ALINE FERNANDES BARRY

CLASSIFICAÇÃO MOLECULAR DE SAPOVÍRUS SUÍNO E DINÂMICA DA INFECÇÃO DE DIFERENTES ESTIRPES EM SUÍNOS LIVRES DE PATÓGENOS ESPECÍFICOS

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Tese apresentada ao Programa de Pós-Graduação em Ciência Animal – área de concentração Sanidade Animal – da Universidade Estadual de Londrina como requisito parcial para a obtenção do título de Doutor em Ciência Animal.

Orientador: Prof. Dr. Amauri Alcindo Alfieri

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“Eu acredito demais na sorte. E tenho constatado que, quanto mais duro eu trabalho, mais sorte eu tenho.”

Thomas Jefferson

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RESUMO

O sapovírus (SaV) foram primeiramente associados a surtos de gastroenterite em crianças. Mais recentemente os SaV tem sido associados a surtos de diarreia em indivíduos de todas as idades. A descrição desses surtos vem também se tornando mais frequente independentemente dos testes diagnósticos utilizados. Em suínos, a frequência dos SaVs é elevada em diversos países, e é sabido que o vírus circula nos rebanhos suínos de todo o mundo. A alta identidade relativa de estirpes de SaV suíno com SaV humano, sugere que o suíno possa ser um reservatório do vírus humano, caracterizando assim uma zoonose, e também que recombinaçoes entre estirpes de SaV humano e suíno possam ocorrer. No presente estudo, foram conduzidos dois trabalhos com o objetivo de esclarecer aspectos ainda obscuros da patogenia e classificação dos SaV. O primeiro estudo, teve por objetivo organizar a classificação dos SaV suínos, utilizando como base os SaV humanos, cuja antigenicidade já é conhecida para algumas estirpes. Desse modo, a classificaçao foi realizada com base no gene da VP1. Uma vez que o gene da polimerase (RpRd) viral é o mais comunmente utilizado para o diagnóstico, a classificação do gene da VP1 foi comparada à filogenia obtida com o gene da RpRd, concluindo que para os SaV suínos, ambos os genes podem ser utilizados para a classificação em genogrupos. Com a classificaçao estabelecida, foi possível verificar que todos os genogrupos de SaV suíno circulam em todo o mundo. No segundo estudo, leitões livres de patógenos específicos (*SPF*) foram inoculados com estirpes de SaV suíno (GVII e GVIII) e uma estirpe de SaV humano (GI.2). As estirpes claramente mostraram diferentes padrões na dinâmica da infecção, incluindo eficiência na replicação e capacidade de invadir outros orgáos fora do trato gastrointestinal. A estirpe de SaV humano não se replicou nos animais, sugerindo que o suíno não é um reservatório do vírus humano e que provavelmente recombinações com estirpes de SaV humano e suíno sejam improváveis de ocorrer uma vez que para o fenômeno seja necessário a infecção concomitante com ambas as estirpes. Em nenhum dos grupos de leitões inoculados houve associação entra infecção por SaV e o desenvolvimento de sinais clínico. Em resumo, com o presente trabalho, uma classificação pode ser estabelecida para os SaV suino e importantes aspectos da dinamica da infecção em suínos foram esclarecidos.

Palavras-chave: Suínos. Leitões. Diarréia. Calicivirus. Sapovirus.

BARRY, A.F. **Molecular classification and dynamics of infection of different sapovirus strains in specific pathogen-free piglets.** 2012. 71p. Thesis (PhD Degree in Animal Science) - Universidade Estadual de Londrina, Londrina. 2012.

ABSTRACT

Sapovirus (SaV) were firstly associated with gastroenteritis in children. Recently, the virus has been associated to diarrhea outbreaks in people of all age. Independently of the diagnostic technique employed, SaV has been more frequently detected in the last few years. In pigs, the frequency is high in several countries, and SaV circulation is described worldwide. The high identity of human and porcine strains of SaV suggests that the pig can be the reservoir of the virus, and also the possibility of recombination. In the present study, two works were developed in order to elucidate aspects of SaV pathogeny and classification. In the first study, with basis on human SaV classification in which the antigenicity is known for some strains, a classification for porcine SaV were proposed. The analysis was performed in the VP1 complete gene, but also in the polymerase gene, that is the most commonly used for diagnosis. It was possible to conclude that both genes can be used for genogroup classification. Additionally, it was described the porcine SaV circulation worldwide. In the second study, SPF piglets were inoculated with porcine SaV (GVII and GVIII), and one human SaV (GI.2). Different dynamics of infection were shown, such as replication efficiency and ability to escape the gastroenteric tract. Human SaV did not replicate in piglets, suggesting that pigs are not SaV reservoir, and that is unlikely recombination between human and porcine SaV in pigs. In any of the inoculated groups was verified association between infection and clinical signs. In summary, with the present work, a classification for porcine SaV porcine was established, and important aspects of the dynamics of infection in pigs were clarified.

Keywords: Swine. Piglets. Diarrhea. Calicivirus. *Sapovirus.*

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1 REVISÃO DE LITERATURA

1.1 SAPOVÍRUS

Características gerais

Os sapovírus (SaV) foram primeiramente descritos em 1976 no Reino Unido em fezes de crianças assintomáticas e com gastroenterite (Madeley e Cosgrove, 1976). A estirpe protótipo do SaV, *Sapporo*, foi descrita em 1977 em um surto de diarreia aguda em crianças de uma creche na cidade de Sapporo, Japão (Chiba et al., 1979). Em suínos, o SaV foi detectado pela primeira em vez em 1980 nos Estados Unidos por meio de microscopia eletrônica do conteúdo intestinal de um leitão de 27 dias de idade com diarréia (Saif et al., 1980). Em 1974, foi proposta a criação da família *Caliciviridae* que hoje compreende os gêneros *Norovirus*, *Sapovirus*, *Vesivirus*, *Lagovirus* e *Nebovirus* (Burroughs & Brown, 1974; ICTV, 2012). A classificação foi aceita em 1979 pelo Comitê Internacional de Taxonomia Viral (Matthews, 1979). Há ainda os gêneros *Recovirus* e *Valovirus* em que a classificação ainda não foi aprovada (Farkas et al., 2008; L'Homme et al., 2009a).

Em seres humanos a sintomatologia decorrente da infecção por SaV é mais comumente observada em crianças, e em muitos casos sem sintomatologia clínica (Akihara et al., 2005; Hansman et al., 2006; Phan et al., 2007). Entretanto, recentemente os SaV vem sendo descritos também em surtos de gastroenterite em adultos (Svraka et al., 2010). Em suínos a associação entre a infecção pelo SaV e sinais clínicos gastrointestinais ainda não é completamente compreendida, mas a inoculação experimental em leitões gnotobióticos produz diarreia e lesões no intestino delgado (Guo et al., 2001a). No Brasil, o SaV foi descrito em seres humanos pela primeira vez em amostras de fezes colhidas nos anos de 1992 a 1994 provenientes de crianças com diarreia no estado do Pará (Nakamura et al., 2006). Poucos anos depois, foi descrita a presença do SaV em fezes de suínos com e sem diarréia (Barry et al., 2008a)

A maioria dos SaV não pode ser isolada em cultivo celular, o que torna difícil o conhecimento da replicação, antigenicidade e patogenia do vírus (Green, 2007). Para os SaV, a compreensão desses aspectos tem como base resultados obtidos com o isolamento em cultivo celular de outros calicivírus, estudos de epidemiologia molecular que utilizam a RT-PCR, a técnica padrão para detecção dos SaV, e a expressão de proteínas recombinantes (Green, 2007; Wang et al., 2007; Hansman et al., 2007e). A partir da produção de *virus like particles* (VLPs) das estirpes GI e GV, foi possível concluir, por exemplo, que diferentemente

dos norovírus (NoV), os SaV não utilizam os carboidratos HBGA como receptores (Shirato-Horikoshi et al., 2007).

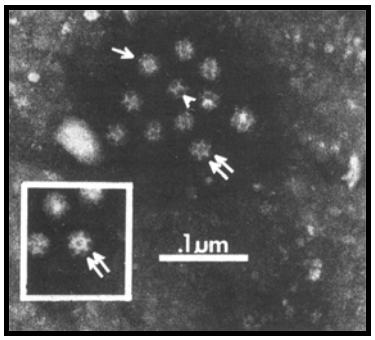
Devido à similaridade genética das estirpes de SaV suíno e humano é sugerido que a infecção por SaV possa ser uma zoonose (Bank-Wolf et al., 2010). Estirpes de SaV detectadas em suínos mas classificadas em genogrupos de SaV humanos já foram descritas, porém, ainda não se pode concluir se seres humanos podem se infectar com o SaV suíno, ou mesmo se o SaV suíno com alta similaridade genética com SaV humanos são capazes de uma infecção produtiva em suínos (Martella et al., 2008ª; Nakamura et al., 2010). Desse modo, além do vírus poder determinar perdas econômicas na cadeia produtiva de suínos, é possível que o suíno represente um reservatório do vírus também para seres humanos.

Características morfológicas e genéticas

Os SaVs não possuem envelope, apresentam 27-35 nm de diâmetro com simetria T=3 icosaédrica formado por uma única proteína, a VP1 (fig.1). O capsídeo é formado por 180 moléculas da proteína VP1 organizada em 90 dímeros (capsômeros). A VP1 apresenta dois domínios chamados de S (*shell*) e P (*protrusion*), esse último sendo ainda subdividido em dois subdomínios P1 e P2. A porção S apresenta a sequência de aminoácidos bastante conservada e estruturalmente forma uma cápsula contínua que envolve o genoma viral e dá a base para a porção P. O domínio P forma uma protrusão, sendo P1 a haste que sustenta P2, e esta forma uma estrutura globular e é a parte mais externa da estrutura do vírus. O subdomínio P2 apresenta a maior variabilidade genética no genoma dos SaV (Chen et al., 2004).

A existência de uma única proteína formando todo o capsídeo viral não é comum em vírus animais. Desse modo, uma única proteína, a VP1, forma o capsídeo viral, reconhece o receptor celular (adsorção viral), e confere ainda a especificidade de hospedeiro, diversidade e antigenicidade da estirpe viral (Chen et al., 2004).

Figura 1 - Microscopia eletrônica de contrastação negativa de partículas virais de sapovírus. Nas setas estão indicadas partículas virais com formato de "Estrela-de-Davi", característica dos calicivírus clássicos.

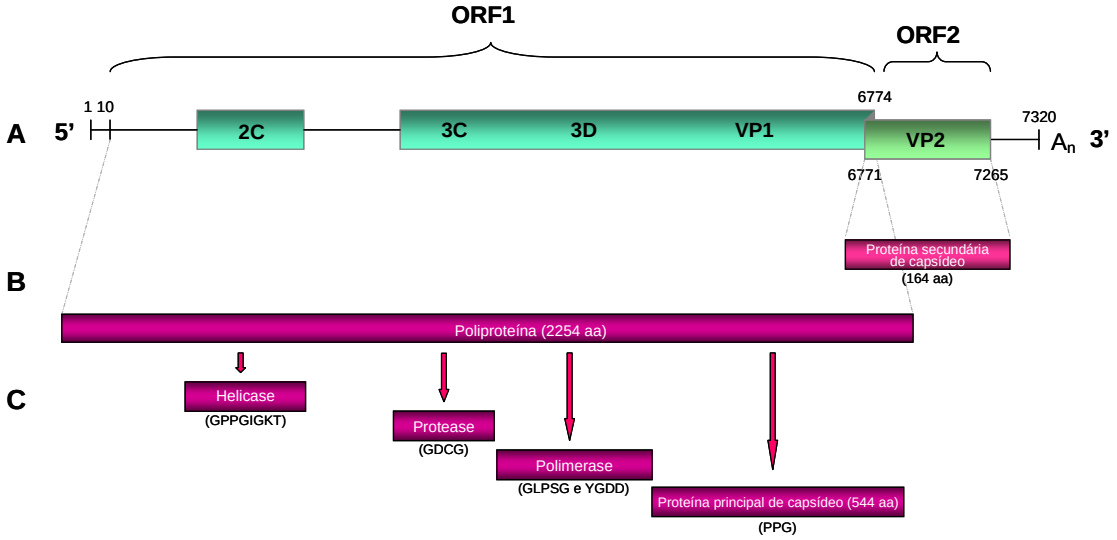


Fonte: Flynn & Saif, 1988.

O genoma do SaVs é composto por uma fita simples linear de RNA de polaridade positiva com aproximadamente 7,5 kb e dividido em dois ou três quadros abertos de leitura (ORFs). A ORF1 é codificada em um grande polipeptídeo que é clivado ao mesmo tempo em que é traduzido dando origem a seis ou sete proteínas não-estruturais, necessárias para a replicação viral, incluindo a helicase, protease e polimerase viral (RNA polimerase RNA dependente – RpRd) (Oka et al., 2005).

A proteína principal de capsídeo ou VP1 é codificada na mesma ORF que as proteínas não-estruturais. A VP1 possui em média 560 aminoácidos (aa) e apresenta ao longo de sua extensão regiões de maior (P2) ou menor (S e P1) variabilidade genética (Chen et al., 2004). Na região 3´ do genoma viral está a ORF2, que codifica uma proteína básica estrutural denominada VP2 (165 aa) que confere maior estabilidade na estrutura da partícula viral e também tem a função de regular a expressão da VP1 (Bertolotti-Ciarlet et al., 2003). Em algumas estirpes humanas de SaV (GI, GVI e GV) pode também haver uma outra ORF sobreposta à região 5' do gene da VP1 chamada ORF3 que codifica uma proteína de 160 aa, ainda com função desconhecida. A existência dessa terceira ORF é sugerida devido à existência da sequência de iniciação *GCAAUGG*. Em ambas as extremidades 5' e 3' do genoma dos SaV há ainda sequências de nucleotídeos (nt) que não são codificadores de proteínas (UTR – *untranslated regions*) (Clarke; Lambden, 2000) (fig.2).

Figura 2 - Esquema representativo do genoma da estirpe Cowden. (A) Representação das duas ORFs, em que os números representam a posição dos nucleotídeos e An a extremidade poliadenilada (B) Proteínas codificadas pelo vírus (C) Produtos da clivagem da poliproteína com seus respectivos sítios conservados entre os calicivírus.



Fonte: Barry et al., 2008b.

