spleens and 10% of sera. Such differences were not statistically significant. On the other hand, PCV2 DNA was detected in 100% of the spleens from PMWS-affected pigs, whereas in non-PMWS affected pigs, PCV2 DNA was detected in spleens in 81.8%. Again, this difference was not statistically significant. These findings confirm that despite the high prevalence of PCMV infections in the swine population examined, no positive or negative association could be inferred from the presence of PCMV DNA and the occurrence of PMWS.

Keywords: Porcine cytomegalovirus, PCMV, SuHV-2, PMWS, PCVAD.

Introduction

Postweaning multisystemic wasting syndrome (PMWS) is a porcine disease which has become a major burden to the swine productive chain [1]. PMWS is one of the more often reported conditions presently known as "porcine circovirus associated diseases" (PCVAD). The virus involved, porcine circovirus type 2 (PCV2), is a member of the genus *Circovirus,* family *Circoviridae* (International Committee on Taxonomy of Viruses – ICTV, 2009). PMWS is characterized clinically by pallor of the skin, respiratory distress, occasional diarrhea, jaundice and, most prominently, wasting in postweaning pigs of about 2-4 months of age [2,3]. PCV2 is necessary, though not sufficient, to induce PMWS [4,5]. Variables affecting the virus, the host – including co-infections – and herd management conditions have shown to influence in development of disease [6]. Moreover, one or more agents may be involved. Several studies have been conducted in search for links between PCVAD and other swine pathogens [3,4,7-14].

Porcine cytomegalovirus (PCMV) is a *Betaherpesvirus* that causes lifelong latent infections in swine; occasionally, it may be associated to inclusion body rhinitis in piglets and reproductive disorders in pregnant sows [15]. The virus is ubiquitous in swine, where it induces latent infections in the host, a common feature between members of the *Herpesviridae* family [16-18]. Infection can be reactivated and viral replication enhanced by immunosuppressive conditions [19-22]. Despite the high prevalence PCMV in swine worldwide [23-26], associations between PCMV and PMWS have not been investigated so far. As PCV2 is an immunosupressing virus, it became of interest to examine whether PCMV would play any role in the development of PMWS.

Evidences pointing out the immunosupressive effect of PVC2 are abundant. PMWS-affected pigs show increased incidence of diseases associated with opportunistic pathogens or unexpected pathogens [27-31]. However, the mechanisms by which immunosuppression occurs are not fully understood. Immune function studies in PMWS-affected pigs revealed extensive depletion of lymphocytes in lymphoid tissues [32] and reduction of circulating B and T cells [33]. Moreover, increased interleukin 10 (IL-10) expression was observed in PCV2 infection suggesting that IL-10-mediated immunosuppression may play an important role in the pathogenesis of PMWS [34,35]. These findings support the involvement of the immune system in PMWS pathogenesis.

The aim of this study was to search for any associations between the detection of PCMV DNA in pigs and the occurrence of PMWS.

Materials and methods

Spleen and serum samples

Spleen and serum samples were received from pig farms in the state of Rio Grande do Sul, Brazil. The case group (PMWS-affected pigs) consisted of 77 spleens from 1-4 months old pigs and 92 serum samples from 1-4 months old piglets. In the case group, the pigs displayed dyspnea, enlargement of superficial inguinal lymph nodes, pallor, jaundice and diarrhea. The diagnosis of PMWS was confirmed by typical macroscopic lesions at necropsy, histopathology and demonstration of PCV2 DNA in tissues by PCR.

The control group (non-PMWS-affected pigs) consisted of eleven samples of spleen tissue from healthy pigs at slaughtering age and serum samples of 119 pigs >6 months old. Additional 24 serum samples from healthy, 1-4 months old piglets, were included in the controls.

DNA extraction

DNA extraction from spleens was performed with sodium iodide (NaI) as previously described [36]. DNA of sera was extracted from 500 μL volumes using a phenol-chloroform method. The extracted DNA was resuspended with 50 μL of TE buffer. The quantity and quality of the extracts was analyzed with the aid of a spectrophotometer (Nanodrop® 1000).

PCR assays for PCMV and PCV2

Primers for PCMV detection were designed by Hamel and collaborators [26]. The primer sequences correspond to nucleotides 37 to 64 and 449 to 420 on the PCMV

DNA polymerase gene. The expected size of the amplification product is 413 bp. The PCR was carried out in 25 μL volumes containing 2 μL of DNA (100 ng for spleen samples and 2 μL of DNA extracted from serum samples), 5 pmol of each primer, 0.8 mM dNTPs, 1.5 mM $MgCl_2$ and 1 U Taq DNA polymerase (Invitrogen). The PCR program consisted of an initial reaction at 94 °C for 3 min, followed by 35 cycles at 94 °C (30s), 61 °C (30s) and 72 °C (30s), with a final extension period of 5 min at 72 °C.

Primers for PCV2 detection were designed by Kim and collaborators [37]. The expected size of the amplification product is 476 bp. The PCRs for PCV2 amplification were performed with 0.8 mM dNTPs, 5 pmol of each primer, 100 ng of DNA, 1 U of Taq DNA polymerase (Invitrogen) and distilled water q.s.p. 25 μL. The amplification conditions were 94°C for 4 min, followed by 35 cycles of 94 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min and a final extension step at 72 °C for 10 min.

Positive controls consisted of reactions with cloned PCMV and PCV2 DNA [36]. PCR products were subjected to electrophoresis on a 1% agarose gel, stained with ethidium bromide and visualized on UV light.

To avoid contamination, separate rooms were used to prepare reaction buffers, to prepare the PCR reactions, to extract DNA, and to examine PCR products. Filter tips were used throughout. Additional controls with ultra-pure water instead of sample DNA were included in every ten PCR tubes.

Possible inhibitory effects of serum DNA on PCR reaction were evaluated by amplification of an unrelated amplicon (Torque teno sus virus; TTSuV) in a separate PCR performed which each serum sample (data not shown).

Sensitivity assay

In order to determine the PCMV PCR sensitivity, an amplicon from a spleen containing PCMV DNA (as later confirmed by sequencing) was cloned in plasmid pCR 2.1 following the manufacturer's protocol (pCR 2.1 TOPO TA Cloning Kit, Invitrogen). The sensitivity of the PCR was determined by amplification of tenfold dilutions of known amounts of plasmid DNA. These experiments were repeated three times.

The PCV2 PCR assay sensitivity was determined as previously described [36] it was shown to amplify PCV2 DNA from as little as 20 molecules of plasmid-cloned containing the full PCV2 genome. The two plasmids containing either the PCMV or the PCV2 genome fragments, were used as positive controls in the PCR assays throughout the study.

Statistical analysis

Statistical analysis was performed applying the χ^2 test available in DagStat [38] to compare the proportion of PCMV and PCV2 detected in the case and control groups. The level of significance was set to $P \leq 0.05$.

Results

Detection of PCMV DNA

The PCR employed to amplify PCMV genome fragments in spleen and sera of PMWS-affected and non-PMWS-affected pigs was shown to amplify a minimum number of genome copies equivalent to 100 molecules/reaction of PCMV DNA, as extracted from the PCMV DNA-containing plasmid (Figure 1).

In spleen tissues, PCMV DNA was detected in 88.3% (68/77) of samples from PMWS-affected pigs and in 72.7% (8/11) from non-PMWS-affected pigs (Table 1). Such differences were not statistically significant (*P≥0.05).*

In sera, PCMV DNA was identified in 7.6% (7/92) of PMWS-affected pigs (Table 1), whereas in non-PMWS-affected pigs PCMV DNA was detected in 8.3% (2/24) in 1-4 months old pigs 11.8% (14/119) of >6 months old pigs (Table 1). Statistical analysis of the frequencies of PCMV DNAemia in PMWS-affected and non-PMWS-affected groups revealed that such frequencies were not significantly different (*P≥ 0.05).*

Detection of PCV2 DNA

All PMWS-affected animals revealed to contain PCV2 DNA in spleen tissues. In spleen samples from non-PMWS-affected pigs, PCV2 DNA was detected at a slightly lower frequency. However, such differences were not significantly different when subjected to statistical analysis (*P≥0.05*).

Discussion

Associations of PCV2 with other swine pathogens have eventually been shown to be play some role in the development of PCVAD. Co-infections with PCV2 and other pig pathogens may lead to the development of PMWS [3]. Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) can act synergistically with PCV2 and lead to PMWS development [10,11]. Porcine parvovirus (PPV) has also been found to play a role in PMWS: co-infections with PCV2 and PPV were detected in approximately 15% of PMWS cases [9]. In addition, experimental co-infections with PCV2 and PPV resulted in PMWS reproduction in gnotobiotic, colostrum deprived pigs [7]. Other pathogens which have been reported to be capable of participating in the establishment of PMWS include *Mycoplasma hyopneumoniae* [39], *Cryptosporidium parvum* [28], Aujeszky's disease virus [40,41], influenza virus and bacterial pneumonias [10,42-44]. Against such a background, a search for a role for PCMV in PMWS, particular in view of the immunossupressive potential of PCV2, would be expected to reveal some sort of interaction. PCV2 is known to induce immunosuppressive effects on the host, predisposing to viral, bacterial and mycotic infections [45]. Opportunistic infections have been often observed in herds with PMWS [27-30,39,42]. Whether the immunosuppressing effect of PCV2 facilitates co-infection or whether a co-infecting pathogen would induce immunosuppression that could trigger PMWS still remains a matter of controversy.

In the present study, a search was made for PCMV in spleens and sera of PMWS-affected and non-PMWS-affected pigs. The presence of PCMV DNA in spleen is indicative of previous infection. This is important in view of the biology of herpesviruses, where latent infections are produced and active virus replication may not

be directly linked to any particular disease. In the present study, virus isolation was not attempted because PCMV recovery by such method has revealed low sensitivity [46-48].

In humans, HCMV DNA in serum was considered a sign of active viral replication, and was associated to clinical disease [49,50]. On the other side, HCMV DNA in leukocytes was considered indicative of latent infections [51]. Here, PCMV DNA was detected in serum in proportions that did not differ significantly between groups of PWMS-affected and non-PMWS-affected groups of pigs. A slightly larger proportion of PCMV DNA-containing samples was found in older, non-PMWS-affected pigs, (Table 1). However, this finding was probably more related to exposure related to aging than to other conditions, since these pigs had no signs of disease. The proportions of PCMV DNA-bearing animals were not significantly different between the two groups, revealing that the presence of PCMV DNA in serum is not associated to the occurrence of PMWS in the sampled population. More likely, PCMV DNA in sera of both PMWS-affected and non-affected pigs to equivalent proportions suggest that serum PCMV DNA is indicative of latent, rather than active PCMV infections.

The results presented here show that PCMV DNA was detected in spleens of a high proportion of the sampled animals – either with or without PWMS. The frequency of detection of PCMV DNA in the sampled population is similar to those reported in previous studies [23-26,52]. Despite the high frequency of PCMV and PCV2 infections in both groups of animals, no association between the presence of PCMV DNA and the occurrence of PMWS could be inferred. Therefore, despite the high prevalences of PCMV and PCV2 in the examined population of pigs, PCMV does not seem to play any significant role in the occurrence of PMWS.

The results reported here show that the prevalence of PCMV infections in the population examined is high in both PMWS-affected and non-PMWS-affected animals. Therefore, PCMV does not seem to play any significant role in the development of PMWS.