Classificação

A classificação mais comumente utilizada para os SaV é feita com base na análise genética (Farkas et al., 2004). Conforme aceito pelo Comitê Internacional de Taxonomia Viral (ICTV), os SaV são classificados em genogrupos (G) que são ainda divididos em genotipos. Devido a descrição de novas estirpes virais, essa classificação vem constantemente sofrendo modificações. A primeira classificação proposta para o gênero *Sapovirus*, constava de três genogrupos, sendo os GI e GII compostos exclusivamente de estirpes de SaV humano e o GIII do único representante do SaV suíno, a estirpe Cowden. Essa classificação foi feita com base em apenas 11 estirpes de SaV e comparativamente aos NoV, que já possuíam uma classificação aceita. Neste estudo, foi utilizada a distância e análise filogenética de nucleotídeos ou aminoácidos entre as estirpes de SaV, considerando cinco regiões diferentes do genoma: o gene completo da VP1, o gene RpRd, a ORF na região 3', a ORF que se sobrepõe ao início da VP1 e a região não codificadora na terminação 3' (Schuffenecker et al., 2001).

No ano seguinte, foi descrita uma nova estirpe de SaV humano que não apresentava similaridade alta de aminoácidos com as estirpes já descritas e foi proposta como

um novo genogrupo, o GIV. Porém, neste estudo apenas um pequeno fragmento do gene da proteína do capsídeo foi analisado (Okada et al., 2002).

Em 2004, novas estirpes foram descritas e uma nova classificação proposta. Com base em um fragmento do gene da RpRd, outros dois genogrupos foram propostos para o SaV humano. A análise em nucleotídeos e aminoácidos do gene completo da VP1 confirmou a classificação proposta mesmo no pequeno fragmento (286 nt) do gene RpRd. Considerando também a classificação anterior proposta em 2001, esses genogrupos foram chamados de GIV e GV. Os SaV suínos continuaram sendo classificados apenas no GIII (Farkas et al., 2004).

Para os SaV humanos, a classificação dos genogrupos não sofreu mais alterações. Entretanto, com o aumento do número de estudos de epidemiologia molecular, mais estirpes foram descritas e classificadas em diferentes genotipos dentro dos quatro genogrupos já estabelecidos. Até o momento são aceitos 7 genotipos no GI e no GII cada e 1 genotipo no GIV e GV (Oka et al., 2012).

Assim como nos estudos epidemiológicos de SaV humano, a pesquisa com SaV suíno também levou a descrição de mais estirpes geneticamente distintas e que não puderam ser classificadas no genogrupo já existente. Nos EUA, um estudo propôs a criação do GVI considerando a filogenia do gene completo da VP1 (Wang et al., 2005). Neste mesmo estudo, foram descritas duas estirpes de SaV detectadas em suínos, mas com maior similaridade com SaV humanos, o que levou a especulação do potencial zoonótico dos SaV. Após sucessivas falhas em amplificar por RT-PCR outras áreas do genoma, essa análise ficou restrita a apenas um fragmento do gene RpRd. No Japão, poucos meses depois, após o sequenciamento do genoma completo de uma estirpe de SaV detectada em suínos, foi proposto o GVII (Yin et al., 2006). Estirpes de SaV com alta similaridade genética com as estirpes norte-americanas que não puderam ser classificadas em nenhum genogrupo foram detectadas na Itália. O gene completo da VP1 dessa estirpe foi sequenciado e a análise filogenética revelou se tratar de um novo genogrupo de SaV suíno, mas com maior proximidade aos genogrupos de SaV humano. Esse genogrupo foi classificado GVIII (Martella et al., 2008a).

Em um estudo recente conduzido por Reuter et al. (2010) foi sugerida a classificação de outros dois novos genogrupos de SaV, GIX e GX, e a subdivisão do GIII em GIII/A e GIII/B. Porém, embora um número significativo de amostras tenha sido incluídas na filogenia, essa análise foi feita apenas com base em um pequeno fragmento do gene RpRd e carece de maior embasamento (Reuter et al., 2010c). Outros dois estudos realizados no Canadá descrevem a existência de dois outros grupos de SaV que não puderam ser

classificados nos genogrupos já descritos (mesmo considerando os GVI, GVII e GVIII). Nesses trabalhos, não foi proposta nenhuma classificação, mesmo após a realização de uma análise do gene completo da proteína do capsídeo (L'Homme et al., 2009b; 2010).

Embora os novos genogrupos de SaV suíno tenham a mesma base de análise molecular e filogenética utilizadas para a classificação dos genogrupos dos SaV humanos, a classificação dos SaV ainda permanece, na maioria dos estudos, considerando apenas a existência de cinco genogrupos no gênero com os SaV suínos sendo classificados apenas no GIII (Quadro 1) (Farkas et al., 2004; Oka et al., 2012).

Quadro 1 - Classificação genética dos SaV com base no gene completo da VP1

Genogrupo	Protótipo	No. de acesso	Origem
I	Sapporo[1]	U65427	humano
II	SLV/Bristol/98/UK	AJ249939	humano
III	Cowden	AF182760	suíno
IV	Hou7-1181	AF435814	humano
V	Arg39	AF405715	humano
VI[2]	Po/OH-JJ681/2000/US	AY974192	suíno
VII[2]	K7/JP	AB221130	suíno
VIII[2]	pig/43/06-18p3/06/ITA	EU221477	suíno

[1]Estirpe protótipo do gênero

[2]Genogrupo com classificação ainda pendente

Recombinações

Com os genogrupos e alguns genotipos de SaV já estabelecidos, começaram a ser descritas também estirpes recombinantes. Nos SaV, a região genômica de junção entre o gene da RpRd e da VP1 apresenta uma sequência de nucleotídeos de aproximadamente 25 nucleotídeos bastante conservada, e por isso permite a recombinação, por *copy choice* (Hansman et al., 2007c). Nesse mecanismo, a recombinação ocorre quando o indivíduo se infecta concomitantemente com duas estirpes distintas de um mesmo vírus e no processo de replicação a polimerase viral, responsável pela síntese da nova fita do genoma, troca de fita molde produzindo assim uma molécula híbrida de RNA (Worobey & Holme, 1999).

A primeira recombinação no gênero *Sapovirus* foi descrita entre duas estirpes do GII, a Mc10 e a C12. Pelo gene da RpRd, essas duas estirpes são classificadas no mesmo genotipo, mas em genotipos diferentes quando realizada a classificação com base no gene da VP1. Na comparação da similaridade das sequências ao longo do genoma foi detectado o ponto de recombinação na junção dos genes da RpRd e do capsídeo viral (junção

pol-cap). Entretanto, não foi possível identificar qual a estirpe recombinante e qual a parental entre Mc10 e C12 (Katayama et al., 2004).

Nos SaV suínos foram descritas recombinações intragenotipo no GIII. Duas estirpes, MM280 e QW270, apresentaram maior similaridade no gene da VP1 quando comparado ao gene da RpRd com a estirpe Cowden, o que é o oposto do fenômeno observado em todos os calicivírus, em que a RpRd apresenta-se mais conservada. Por essa razão, foi realizado o sequenciamento de uma região contínua de 3 kb contendo ambos os genes e foi concluído que menos passos eram necessários para o surgimento de MM280 e QW270 se mutações e recombinações acontecessem juntas do que exclusivamente mutações (8.207 x 11.352, respectivamente). Assim como para as estirpes de SaV humano Mc10 e C12, o ponto de recombinação também ocorreu na junção pol-cap (Wang et al., 2005).

Em 2005, foi descrita a primeira recombinação intergenogrupo. Após o sequenciamento do genoma completo de quatro estirpes de SaV humano foi realizada a análise filogenética. As estirpes Ehime1107 (Japão) e SW278 (Suécia) apresentaram polimerase do GII e VP1 do GIV com a recombinação ocorrendo também na junção pol-cap. A descrição de uma recombinação entre genogrupos diferentes forneceu mais dados para se especular sobre recombinações entre estirpes de SaV humano e suíno que pertencem a genogrupos diferentes, e o potencial zoonótico do vírus (Hansman et al., 2005a).

Embora fosse sugerido que recombinações envolvendo o GII fossem mais comuns, também foram descritas recombinações intragenogrupo I. A estirpe recombinante HU/5862/Osaka/JP apresenta polimerase GI.1 e capsídeo GI.8 (genotipo ainda com classificação pendente). Assim como as estirpes do GII e III, a recombinação também ocorreu na junção pol-cap. Nesse estudo foi sugerido que a classificação de uma estirpe fosse realizada com base em ambos os genes, RpRd e VP1 (Phan et al., 2006b). No mesmo GI e genotipo 1, também foi descrita uma recombinação intragenotipo, entretanto a classificação nos subgenotipos que servem como base para o estudo, não é utilizada universalmente. O mesmo autor sugere que a região de maior ocorrência das recombinações, a junção pol-cap, seja espécie especifica e que, portanto, a probabilidade de recombinações entre estirpes de SaV humano e suíno seja improvável de acontecer (Phan et al., 2007).

Outras recombinações intragenotipo e intragenogrupo também foram descritas para SaV humano e suíno (Phan et al., 2006c; Nguyen et al., 2008; Dey et al., 2011). Embora em alguns estudos a confirmação da ocorrência da recombinação seja insuficiente, esse processo de evolução para os SaVs parece relativamente comum (Jeong et al., 2007; Phan et al., 2007; Dos Anjos et al., 2011). A rápida evolução de algumas estirpes utilizando esse

processo parece também encontrar uma população de hospedeiros mais susceptível e como consequência causar surtos de gastroenterite. A distribuição geográfica de estirpes recombinantes foi descrita mais frequentemente na Ásia, mas existem relatos em todo o mundo (Hansman et al., 2007b).

Antigenicidade

Como citado anteriormente, apenas a estirpe Cowden, protótipo do GIII, foi isolada em cultivo celular (Flynn & Saif, 1988; Parwani et al., 1991; Chang et al., 2004). A ausência de um sitema de cultivo e isolamento eficiente para os SaV limita, em parte, um melhor conhecimento sobre a antigenicidade das diferentes estirpes existentes. Até o momento, poucos estudos foram conduzidos nessa área, mas apenas com SaV humanos (Jiang et al., 1997; Hansman et al., 2007e).

No primeiro estudo em que foi sugerida a diversidade genética dos SaV, que anteriormente eram classificados em um único grupo dentre os calicivírus humanos, também foi demonstrada a relação antigênica das estirpes analisadas (Jiang et al., 1997). Uma região de 3.2 kb do genoma de três estirpes de SaV humano responsável por surtos de gastroenterite em crianças foi sequenciada e o resultado revelou haver variabilidade genética. Com o objetivo de avaliar a antigenidade das estirpes detectadas, as amostras foram testadas em um ELISA previamente desenvolvido utilizando partículas purificadas da estirpe protótipo do gênero, Sapporo (Matson et al., 1989). O resultado sugeriu que a diversidade verificada na sequência no genoma das estirpes poderia ser biologicamente significativa, pois estirpes classificadas em genogrupos diferentes e mesmo estirpes pertencentes ao mesmo genogrupo (GI), mas a genotipos diferentes, não apresentaram reação cruzada. (Jiang et al., 1997).

Utilizando diferentes sistemas de expressão em células de insetos ou mamíferos e baculovírus como vetor, verificou-se que a proteína recombinante da VP1 dos SaV resulta da formação de VLPs, ou seja, estruturas semelhantes à partículas virais, mas sem a presença do material genético. Essas partículas sofrem auto-montagem e após sua formação apresentam tamanho e conformação muito similares a partícula viral (Numata et al., 1997; Guo et al., 2001b; Hansman et al., 2005b; Oka et al., 2009). Já com os cinco genogrupos de SaV humano estabelecidos e dispondo do sistema de expressão para a VP1 foi desenvolvido um *Western blotting* e um ELISA a partir de proteínas recombinantes de capsídeo de estirpes dos genogrupos I, II e V. Foi verificado que, apesar de pertencerem a genogrupos diferentes e distantes na análise filogenética, algumas estirpes de SaV humano apresentam reação cruzada

no *Western blotting* (Hansman et al., 2005b). Entretanto, meses depois, o mesmo grupo desenvolveu um ELISA com VLPs de uma estirpe do GV e testou com anti-soro de estirpes do GI, GII e GV, demonstrando que a reação é genogrupo-específica (Hansman et al., 2005c).

Em um estudo mais abrangente foram desenvolvidos ELISAs para anticorpos e antígenos a partir de cinco estirpes de SaV humano incluindo os quatro genogrupos de SaV humano e dois genotipos do GI. No teste direto (Ag ELISA), nenhuma reação cruzada foi verificada. Já no ELISA indireto, as duas estirpes do GI apresentaram fraca reação cruzada entre elas e também com a estirpe do GII, entretanto, a reação com estirpes homólogas se mostrou significativamente mais alta. As estirpes dos GII, GIV e GV demontraram apenas reação com a estirpe homóloga (Hansman et al., 2007e).

A elaboração de dois sistemas de ELISA para detecção de partículas virais utilizando a expressão em células de mamíferos e incluindo uma região adicional anterior ao códon de iniciação da VP1 permitiu verificar que estirpes do mesmo genogrupo (GII), mas genotipos diferentes (GII.2 e GII.3) apresentam antigenicidade diferentes (Oka et al., 2009). Embora não tenha sido discutido nesse estudo, uma importante conclusão pode ser extrapolada para a seleção do gene de classificação dos SaVs. As estirpes utilizadas para a elaboraçao dos testes de ELISA são recombinantes e pela filogenia do gene RpRd pertencem ao mesmo genotipo no GII. Já na filogenia do gene da VP1, pertencem a genotipos diferentes (também no GII). Desse modo, e considerando que a reação cruzada não foi significativa, é prudente afirmar que a classificação com base do gene da VP1 apresenta maior fundamento em ser utilizada, pois será a responsável pela indução da resposta imune do hospedeiro independentemente da polimerase viral.

Em resumo, esses estudos em conjunto confirmam e conferem mais embasamento prático para a classificação em genogrupos e genotipos proposta com base na filogenia da sequência do gene da VP1. O Quadro 2 demonstra as reações antigênicas entre algumas estirpes de SaV humano.