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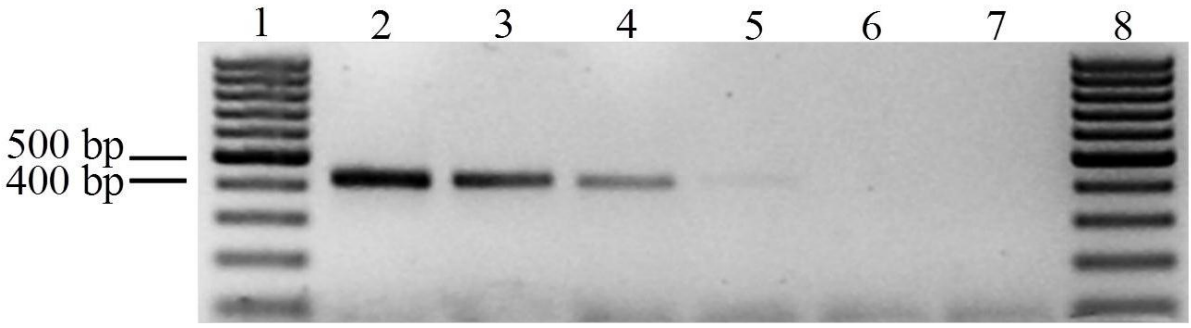
Figure 1. Determination of PCR sensitivity for detection of PCMV. Known copy numbers of the plasmid containing the PCR- targeted region of the PCMV DNA polimerase (*DPOL)* gene (lanes 2 to 6: 10^5 to 10^1 molecules per reaction, respectively) were submitted to PCR amplification (for details refer to methods). The detection limit of the PCR was 100 molecules/reaction. Lanes 1 and 8, molecular size marker; lane 7: negative control.

Table 1. Porcine cytomegalovirus **(**PCMV) DNA detection in spleen and sera from PMWS-affected and non-PMWS-affected pigs.

	Groups	No. of samples tested	Samples with PCMV DNA
Spleen	PMWS-affected pigs (1-4 months old)	77	68 (88.4%)
	Non-PMWS-affected pigs (> 6 months old)	11	8 (72.7%)
	Total	88	76 (86.4%)
Serum	PMWS-affected pigs (1-4 months old)	92	7 (7.6%)
	Non-PMWS-affected pigs (>6 months old)	119	14 (11.8%)
	Non-PMWS-affected pigs (1-4 months old)	24	2 (8.3%)
	Total	235	24 (10.2%)

Figure 1.



CAPÍTULO 2: The role of Porcine bocaviruses in postweaning multisystemic wasting syndrome (PMWS)

*Artigo a ser submetido ao periódico PLoS ONE.

The role of Porcine bocaviruses in postweaning multisystemic wasting syndrome (PMWS)

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Abstract

The present study was conducted in search for DNA of porcine bocavirus (PoBV) 1, 2, 3 and 4 in tissues (lungs, kidneys, livers, spleens and lymph nodes) and sera of pigs with post-weaning multisystemic wasting syndrome (PMWS) and non-PMWS-affected pigs. The samples were analyzed by PCR, targeting a high conserved region of each PBoV cluster. A positive association was found among the frequency of detection of PBoV2 and PBoV3 genomes and the occurrence of PMWS, whereas no association was in detection of PBoV1 or PBoV4 genomes. High rates of adult healthy animals carry genetic material of PBoV2 and 4 in tissues and PBoV1, PBoV2 and PBoV4 in sera. These results suggest that PCV-2 co-infection with PBoV2 and PBoV3 may play an important role in PMWS and detection of viral DNA in healthy adult subjects could represent long-time virus shedding following an asymptomatic infection during adulthood.

Keywords: porcine bocavirus, PBoV1, PBoV2, PBoV3, PBoV4, PPV4, PBo-likeV, PMWS, PCVAD.

Introduction

The family *Parvoviridae* comprises viruses with a linear, single stranded genome of about 4000-6000 nucleotides, with either positive or negative polarity, surrounded by a naked capsid with icosahedral symmetry [1]. The family is presently subdivided in two subfamilies: *Parvovirinae*, which includes viruses that affect vertebrates, and *Densovirinae*, whose members infect arthropods (International Committee on Taxonomy of Viruses, ICTV 2009) [2]. While some viruses of the *Parvoviridae* are known to cause mild disease or asymptomatic infections, others have been linked to fetal death and abortions in animals and humans [3-5].

The *Parvovirinae* subfamily comprises five genera: *Amdovirus, Dependovirus, Erythrovirus, Parvovirus and Bocavirus*, plus *a* newly proposed genus, *Hokovirus* (International Committee on Taxonomy of Viruses, 2009) [2]. Bocaviruses have been associated to respiratory and gastrointestinal tract infections, particularly in the young. The genome of members of the *Bocavirus* genus can be distinguished from viruses from other genuses of the family by the presence of a third open reading frame (ORF) located between the non-structural and structural protein-coding regions [6]. Such ORF encodes an additional non-structural protein, named NP1, whose function is unknown. Recently, many PBoVs have been detected in swine [7-11]; these were subdivided in four species, PBoV1, 2, 3 and 4 [12]. Porcine bocaviruses exhibit the greatest genetic diversity among all bocaviruses so far identified [12].

Associations between PBoVs and disease have been investigated. Porcine bocavirus type 1 (PBoV1, previously named “porcine boca-like virus”, or “PBo-likeV”) was reported to be significantly more prevalent in weanling piglets with respiratory signs than in healthy animals [13]. In addition, PBoV1 DNA was detected in PMWS-

affected pigs twice more often than in healthy animals, [10,14]. However, others found no association between PBoV1 and disease [12].

Porcine bocavirus type 2 (PBoV2), also referred to as porcine parvovirus type 4 or *PPV4*, was identified in lung washings of pigs with *porcine circovirus associated disease* (PCVAD) [7]. Phylogenetically, PBoV2 is more closely related to bovine parvovirus 2 (BPV2), which is not currently included in the *Bocavirus* genus. PBoV2 encodes an ORF3 in the intermediate region of the viral genome that resembles the genomes of members of the *Bocavirus* genus [15]. The other two porcine bocavirus types, PBoV3 and PBoV4, were detected in stools of healthy pigs [8,9]. More recently, two distinct PBoV4 genotypes were isolated from pigs with post weaning wasting syndrome (PMWS) [11].

The condition known as PMWS is presently regarded as the major infectious cause of losses in the pig productive chain [16]. It is the major *porcine circovirus associated disease* (PCVAD), a term which encompasses different syndromes where infection with porcine circovirus type 2 (PCV2) is necessary - though not sufficient - to induce disease [17-20]. Development of PMWS may be influenced by the viral strain, herd management, host factors and co-infections with other pathogens [21].

In view of the significance of PMWS and the possible role for other agents in its pathogenicity, the present study was performed in search for possible associations between PBoVs infections and PMWS. The frequency of detection of PBoVs genomes in pigs and viral loads were determined in different tissue samples and sera of PMWS-affected and non-PMWS-affected pigs.

Results

Sensitivity of PCRs to detect PoBV1, PBoV4 and a duplex PCR to PBoV2 and PBoV3

The sensitivity of the PBoV1-4 PCRs were determined by assaying serial tenfold dilutions of known quantities (10^4 to 10^0 molecules) of the plasmid containing the cloned PBoV1, PBoV2, PBoV3 or PBoV4 fragment. To be confident that genomic PBoV1 DNA could be amplified in the presence of isolated tissue and serum DNA, an internal control molecule was constructed that could be amplified by the same primers as the PBoV1 viral DNA and was added to each PCR reaction. To differentiate between the viral product and the internal control product the amplified region of the internal control construct was made 133 bp longer than the viral product (496 bp) (see methods). The inhibition rate of the PCR PBoV1 was approximately 2% (data not shown). Only DNA that did not inhibit the PBoV1 reaction was used in detection of other PBoVs.

The products of a hundred of molecules of IC and a hundred molecules of the cloned PBoV1 segment could still easily be visualized in ethidium bromide-stained agarose gels (Figure 2A).

The duplex PCR, designed to detect genomes of PBoV2 and PBoV3 simultaneously, as well as PBoV4 PCR, were able to detect approximately 100 molecules of correspondent cloned PBoV fragment (Figure 2B and 2C).

Detection of PBoV1-4 genomes in tissues

The frequency of detection of PBoVs genomes in spleens, lungs, livers, kidneys and lymph nodes are shown in Figure 3. The animal was considered positive in the presence of viral genome fragment in at least one of the organs analyzed.

PBoV1 genomes was detected solely in PMWS-affected tissues samples and was three times higher in lymph nodes than in other tissues examined, it seems the virus is not uniformly distributed in the organs tested (Figure 4A).

PBoV2 had a higher detection rate in healthy animals (at slaughtering age) than in PMWS-affected animals (27.7% vs. 54.5%). There was no difference on detection of PBoV2 genomes among different organs analyzed (Figure 4).

The PBoV3 genomes detection rates were the lowest among the porcine bocavirus viruses studied (Figure 3). It could only be detected in PMWS-affected animals, still with a low frequency (5.3%) and was not detected at all in healthy adult animals. PBoV3 genomes were detected solely in samples of spleen tissues (Figure 4A).

PBoV4 DNA was detected to high frequencies in both healthy and PMWS-affected pigs (Figure 3). The rate of detection of the PBoV4 was approximately the same found in animals with or without the syndrome. As well as PBoV2, the PBoV4 genomes were detected in adult animals (Figure 3). The detection of PBoV4 varied among the organs examined, being more frequently in samples from lymph nodes tissues (Figure 4).

Detection of PBoV1-4 in sera

The results on the detection of PBoV genomes in serum samples from PMWS-affected and non-PMWS-affected animals are summarized in Table 2. Statistical analysis showed that frequencies of PBoV1 DNAemia in PMWS-affected and non-PMWS-affected groups not significantly differ (32.6% in PMWS-affected and 33.3%in healthy pigs at same age). Thus, no association was found between the detection of PBoV1 genomes in serum and the occurrence of PMWS (Table 2).

A six-fold higher frequency of PBoV2 DNAemia was detected in PMWS-affected pigs when compared with controls animals at the same age (41.2% vs. 6.7%, respectively). This reveals a strong association between the occurrence of PMWS and PBoV2 DNAemia ($P \leq 0.05$). However, the frequency of detection of PBoV2 in serum in adult healthy was as high as that detected in PMWS-affected pigs (Table 2).

Regarding PBoV3, no copies of the viral genome were detected in serum samples. The results of the search for PBoV4 genomes revealed a low frequency of detection of this virus type in serum samples (Table 2). A non-significant difference was detected between the frequencies of detection of PBoV4 DNA in PMWS-affected pigs and non-affected pigs.

Detection of PCV2 DNA in tissues

All PMWS-affected animals contained PCV2 DNA in all tissue samples examined (spleen, lymph nodes, liver, lung and kidney), whereas in non-PMWS-affected pigs, PCV2 DNA was detected at a slightly lower frequency (82%).

Unlike the PMWS-affected animals the rate of detection of PCV2 in healthy animals varied among the tissues analyzed. The rates of detection of PCV2 were: spleen (63.6%), kidney (63.6%), lung (45.6%), liver and lymph node (27.3%).

Discussion

Animal bocaviruses have been known since the early 1960s [22,23]. So far, the ICTV recognizes only two species of animal bocavirus: CMV (Canine minute virus) and BPV1 (Bovine parvovirus type 1). The genus *Bocavirus* gained special attention when a human bocavirus genome (HBoV) was detected in pooled specimens from the respiratory tract of children with respiratory disease [24]. Since then, bocaviruses were identified in gorillas (GBoV1) [25], pigs [7-11], as well as another three HBoVs in humans [22-24,26-28]. Whether any of the four HBoV species causes human disease is still undetermined [29-31].

Although some association studies of porcine bocaviruses with swine diseases have been conducted, the exact clinical pictures a wait determination. The present study was performed to examine the frequency of distribution of PBoV1, 2, 3 and 4 in PMWS-affected (age range 1-3 months old) and non-PMWS-affected pigs in two age groups (1-2 months old and 6-19 months old pigs) in order to search for possible associations between the occurrence – or absence – of disease.