Quadro 2 - Reação antigênica entre estirpes de SaV humano

Estirpe viral - Ag (G)	Soro - Ac (G)	Reação	Teste	Referência
Sapporo/82 (GI.1)	Houston/86 (GI.1)	+	ELISA (partículas purificadas)	Jiang et al., 1997
	Houston/90 (GI.2)	-		
	London/92 (GII.1)	-		
Mc114 (GI.1)	Mc114 (GI.1)	+	Western blotting (rVP1)	Hansman et al., 2005a
	C12 (GII)	+ fraco		
	NK24 (GV.1)	-		
C12 (GII)	Mc114 (GI.1)	-		
	C12 (GII)	+		
	NK24 (GV.1)	-		
NK24 (GV.1)	Mc114 (GI.1)	+		
	C12 (GII)	-		
	NK24 (GV.1)	+		
Mc114 (GI.1)	Mc114 (GI.1)	+	Ag ELISA	Hansman et al., 2007b
	Yokote1 (GI.5)	-		
	Syd53 (GII.3)	-		
	Syd3 (GIV.1)	-		
	NK24 (GV.1)	-		
Yokote1 (GI.5)	Mc114 (GI.1)	-		
	Yokote1 (GI.5)	+		
	Syd53 (GII.3)	-		
	Syd3 (GIV.1)	-		
	NK24 (GV.1)	-		
Syd53 (GII.3)	Mc114 (GI.1)	-		
	Yokote1 (GI.5)	-		
	Syd53 (GII.3)	+		
	Syd3 (GIV.1)	-		
	NK24 (GV.1)	-		
Syd3 (GIV.1)	Mc114 (GI.1)	-		
	Yokote1 (GI.5)	-		
	Syd53 (GII.3)	-		
	Syd3 (GIV.1)	+		
	NK24 (GV.1)	-		
NK24 (GV.1)	Mc114 (GI.1)	-		
	Yokote1 (GI.5)	-		
	Syd53 (GII.3)	-		
	Syd3 (GIV.1)	-		
	NK24 (GV.1)	+		
Mc10 (GII.2)	Mc10 (GII.2)	+	Ag ELISA	Oka et al., 2009
	C12 (GII.3)	-		
C12 (GII.3)	Mc10 (GII.2)	-		
	C12 (GII.3)	+		

Ag - antígeno; Ac - anticorpo; G - genogrupo

Epidemiologia

A transmissão dos SaV ocorre via fecal-oral direta ou indiretamente da pessoa ou animal infectado, por meio da ingestão de água ou alimentos contaminados, ou por aerossóis (provenientes do vômito) (Green, 2007). Além das fezes de indivíduos infectados, a presença do material genético do SaV também já foi detectada em água de esgotos tratados e

não tratados e águas de rios no Japão. A análise molecular revelou a presença de diferentes estirpes de SaV pertencentes aos GI e GV (Hansman et al., 2007d). Também já foram descritos surtos de gastroenterite ocasionados pelo consumo de ostras cruas em que foram identificadas diversas estirpes de SaV pertencentes a genogrupos (I e II) e genotipos distintos (Nakagawa-Okamoto et al., 2009).

Em seres humanos, a distribuição do SaV é mundial. Entretando, devido ao uso de diferentes técnicas diagnósticas com diferente sensibilidade para detecção do vírus e à diferentes características das populações avaliadas, a real prevalência da infecção não é precisa. Há porém consenso que crianças menores de cinco anos são mais frequentemente infectadas que adultos, e que ambientes que favoreçam a aglomeração como creches e orfanatos são fatores de risco para a infecção/transmissão (Hansman et al., 2007c). Casos esporádicos ou surtos de gastroenterite ocasionados pela infecção por SaV tem se tornado mais frequente, entretanto, a infecção em indivíduos assintomáticos também é comum. Por outro lado, recentemente mais casos de infecção com sintomas clínicos tem sido reportados em populações de todas as idades (Phan et al., 2006a; Hansman et al., 2007a; 2007c; Svraka et al., 2010).

Em um estudo conduzido em 2010, foi descrito que surtos de gastroenterite por SaV tem se tornado mais frequente nos últimos anos comparativamente a anos anteriores. Indivíduos de todas as idades (0 a mais de 100 anos) foram acometidos. Neste estudo, 4% dos surtos de diarreia em um período de aproximadamente um ano foram causados por SaV. Com o objetivo de excluir a hipótese de melhores testes diagnósticos utilizados atualmente, amostras de surtos anteriores foram re-testadas confirmando que no período mais recente (2007-2009) houve aumento significativo nos surtos por SaV. No mesmo estudo, o mesmo padrão foi observado para outras regiões do mundo, (Svraka et al., 2010).

Segundo Svraka et al. as estirpes de SaV mais frequentemente associadas a surtos de gastroenterite pertencem ao GI.2. É possível que as estirpes pertencentes a esse genotipo apresentem mecanismos de persistência na população como por exemplo baixa dose infectante, alta infectividade, alta excreção viral pelo hospedeiro, alta persistência ambiental, múltiplas vias de transmissão e excreção prolongada após recuperação clínica (Svraka et al., 2010). Entretanto, surtos em que outros genotipos estão envolvidos também são descritos (Akihara et al., 2005; Phan et al., 2006a).

Em suínos, a circulação dos SaV já foi descrita também em diversos países em todo o mundo (Martinez et al., 2006; Wang et al., 2006; Martella et al., 2008b; L´Homme et al., 2009; Nakamura et al., 2010; Reuter et al., 2010c). Do mesmo modo que em seres

humanos, diferentes técnicas diagnósticas são utilizadas e por isso a frequência de infecção em diferentes populações também varia, sem necessariamente representar a real prevalência. Os dados apresentados variam de 4.9 a 62% (Kim et al., 2006; Wang et al., 2006). No Brasil, em estados produtores de suínos, foi descrita a frequência de 30,1% de SaV em leitões (Barry et al., 2008a). Assim como ocorre em seres humanos, a infecção por SaV suíno é mais frequentemente descrita em indivíduos mais jovens em idade de início de creche (Wang et al., 2007; Barry et al., 2008a).

O teste diagnóstico mais utilizado para detectar SaV em populações suínas é a RT-PCR, que permite, pelo sequenciamento, analisar e identificar a estirpe infectante. Entretanto, devido ao baixo número de amostras sequenciadas na maioria dos estudos, há apenas uma estimativa da prevalência por genogrupo de SaV suíno. Acredita-se que a estirpe mais prevalente de SaV suíno pertença ao GIII. Em trabalhos mais recentes, e com base ainda em uma classificação pendente, é possível que os SaV GVII e GVIII também circulem em todo o mundo. O GVI é o genogrupo menos frequentemente detectado até o momento (Reuter et al., 2010; Svraka et al., 2010).

Patogenia

Pouco se sabe ainda sobre a patogenia da infecção por SaV em seres humanos. O único estudo com inoculação experimental foi conduzido em leitões gnotobióticos com uma estirpe suína de SaV: a estipe Cowden, protótipo do GIII. Os leitões inoculados com a estirpe selvagem apresentaram diarreia por 2 a 5 dias e excreção viral por pelo menos 7 dias. O vírus foi detectado por ELISA, RT-PCR e microscopia eletrônica. Na histopatologia foi detectada atrofia moderada a severa e fusão das vilosidades de duodeno e jejuno. Por imunofluorescência, o antígeno viral também pode ser detectado na superfície dos enterócitos do intestino proximal. Viremia também foi detectada nos leitões inoculados (Guo et al., 2001).

Diagnóstico

As primeiras estirpes de SaV foram detectadas por meio de microscopia eletrônica, entretanto, esse método de diagnóstico é laborioso e de baixa sensibilidade (Green, 2007). O cultivo celular é uma técnica muito utilizada para o isolamento de diversos vírus. Embora não seja utilizado como método de diagnóstico na maioria dos casos devido ao tempo

necessário para o isolamento viral, ele possibilita a execução de outros testes para detecção viral como, por exemplo, a soroneutralização. Entretanto, não foi descrito até o momento um sistema de cultivo celular eficiente para a replicação de todos os SaV humanos e animais (Green, 2007). Apenas a estirpe Cowden, já foi replicada com sucesso em cultivo celular, porém, com a necessidade de condições específicas para o isolamento do vírus (Flynn & Saif, 1988; Parwani et al., 1991; Chang et al., 2004). Alguns testes como ELISA já foram desenvolvidos utilizando proteínas recombinantes, mas os estudos tiveram como objetivo avaliar antigenicidade viral e não foram aplicados em grande número de amostras para diagnóstico e, portanto, não realizado é rotineiramente (Atmar; Estes, 2001; Hansman et al., 2007e; Oka et al., 2009).

Devido às limitações dos testes comumente utilizados em virologia, a RT-PCR é a técnica mais utilizada tanto para o diagnóstico da infecção, quanto para a identificação e classificação das estirpes de SaV. Todavia, a grande variabilidade genética, principalmente dos SaV suínos, torna difícial uma técnica diagnóstica que permita detectar e classificar com base na VP1 os SaV. Desse modo, a detecção é rotineiramente realizada utilizando como alvo o gene da RpRd, que é relativamente conservado no genoma dos SaV. Muitos estudos utilizam a filogenia dessa região como suficiente para determinar o genogrupo da estirpe viral. Porém, essa classificação ainda necessita de mais estudos para comprovar a sua legitimidade (Wang et al., 2007).

Em resumo, o aumento da pesquisa por SaV em populações suínas e humanas tem demonstrado que a frequência de infecção do vírus é mais alta do que inicialmente acreditava-se. Pouco se sabe ainda sobre a real prevalência, patogenia e muitos aspectos epidemiológicos da infecção por SaV. Estudos moleculares e filogenéticos vêm demonstrando que os SaV apresentam grande variabilidade genética e que recombinações são processos relativamente freqüentes. Desse modo, mais estudos são necessários para entender o processo evolutivo do vírus, o papel dos diferentes hospedeiros, em particular a relação entre as estirpes de SaV de seres humanos e suínos, e a real relação causa e conseqüência das infecções por SaV.

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2 OBJETIVOS

2.1 GERAL

- Propor uma classificação molecular para as estirpes de sapovírus (SaV) suíno detectadas em todo o mundo e definir a dinâmica da infecção por SaV humano e suíno por meio da inoculação em leitões livres de patógenos específicos (*SPF*).

2.2 ESPECÍFICOS

- Por análise filogenética do gene da VP1, propor uma classificação para as estirpes de SaV suíno e verificar se a mesma classificação pode ser aplicada utilizando-se o gene da RNA polimerase viral;
- Identificar e classificar nos genogrupos determinados pela classificação proposta as estirpes de SaV suíno circulantes em todo o mundo;
- A partir de estirpes de SaV classificadas em diferentes genogrupos e provenientes de amostras de fezes, reproduzir a infecção por SaV suíno (GVII, GVIII) e humano (GI.2) em leitões *SPF*;
- Caracterizar particularidades da infecção por SaV dos genogrupos GI.2, GVII e GVIII em leitões *SPF*;
- Verificar a associação entre as diferentes estirpes de SaV suíno e humano inoculadas e sinais clínicos desenvolvidos nos leitões;
- Comparar a dinâmica da infecção de estirpes dos GVII e GVIII do SaV suíno e do GI.2 do SaV humano em leitões experimentalmente infectados.

3 ARTIGOS PARA PUBLICAÇÃO

SHORT COMMUNICATION

Genetic classification and geographical distribution of porcine sapovirus

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Abstract

Sapovirus (SaV) has been described as a cause of gastroenteritis worldwide. In swine, SaV are also described in several countries. In most of the studies virus detection is performed by RT-PCR followed by sequencing and molecular analysis. As a consequence, several strains have been identified, but most of the porcine SaV remains without classification. In the present study a classification for porcine SaV using phylogenetic analysis and pairwise distance among all the available complete VP1 gene of SaV is proposed. Since the RNA-dependent RNA polimerase (RdRp) is the most common gene employed in the diagnostic of SaV, the correlation with this gene was also performed. Additionally, based on the phylogenetic analysis of this RdRp fragment we verify the world distribution of SaV.

Keywords: Pig, Calicivirus, Sapovirus, genetic diversity, phylogenetic analysis

Introduction

Viral gastroenteritis is a major public health problem worldwide, and the enteric caliciviruses (norovirus – NoV and sapovirus – SaV) are responsible for most of the reported cases (Frankhauser et al., 2002; Lopman et al., 2003). NoV is the leading cause of outbreaks, but more recent studies have described also an important role of SaV infection in cases of gastroenteritis (Hansman et al., 2007a; Svraka et al., 2010). The epidemiology of SaV

infections is not well established, and since SaV can also infect animals, and that porcine and human SaV strains present high genetic identity, the zoonotic potential of SaV is still being investigated (Bank-Wolf et al., 2010).

SaV infection in pigs were described in several countries and it is possible its circulation worldwide. The infection rate ranges from 4.9 to 62% in diarrheic and asymptomatic pigs (Kim et al., 2006; Wang et al., 2006; Reuter et al., 2010). It is also described that the infection can be age-related and the young animals (before weaning) are more frequently infected than older pigs (Wang et al., 2006; Barry et al., 2008).

Sapovirus is a genus of *Caliciviridae* family which also includes other genera (*Norovirus*, *Lagovirus*, *Vesivirus*, *Nebovirus*, *Recovirus* and *Valovirus*) (Green et al., 2000; Smiley et al., 2002; Farkas et al., 2008; L'Homme et al., 2009). Viral particles are non-enveloped with 27 to 35 nm in diameter and icosahedral symmetry (Guo et al., 1999). The genome is a linear positive-sense, single-stranded polyadenilated RNA molecule with 7.3 kb in length organized in two or three open reading frames (ORFs). ORF1 encodes a polyprotein that is simultaneously synthesized and cleaved in the viral non-structural proteins and the main capsid protein (VP1) (Oka et al., 2005). Another structural protein is codified by ORF2 and is responsible for stabilization of the viral particle and also regulation of VP1 expression (Bertolotti-Ciarlet et al., 2003). Some human SaV strains posses another ORF that overlaps the 5' of VP1 gene (Clarke; Lambden, 2000).

The cell propagation of porcine sapovirus was already described, but the virus requires special conditions to be able to replicate, and the isolation is not routinely employed for diagnosis (Chang et al., 2004). As a consequence, most studies perform SaV screening by RT-PCR followed by sequencing, which allows the molecular identification and phylogenetic analysis among strains circulating in populations of different geographical areas (Reuter et al., 2010). However, since the virus presents high genetic variability and is constantly evolving, the use of an unique efficient (sensitive and specific) PCR to detect all the SaV genogroups is not possible, being necessary the employment of one assay for screening (usually targeting the RNA-dependent RNA polimerase – RdRp that is highly conserved) and another one, targeting a more variable region (VP1) of the genome, for the identification and classification of the strain (Jiang et al., 1999; Oka et al., 2012).