In this study, PBoV1 DNA in serum was detected at high frequency in both groups of animals, affected or not by PMWS (33.3% and 34.7%, respectively). The frequency of detection did not differ statistically between groups ($P \geq 0.05$). This result suggests that the presence of PBoV1 DNAemia does not contribute to the PMWS development.

When tissue samples from PMWS-affected pigs were analyzed for the presence of PBoV1 DNA, 40% of the animals were positive for at least one organ investigated. However, it was observed a tissue-specific distribution PBoV1. In lymph nodes the rate of virus detection was 3 times higher than the other tissues examined. This result

provides insights into PBoV1 tropism for immune cells as well as important input on the choice of best tissue to investigate the presence of PBoV1 in pig organs.

PBoV1 DNA was not found in tissues from adult animals (slaughter-age), in any of the analyzed tissues. Interestingly, the viral DNA was detected in approximately 10% of serum samples from adult animals. This may be because the age of these animals varied between 6 and 19 months, and these younger animals could still be harboring the virus.

PBoV1 genomes was reported to be more prevalent in PMWS-affected (88%) than in non-PMWS- affected (46%) pigs [14], indicating that the PBoV1 may have some relationship with PMWS. Interestingly, our study no found this association. This variation may arise from different management conditions or other factors related to the geography of the regions where sampled pigs were raised.

In addition, PBoV1 genomes were detected in bronchoalveolar washings from pigs suffering with respiratory disease to significantly higher rates than in healthy animals [13]. These results led the authors to propose that PBoV1 is an emerging virus associated to diseases of the porcine respiratory tract. However, two studies recently conducted in China, reported the prevalence of PBoV1 in more than 30% of clinically healthy animals, found no significant difference in the distribution of PBoV1 in sick and healthy animals sampled [12,32].

Our findings reveal a high prevalence of PBoV2 tissue and serum samples in both affected and non-PMWS-affected animals. However, the frequency of detection varies greatly when analyzing animals of different ages. In addition, the present data shown that PBoV2 were detected at 42.1% in serum of PMWS-affected animals, while in healthy animals with the same age, the virus was detected six times less (frequency of detection of 6.7%). These findings suggest an involvement of PBoV2 on PMWS. In

tissue samples from PMWS-affected animals, the virus was detected in 27.7% of the sampling pigs. Interestingly, approximately 50% of adult animals showed PBoV2 genomes in tissues and 40% in serum samples. These data suggest some kind of viral persistence PBoV2 (discussed below). Unlike PBoV1, no target tissue has been shown to detect PBoV2.

The PBoV2 was identified primarily in animals co-infected with porcine circovirus type 2 (PCV2) from field cases of severe PCV2-associated disease. The virus, identified by metagenomics techniques, has been associated with severe respiratory disease [7]. In a Chinese report [33], the PBoV2 was detected at a rate of 2.1% (12/573) among the clinical samples examined (7 sows about seven months and 3 boars about four months old and two from a dead piglet about 8 weeks old) and 0.8% among the samples taken from healthy animals (1/132). The adult pigs were diagnosed with reproductive failure, while the sick piglet had displayed fever and neurologic symptoms. Zhang et al. found higher rates infections in healthy animals (~ 16%) than in diseased animals (~ 4%) [12].

The PBoV3 was the bocavirus with lowest rate of detection in this work. No animals (healthy or PMWS-affected) presented PBoV3 DNAemia. When analyzed tissue samples from healthy animals, any sample were positive. The PBoV3 was found solely in 6.4% of spleen samples from PMWS-affected animals. Zhang et al. detected the PBoV3 with a higher frequency in diseased animals than in healthy animals [12]. Our results suggest a possible association of PBoV3 and PMWS, although the detection rate was very low. Therefore, further studies are needed in order to elucidate this possible association.

The PBoV4 was the most prevalent porcine bocavirus in tissues analyzed. The frequency of detection of this agent was close to 100% in tissues of healthy adult

animals. In PMWS-affected animals, the detection was approximately 70%. The PBoV4 DNAemia was low in adult animals (2%), while in young animals was slightly higher (4.9% in PMWS-affected and 11.2% in healthy pigs with the same age). These results corroborate with the findings of Zhang et al. [12]. Like PBoV1 the PBoV4 was detected more frequently in lymph node tissues.

Interestingly, a high rate of adult animals, with no signs of disease showed presence of genetic material of PBoVs in tissues and in serum. Furthermore, we cannot rule out some kind of viral persistence in these animals, which should be investigated. In HBoV, recent findings suggest that persistent infection may occur in mucosal lymphocytes [34,35]. Moreover, the virus was shown to persist in host cells by forming extra-chromosomal closed circular (episomal) [36,37]. The observations that HBoV genome exist as head-to-tail monomer in infected tissue either reflects the likely evolution of alternative replication mechanism in primate bocaviruses or a mechanism of viral persistence in their host. These mechanisms of viral persistence of HBoV should be studied in PBoVs, since the persistence of the viral DNA may be a special viral advantage to reactivation or a role in viral pathogenesis.

In summary, we found a positive association between the detection of PBoV2 and PBoV3 genomes and the occurrence of PMWS and no association was found in detection PBoV1 or PBoV4 genomes in PMWS animals. Interestingly, clinically healthy adult animals showed PBoV1, PBoV2 and PBoV4 genomes in serum samples while in tissue samples only the PBoV2 and PBoV4 were found. Therefore, it is quite clear the need for more studies to delineate the potential to cause disease of newly discovered PBoVs and that can have the interactions with PMWS and other co-infections.

Materials and methods

Tissue, serum samples and DNA extraction

Tissues and serum samples were received from pig farms from the state of Rio Grande do Sul, Brazil. PMWS-affected pigs consisted of 376 tissue samples (73 spleens, 76 livers, 73 lymph nodes, 73 lungs and 81 kidneys) from 94 pigs (2-4 months old). In addition, 102 serum samples from PMWS-affected, 1-3 months old piglets, were included in the study. Samples from pigs with PMWS were collected at necropsy, which was performed when the animals were displaying clinical signs of PMWS (dyspnea, enlargement of superficial inguinal lymph nodes, pallor, jaundice and diarrhea). The diagnosis of PMWS was confirmed by identification of typical macroscopic lesions at necropsy, histopathology and demonstration of PCV2 DNA in tissues by PCR [38,39].

The non-PMWS-affected group consisted of eleven 55 tissue samples (spleen, liver, kidney, lymph nodes and lung tissue from healthy pigs, collected at slaughtering age). Additional serum samples from 98 pigs, 6 to 19 months old, plus 89 serum samples from healthy, 1-2 months old piglets, were included as controls.

Tissue and serum samples DNA extraction

DNA extraction from tissues was performed with sodium iodide (NaI) as previously described [40]. DNA of sera was extracted from 500 μL volumes using a universal phenol-chloroform extraction method [41]. The extracted DNA was resuspended in 50 μL of TE buffer. The quantity and quality of the extracts was analyzed with the aid of a spectrophotometer (Nanodrop® 1000).

PBoVs primers design

For the detection of PBoV1, the primers used were described previously [13].

The primers for detection of the other three PBoV types were designed as follows: all available nucleotide sequences of PBoV deposited in GenBank were downloaded (Supplementary Table 1) and aligned with the MUSCLE software [42], with occasional (manual) editions, when necessary. A phylogenetic analysis was inferred by Neighbor-Joining and the Kimura 2-parameter methods in MEGA5 [43]. The phylogenetic tree so designed contained four large clusters, I (PBoV1), II (PBoV2), III (PBoV3) and 4 (PBoV4) (Figure 1). For detection of PBoVs of each cluster (with exception of PBoV1, or PBoV cluster I, as already mentioned), primers were designed in *Geneious* program, looking for a highly conserved region among the sequences that make up each cluster [44]. All primers for PBoVs and also to detect PCV2 were listed in Table 1.

PBoV1 and PBoV4 PCR

For PBoV1 and PBoV4 PCRs, assays were prepared in 25 μL volumes containing 2 μL of extracted DNA (100 ng for tissue samples and 2 μL of DNA extracted from serum samples), 5 pmol of each primer, 0.8 mM dNTPs, 1.5 mM $MgCl_2$ and 1 U Taq DNA polymerase (Invitrogen). The PBoV1 PCR program consisted of an initial denaturation step at 94 °C for 3 min, followed by 35 cycles at 94 °C (30s), 58 °C (30s) and 72 °C (30s), with a final extension period of 5 min at 72 °C. The PBoV4 PCR program was the same of PBoV1, except for the annealing temperature, which was 65 °C. Positive controls consisted of reactions with genome fragments of PBoV1 and PBoV4 previously cloned (using the pCR2.1 TOPO TA system, Invitrogen).

PBoV2 and PBoV3 duplex PCR

For detection of PBoV2 and PBoV3, a duplex PCR was performed. The primers, designed as outlined above, are listed in Table 1. The reaction was carried out in 20 μL volumes containing 2 μL of DNA (100 ng for tissue samples and 2 μL of DNA extracted from serum samples), 5 pmol of each primer, 0.8 mM dNTPs, 4 μL of 5X Phusion HF Buffer and 0.4 U Phusion High-Fidelity DNA polymerase (Finnzymes). The PCR program consisted of an initial denaturation step at 98 °C for 1.5 min, followed by 35 cycles at 98 °C (10s) and 72 °C (20s), with a final extension period of 1.5 min at 72 °C. Positive controls consisted of reactions with genome fragments of PBoV2 and PBoV3 previously cloned (using the pCR2.1 TOPO TA system, Invitrogen).

PCV2 PCR

For PCV2, the primers used were those designed by Kim and collaborators [45] (listed in Table 1). The expected size of the amplification product is 476 bp. The PCRs for PCV2 were performed in 25 μL volumes, with 0.8 mM dNTPs, 5 pmol of each primer, 100 ng of DNA and 1 U of Taq DNA polymerase (Invitrogen). The amplification conditions were: denaturation at 94°C for 4 min, followed by 35 cycles of 94 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min and a final extension step at 72 °C for 10 min. Positive controls consisted of cloned PCV2 DNA in pCR2.1 vector (Invitrogen) [40]. PCR products were subjected to electrophoresis in a 1% agarose gel, stained with ethidium bromide and visualized on UV light [41].

To avoid contamination, separate rooms were used to extract DNA, to prepare reaction buffers, to prepare the PCR reactions, to extract DNA, and to examine PCR

products. Filter tips were used throughout. Additional controls with ultra-pure water instead of sample DNA were included in every ten PCR tubes.

Sensitivity of the assays

In order to determine the PBoV1-4 PCR sensitivities, amplicons obtained from samples containing PBoV1, 2, 3 and 4 DNA were cloned in plasmid pCR2.1 following the manufacturer's protocol (pCR2.1 TOPO TA, Invitrogen). The sensitivity of the PCRs was determined by amplification of tenfold dilutions of known amounts of plasmid DNA. These experiments were repeated three times, in three different days.

The PCV2 PCR assay sensitivity was determined as previously described [40]. The assay was shown to amplify as little as 20 molecules of PCV2, as determined by amplification of a plasmid containing the full PCV2 genome. The five plasmids containing either the PBoV1, 2, 3, 4 or the PCV2 fragments, were used as positive controls in the PCRs throughout the study.