For SaV, and mainly porcine SaV, there is not a consensus yet to classify strains. Some studies use the classification based on RdRp gene only, and others on the partial or complete VP1 gene (Farkas et al., 2004; L'Homme et al., 2009; Reuter et al., 2010). Within most used classifications, SaV are classified in five genogrups (G), GIII being the only one formed by

porcine SaV strain (Farkas et al., 2004). Based on analysis of the complete genome or at least the complete VP1 gene, GVI, GVII and GVIII are also proposed (Wang et al., 2005a; Yin et al., 2006; Martella et al., 2008). However, this classification is not universally accepted.

The goal of a classification system is to provide a useful and viable method to identify the strains using solid bases such as the differences of antigenicity between strains, which reflect the way the host will produce the immune response to the antigen (Green et al., 1995). Based on expression of recombinant VP1 proteins of human SaVs, it was described that strains genetically classified in different genogroups and even genotypes do not present cross reactivity, which indicates a good correlation between genetic classification and immunologic response by the host (Hansman et al., 2007b).

The objective of the present study is to establish a classification for porcine SaV based on phylogenetic analyses of the complete VP1 gene of human SaV and to correlate this classification with the classification based on partial RdRp gene. Additionally, the phylogenetic analysis of porcine SaV detected worldwide was performed.

Materials and methods

SaV RdRp and VP1 gene sequences

The initial SaV sequences search included more than 2.000 files, but only 142 could be included in the complete VP1 gene analysis. Of these sequences, 111 are human SaV, 28 porcine, 1 from a sea lion, 1 from a dog, and another from a mink. Since most of the studies performed with porcine SaV use the molecular analysis of a partial region (331 bp amplified by the primers designed by Jiang et al., 1999) of the RdRp gene, all sequences used in the complete VP1 gene analysis in which the RdRp fragment were also available was used in the phylogeny and pairwise distance analyses. For the analysis of porcine SaV circulating in pigs worldwide, the first analysis was performed separately for each country, and then for continent. The objective was to include in the final tree at least one representative strain per genogroup of each country. In total 322 sequences were available at the present date, being 88, 120 and 114 from America, Asia and Europe, respectively. All the sequences used in this study were obtained from GenBank.

Molecular analysis

Multiple alignments with ClustalW (version 1.4) was performed at MEGA version 5. After the alignment, VP1 incomplete sequences and fragments of different regions of the RdRp had to be removed for the general analysis.

Phylogenetic analysis

The phylogenetic trees for the VP1 gene and for the RdRp fragment were reconstructed by Neighbor-joining method based on Maximum Composite Likelihood model. The RdRp tree with sequences from all countries in which SaV was detected was generated using the Tamura Nei model. For all the dendrograms bootstrap of 1000 replicates were used at MEGA version 5.

Pairwise distance

Pairwise distances between human SaV strains were calculated in MEGA version 5 using the Maximum Composite Likelihood model.

Results and discussion

SaV VP1 phylogeny

The dendrogram reconstructed with the SaV VP1 gene showed the four already well defined human SaV genogroups: GI and GII composed by 7 genotypes each, and GIV and GV composed by only one genotype each (Fig.1). The porcine SaV strains were organized in GIII, in which the Cowden strain is the prototype; a branch (GVIII) formed by only porcine SaVs but closer to human SaVs than to other porcine strains; and also another big branch with porcine SaVs in which a definitive classification is still lacking even in the original study where they were described (Wang et al., 2005a; Yin et al., 2006; Martella et al., 2008; L´Homme et al., 2009). Other two strains detected in pigs in Japan were closely related to human SaV GV (Nakamura et al., 2010).

Despite de fact that some porcine SaV groups are well defined in the phylogenetic tree, the exclusively use of this analysis is not enough to define if the porcine SaV organized in the big branch (swine SaV) belong to different genogroups, or if they are all part of the same group and should be classified in different genotypes.

Genogroups definition

For human SaV, it was verified that the genetic classification and subdivision in genogroups and genotypes correlates well with the host immunological response (Hansman et al., 2007b; Oka et al., 2009). Since the antigenicity is only know for human SaV and in order to avoid arbitrary classification based on small fragments of different genes, a standardization of porcine SaV genogroups should be performed based on sequences of human SaV strains.

The pairwise distance analysis performed based on the VP1 gene of human SaV showed the intragenotype, intragenogroup and intergenogroup distances from 0.000-0.225; 0.282-0.841 and 1.378-1.949, respectively. The distance values do not overlap, and porcine SaV groups of strains can be defined based on this values, i.e., classified in the same genotype, genogroup or a new genogroup created if the distance is higher than what established.

In this way, it can be concluded that based on complete VP1 gene SaV GIII is composed only by porcine SaV strains and posses only one genotype. Twelve of the 28 porcine SaV sequences, including the prototype Cowden strain, were classified in GIII. Only a few studies have been using the classification of other porcine SaV genogroups (L´Homme et al., 2009; Reuter et al., 2010). However, as proposed in the original study in which the strains were described and also in the present study, GVI and GVII strains formed other genogroups: OH-JJ681 is the only and prototype strain of GVI; and four strains form the GVII, which the prototype is K7/JP (Wang et al., 2005a; Yin et al., 2006). The two groups formed by porcine SaV strains more closely related to human SaV were classified differently. One group, composed by TYMPo31 and TYMPo239, with a short distance from human SaV strain GV, probably constitutes a new cluster (genotype) in the GV (Fig.1). In this case, even more closely related to human SaV strains, the distance is not short enough to suggest interspecies transmission (Nakamura et al., 2010). The other strains formed a new genogroup and as suggested by Martella et al. (2008) and respecting the nomenclature proposed by the author, are classified as GVIII, in which the strain 06-18p/ITA is the prototype (Martella et al., 2008).

The pairwise distance analysis of the strains placed in the swine SaV branch (Fig.1) showed that the sequences can not be classified in the same group. The new genogroups proposed in the present study were named GIX, GX, GXI, GXII and GXIII. The prototype strain of each group and the distance among each of these prototypes are indicated (Tables 1 and 2). The prototypes of GIX and GXI presented lower pairwise distance with the prototype of GVII (1,220 and 1,073, respectively) and to each other (1,042) than the intergenogroup distance (1.378-1.949) for calculated for human SaV. However, in the phylogenetic analysis this group can not be placed together and also, this distances are higher than what established as intragenogroup distance (0.282-0.841). The nomenclature GIX and GX were already proposed by Reuter et al. (2010), however, in this previous study, the classification was based exclusively on the phylogenetic analysis of a small fragment (286 nt) of the RdRp gene and do not provide sufficient basis to create new genogroups (Reuter et al., 2010).

In the last years, several porcine SaV strains that were described were just merely deposited in GenBank or classified as “G?” (Barry et al., 2008; L´Homme et al., 2009; Reuter

et al., 2010). For the GVI, GVII and GVIII in which the classification is well supported, the lack of a unique study to analyze the information of all genogroups together leads to continue to classify SaV in only five genogroups even in most recent studies (Wang et al., 2005a; Yin et al., 2006; Martella et al., 2008). Contrary to NoV in which porcine viruses strains seem to be more stable and are classified only in three genotypes in one genogroup, SaV is composed mostly of porcine virus strains than human virus (Wang et al., 2005b; Oka et al., 2012). In total there are nine genogroups of porcine SaV, and based on pairwise distance they are even more different than the most distant human SaV strains, suggesting that the virus evolution have been occurring for many years. All the porcine genogroups herein described are formed exclusively by porcine SaV strains.

Correlation between RpRd and capsid genes

The high genetic variability demonstrated in the VP1 gene also justifies the difficulty in only one method to detect all the strains of porcine SaV using the same protocol. As a consequence, the classification of SaV was often performed using only the polymerase gene (Reuter et al., 2010). However, a validation for this method is still lacking.

The RT-PCR described by Jiang et al. (1999) amplifies a fragment (331 bp for SaV) of the RdRp gene and is the most commonly protocol used for the diagnosis of porcine SaV (Jiang et al. 1999; Martinez et al., 2006; Barry et al., 2008). We performed a phylogenetic analysis to verify if the same organization is maintained using the VP1 gene and this RdRp fragment, and if the genogroups are clearly distinct from each other, i.e., to verify if the RdRp phylogenetic analysis is also valid for SaV genogroup classification.

In this and also in previous studies, the classification of human SaV could not be performed based on phylogenetic analysis of RdRp. The prototype strains were not placed separately in individual groups, e.g. GIV is grouped with GII strains, and not in different groups such as in the complete VP1 gene analysis (Oka et al., 2012). However, the phylogenetic analysis of the RdRp gene showed that the porcine SaV genogroups are clearly placed separately in distinct groups such as in the VP1 gene analysis (Fig.2). In order to verify the results of the phylogenetic analysis, the pairwise distance were also calculated in this fragment of RdRp gene. The intragenogroup distance for porcine SaV was 0.015 until 0.328, and 0.444 until 1.023 among the genogroups, showing that the values did not overlap. Unfortunately not all porcine SaV sequences in which the VP1 gene was analyzed could be used in the RdRp gene analysis since different fragments or no sequence of this gene was

available for some strains. This was also the case for the prototype of GXIII (Po/2053P4/Brazil).

These results show that the polymerase fragment amplified by the primers p289/290 that are the most commonly used to detect SaV can also be used to classify the porcine SaV genogroups (Jiang et al. 1999). However, to define the distance values range in which a strain can be placed in one genotype or genogroup such as in the study performed by Zheng et al. (2006), more strains of porcine SaV must be described to a higher number of sequences be included in the analysis (Zheng et al., 2006). On the other hand, a study in which the values are fixed is provisory since the virus is constantly evolving and mutating, and the reference strains need to be often updated.

Porcine SaV circulation

In order to determine the geographic distribution of porcine SaV, a phylogenetic tree was reconstructed with the human (*n*=4) and porcine (*n*=8) SaV prototypes (Table 1) and all the porcine SaV available sequences (data not shown). The prototype strain of GXIII (Po/2053P4/Brazil) could not be included in this analysis since the available fragment was smaller than the fragment amplified by p289/290 primers (Jiang et al., 1999). According to the phylogenetic analysis of the RdRp gene fragment, all genogroups of porcine SaV defined in this study circulate worldwide, i.e., America, Asia and Europe (Fig.3). Using the classification proposed in the study of Reuter et al., (2010) the porcine SaV was classified on six genogroups. However, in the present study, it was possible to verify eight genogroups of porcine SaV also based on partial polymerase gene.

In conclusion, in the present study a classification for porcine SaV based on molecular and phylogenetic analysis of the complete VP1 gene was proposed. The classification described in previous studies was respected and new genogroups proposed. It was verified that the same classification can be used also for a fragment of RdRp gene, and based on this fragment, was shown that all the genogroups of porcine SaV established in this study circulates worldwide.

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Figura 1 - Neighbor-joining phylogenetic tree (1000 replicates) reconstructed with Maximum Composite Likelihood model based on the complete VP1 gene (nucleotides). (A) All available sequences (*n*=142, 17/02/2012) from GenBank. (B) Prototype strains of human SaV and porcine SaV. Genogroup denomination is indicated, and porcine SaV groups are filled in grey.

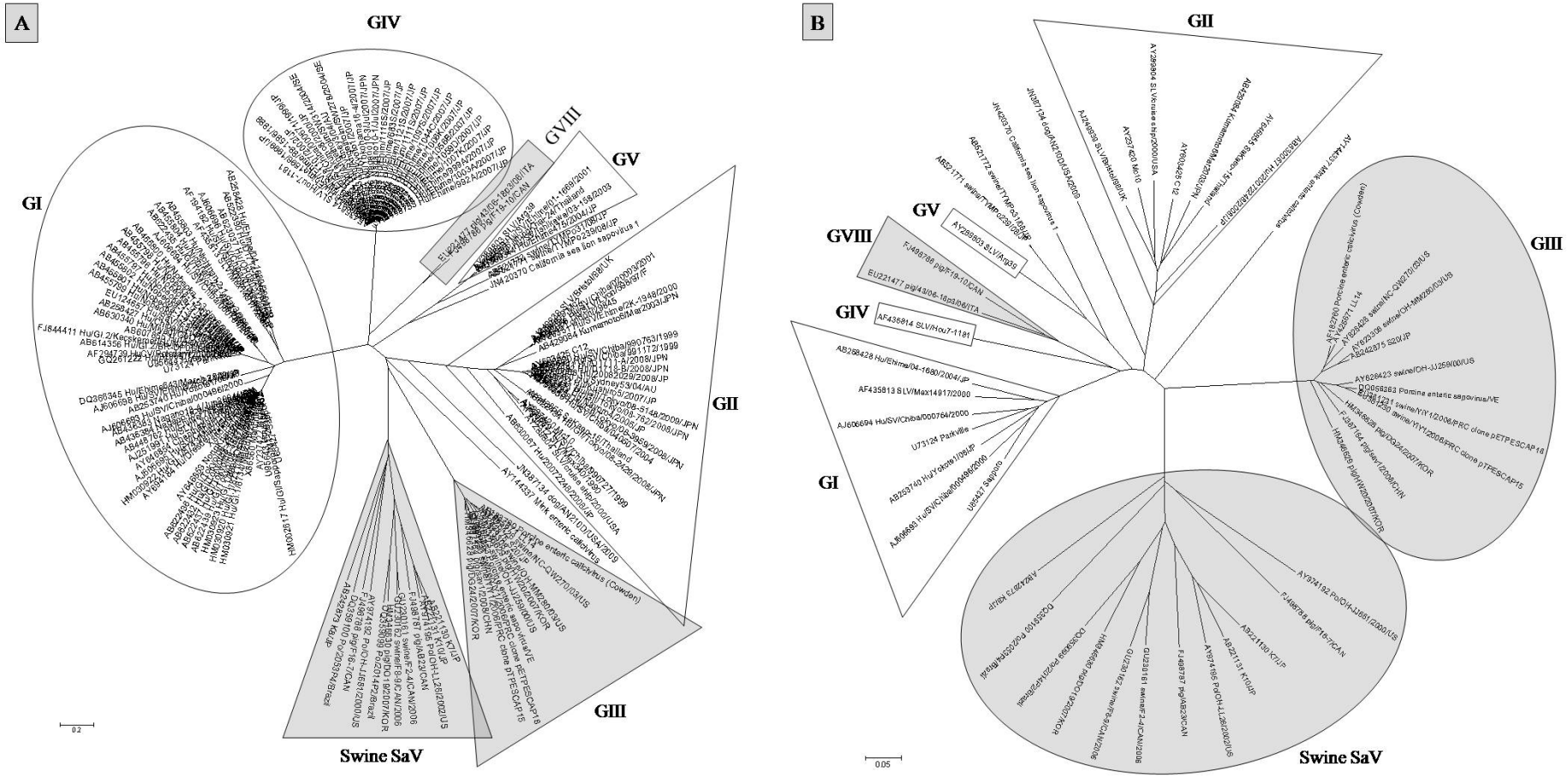

Figura 2 - Phylogenetic analyses of partial (286 nt) RNA-dependent RNA polymerase (RdRp) gene using the same sequences in which the complete VP1 gene is available. Nomenclature proposed for porcine genogroups (G) is indicated. GXIII is proposed in complete VP1 classification, but the RdRp fragment is not available.