PCR internal control

In order to detect the possible presence of PCR inhibitors, an internal control (IC) for PCRs was constructed as follows: a PCR with the same primers used for PBoV1 amplification was performed with total DNA extracted from swine lung tissue samples under very low stringency conditions (i.e. annealing temperature 40 °C). The obtained product was examined by electrophoresis and stained with EtBr as above. An amplicon corresponding to a DNA band of 630 bp (as later determined) was excised from the gel, cloned (in pCR2.1 TOPO TA system, Invitrogen) and sequenced. The plasmid containing the internal control (IC) was quantified and 100 molecules were added to each PBoV1 PCR tube. The results of reactions were recorded as positive

whenever the amplicon of the expected size was present concomitantly with the amplicon corresponding to the IC. The absence of an IC band was considered indicative of failure in the process; whenever it happened, procedures were checked and repeated until consistent results were obtained.

Statistical analysis

Statistical analysis was performed by applying the χ^2 test available in the DagStat software [46] to compare the proportion of PBoVs and PCV2 detected in PMWS-affected and non-affected animals. The level of significance was set to $P \leq 0.05$.

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Table 1. Primers to detect PCV2, PBoV1, 2, 3 and 4 used in this study.

Swine vírus	Primer name	Sequence (5'-3')	Amplification size (bp)	References
Porcine bocavirus cluster I	Sboca-F	GGGCGAGAACATTGAAGAGGT	496	Zhai et al., 2010
	Sboca-R	TTGTGAGTATGGGTATTGGTG		
Porcine bocavirus cluster II	PBoV2-F	TTCGGCAGGCGGAGGCTTTG	160	This study
	PBoV2-R	CGCGGAGTACCAGCGGACAC		
Porcine bocavirus cluster III	PBoV3-F	GGAGGTCGATGGCACCCACG	257	This study
	PBoV3-R	GCCCTTTCCGATCCACCCGC		
Porcine bocavirus cluster IV	PBoV4-F	AAAGCCGACGAGGCCGCAAA	356	This study
	PBoV4-R	GCCTCACCCGCYCCTTGTTC		
Porcine circovirus	1094F	CGGATATTGTAGTCCTGGTCG	476	Kim et al., 2001; Dezen et al., 2011
	1569R	ACTGTCAAGGCTACCACAGTCA		

Table 2. Porcine bocaviruses DNAemia. Two microliters of the extracted DNA was submitted to PCR amplification with specific primers to PBoV1, 2, 3 and 4. The frequency of detection of PBoVs DNAemia in PMWS-affected and non-PMWS-affected pigs (young and adults) were shown.

			Prevalence (%)			
	Age	No. samples tested	PBoV1	PBoV2	PBoV3	PBoV4
Healthy	Young (1-4 months old)	89	32.6	6.7	0.0	11.2
	Adult (> 6 months old)	98	9.2	40.8	0.0	2.0
PMWS-affected	1-4 months old	102	33.3	41.2	0.0	4.9

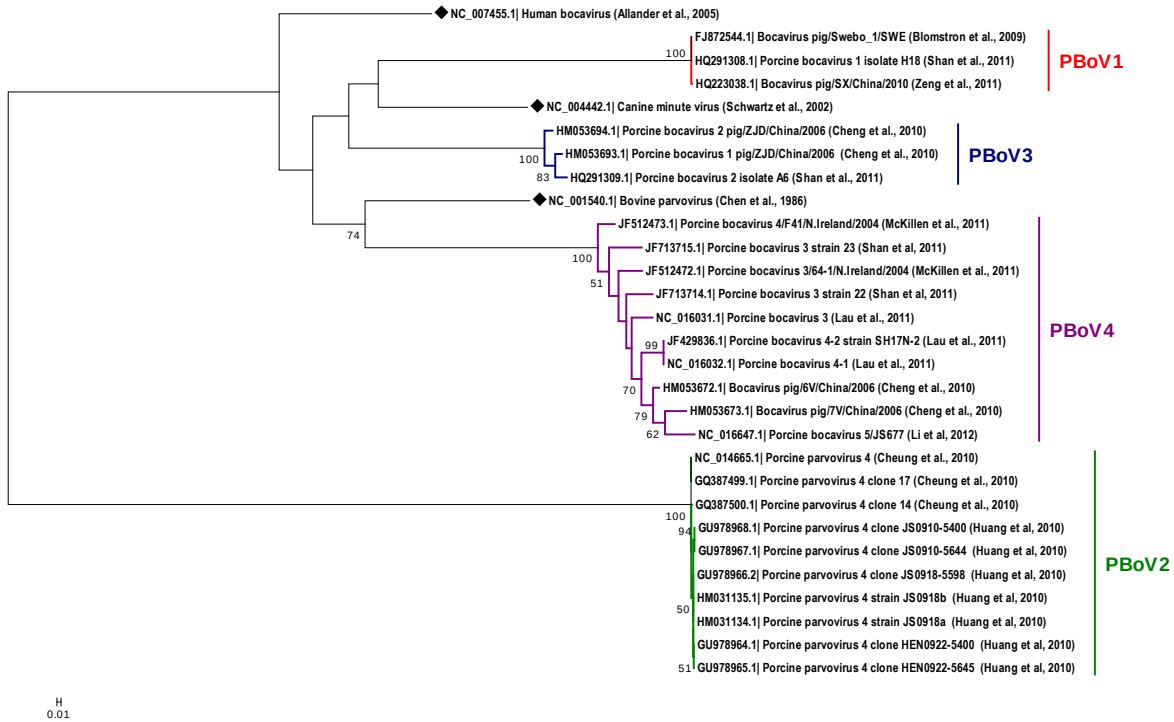

Figure 1. Phylogenetic analysis of complete genomes of porcine bocaviruses available on Genbank. Phylogenetic analysis was inferred using the Maximum Likelihood based on the Hasegawa-Kishino-Yano model. Evolutionary analyses were conducted in MEGA5.

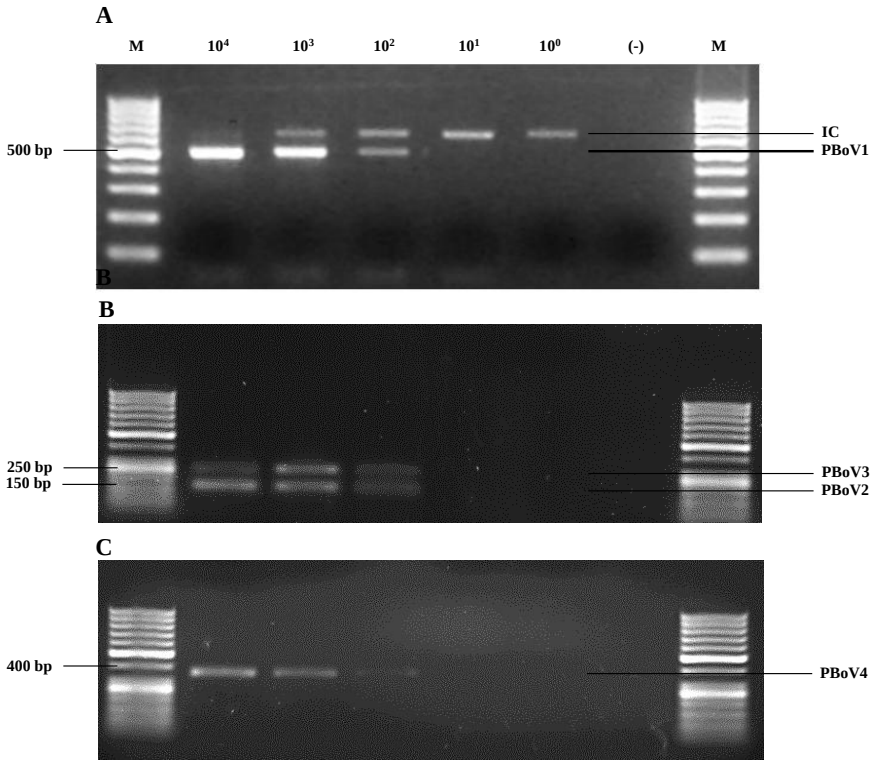

Figure 2. Determination of PCR sensitivity. Known copy numbers of plasmids containing the PCR-targeted region of the PBoV1, 2, 3 and 4 were submitted to PCR amplification. **2A.** Known copy numbers of the plasmid containing the PCR-targeted region of the PBoV1 were amplified concomitantly with 100 molecules of the internal control (IC; refer to methods for details). The detection limit of the PCR was 100 molecules/reaction (lanes 2 to 6: 10^4 to 10^0 molecules per reaction, respectively). Lanes 1 and 8, 100 bp molecular size marker. **2B.** PBoV2 and 3 multiplex PCR sensitivity. Lane 1 and 8, 50 bp molecular size marker. **2C.** PBoV4 PCR sensitivity. Lane 1 and 8, 50 bp molecular size marker.

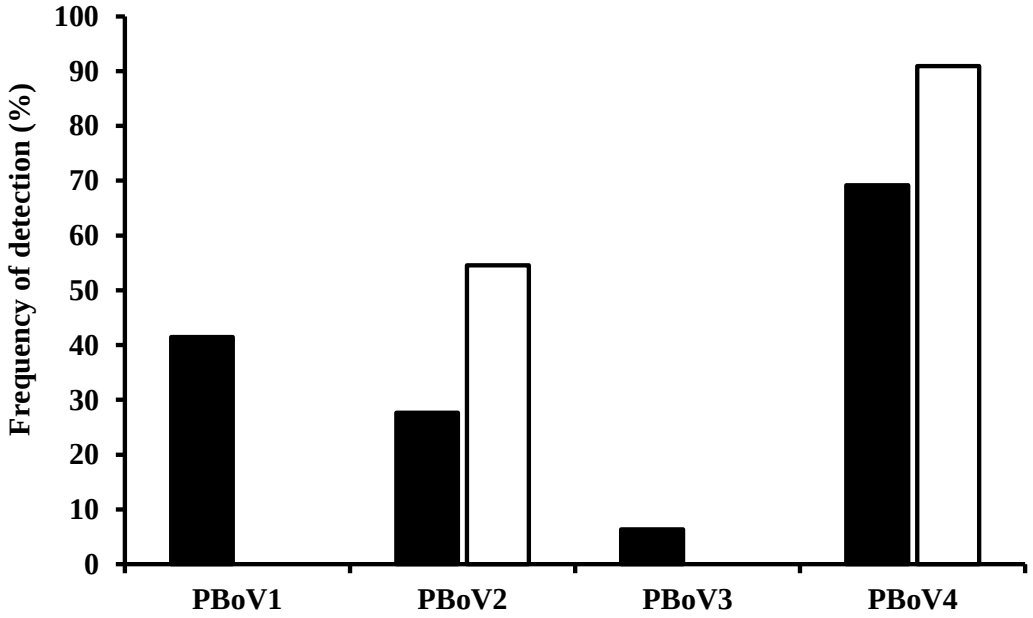

Figure 3. Frequency of detection of PBoVs in PMWS-affected pigs (black bars) and healthy adult pigs (empty bars). Ninety-four PMWS-affected animals and eleven healthy adult pigs were tested for the presence of PBoV1, 2, 3 and 4 in five tissue samples (lymph nodes, spleen, liver, lung and kidney). Animals with one or more tissue samples containing viral genomes were considered positive.

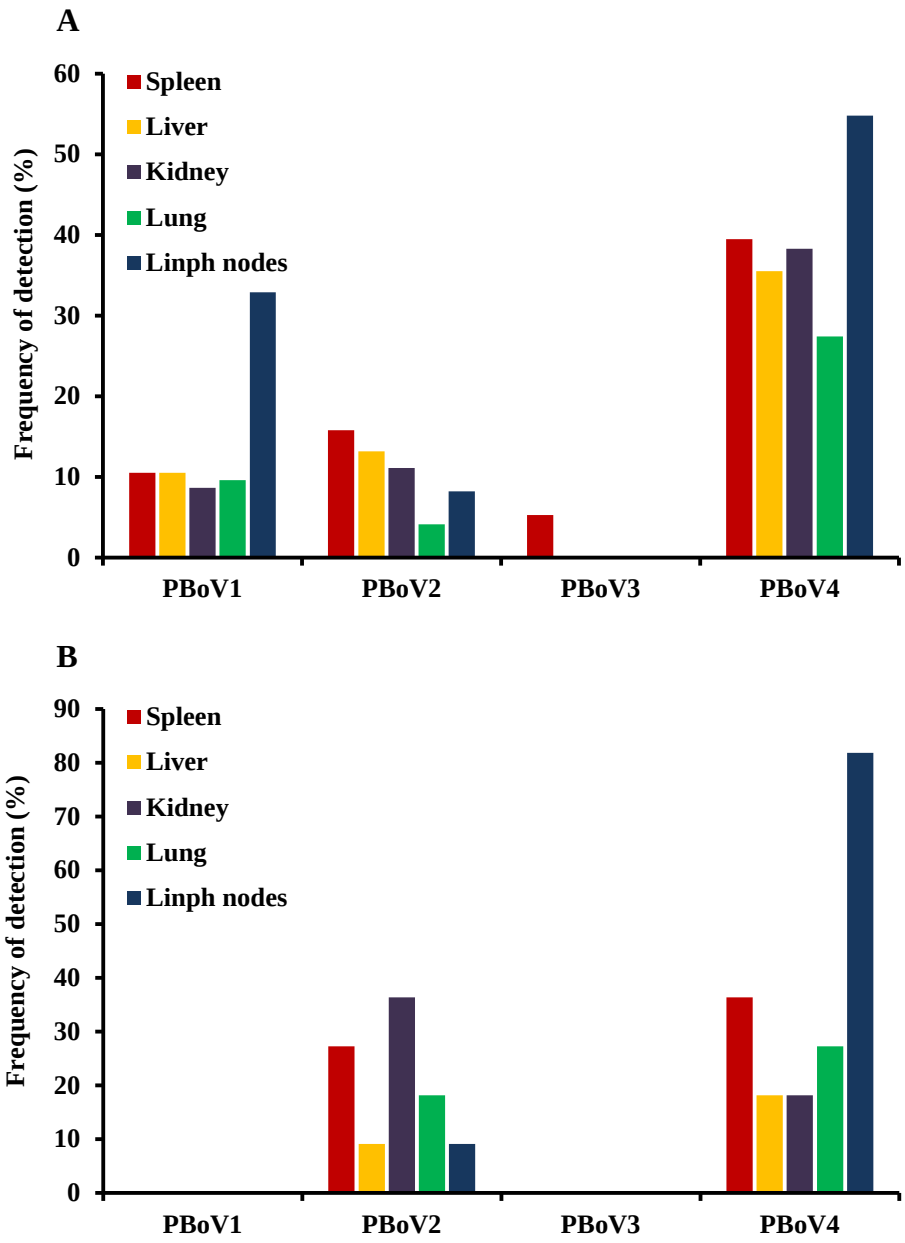

Figure 4. Tissue distribution of PBoV1, 2, 3 and 4 in sampled pigs. Ninety-four PMWS-affected animals (**4A**) and eleven healthy adult animals (**4B**) were tested for the presence of PBoV1, 2, 3 and 4 in five tissues (lymph nodes, spleen, liver, lung and kidney). The results were expressed in percentage of genome detection.

Table S1. Sequences of porcine bocaviruses used in primers design.

Genbank accession No.	Sequence name
FJ872544.1	Bocavirus pig/Swebo_1/SWE
GQ387499.1	Porcine parvovirus 4 clone 17
HM053672.1	Bocavirus pig/6V/China/2006
HM053673.1	Bocavirus pig/7V/China/2006 ds
HM053693.1	Porcine bocavirus 1 pig/ZJD/China/2006
HM053694.1	Porcine bocavirus 2 pig/ZJD/China/2006
GU902967.1	Bocavirus pig/sw-18/SWE/2010
GU902968.1	Bocavirus pig/sw-90_1/SWE/2010
GU902969.1	Bocavirus pig/sw-92_2/SWE/2010
GU902970.1	Bocavirus pig/sw-107/SWE/2010
GU902971.1	Bocavirus pig/sw-A1/SWE/2010
HQ223038.1	Bocavirus pig/SX/China/2010
JF512472.1	Porcine bocavirus 3/64-1/N.Ireland/2004
JF512473.1	Porcine bocavirus 4/F41/N.Ireland/2004
HQ291308.1	Porcine bocavirus 1 isolate H18
HQ291309.1	Porcine bocavirus 2 isolate A6
GU978966.2	Porcine parvovirus 4 clone JS0918-5598
HM031135.1	Porcine parvovirus 4 strain JS0918b
HM031134.1	Porcine parvovirus 4 strain JS0918a
GU978968.1	Porcine parvovirus 4 clone JS0910-5400
GU978967.1	Porcine parvovirus 4 clone JS0910-5644
GU978965.1	Porcine parvovirus 4 clone HEN0922-5645
GU978964.1	Porcine parvovirus 4 clone HEN0922-5400
GQ387500.1	Porcine parvovirus 4 clone 14
GQ387499.1	Porcine parvovirus 4 clone 17

CAPÍTULO 3: Porcine Bocavirus 2 (PBoV2) DNAemia in healthy and postweaning multisystemic wasting syndrome-affected pigs

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Porcine Bocavirus 2 (PBoV2) DNAemia in healthy and postweaning multisystemic wasting syndrome-affected pigs

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Abstract

In this study, a SYBR Green-based real-time polymerase chain reaction (qPCR) to detect porcine bocavirus 2 (PoBV2) genomes was developed. The through level of the assay was 10 copies of the PoBV2 replicase gene, cloned in a recombinant plasmid. The dynamic range of the assay was of eight orders of magnitude (10^8-10^1copies). The test was applied to search for PBoV2 DNAemia in sera of 1 to 4 months old pigs displaying signs of postweaning multisystemic wasting syndrome (PMWS), in sera from healthy swine at equivalent age and in sera from older, healthy animals (>6 months old). High levels of PoBV2 DNA were detected in PMWS-affected pigs (84.0%, 42/50) in comparison to healthy pigs at the same age (40.4%, 19/47) ($P \leq 0.001$). PBoV2 DNA was also detected to high frequency (81.8 %) in sera from older healthy animals (36/44). The mean viral DNA load in PMWS-affected pigs was 5.2×10^7 copies/mL, whereas in young healthy pigs it was 1.4×10^5 copies/mL ($P \leq 0.05$). The PBoV2 genome load in PMWS-affected pigs was not significantly different from the viral loads detected in older animals (4.2×10^7 copies/mL). However, average DNAemia in older pigs was significantly higher than that of young healthy pigs (1.4×10^5 copies/mL). It is concluded that high levels of PBoV2 DNAemia are expected in adult pigs with no apparent signs of disease. It is possible that the early, higher viral load observed in young, PMWS-affected pigs, might be consequent to the immune impairment associated to PMWS. Nonetheless, these findings reveal that PBoV2 is highly prevalent in the examined swine herds, suggesting that such infections are widespread among swine.

Background

Bocaviruses (from the Bocavirus genus of Parvovirinae subfamily within the Parvoviridae family) are known to cause respiratory and gastrointestinal tract infections, particularly in young hosts, whereas in adults subclinical infections are common [1, 2]. The Bocavirus genus presently comprises at least seven viruses, including bovine parvovirus 1 (BPV-1), canine minute virus (CMV), gorilla bocavirus (GBoV1) and human bocaviruses (HBoV 1 to 4) [3-9].

Recently, many bocaviruses were identified in swine [10-15]. The grouping of the various new porcine bocaviruses into 4 types (PBoV1, 2, 3 and 4) has been proposed [16]. Porcine bocavirus 1 (PBoV1) was first described co-infecting animals with porcine circovirus 2 (PCV2) [13] but associations with swine disease are controversial [16-20]. Porcine bocaviruses types 3 and 4 (PBoV3 and PBoV4) were discovered by metagenomics and so far have not been linked to any disease [12]. Porcine bocavirus type 2 (PBoV2), also named "*porcine parvovirus type 4*" (PPV4) [10], was first identified in lung washings from pigs with PMWS [10]. Phylogenetically, PBoV2 is more closely related to bovine parvovirus 2 (BPV2), than the other PBoVs [10, 16, 21]. Regarding to genome structure and organization, PBoV2 encodes an ORF3 in the middle of the viral genome, like other members of the *Bocavirus* genus [21]. Postweaning multisystemic wasting syndrome (PMWS) [22-24] is a major cause of losses to the swine productive chain [10, 25]. The syndrome is the most important "*porcine circovirus associated disease"* (PCVAD), a collective term that describes a number of conditions associated to porcine circovirus type 2 infections [22-24, 26-28]. Considering the significance of PMWS for swine production worldwide

and the discovery of PBoV2 in bronchoalveolar washings from animals co-infected with PCV2, the search for links between PMWS and PBoV2 has become naturally expected.

The present work was concentrated on examining the status of PBoV2 infections in swine sera in view that, in our preliminary searches for PBoV2 in sera and pig tissues, PBoV2 was detected to higher frequency in PMWS-affected animals than in healthy ones (unpublished data). Thus, it became of interest to examine the PBoV2 distribution in herds and whether the virus might be associated to the occurrence of PMWS.

In order to achieve this goal, a highly sensitive, specific, SYBR Green-based real-time quantitative PCR (qPCR) was developed for detection and quantitation of PBoV2 genomes in serum samples. The test was then applied to quantitatively search for search PBoV2 DNA on healthy pigs in different age groups and in PMWS-affected pigs.

Results

Quantitative PBoV2 real-time PCR (qPCR) standardization

Serial tenfold dilutions of pJET1.2-PBoV2 (see methods) were used to construct a standard curve by plotting the plasmid copy number (in $\log_{10}$) against the threshold cycle (Ct) (Figure 1A and 1B). The standard curve revealed a wide dynamic range (10^8-10^1 copies per reaction) with a linear correlation (R^2) of 0.997-1.0 between Ct values and the plasmid copy numbers (expressed in $\log_{10}$). The quantitative analysis evidenced a detection limit of approximately 10 copies of viral DNA by reaction (Figure 1A and 1B).

When DNA extracted from PBoV2-negative serum samples (as previously screened by conventional PCR), was tested by qPCR, no significant fluorescence signal was detected. Negative controls did not give rise to any amplification product, as revealed by the absence of spurious peaks on dissociation curve (Figure 1C). The dissociation temperature of the 160 bp amplicon was 82.63 °C ± 1 °C (Figure 1C).

Frequency of detection of PBoV2 genomes and determination of viral load

The frequency of detection of PBoV2 genomes in PMWS-affected pigs was twice superior (84% vs. 40.4%, respectively) and significantly higher ($P \leq 0.001$) than in healthy animals within the equivalent age group (Figure 2). However, when older, healthy animals were examined, PBoV2 DNA was detected in sera of 81.8% of the animals, a frequency not significantly different from that detected in young, PWMS-affected pigs. Thus, whereas the frequency of occurrence of PBoV2 DNAemia in healthy young healthy pigs was low, in

PMWS-affected pigs, it was as high as those detected in adult (>6 months old), healthy animals (Figure 2).

An analysis was then carried out to determine whether there would be any relationship between the occurrence of PMWS and PoBV2 DNA loads. The mean viral DNA load in PMWS-affected pigs was 5.2×10^7 copies/mL, whereas in young healthy pigs it was 1.4×10^5 copies/mL (Figure 3). Thus, a viral DNA load was significantly higher in PMWS-affected pigs than in healthy young animals ($P\leq0.05$). However, no significant differences in genome loads were detected in adult healthy pigs (average 4.2×10^7 copies/mL) when compared to those of PMWS-affected animals (Figure 3). Therefore, PBoV2 genomes were detected to high frequencies and at higher viral loads in adult than in young healthy animals, whereas PMWS-affected pigs displayed PoBV2 DNAemia earlier and in higher amounts than those detected in healthy pigs at the same age.