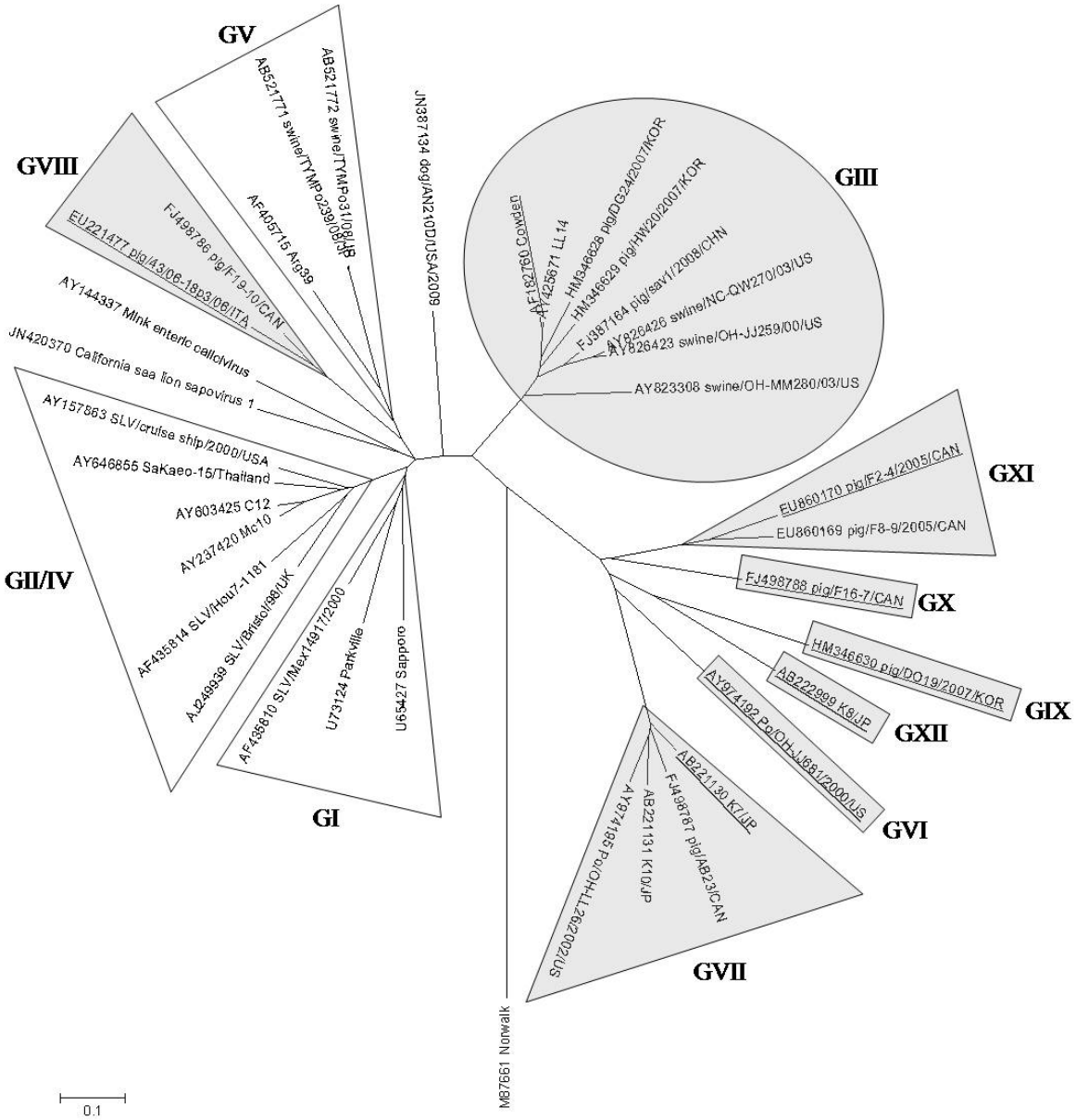

Figura 3 - World distribution of porcine sapovirus based on phylogenetic analysis of partial (286 nt) RdRp gene proposed in this study.

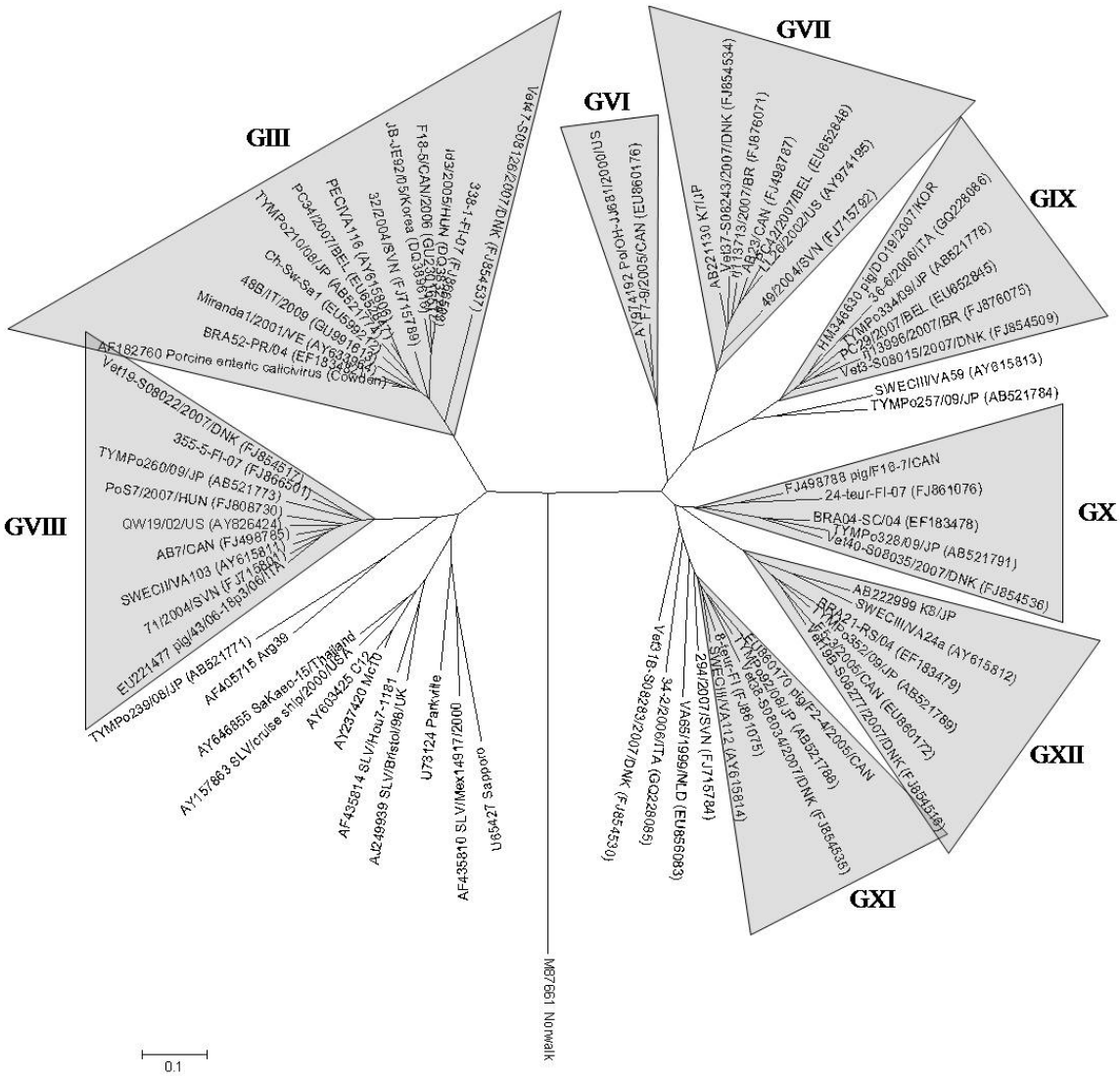

Table 1- Genetic classification based on the complete VP1 gene and prototype strains

Genogroup	Strain	Access no.	Origin
I	Sapporo	U65427	human
II	SLV/Bristol/98/UK	AJ249939	human
III	Cowden	AF182760	porcine
IV	Hou7-1181	AF435814	human
V	Arg39	AF405715	human
VI	Po/OH-JJ681/2000/US	AY974192	porcine
VII	K7/JP	AB221130	porcine
VIII	pig/43/06-18p3/06/ITA	EU221477	porcine
IX	pig/DO19/2007/KOR	HM346630	porcine
X	F16-7/CAN	FJ498788	porcine
XI	pig/F2-4/2005/CAN	EU860170	porcine
XII	K8/JP	AB222999	porcine
XIII	Po/2053P4/Brazil	DQ359100	porcine

Table 2 - Nucleotide pairwise distance based on complete VP1 gene (lower half) and 286 nt fragment of RdRp gene* (upper half) among prototype strains of porcine sapovirus

Strain (genogroup)	Cowden	JJ681	K7/JP	06-18p3	DO19	F16-7	F2-4	K8/JP
Cowden (GIII)	-	0,726	0,827	0,628	0,821	0,795	0,852	0,848
Po/OH-JJ681/2000/US (GVI)	2,315	-	0,457	0,875	0,571	0,519	0,549	0,542
K7/JP (GVII)	2,320	1,691	-	0,844	0,557	0,541	0,632	0,646
pig/43/06-18p3/06/ITA (GVIII)	2,186	2,366	2,402	-	0,958	0,770	0,831	0,899
pig/DO19/2007/KOR (GIX)	2,343	1,708	1,220	2,425	-	0,553	0,534	0,472
F16-7/CAN (GX)	2,354	1,448	1,718	2,361	1,588	-	0,444	0,465
pig/F2-4/2005/CAN (GXI)	2,424	1,586	1,073	2,399	1,042	1,531	-	0,554
K8/JP (GXII)	2,264	1,547	1,738	2,317	1,690	1,578	1,667	-
Po/2053P4/Brazil (GXIII)	2,309	1,599	1,626	2,510	1,655	1,637	1,566	1,592

*this fragment is not available for Po/2053P4/Brazil

3.2 Dynamics of Infection by Porcine and Human Sapovirus in Specific Pathogen-Free Piglets

ORIGINAL RESEARCH PAPER

Dynamics of infection by porcine and human sapovirus in specific pathogen-free piglets

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Abstract

Sapoviruses (SaV) are enteric virus responsible for gastroenteritis in humans. In swine, the prevalence of infection can be high in some populations. However, the role of swine as reservoirs for human SaV or as etiologic agent of diarrhea in pigs is not well elucidated. In the present study, three groups of specific pathogen-free piglets were inoculated with SaV genogroups GVII, GVIII and GI.2. The two groups inoculated with the porcine SaV (GVII and GVIII) replicated the virus and shed viral particles in feces. Animals inoculated with the human SaV (GI.2) only shed the virus in feces, but no viral load increase was verified. Viremia was detected in piglets inoculated with GVII and GI.2, and also the virus could be detected in extra intestinal organs. The SaV GVIII, in which viremia was not present, could not be detected in other organs. Although clinical signs or histopathological alterations in the intestine were not observed in any group, the functional alterations in enterocytes can not be excluded. The results present in this study show the differences in replication efficiency of two newly described genogroups of SaV. The lack of a GI.2 strain (the most common human SaV circulating worldwide) ability in replicate the virus and generate a productive infection in pigs suggests that pigs are not a possible reservoir for this strains of human SaV.

Keywords: Pig, Experimental infection, Diarrhea, Histopathology, Zoonosis, Calicivirus, Sapovirus

Introduction

Sapoviruses (SaVs) are enteric viruses classified in *Sapovirus* genus of *Caliciviridae* family (Atmar, Estes, 2001). These viruses can infect humans and cause sporadic cases of diarrhea or outbreaks (Hansman et al., 2007; Svraka et al., 2010). In pigs, SaV are frequently detected in single or mixed infection, but the association of the disease is not clearly understood (Wang et al., 2007).

SaVs are small, non-enveloped with 27 to 35 nm in diameter and icosahedral symmetry viral particles. The genome is a linear positive-sense, single-stranded polyadenilated RNA molecule with 7.3 kb in length organized in two or three open reading frames (ORFs) (Guo et al., 1999). ORF1 encodes a polyprotein that is cleaved simultaneously with the synthesis in the viral non-structural proteins and the main capsid protein (VP1). Another structural protein is codified by ORF2 and is responsible for stabilization of the viral particle and regulation of VP1 expression (Bertolotti-Ciarlet et al., 2003). In some human SaVs strains another ORF that overlaps the 5' of VP1 gene is present (Clarke; Lambden, 2000).

Based on molecular and phylogenetic analyses of the main structural protein gene, SaVs are classified in eight genogroups (G) (Farkas et al., 2004; Yin et al., 2006; Martella et al., 2008). There are also other strains detected in pigs that did not belong to any of these groups in which a new classification is still lacking (Reuter et al., 2010; L'Homme et al., 2009; 2010). The SaV strains detected in humans are classified as GI, GII, GIV and GV; swine SaV strains as GIII, GVI, GVII and GVIII. The strains detected in pigs posses high genetic variability and strains from the GVIII are closely genetically related to the human strains of SaV, which raises public health concerns.

Besides experimental inoculation and cell culture studies with the Cowden strain, the prototype of GIII swine SaV and the first porcine SaV described, the pathogenesis of other swine SaV are unknown (Guo et al., 2001; Chang et al., 2004). The association of SaV and gastroenteric clinical signs in all SaV studies were performed using the results obtained with generic primers that detect all SaV genogroups, i.e., considering that the different genogroups of SaV have the same pathogenesis and lead to similar outcomes (Wang et al., 2007).

In the present study, two strains of porcine SaV classified in distinct genogroups were inoculated in specific pathogen-free (SPF) piglets in order to compare the dynamics of SaV

infection in pigs. Additionally, one group of piglets was inoculated with a human SaV strain to verify the hypothetical zoonotic potential of this SaV strain.