Another comparison was performed with basis on the number of PoBV2 genome copies and the PMWS status of the animals. Viral DNA load levels were arbitrarily divided in "high" ($\geq10^3$ copies per mL), and "low" ($\leq10^3$ copies per mL) (Figure 3). Using such arbitrary scale, 14% of the PMWS-affected animals displayed high viral DNA loads, against 4% of the healthy animals at the same age. In adult animals, 23% of the animals had a viral DNA load $\geq10^3$ copies per mL, significantly higher than the average viral DNA load detected in young healthy animals (Figure 3). This analysis provides further evidence to substantiate the fact that PMWS-affected pigs, as well as older healthy animals, bear higher PoBV2 genome loads than healthy young pigs. The group formed by the older healthy animals presented the largest number of animals with viral loads $\geq10^3$

molecules per mL (23%), indicating that the frequency of detection of PoBV2 DNAemia is associated to ageing in the sampled population.

Discussion

Bocaviruses are emerging pathogens whose association with disease have been investigated in human and animal diseases [1, 7-9, 29]. In recent years, a number of bocaviruses have been detected in pigs [10-14]. Among the four porcine types identified (PBoV1 to 4), PBoV2 is the type which seems most consistently present, either in diseased animals [10, 25]. However, in the few occasions where associations between PoBV2 and disease have been investigated, results obtained have given rise to conflicting findings [16, 25].

In preliminary studies on pig tissues, our group had detected a high frequency of PBoV2-bearing samples from diseased pigs (unpublished data). As PMWS is currently a major problem for the swine productive chain and is a multifactorial disease [24], it became of interest to examine whether there would be any relationship between the occurrence of PBoV2 in PMWS. A qPCR was initially developed to detect PoBV2 genomes. The validation of the test revealed that is was capable of detecting as little as 10 copies of PoBV2 genomes per reaction, as determined by quantitating a recombinant plasmid containing the targeted region of the PBoV2 fragment genome. The test was shown to be very specific, since no spurious amplifications were detected in positive and negative control samples (the latter consisting of DNA extracted from PBoV2-negative sera previously screened by conventional PCR). The qPCR so developed was applied to examine the frequency of distribution of PoBV2 genomes in young, PMWS-affected animals (age range 1-4 months old) as well as in non-PMWS-affected pigs in two distinct age groups (1-4 months old and >6 months old pigs). In PMWS-affected animals, PoBV2 was detected at a frequency twofold higher

in PMWS-affected animals (84.0%) than in healthy pigs at the same age (40.4%) (*P≤0.001*). When the analysis was performed in samples from healthy adult animals (age range 6-19 months), these also revealed to bear PoBV2 DNA to a frequency which did not differ significantly from that detected in PMWS-affected young pigs. Besides de similar frequencies, the viral DNA loads in samples from older healthy animals also were not significantly different from those in PMWS-affected young pigs (81.8%). These observations suggest that PBoV2 infections are endemic and do not seem to be related to the occurrence of PMWS. Such conclusion can be drawn from verifying the high incidence of PBoV2 DNA-carrying healthy adult pigs. The fact that young healthy animals also bear PBoV2 DNA, despite that at frequencies significantly lower than do PMWS-affected animals, suggest that such infections star to occur early in life and tend to increase in frequency with aging. The significantly higher levels of PBoV2 DNAemia in PMWS-affected pigs when compared to healthy pigs at the same age suggest that PMWS may be altering the response to PBoV2 infection, perhaps allowing PoBV2 to multiply to higher copy numbers due to the immunosuppressive effects of PCV2 [23, 24, 30-32]. As PMWS-affected pigs are young animals, it is also possible that passively acquired, maternally derived immunity may wane sooner in PMWS-affected pigs than in healthy ones, thus leaving piglets unprotected and prone to higher levels of PBoV2 DNAemia. The exam of such possible explanations was beyond the scope of the present study though will certainly deserve some attention in the future.

Regardless of the reasons which led to the significantly higher frequency of occurrence and higher PBoV2 DNA loads in PMWS-affected pigs, the quantitation of PBoV2 genomes appear to provide additional evidence on the

PMWS status of the pig population examined. As postulated previously, the diagnosis of PMWS is based on the occurrence of clinical signs, characteristic histopathological lesions and a PCV2 viral load $\geq 10^7$ copies of PCV2 DNA per mL. An additional subsidy for the establishment of the diagnosis may be provided by the detection of PBoV2 DNAemia to levels $\geq 10^3$ copies per mL.

Conclusions

The SYBR Green-based real-time qPCR designed here to detect PBoV2 DNA in pig serum was shown to be a sensitive and specific method for detection and quantification of PBoV2 DNA in serum samples. Viral genomes were detected to higher frequencies (84%) and at higher copy numbers (5.2×10^7, respectively) in PMWS-affected pigs than in healthy pigs at the same age (42%; 1.4×10^5 copies per mL). In addition, older healthy adult animals were also found to carry PoBV2 genomes in serum, to frequencies and copy numbers that did not significantly differ from those found in younger, PMWS-affected animals. However, these were significantly different from the frequency detected in young healthy pigs. Such findings indicate that PoBV2 infections are ubiquitous in pigs; infection probably begins early in life and frequency seems to increase with aging, since older animals tend to carry PoBV2 DNA with higher frequency and to higher loads than healthy, younger animals. In PMWS-affected animals, however, PoBV2 genomes can be detected more frequently and with higher viral DNA loads than that observed in healthy pigs at the same age. These findings may be consequent to some sort of immunossupression, known to occur in PMWS-affected pigs.

Materials and Methods

Primer design

Conserved regions of the PBoV2 *replicase* gene were identified by nucleotide sequence alignment (data not shown). The primers used for the SYBR Green real-time qPCR were designed using the *Geneious* software (version 5.4). The designed primers were: PBoV2-F (5'-TTCGGCAGGCGGAGGCTTTG-3') and PBoV2-R (5'-GCGGAGTACCAGCGGACAC -3'), and were complementary to nucleotides 761–780 and 901–920 along the genome of *Porcine parvovirus 4 clone JS0918-5598* (GenBank accession No. GU978966.2).

Construction of the plasmid DNA standard curve and sensitivity assay

The PBoV2 replicase gene fragment was amplified using the forward and reverse primers (PBoV2-F and PBoV2-R) in a conventional PCR. The product was cloned into the prokaryotic plasmid vector pJET1.2 (Fermentas) and confirmed by sequencing. The recombinant plasmid pJET1.2-PBoV2 was quantified using a Qubit fluorimeter (Invitrogen). Serial 10-fold dilutions of pJET1.2-PBoV2 were prepared in 10 mM Tris-EDTA buffer (pH 8.0) plus 2 $ng.mL^{-1}$ of Lambda phage DNA to generate the standard curve.

To determine the detection limit and efficiency of the assay, the standard plasmid DNA (pJET1.2-PBoV2) was used as a template and diluted serially in tenfold steps to produce 1×10^{0} to 1×10^{8} copies/reaction. The sensitivity test was performed three times, in three independent assays.

Real-time qPCR for PBoV2

The SYBR Green based real-time qPCR was performed in 12.5 μL volumes, with mixtures containing 3 μL of extracted DNA (diluted 1:4) or standard plasmid, 6.25 μL of 2X SYBR Green I ROX Plus Master Mix (LGC Biotecnologia) and 170 nM each of the forward and reverse primers. Amplification and detection were performed in a StepOne system (Life Technologies) under the following conditions: PCR activation at 95 °C for 15 min followed by 40 cycles of amplification (15 s at 95 °C and 30 s at 63 °C). All quantitative real time reactions (standards, test samples, and controls) were performed in triplicate. The reported results are averages of such triplicates. 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.

Clinical specimens – serum samples and DNA extraction

Serum samples were received from pig farms from the state of Rio Grande do Sul, Brazil. The case group (PMWS-affected pigs) consisted of 50 serum samples from 1-4 months old piglets. In the case group, evident clinical signs were displaying dyspnea, enlargement of superficial inguinal lymph nodes, pallor, jaundice and diarrhea. The PMWS diagnosis was confirmed by typical macroscopic lesions at necropsy, histopathology and demonstration of PCV2 DNA in tissues by PCR [33, 34]. The control group (non-PMWS-affected pigs) consisted of serum samples of 47 pigs 6-19 months old (adult asymptomatic pigs) and 50 serum samples from healthy, 1-4 months old piglets.

DNA of sera was extracted from 500 μL volumes of sera using a phenol-chloroform method [35]. Extracted DNA was resuspended in 50 μL of TE buffer.

The quantity and quality of the extracts were analyzed with the aid of a spectrophotometer (Nanodrop® 1000).

Statistical analysis

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

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SPC, APMV and HFS carried out the DNA extractions. SPC, CMS, TFT and APMV carried out the qPCRs. ACF and TFT performed cloning experiments and performed the statistical analysis. SPC, PMR and ACF conceived of the study, participated in its design. All authors aided in writing, read and approved the final manuscript

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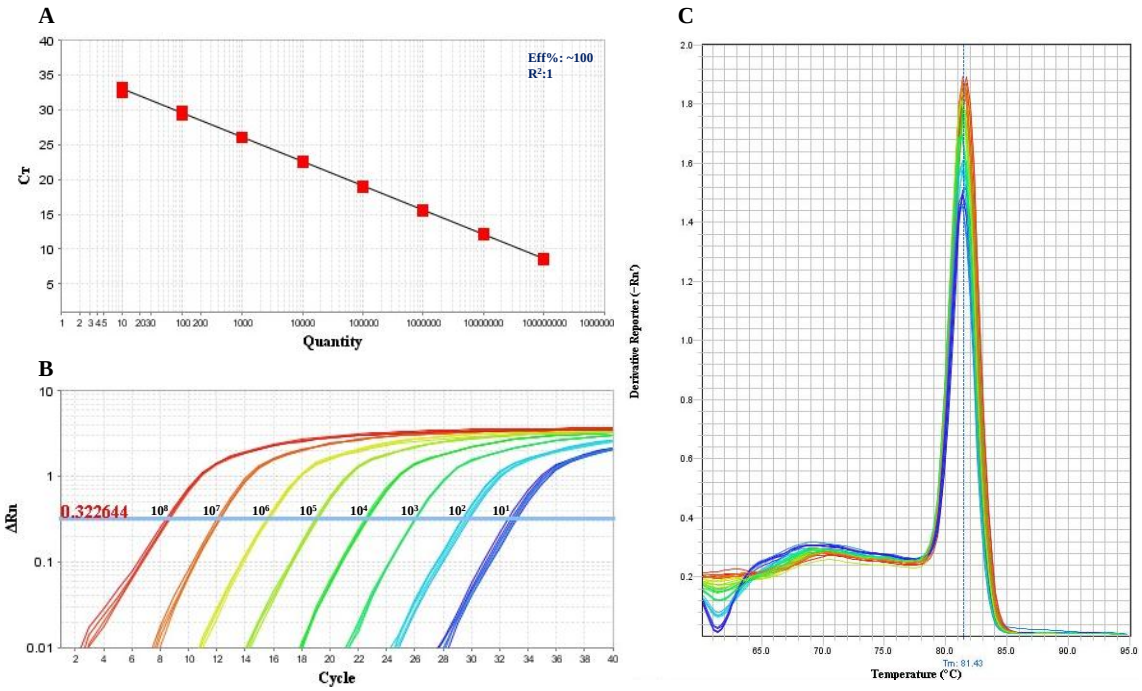

Figure 1. A. Standard curve constructed based on the amount of *Porcine bocavirus 2* (PBoV2) DNA copies versus the threshold cycle (Ct) obtained on SYBR Green real-time polymerase chain reaction (qPCR). A 10-fold dilution series of plasmid pJET1.2-PBoV2 was used as PBoV2 DNA template. Each point in the graph represents the mean Ct value of a triplicate measurement. The amounts of pJET1.2-PBoV2 molecules are given on a $\log_{10}$ scale. **B.** Amplification curves of the PBoV2 dilution series. **C**. Dissociation curves of the SYBR Green real-time PCR products from PBoV2 serum samples.