Materials and methods

Inocula

For the preparation of the two porcine SaV inocula, fecal samples of pigs previously tested as positive by RT-PCR for SaV and classified by phylogenetic analyses as SaV GVII and GVIII were used. To assure that after the inoculation all the symptoms were caused by the SaV infection, which is frequently detected in mixed infections, the samples were also tested by RT-PCR for rotavirus A, B and C, and porcine coronaviruses (transmissible gastroenteritis - TGE and porcine epidemic diarrhea - PED). The samples that tested negative for the viruses described above, were used for the inocula preparation. The porcine fecal samples used in this study were kindly provided by Dr Yvan L'Homme from Canadian Food Inspection Agency.

The human SaV inoculum was prepared with a human fecal sample positive for SaV GI.2 and negative for Adenovirus, Aichi virus, Astrovirus, Rotavirus, Norovirus GI and GII, and SaV GIV. Dr. Jan Vinjé (CDC) kindly provided and previously tested this fecal sample.

Fecal suspensions were prepared at 10% in MEM. Forty µL/mL of Pen Strep (Invitrogen, Breda, The Netherlands) was added for antibiotic treatment. The suspensions were centrifuged at 4,000 rpm/10 min and filtered in 5 and 1.2 µm pore size filters. Before inoculation, inocula were cultured on blood agar at 37°C for 48 hours. In this procedure, no growth of bacteria was observed.

Animals and groups

Prior to the execution, the infection experiment was approved by the ethics committee of the Animal Sciences Group, Wageningen University and Research Centre (reference number 2011007/2009153.c/EXICAV, approval date Feb 1st 2011). The review of animal experiments by this body is fully in compliance with European ethical requirements for animal experiments. Piglets were derived by caesarean section, colostrum deprived, and maintained in sterile isolator units as described previously (Meyer et al., 1964). The experiment was performed in a BSL-2+ facility.

Seventeen SPF piglets were housed in one room in separate compartments. Group T02 was inoculated with porcine SaV GVII, T03 with porcine SaV GVIII, and group T04 with human SaV GI.2. The SaV inoculated groups were composed of four piglets each, including one sentinel (non-inoculated), and the control group (T05) of five animals being two sentinels.

The inoculation was performed at 6 days of age with 2 mL of the inocula administered intravenously and 2 mL orally. Two routes of inoculation were employed in order to assure the infection. The piglets in the control group were inoculated with minimum essential medium (MEM) (Gibco, Breda, The Netherlands) also by both routes.

All piglets were subjected to a clinical examination daily and an individual clinical score was attributed to each piglet every day. The score items included depression (1), loss of appetite (3), vomit (3), and diarrhea (3). Animals that presented score from 0 to 3 were considered to have mild disease, score from 4 to 6 moderate disease, and from 7 to 10, severe disease.

Samples

Prior to inoculations, at day -1, fecal and blood samples of all piglets were collected and tested for SaV GVII, GVIII or GI.2, depending on the group the piglets were in. To avoid contaminations, animal manipulations and collection of samples was started in the control group every day. Fecal samples were collected daily and directly from the pig rectum. Blood samples were collected every three days. The euthanasia was performed at six or seven days after inoculation to make sure the piglet intestine were not recovered of infection. At necropsy, organs (lungs, liver, spleen, stomach, duodenum, jejunum, ileum, colon, kidney and brain), gastric content, and liquor were collected and stored at -80°C. For the histopathology, fragments of the intestine (duodenum, jejunum, ileum, colon) were collected and stored in 10% buffered formalin.

Nucleic acid extraction

For nucleic acid extraction, fecal samples were prepared in 10% PBS and centrifuged at 3,000 rpm during 10 min (1000 × g). For the organ suspensions, 500 mg of each organ was macerated manually, diluted in 1 mL of PBS, vortexed and centrifuged at 3,000 rpm during 5 min (1000 × g). To avoid contamination with feces in the intestine fragments, they were firstly washed in 5 mL of PBS before the maceration.

Aliquots of 200 µL of the inocula, sera and the prepared suspension (feces/organs) were used for RNA extraction that was performed using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany) according to the manufacturer instructions.

Real Time PCR (qPCR) porcine SaV (GVII and GVIII)

For the detection of SaV GVII a TaqMan protocol was employed. The primers pSGVIII-fwd (5' – ATT GAC ATY TTG GGT GAG TTY ATT GA – 3') and pSGVII-rev (5' – GGC ART GAG ATG TCA AAY ACA AG – 3') and the probe pbSGVII (5' – ACG ATA CAA TCT GTG CTC AA – 3') were designed. The reaction was performed with 1x Reaction mix (5x), 1x enzyme mix, 500 nM of each forward and reverse primers, 250 nM of the probe, 1 µL of the RNA, and ultra pure water to a final volume of 20 µL using the RNA UltraSense™ One-Step Quantitative RT-PCR System (Invitrogen, Breda, The Netherlands). Cycling conditions were: reverse transcription at 50°C/20 min, denaturation at 95°C/7 min, and 55 cycles of PCR at 95°C/20 sec and 53°C/45 sec. If cycle threshold (Ct) values were not similar in both results, the reaction was repeated other two times.

The same protocol developed for SaV GVII showed to be efficient in the detection of SaV GVIII and was also used to test samples from piglets inoculated with this genogroup.

qPCR Human SaV (GI.2)

The detection of human SaV was performed by using the protocol described by Oka et al. (2006) with slight modifications.

qPCR Control group

The fecal and serum samples from the five piglets in the control group were tested with the qPCR for detection of SaV GVII/GVIII and human SaV.

Histopathology

Formalin-fixed sections of the duodenum, jejunum, ileum, and colon were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically. The histologic evaluation was done blindly, and a comparison was made with tissues from piglets in the control group.

Statistical analyses

Data analyses were performed by chi square ($\chi 2$) or Fisher exact with confidence limits of 95%, $p < 0.05$ using EpiInfo version 7.

Results

SaV GVII

Two of the four piglets inoculated with SaV GVII presented an increase in body temperature that lasted for 1 and 3 days. Two piglets showed clinical signs. One of them presented moderate gastroenteric sign during one day and the other (sentinel) four non-consecutive days of depression and apathy (Table 1).

In the fecal samples of the four animals in this group, a high increase (average of 18.8 in the Ct) of viral load was detected (qPCR) compared with the first day of virus shedding. The virus could be detected during 4 to 7 days, but the peak of viral shedding was different in each animal. In the sentinel piglet, virus shedding started at 1 day post-inoculation (dpi) and an increasing viral load was observed until 5 dpi, after which the viral load started to decrease (Fig. 1A). The same two piglets with fever presented the highest increase in viral load in feces.

In the organ fragments from the gastric enteric system, SaV GVII presence was verified in duodenum, jejunum, ileum and colon, but not in the gastric content (collected at necropsy) of the four piglets. The highest viral loads were detected at the duodenum of three piglets. Viral loads were the lower in the ileum.

No significant histopathological alterations were detected in the duodenum, jejunum, ileum and colon of any animal in this group.

Viremia was detected in all four piglets. At the first day (3 dpi) of blood sampling, the virus was not detected in any of the inoculated piglets. At 6 dpi, the second day of blood sampling, the three inoculated animals were positive. The sentinel animal also presented the virus in the blood, but this was detected by 3 dpi, and not in any other day of blood sampling.

Extraintestinal organs (lungs, stomach, liver, spleen, kidney, and brain) of the piglet with the higher viral load in feces in the group were also tested for the presence of SaV. All of these tissues were tested SaV positive except the brain. No virus was detected in the liquor of the four piglets.

SaV GVIII

One piglet inoculated with SaV GVIII, and also the sentinel showed increase in body temperature during 1 day. The same animals also presented clinical signs. One of them showed one day (5 dpi) of depression and apathy and the other (sentinel) moderate gastroenteric signs (anorexia and diarrhea) during 3 days starting on 3 dpi (Table 1).

Increases (average of 7.59 in the Ct) in viral loads in feces were detected in the four piglets (Fig. 1B). The two animals with the highest increase in virus shedding did not show clinical signs. The virus was detected during 4 to 5 days, however the peak of virus shedding was detected in the euthanasia day in three piglets, and it is uncertain if they would continue to eliminate the virus.

SaV GVIII was detected in the intestine fragments of all four piglets, and, similar to the piglets inoculated with SaV GVII, higher viral loads were detected in the duodenum and lower in the ileum. The stomach contents of the four piglets were negative.

Same as the piglets inoculated with SaV GVII, the piglets inoculated with SaV GVIII did not present histopathological changes in the duodenum, jejunum, ileum or colon.

Viremia was not detected in any animal.

In the organs (lungs, stomach, liver, spleen, kidney, and brain) of the piglet with higher viral shedding in feces in the group, no virus was detected. The liquor of the four animals of this group also tested negative for SaV.

Human SaV GI.2

In the group of piglets inoculated with the human SaV, two piglets presented fever during 1 to 2 days and three showed mild clinical signs during 1 to 3 days.

None of the inoculated animals showed increases of viral loads in feces collected consecutively (Fig. 1C). In these piglets, viral shedding stopped 2 to 5 days after the first detection and fecal samples of all the four piglets were negative at the euthanasia.

The four fragments of intestine and the gastric content of the four piglets were also negative by qPCR.

No histopathological alterations were verified in the intestine.

Despite the fact that there was no clear viral replication observed (no increasing of viral load in feces), in two of these piglets viremia was detected at 6 and 7 dpi. This viremia was not detected at the first day of blood sampling (3 dpi), which indicates that the virus in the serum was not from the inoculum.

The organs of the piglet with higher viral load in feces in the group were also tested. SaV was detected in the lungs, liver and spleen, but not in stomach, kidney and brain. This piglet with SaV in the extraintestinal organs also presented viremia.

Control group

None of the piglets in the control group presented fever. Two animals presented mild and one moderate clinical signs.

The fecal samples from the piglets in the control group were all negative for human SaV. At 6 and 7 dpi, one control animal was tested positive for porcine SaV. The other four control piglets remained negative for porcine SaVs during the entire experiment.

All serum samples of piglets in the control group were tested negative for both human and porcine SaV.

No histopathological alterations were verified in the intestine of piglets in this group.

Discussion

The frequent detection of SaV in porcine swine herds has been raising questions relative to the virus pathogenicity. The genetic classification allowed to identify that different SaV strains have been infecting pigs. The possible presence of a potentially zoonotic strain of SaV in infected pigs has been hypothesized repeatedly (Hansman et al., 2005; Wang et al., 2005). However, this hypothesis was always based exclusively on the genetic similarity of human and swine SaV strains.

In the present study, in order to clarify the association between SaV infection and diarrhea, SPF piglets were inoculated with two different strains of porcine SaV, and also with a human SaV strain. Through the inoculation of one group of piglets with a human SaV (GI.2) obtained from a human fecal sample it could be verified if scientific evidence can be found for the hypothetical zoonotic origin of such SaV.

The results obtained with the three groups of piglets inoculated with porcine and human SaVs suggest that SaV infection, which presents high prevalence worldwide, are not associated (p=0.6) with clinical signs (apathy, depression and/or gastroenteric signs). In the SaV inoculated piglets, fever was more frequent in the infected animals (50%) than in the control group, however there was no association (p=0.075). Mild to moderate gastroenteric signs were present in the inoculated piglets but also in the control group (p=0.68). Factors such as post birth stress, non adaptation to milk replacer, excess of milk ingestion might explain the vomit and diarrhea showed by the piglets. In most of the previous studies of molecular epidemiology, association between SaV infection and diarrhea was not verified (Wang et al., 2006; Barry et al., 2008). The experimental inoculation performed in the present study, confirmed these observations.

In a study conducted with gnotobiotic piglets, the prototype of SaV GIII, Cowden strain, caused diarrhea and mild to severe lesions in the intestines of the inoculated piglets (Guo et al., 2001). However, the immune system of these piglets is not comparable to the immune system of piglets raised in pig farms, and the results of this previous study should not be extrapolated to the infections in pigs in the field. On the other hand, the absence of severe clinical signs after the inoculation of SaV in SPF piglets suggests it is not likely that this infection leads to the development of important disease in raised piglets. The economic impact of SaV infection for pig farmers may not be very high.

In the two groups inoculated with porcine SaV the increase in viral load in feces when compared with the first day of virus detection indicates virus replication. The same increasing was verified in all the four piglets in each group. The comparison of the results of these two groups clearly showed differences in the dynamics of infection by different porcine SaV strains. SaV GVII and GVIII strains have been detected exclusively in pigs and, by genetic classification, are closely related to other genogroups of porcine SaV and human SaV, respectively (Yin et al., 2006; Martella et al., 2008). Comparing the increases in virus shedding between the piglets inoculated with SaV GVII (average of 18.8) and the group inoculated with SaV GVIII (average of 7.59), the virus were more efficiently (2.48 times) replicated in the first group, suggesting that SaV GVII strains are more adapted to the host (pig) than SaV GVIII strains.

In opposition to virus replication clearly demonstrated in the piglets inoculated with porcine SaV strains this was not verified in the group inoculated with human SaV strain. The piglets presented the virus and shed it in the feces during 2 to 5 days. However, in this group, the viral load was decreasing until it was not detected anymore, indicating a clearance of the virus possibly without replication. An inability of this human SaV virus to replicate would suggest that porcine intestinal cells are not permissive to this human SaV strain or the replication could be incomplete. To understand and identify the exact step in which the replication in such case was not completed, other studies will be necessary. Since pigs could not replicate the human SaV strain, the hypothesis of a zoonotic swine origin of SaV is not supported, at least for GI.2 strains, which is the most commonly detected in humans.

Porcine SaV was detected with different viral loads in the samples from the gastro enteric tract. All piglets in the two groups (T02 and T03) were negative for SaV in the stomach content. The infection of the sentinel animals in the first days of the experiment in both groups strongly suggests that the transmission occurred by viral particle presented in the vomit of the inoculated piglets, since they were not shedding the virus in feces yet. These

observations together indicate that the elimination via vomit can occur only in the initial stage of the infection.

In the intestine fragments of the piglets from groups T02 and T03 (porcine SaV) the levels of virus detection were different. In the small intestine, six of the eight piglets (including the sentinels) in both groups presented the highest level of SaV in the duodenum. In one of the piglets, in which the duodenum was not the intestine fragment with higher SaV level, the start of viral shedding in feces was 1 dpi, and possibly the replication was lower in the duodenum in the day of organs sampling (7 dpi). In seven from the eight piglets, the lower SaV detection was in the ileum, indicating that the replication in this portion of the intestine, if present, is not efficient as in the initial parts. These findings suggest that the replication preferably occurs in the proximal small intestine and is very low in the ileum. A previous study described similar results, with the duodenum being the most active small intestine portion for SaV antigen presence, followed by jejunum. In the same study, the ileum was also the portion of the small intestine with less antigen (Guo et al., 2001).

In the group inoculated with human SaV no virus was detected in any intestine fragment, which reinforces the absence of human SaV replication in pigs.

The absence of histopathological alterations did not exclude the possibility of functional alterations in the enterocytes. If this is the case, is possible that the conversion rate in infected piglets is higher, but the impact of SaV is not comparable to the economical losses due to viruses that clearly cause diarrhea, like rotavirus and coronavirus.

In the piglets inoculated with SaV GVII viremia was just detected at the end of the experiment (6 dpi), showing that the viruses reached the blood stream. The sentinel piglet that was not inoculated by any route, viremia was detected on 3 dpi. The absence of virus in the blood in the first sampling, excludes the possibility that the viral particles came from the inocula. The possible route for the virus to reach the blood stream is the intestinal lymphoid cells, present in the ileum specifically. This part of the intestinal tract showed the relatively lower viral loads in three piglets, but detectable viral particles in the four animals. The two piglets with higher viral load in the ileum, also presented higher viral loads in the sera, reinforcing this hypothesis. Viremia was not detected in SaV GVIII group and the testing of the extraintestinal organs did not show the presence of the virus either. The presence of SaV GVII in the sera and extraintestinal organs of piglets in this group, and the absence of viremia in the piglets inoculated with SaV GVIII and also in organs other the intestine, strongly suggests that the virus spread through the entire body by the circulatory system.

In the group inoculated with human SaV, the presence of the virus in the serum at 6 and 7 dpi (but not in 3 dpi) for two animals indicates that, presuming the virus did not replicate, this is a passive process of the virus. In the same way observed for SaV GVII strains, may be a consequence of circulating cells of the immune system.

In the groups T02 (SaV GVII) and T04 (human SaV), SaV was detected in extraintestinal organs such as lungs, liver, spleen and kidney. Probably, the virus reached the organs out of the gastroenteric tract by blood stream. However, since these organs could be sampled only once, at necropsy, it was not possible to verify if the virus was replicating or just circulating passively. By comparison of the viral load in each organ and also the constant increasing of viral shedding in feces and very low level of virus in the serum samples, it is suggested that the intestines are the main organ for virus replication, with the feces being the main excretion route for SaVs, but also intruding other organs.

Contrary to what has been described for norovirus and rotavirus, for which its detection in brain raised questions about development of neurological disorders as a result of the infection, in these SaV inoculated piglets the virus was not detected in liquor or brain (Kobayashi et al., 2010; Obinata et al., 2010).

In summary, this study provided for the first time, conclusive information about the development of symptomatology of SaV infection in pigs. Different strains of porcine SaV present different dynamics when infecting piglets, but even the most adapted genogroup (GVII) did not induce significant clinical signs. Human SaV did not replicate in pigs and consequently did not lead to disease. SaV has passively circulated in all the organism, being viremia necessary for this spread. In conclusion, some aspects of SaV infection were elucidated and can be extrapolated for the infection in field. SaV infect piglets in high rates, however it is unlikely that represent significant economic losses for pig producers.

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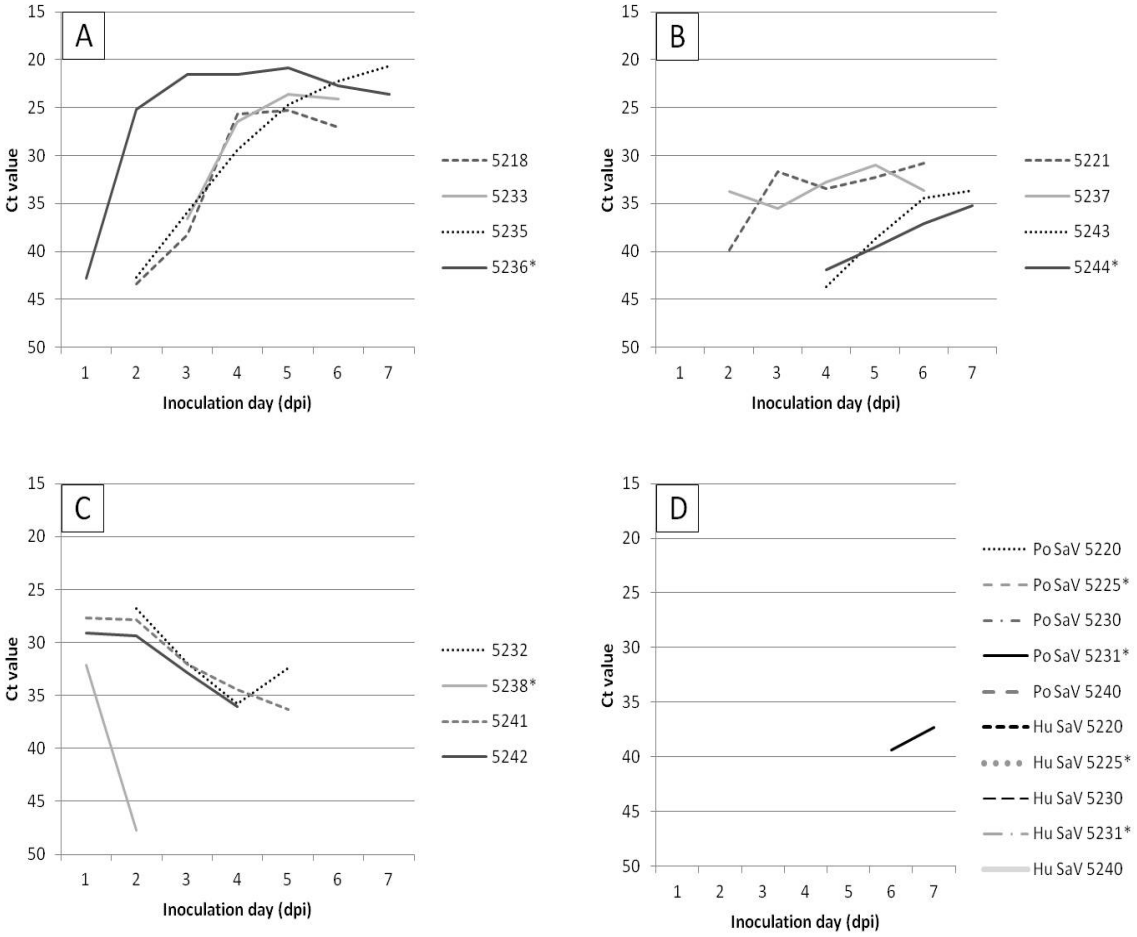
Table 1 - Summary of the results of the SaV experimental inoculation in specific pathogen-free piglets

Group / Inoculated strain	Animal	Clinical sign		Increased rectal temperatures (day)	Feces Ct		Viremia (dpi)
		score**	duration		viral replication***	Decrease value	
T02/SaV GVII	5218	++	2 days	-	+	18.15	+ (6)
	5233	-	-	-	+	13.02	+ (6)
	5235	-	-	40°C (7)	+	22.09	+ (6 and 7)
	5236*	+	5 days	40.3°C (6)	+	21.97	+ (3)
T03/SaV GVIII	5221	-	-	-	+	9.07	-
	5237	+	4 days	39.7°C (5)	+	4.54	-
	5243	-	-	-	+	10.03	-
	5244*	++	2 days	39.7°C (7)	+	6.71	-
T04/SaV GI.2	5232	-	-	-	-	-	-
	5238*	+	4 days	40.7°C (5)	-	-	-
	5241	+	2 days	40° (4)	-	-	+ (6)
	5242	+	1 day	-	-	-	+ (6 and 7)
T05/control	5220	+	4 days	-	-	-	-
	5225*	++	4 days	-	-	-	-
	5230	+	2 days	-	-	-	-
	5231*	-	-	-	+ (porcine SaV)	-	-
	5240	-	-	-	-	-	-

*sentinel piglet (non-inoculated)
**Clinical score: mild (+), moderate (++), severe (+++)
***Ct (cycle threshold) decrease relative to the first day of virus detection

Figura 1 - Viral load detected by real time PCR from fecal samples collected consecutively from piglets inoculated with SaV GVII (A), SaV GVIII (B), human SaV strain GI.2 (C), and control group tested for porcine (Po SaV) and human SaV (Hu SaV) (D).



5 CONCLUSÕES

- A análise filogenética com base no gene completo da VP1 das estirpes de sapovírus (SaV) circulantes no mundo todo revelou que o SaV suíno apresenta maior variabilidade genética que o SaV humano, sendo classificado em pelo menos nove genogrupos;

- Embora para os SaV humanos a análise do gene da RpRd viral não possa ser utilizada para a classificação em genogrupos, no SaV suíno houve concordância na classificação do gene da VP1 com a classificação no fragmento do gene da RpRd mais comumente utilizado para detecção do vírus;

- Utilizando a classificação para SaV suíno estabelecida neste estudo foi verificado que estirpes dos diferentes genogrupos de SaV suíno circulam em todo o mundo;

- As estirpes de SaV suíno (GVII e GVIII) utilizadas na inoculação experimental em leitões livres de patógenos específicos (*SPF*) apresentam diferenças em vários aspectos como eficiência na replicação e capacidade de fazer viremia e se espalhar para outros órgãos fora do trato gastrointestinal, o que sugere diferenças na adaptação do vírus ao hospedeiro suíno;

- A detecção de maior carga viral nos fragmentos proximais (duodeno e jejuno) do intestino sugere que a replicação dos SaV suíno ocorra nessas porções intestinais. Entretanto a ausência de lesões na histopatologia sugere que haja apenas lesões funcionais e que provavelmente o SaV não seja causa de grandes perdas econômicas e criações de suínos;

- Uma vez que a inoculação do SaV humano (GI.2) nos leitões *SPF* não gerou infecção produtiva verificada pela ausência de replicação, é possível que o suíno não seja um carreador do vírus humano e portanto não represente um reservatório do vírus.

APÊNDICES

APÊNDICE A
Artigo publicado

Durante a pesquisa dos sapovírus (SaV) em amostras de fezes de suínos e utilizando o par de *primers* p289/290 descritos por Jiang et al. (1999) foi amplificado um produto de aproximadamente 300 pares de bases. Após o sequenciamento e análise molecular e filogenética, esse fragmento foi identificado como kobuvírus em uma amostra previamente testada como positiva para SaV. Com consulta à literatura, observamos se tratar de um vírus ainda pouco descrito e nunca detectado no Brasil. *Primers* descritos em literatura foram solicitados e um maior número de amostras de suínos e ovinos testado no Brasil. Do mesmo modo foi detectado o kobuvírus em rebanhos suínos e bovinos holandeses. Os dados foram então analisados em conjunto e foi redigido um artigo que se encontra a seguir.

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Short communication

First detection of kobuvirus in farm animals in Brazil and the Netherlands

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Animal kobuviruses have been described in pigs, cattle, sheep and bats in countries in Asia and Europe. The virus can be detected in fecal and serum samples of infected animals with or without diarrhea, but most of the clinical as well as epidemiological features of kobuvirus infection are still unknown. This study reports the first detection of kobuvirus in farm animals from Brazil and the Netherlands and the molecular analysis of the detected strains. In Brazil, 53% (61/115) of the pigs (suckling, weaned and sows) were shedding porcine kobuvirus in feces, while in the Netherlands 16.7% (3/18) of the tested weaned pigs were infected. Kobuviruses detected in fecal samples of pigs in Brazil showed association ($p = 0.0002$) with diarrhea. In pig serum, kobuvirus was detected at different ages (3, 21, 36, 60, 75, and 180 days), with an overall rate of 76.7% (23/30). The sequencing of amplicons detected in serum of pigs of different ages suggested reinfection and no persistent infection. Kobuvirus was also detected in sheep and cattle feces from Brazil and the Netherlands, respectively. Phylogenetic analyses of Brazilian and Dutch kobuviruses from pig, cattle and sheep revealed genetic variability, particularly in one strain detected in sheep feces, which was more closely related to human Aichi virus. The molecular and phylo-genetic analyses performed with other published kobuvirus strains and the strains presented in this study, showed that, in most of the cases, kobuvirus seems to group according to host species, but not to geographical region of origin. The data presented in this study contribute to the comprehension of kobuvirus epidemiology and also to the molecular identification of kobuvirus strains circulating worldwide.

1. Introduction

Kobuvirus was first detected in fecal samples from humans with gastroenteritis after the consumption of raw oysters (Yamashita et al., 1991). Later, similar viruses were described in cattle, pigs, sheep, and bats (Yamashita et al., 2003; Reuter et al., 2008, 2010a; Li et al., 2010).

Kobuvirus is a genus of the Picornaviridae family that also in-cludes 11 other genera: Aphtovirus, Avihepatovirus, Cardiovirus, Enterovirus, Erbovirus, Hepatovirus, Parechovirus, Sapelovirus, Sene-cavirus, Teschovirus and Tremovirus. Kobuvirus has two species: Ai-chi virus, which can infect humans, and Bovine kobuvirus that infects cattle and sheep. The porcine kobuvirus is a candidate spe-cies (Reuter et al., 2011).

Kobuviruses are non-enveloped with a 27–30 nm diameter and an icosahedral symmetry. The genome is a linear positive-sense, single-stranded RNA molecule of 8.2–8.4 kb with a VPg linked to

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the 5^0 end and a poly (A) tail at 3^0. UTR at both extremities are also present in the genome of kobuviruses (Reuter et al., 2011). The virus has only one open reading frame (ORF) encoding for a single polyprotein that is cleaved in structural (VP0, VP3 and VP1), and non-structural (2A–2C, 3A–3D) proteins (Yamashita et al., 1998).

The detection of kobuvirus in pig feces has been reported from a few countries in Europe and Asia (Reuter et al., 2008; Yu et al., 2009; Khamrin et al., 2009, 2010; Park et al., 2010), and also in pig sera (Reuter et al., 2010b). The prevalence of infection in pigs ranges from 30% to 99%. This large variation may be due to different ages of evaluated populations, presence or absence of gastroenteritis, and perhaps other factors. Also, in cattle just a few studies have been performed and most of these were conducted in a limited number of samples, which just allows the conclusion that the virus can be de-tected in fecal and serum samples (Yamashita et al., 2003; Khamrin et al., 2008; Reuter and Egyed, 2009; Mauroy et al., 2009; Park et al., 2011). In a recent study in cattle, the association between infection and age was also raised, as described in pigs (Jeoung et al., 2011). In both sheep and bat, kobuvirus was described in only one study (Li et al., 2010; Reuter et al., 2010a). Interspecies transmission between pig and cattle and vice versa has been suggested in two independent studies, however, the possibility of a passive infection could not be excluded (Khamrin et al., 2010; Park et al., 2011).