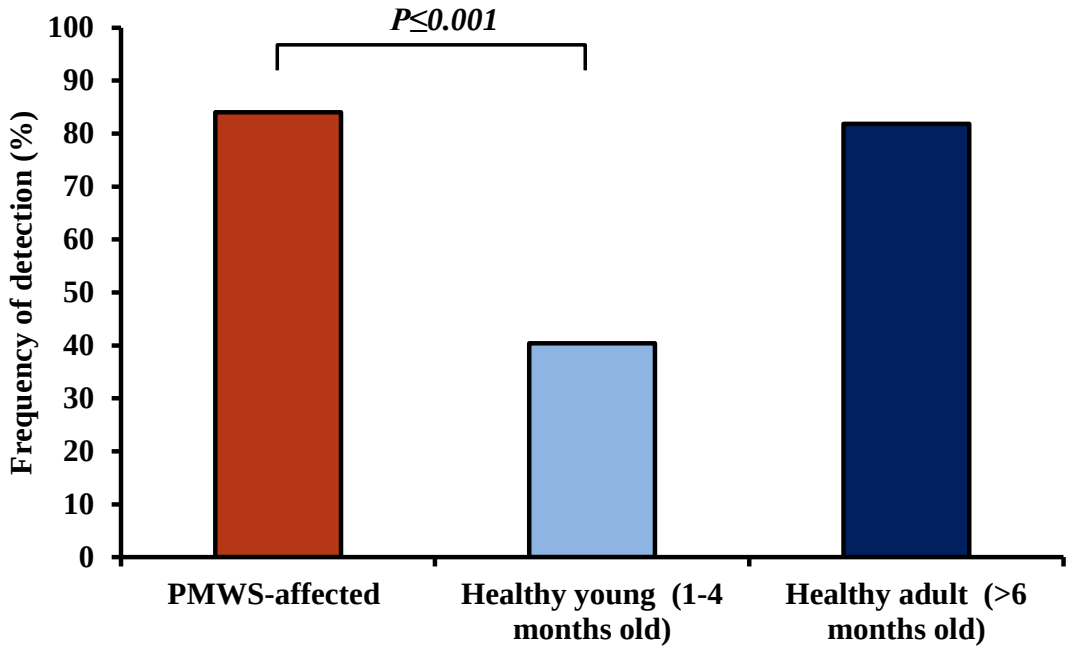

Figure 2. Distribution of PBoV2 in serum samples from PMWS-affected, non-PMWS-affected and healthy adult pigs. PBoV2 was detected twice more in animals affected by syndrome than in healthy animals with the same age. Adult animals showed a high prevalence of PBoV2 in serum samples analyzed.

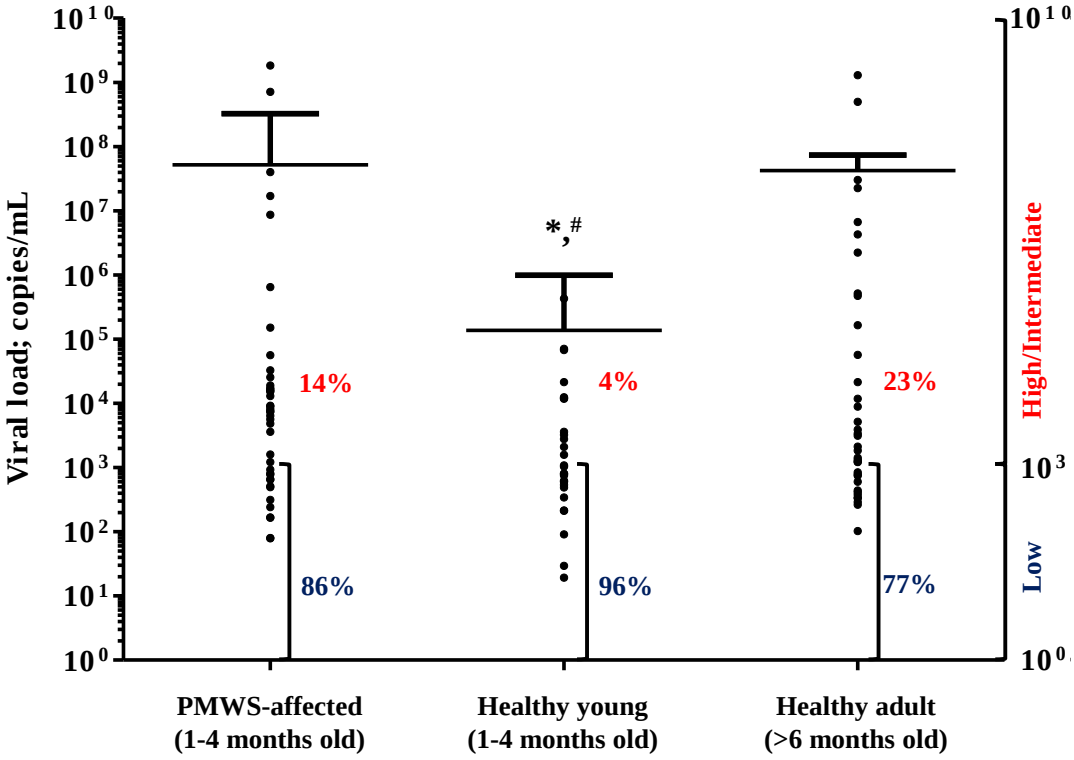

Figure 3. Dot plot and median serum viral load of PBoV2 in PMWS-affected, non-PMWS-affected and healthy adult pigs. Bars represent standard deviation. The percentages show the division performed to separate the animals according to viral load (greater than 10^3: intermediate/high, less than 10^3: low). The median of viral load among tested groups significantly differ among PMWS-affected pigs and healthy pigs of equal age ($^{*}P \leq 0.05$) and to healthy young (1-4 months old) and healthy adults (>6 months old) ($^{\#}P \leq 0.05$). The statistical analysis and graphs was performed on GraphPad Prism 5.

7. DISCUSSÃO GERAL

Infecções polimicrobianas e seu impacto na SMDS

Como uma doença multifatorial, condições ambientais, estratégias de manejo, além de fatores genéticos do hospedeiro estão envolvidos na gênese da SMDS. Vários são os estudos que relacionam a presença de patógenos suínos com o desenvolvimento da SMDS (ALLAN e ELLIS, 2000; OPRIESSNIG, GIMENEZ-LIROLA, *et al.*, 2011; OPRIESSNIG e HALBUR, 2011).

Infecções pelo PCV2 são reconhecidas por seus efeitos imunossupressores no hospedeiro, o que predispõe a infecções virais, bacterianas e micóticas (SEGALES, DOMINGO, *et al.*, 2004). Tendo em vista o potencial imunossupressivo do PCV2, algum tipo de interação entre patógenos suínos e o desenvolvimento da SMDS pode ser esperado. Infecções oportunísticas têm sido frequentemente observadas em rebanhos com SMDS (CARRASCO *et al.*, 2000; KIM *et al.*, 2002; PALLARES *et al.*, 2002; NUNEZ *et al.*, 2003; CAVALLINI SANCHES *et al.*, 2006; ZLOTOWSKI *et al.*, 2006). Entretanto, não está claro se o efeito imunossupressor do PCV2 facilitaria as co-infecções, ou se estas atuariam como fatores desencadeantes da SMDS.

O vírus da síndrome respiratória e reprodutiva dos suínos (PRRSV), ainda exótico no Brasil, parece capaz de atuar sinergisticamente com o PCV2 para o desenvolvimento da SMDS (ALLAN, MCNEILLY, ELLIS, *et al.*, 2000; HARMS *et al.*, 2001; PALLARES *et al.*, 2002; POGRANICHNIY *et al.*, 2002; ROVIRA *et al.*, 2002; SEGALES, CALSAMIGLIA, *et al.*, 2002; DROLET *et al.*, 2003; ROSE *et al.*, 2003; WELLENBERG, STOCKHOFE-ZURWIEDEN, BOERSMA, *et al.*, 2004; DORR *et al.*, 2007; GRAU-ROMA *et al.*, 2007; MORANDI *et al.*, 2010). O parvovírus suíno tipo 1 também é associado ao desenvolvimento da SMDS, sendo que infecções experimentais do vírus com PCV2 levaram ao desenvolvimento da síndrome (ALLAN *et al.*, 1999; ELLIS *et al.*, 1999; ALLAN, MCNEILLY, MEEHAN, *et al.*, 2000; CHOI *et al.*, 2000; ELLIS *et al.*, 2000; KENNEDY *et al.*, 2000; KRAKOWKA *et al.*, 2000; ROSE *et al.*, 2003; ALLAN *et al.*, 2004; HA *et al.*, 2008; ROSE *et al.*, 2009; HA *et al.*, 2010; ANDERSSON *et al.*, 2011). Os *Anellovirus* suínos (TTSuV1 e TTSuV2) também podem estar associados à SMDS, embora os resultados de muitas pesquisas sejam bastante conflitantes (KEKARAINEN *et al.*, 2006; TEIXEIRA, 2008; ARAMOUNI *et al.*,

2011). Outros patógenos que foram descritos com maior frequência nos casos de SMDS foram o *Mycoplasma hyopneumoniae* (PALLARES *et al.*, 2002), *Cryptosporidium parvum* (NUNEZ *et al.*, 2003), o vírus da pseudoraiva (QUINTANA *et al.*, 2001; MALDONADO *et al.*, 2005), influenza suína e pneumonias bacterianas (KIM *et al.*, 2002; POGRANICHNIY *et al.*, 2002; DORR *et al.*, 2007; WEI *et al.*, 2010).

Com os avanços recentes nos métodos de detecção de patógenos, a importância das doenças polimicrobianas tornou-se mais evidente, e a identificação de interações de patógenos e seus mecanismos de potenciação de enfermidades se tornou um tema de grande interesse (OPRIESSNIG, GIMENEZ-LIROLA, *et al.*, 2011). Alguns mecanismos conhecidos na interação de patógenos suínos incluem: o dano da mucosa ciliada nos tecidos do trato respiratório, facilitando a instalação e proliferação de bactérias (POL *et al.*, 1997; LOVING *et al.*, 2010); a indução de imunossupressão (RENUKARADHYA *et al.*, 2010); a alteração da expressão de citocinas no hospedeiro (THANAWONGNUWECH *et al.*, 2004), ou ainda afetando a função macrofágica (CHIOU *et al.*, 2000).

A infecção pelo PCV2 raramente resulta em doença clínica, no entanto, a SMDS pode ser iniciada, acelerada, prolongada ou reforçada por co-infecções virais ou bacterianas. Devido ao seu efeito sobre o sistema imunológico, animais infectados pelo PCV2 ficam mais susceptíveis a infecções fúngicas, protozoárias e metazoárias.

Muitos estudos retrospectivos ou transversais têm investigado a presença e prevalência de vários agentes infecciosos associados à SMDS em condições de campo. Os modelos experimentais confirmam que a replicação e as lesões associadas ao PCV2 são aumentadas por infecção simultânea com outros vírus ou bactérias. Os mecanismos exatos pelos quais os patógenos concorrentes aumentam o potencial patogênico do PCV2 ainda são desconhecidos. Co-infecções podem promover a infecção por PCV2, por aumentar a replicação celular do hospedeiro, e por consequência a replicação do PCV2. Também tem sido proposto que co-infecções podem interferir com a depuração do PCV2 pela alteração da produção dos perfis de citocinas. Dada a atual base de conhecimentos, é importante se fazer uma investigação minuciosa de diagnóstico de co-infecções em rebanhos onde a SMDS é um problema recorrente, a fim de

implementar estratégias de intervenção mais adequadas e eficazes e identificar agentes que possam estar contribuindo para a gênese e/ou patogênese da SMDS.

O papel do PCMV durante a SMDS

Nesse estudo, o DNA de PCMV foi procurado em animais saudáveis e afetados pela SMDS. A presença de DNA de PCMV em amostras de DNA extraída de baços foi considerada indicativa de infecção prévia, já que os betaherpevírus induzem latência em células linfóides (EIZURU, 2006; SINCLAIR *et al.*, 2006).

Em humanos, DNA de HCMV em soro é considerado indicativo de infecção ativa (SPECTOR *et al.*, 1992; WOLF *et al.*, 1993), enquanto DNA de HCMV em leucócitos é considerado indicativo de infecção latente (ISHIGAKI *et al.*, 1991). Além disso, a detecção de genomas de HCMV em amostras de soro apresenta um grau de correlação com doença clínica maior do que ensaios quantitativos com qPCRs (SHINKAI *et al.*, 1997).

Os resultados aqui apresentados mostram que genomas de PCMV foram detectados em baços em altas proporções, em ambos os grupos de animais avaliados (com e sem a síndrome). A frequência de detecção de DNA do PCMV na população amostrada foi semelhante àquela relatada em estudos anteriores (RONDHUIS *et al.*, 1980; ASSAF *et al.*, 1982; TAJIMA *et al.*, 1993; HAMEL *et al.*, 1999; GOLTZ *et al.*, 2000). Apesar da alta frequência de infecções pelo PCMV e PCV2 em ambos os grupos de animais, nenhuma associação entre a presença de genomas de PCMV e a ocorrência de SMDS pode ser inferida.

Quando amostras de soro foram analisadas, frequências semelhantes de DNAemia por PCMV foram verificadas em ambos os grupos, afetados ou não pela síndrome, sugerindo um não envolvimento do agente no desenvolvimento da SMDS. Os resultados aqui apresentados mostram que a prevalência de infecções PCMV na população examinada é alta em ambos os grupos de animais, tanto saudáveis como afetados pela SMDS. Portanto, o PCMV não parece desempenhar qualquer papel significativo no desenvolvimento da SMDS.

A presença dos recém-descobertos bocavírus suínos em animais saudáveis e afetados pela SMDS

Bocavírus que infectam animais são conhecidos na medicina veterinária desde os anos 1960 (ABINANTI *et al.*, 1961; BINN *et al.*, 1970). O gênero *Bocavirus* ganhou atenção especial quando um genoma de bocavírus humano

(HBoV) foi detectado em amostras do trato respiratório de crianças com doença respiratória (ALLANDER *et al.*, 2005). Desde então, bocavírus foram identificados em gorilas (GBoV1) (KAPOOR, MEHTA, *et al.*, 2010), suínos (BLOMSTROM *et al.*, 2009; CHENG *et al.*, 2010; CHEUNG *et al.*, 2010; MCKILLEN *et al.*, 2011; SHAN, LAN, *et al.*, 2011), bem como mais três novos HBoVs em seres humanos (ABINANTI *et al.*, 1961; BINN *et al.*, 1970; ALLANDER *et al.*, 2005; ARTHUR *et al.*, 2009; KAPOOR *et al.*, 2009; KAPOOR, SIMMONDS, *et al.*, 2010). Entretanto, esses trabalhos, falham no quesito associação desses novos agentes com doenças. Nesse estudo, apresentamos o desenvolvimento de ensaios baseados em PCR para a determinação da frequência de detecção de genomas dos recém-descobertos PBoVs.

PBoV1

Diferentemente de outros autores, nenhum tipo de associação com SMDS e a frequência de detecção do PBoV1 foi encontrado (BLOMSTROM *et al.*, 2009; BLOMSTROM *et al.*, 2010; ZHAI *et al.*, 2010), quando analisadas amostras de soro. A taxa de detecção desse agente foi bastante alta (aproximadamente 30%), em ambos os grupos de animais (afetados ou não pela SMDS), não havendo diferença estatisticamente significativa ($P \geq 0,05$). Quando amostras de tecidos foram analisadas, aproximadamente 40% foram positivas para PBoV1 em animais afetados pela SMDS. Em animais não afetados em nenhuma amostra foi verificada a presença do agente. Entretanto, a idade dos animais não afetados pela síndrome era superior à dos animais acometidos pela síndrome. Portanto, não se pode realizar nenhum tipo de inferência de associação com doença. A frequência de detecção desse agente em aproximadamente 1/3 dos animais analisados é compatível com trabalhos realizados em outros países (CHENG *et al.*, 2010; CADAR *et al.*, 2011; SHAN, LAN, *et al.*, 2011; ZENG *et al.*, 2011; ZHANG *et al.*, 2011). Entretanto, no único trabalho que analisou a presença desse agente em animais afetados pela SMDS (BLOMSTROM *et al.*, 2010), a frequência de detecção do PBoV1 foi de aproximadamente 80%, superior a encontrada neste trabalho (que foi de aproximadamente 40% em amostras de tecidos). Curiosamente, 10% dos animais adultos testados apresentaram DNAemia para PBoV1, fato nunca antes reportado para os bocavírus suínos. Recentemente, em

humanos, foi reportada a presença de genomas de HBoV em doadores de sangue clinicamente saudáveis (BONVICINI *et al.*, 2011), e mecanismos de persistência do vírus vem sendo explorados (KAPOOR *et al.*, 2011; LUSEBRINK *et al.*, 2011). Outro fato interessante foi a distribuição do PBoV1, sendo detectado com maior frequência em tecidos de linfonodos.

PBoV2

Quanto à presença de DNAemia de PBoV2, houve uma diferença altamente significativa entre animais acometidos pela síndrome e animais saudáveis com a mesma idade. Animais acometidos pela síndrome tiveram uma frequência de detecção seis vezes superior aos não acometidos pela SMDS (40% vs. 6%). Isso indica uma possível associação desse agente com a SMDS. Em amostras de tecidos, o PBoV2 foi encontrado em aproximadamente 30% das amostras. Entretanto, em animais adultos o PBoV2 foi detectado em 60% das amostras.

Como a taxa de detecção do PBoV2 pareceu estar relacionada ao desenvolvimento da SMDS, optou-se pelo desenvolvimento de um PCR, do tipo quantitativa, para a determinação da carga viral em animais afetados ou não pela SMDS, em diferentes idades. A qPCR baseada na química do SYBR Green-I se mostrou altamente sensível (com um limite de detecção de aproximadamente 10 cópias por reação). Nenhum tipo de amplificação inespecífica foi evidenciada (verificada pela curva de dissociação realizada após cada reação).

Genomas de PBoV2 foram detectados em alta frequência e em maior números de cópias em animais afetados pela SMDS (84% e 5.2×10^7 cópias por mL, respectivamente) do que em animais saudáveis da mesma faixa etária (42%), diferindo estaticamente ($P\leq0,05$) . Surpreendentemente, animais adultos, sem sinais clínicos de nenhuma doença, apresentaram uma alta taxa de detecção, bem como uma alta carga viral.

Nossos achados mostram que o PBoV2 é um vírus ubíquo e que animais adultos mantêm genomas virais circulantes e também nos tecidos. Em animais afetados pela SMDS, a infecção pelo PBoV2 parece ocorrer mais cedo do que em animais saudáveis com a mesma idade. Esse achado pode ser associado ao fato de que animais afetados pela síndrome têm um forte comprometimento do sistema imune, deixando os animais mais propícios à aquisição de patógenos.

PBoV3

O PBoV3 foi encontrado somente em animais acometidos pela SMDS, indicando uma possível associação com a síndrome. Genomas desse vírus foram relatados primariamente em amostras de fezes de animais saudáveis (CHENG *et al.*, 2010; SHAN, LAN, *et al.*, 2011), sendo que a associação da detecção de genomas de PBoV3 em tecidos de suínos com doenças é relatada (ZHANG *et al.*, 2011).

PBoV4

O PBoV4 foi o vírus com uma maior frequência de detecção nesse trabalho. Genomas de PBoV4 foram detectados em animais acometidos ou não pela SMDS, com frequências que não diferiram significantemente. A taxa de detecção variou em animais jovens *vs.* adultos, sendo que nesses últimos, a presença de genomas foi superior, chegando perto dos 90%.

Em amostras de soro, o PBoV4 foi identificado com uma frequência baixa, em ambos os grupos, afetados ou não pela SMDS. Em soros de animais adultos, o vírus foi detectado em 2% das amostras, enquanto em animais jovens a frequência variou de 5% nos animais afetados pela SMDS e 11% nos animais saudáveis com idade equivalente. Esses resultados sugerem que com o avanço da idade os animais tendem a não ter genomas virais circulantes no sangue. Entretanto, a frequência de detecção de genomas em tecidos aumenta de forma significativa em animais adultos.

A frequência de detecção do PBoV4 varia de acordo com o tecido analisado. Em ambos os grupos analisados (com ou sem a SMDS), genomas de PBoV4 foram detectados com uma maior frequência em tecidos de linfonodos.

8. CONCLUSÕES

- A PCR para detecção de PCMV, desenvolvida por Hamel e colaboradores (HAMEL *et al.*, 1999) e repadronizada em nosso laboratório, demonstrou alta sensibilidade e especificidade para a detecção de genomas de PCMV em amostras de tecido e de soro; no entanto, não foi possível inferir associação significativa entre PCMV e a SMDS;
- O segundo estudo desenvolvido permitiu relatar, pela primeira vez, a presença dos recém-descobertos bocavírus suínos (PBoV1, PBoV2 ou PPV4, PBoV3 e PBoV4) no Brasil;
- Assim como para o PCMV, os resultados obtidos não revelaram associação entre a detecção de PBoV1 e PBoV4 e a presença de SMDS. O mesmo não ocorreu para PBoV2 e PBoV3, onde uma associação positiva pode ser observada;
- Animais adultos clinicamente saudáveis apresentam genomas circulantes de PBoV1, 2 e 4; também foi detectada a presença de PBoV 2 e 4 em amostras de tecidos de animais adultos clinicamente saudáveis;
- Por fim, o desenvolvimento da qPCR, baseada em uma região conservada do gene *rep*, possibilitou detectar e quantificar genomas de PBoV2 e mostrou ser um método de diagnostico molecular altamente específico, preciso e rápido; em virtude da padronização e detecção ter sido realizada a partir de amostras de DNA extraídas de soro, a técnica representa uma valiosa ferramenta para estudos de viremia e monitoramento da infecção em granjas de suínos;
- A técnica de qPCR permitiu demonstrar que animais acometidos pela SMDS apresentam uma carga viral significantemente superior a dos animais clinicamente saudáveis, com idade equivalente e que uma alta taxa de animais adultos apresentam elevado número de genomas de PBoV2 circulantes no soro;
- As PCR desenvolvidas no estudo mostraram ser importantes ferramentas para detecção de genomas virais dos diferentes bocavírus suíno (PBoV1, 2, 3 e 4), de citomegalovírus suíno (PCMV ou SuHV-2) e de circovírus suíno 2 (PCV2); no entanto, mais estudos são necessários para entender a

evolução e a epidemiologia dos recém-descobertos bocavírus em rebanhos suínos, assim como, para elucidar o envolvimento desses novos agentes na gênese e/ou patogênese da SMDS.

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