The present study describes the presence of kobuviruses in feces of pigs, cattle, and sheep from Brazilian and Dutch herds and detection of porcine kobuvirus in serum of pigs of different ages. Additionally, a phylogenetic analysis of the detected strains was performed.

2. Materials and methods

2.1. Specimen collection

In Brazil, 115 fecal samples were collected from June/2009 to February/2010 from one pig herd, including suckling, weaned, and adults animals, with (n = 37) or without (n = 78) diarrhea. From the same pig farm, successive serum samples were collected from five animals of 3, 21, 36, 60, 75, and 180-day-old.

In July 2010, 23 fecal samples were collected from diarrheic (n = 9) and non-diarrheic (n = 14) sheep in one Brazilian sheep herd. The animals were from 1 to 7 months old.

In the Netherlands, fecal samples from 18 weaned piglets with (n = 5) and without diarrhea (n = 13) were collected from 12 herds in 2008. In 2007, fecal samples (n = 9) of 12- to 14-day-old asymp-tomatic calves were collected from five herds.

2.2. Kobuvirus detection

The nucleic acid extraction of the Brazilian samples was per-formed according to Boom et al. (1990). For the Dutch samples, the QIAamp MinElute Virus Spin kit (QIAGEN, Venlo, The Neth-erlands) was used. The RT-PCR was performed using the primers UNIV-kobu-F/R, which had been designed based on human, bovine, and porcine kobuvirus strains, and target a region of the RdRp gene (Reuter et al., 2009). Electrophoresis was performed in ethidium bromide stained 2% agarose gel and RT-PCR products were visual-ized under UV light.

2.3. Statistical analysis

Data analyses were performed by chi-square (v^2) with confi-dence limits of 95%, $p < 0.05$ in EpiInfo version 3.3.2.

2.4. Sequencing and phylogenetic analyses

Purification was performed with GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Pittsburgh, USA) and Zymoclean™ Gel DNA Recovery Kit (Zymo Research, CA, USA) for the Brazilian and Dutch amplicons, respectively. Sequencing was performed in both directions using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) and run in ABI 3730 DNA Ana-lyser. BioEdit vs7.0.9 and MEGA 4.1 software were used for the molecular and phylogenetic analyses, respectively.

3. Results and discussion

From the Brazilian porcine fecal samples, 61 (53%) of 115 were positive for kobuvirus (Table 1). There was association (p = 0.0002) between infection and diarrhea, with 29 (78.4%) of 37 diarrheic and 32 (41%) of 78 non-diarrheic samples presenting the virus. How-ever, a causal relationship could not be proven, since 27 (44.3%) of the 61 kobuvirus infected pigs presented mixed infections including other enteric viruses (rotavirus, picobirnavirus and/or sapovirus – data not show). The highest (56/64; 87.5%) kobuvirus detection was from nursing piglets (until 21-day-old) indicating a high susceptibility of young animals to infection, possibly due to an inefficient immune response or other intrinsic age-related factors. However, further studies are necessary to support any idea

Table 1
Frequency of kobuvirus RNA in biological samples evaluated by RT-PCR assay.

Country	Host	Sample	Age	Positive/ tested	Total
Brazil	Pig	Feces	4–21 days	56/64 (87.5%)	61/115 (53%)
			28–60 days	3/34 (8.8%)	
			>1 year (sows)	2/17 (11.8%)	
		Serum*	3 days	5/5 (100%)	23/30 (76.7%)
			21 days	1/5 (20%)	
			36 days	5/5 (100%)	
			60 days	4/5 (80%)	
			75 days	4/5 (80%)	
			180 days	4/5 (80%)	
	Sheep	Feces	1–7 month		9/23 (39.1%)
The Netherlands	Cattle	Feces	12–14 days		7/9 (77.8%)
	Pig	Feces	Weaned		3/18 (16.7%)

* Serum samples collected from five pigs with different ages.

on risk factors for this infection. Higher frequency rates in young piglets were also described elsewhere (Reuter et al., 2009; Park et al., 2010; An et al., 2011). In contrast, only two (11.8%) of the 17 tested sows were shedding the virus in feces at the time of sam-pling, which could indicate that young animals more often demon-strate productive infection, or that the fecal–oral route may not be the only mode of viral transmission from the mother to piglets in early life.

In pig serum samples, kobuvirus was detected in all age groups (Table 1). This finding is in agreement with other studies per-formed with pig serum samples (Reuter et al., 2010b). All piglets were infected at the first sampling (3 day-old), but since only 11.8% of the sows were shedding the virus in the feces, this may indicate an alternative route of infection, for example through milk, blood (handling procedures), urine, saliva, or even aerosols as for other picornaviruses (Reuter et al., 2010b). At 21 days of age, only one of the five piglets presented kobuvirus in serum. This viral clearance indicates that the viremia of the first infection did not last until 21 days of age. The justification for such needs further study of immune-development in kobuvirus infection. The ob-served viral clearance also demonstrates that the animals were not persistently infected, i.e. kobuvirus not circulating in the blood during a pigs entire life. The 100% kobuvirus frequency at 36 day-old, showed kobuvirus reinfection in four (80%) of the five piglets. Weaning in Brazil is normally performed at 21 days, when the pig-lets go to the nursery with animals from other litters. The stress caused by changes in feeding and environment could lead to immunosuppression and predisposition for infections. In contrary to the high frequency in serum, and such as verified in sows, only 8.8% (3/34) of the weaned piglets tested positive for kobuvirus in feces. A possible explanation for this finding may be that some pro-tection obtained after the first infection was preventing the shed-ding of the virus in feces, such as described in other enteric virus studies (Hodgins et al., 1999). These results also suggest the exis-tence of another source of virus elimination. In this case, milk and blood could be excluded since the animals were not weaning and did not undergo any procedure for blood transfer. The impor-tance of kobuvirus detection in serum needs further investigation since it has not been defined yet if the intestines are the main or-gan for virus replication and if the virus escaped from the intestinal tract to blood or was just eliminated via feces with replication occurring in other organs.

To confirm the possibility of reinfection, amplicons obtained from serum samples from one pig, but at different ages, were se-quenced. The animal was negative at 21 and 60 days. The molecu-lar analysis of three amplicons revealed that the animal was infected with the same strain at days 3 and 180, but with a differ-ent strain at 36 days of age. These results indicate at least three

different infections. Since the animal was re-infected with the same strain later in life, it is possible that kobuvirus immunity is not long-lasting. Co-infection with two different strains could also have occurred at 36 days, but in this case only one strain was de-tected in the RT-PCR. Since the identification of the strain was based on a fragment of the RdRp gene, which was shown to be the more conserved gene in porcine kobuvirus, it is very likely that these two strains are different and do not just result from the con-stant changes in the RNA genome of kobuvirus. In a recent study, it was shown that the estimated mean substitution rate in this part of RdRp gene of porcine kobuvirus is 1.3 10^{2} substitutions/ site/year, which indicates that the two infections at 3 and 36 days (33 days apart) were not resulting from virus mutations (Park et al., 2011).

The sequencing of 15 and eight amplicons from porcine fecal and serum samples, respectively, confirmed the RT-PCR specificity. Despite the fact that all samples were obtained from the same pig herd, the porcine kobuvirus detected in Brazil showed genetic var-iability (90.9–99.3%). The sequences from fecal samples appeared to be more variable, compared to the serum samples: 91.5–100% versus 97.2–100%. This result reveals the circulation of different strains at the same time. On the other hand, the detection of 18 identical sequences (six of eight serum and 12 of 15 fecal samples), and just a few different ones, suggests the predominance of one strain. In the phylogenetic analysis, all sequences clustered with other porcine kobuvirus strains including the prototype S-1-HUN (Fig. 1).

In sheep, the 216 bp RT-PCR fragment was amplified in nine (39.1%) of the 23 stool samples evaluated. Molecular and phyloge-netic analyses were performed using three sequences. Two se-quences grouped with bovine kobuvirus strains and with the TB3 strain, the only other sheep kobuvirus strain described (Reuter et al., 2010a). However, these sequences showed a relatively high (71.6–95.8%) genetic variability to each other. BRA11-sheepKobu was distant from bovine/sheep strains and formed a cluster with Aichi virus, the prototype of human kobuvirus (Fig. 1). Unlike other studies which describe the detection of bovine kobuvirus in a pig fecal sample and vice versa, the grouping of BRA11-sheepKobu (from sheep) with Aichi virus, does not suggests interspecies trans-mission (Khamrin et al., 2010; Park et al., 2011). The pairwise dis-tance between BRA11-sheepKobu and Aichi (0.334) and TB3 (0.309) strains, and the amino acid phylogenetic analysis that clus-tered these strains separately, indicate that BRA11-sheepKobu pos-sibly evolved differently from other bovine/sheep kobuvirus strains. However, further studies including the capsid gene must be performed to identify the strain origin.

In the Netherlands seven (77.8%) of the nine calf stool samples presented the virus, and three (16.7%) of the 18 pig fecal samples tested positive (Table 1). The kobuvirus frequency in bovines was higher than described in other studies from Japan (16.7%), Thailand (8.3%), Hungary (6.25%), and Korea (34.6%) (Yamashita et al., 2003; Khamrin et al., 2008; Reuter and Egyed, 2009; Jeoung et al., 2011). However, since only a few samples were tested, this may not re-flect the real prevalence of infection in Dutch cattle. In swine, the rate of kobuvirus infection was lower compared to the overall Brazilian frequency. However, taking into account only weaned pigs, the rate of kobuvirus infection in the Netherlands (16.7%) was higher than what was found (8.8%) in Brazilian weaned piglets. In order to perform the identification of the kobuvirus strains, two amplicons of each host species were sequenced. The bovine kobu-virus sequences showed genetic variability (92.3%), but both grouped with the bovine prototype U-1. The porcine strains were also different (90.2%) but in the phylogenetic analysis grouped with other porcine kobuvirus strains (Fig. 1).

In general, a phylogenetic analysis of just a small fragment of a conserved gene of a virus, such as RdRp, is not enough to character-ize a strain. For such, the sequencing of larger gene parts encoding structural protein would be preferable. However, the grouping of

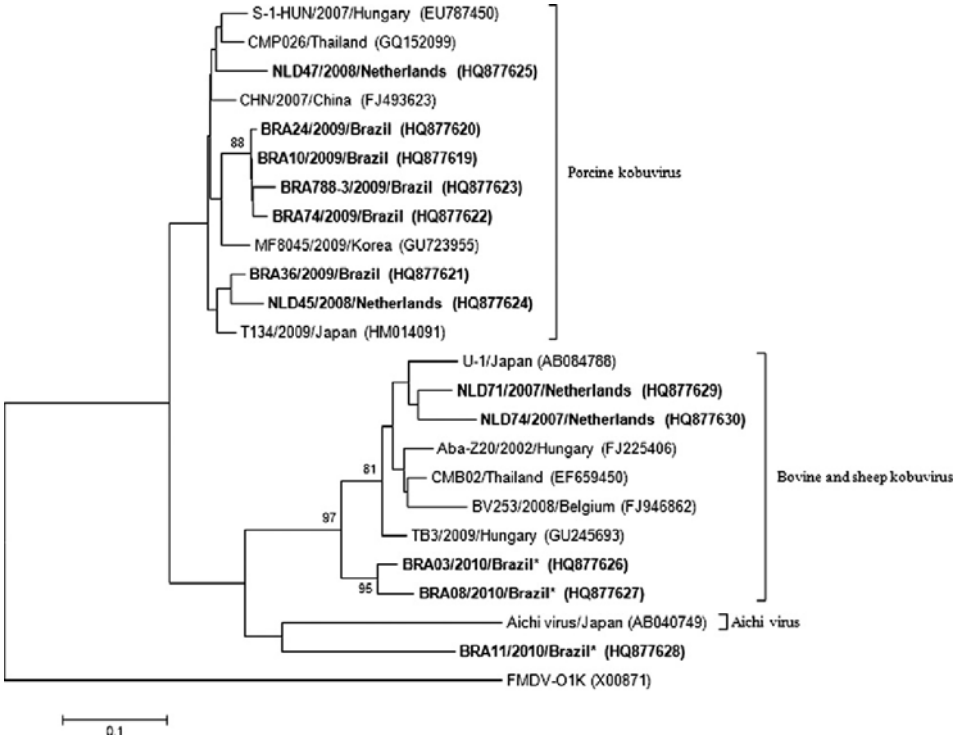

Fig. 1. Neighbor-joining phylogenetic tree reconstruction using the Tamura–Nei model based on partial (141 nt) polymerase 3D gene. Bootstraps values (1000 replicates) higher than 70% are shown. GenBank accession numbers of reference strains, Brazilian and Dutch kobuvirus are indicated between parentheses. New sequences from Brazil and the Netherlands are in boldface. /Kobuvirus detected in sheep.

kobuvirus strains according to host species and the relatively low number of interspecies transmissions described in other studies, suggests that kobuvirus infections are not species-specific but seem to be rather well-adapted to their hosts. Evolutionary analy-sis of different regions of the kobuvirus genome also indicates host adaptation (Reuter et al., 2010b). The porcine strains show less var-iability than bovine/sheep kobuvirus. For the latter group it has re-cently been proposed to classify it into at least four clusters (Jeoung et al., 2011). Kobuvirus strains presented in this study were from America and Europe and in the phylogenetic analysis grouped with strains from Asia and other European strains. From this observation it can be concluded that the strains do not seem to group according geographical regions.

This is the first description of kobuvirus in the American conti-nent and in the Netherlands. Since the virus was detected in differ-ent animal species from two distinct and not related countries, it is possible that kobuviruses circulates worldwide. The successive detection of the virus in sera from pigs in different ages and the high rate of viral shedding only in suckling piglets can contribute to the understanding of kobuvirus epidemiology. The second description of kobuvirus in sheep in the world and its genetic var-iability reinforces the need of prevalence studies in other animal species than pigs and cattle. However, the real importance of kobu-virus for animal health is still largely unknown and need further clinical and epidemiological studies.

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