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

PESTIVÍRUS EM SUÍNOS E BOVINOS: UMA ABORDAGEM SOROLÓGICA, MOLECULAR E EVOLUTIVA

ANA CRISTINA SBARAINI MÓSENA

Porto Alegre

2021

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PESTIVÍRUS EM SUÍNOS E BOVINOS: UMA ABORDAGEM SOROLÓGICA, MOLECULAR E EVOLUTIVA

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Tese apresentada como requisito parcial para a obtenção do grau de Doutor em Ciências Veterinárias

Orientador: Cláudio Wageck Canal
Co-orientador: Matheus Nunes Weber

Porto Alegre
2021

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

CIP - Catalogação na Publicação

Mosena, Ana Cristina Sbaraini
Pestivírus em suínos e bovinos: uma abordagem sorológica, molecular e evolutiva / Ana Cristina Sbaraini Mosena. -- 2021.
128 f.
Orientador: Cláudio Wageck Canal.

Coorientador: Matheus Nunes Weber.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Veterinária, Programa de Pós-Graduação em Ciências Veterinárias, Porto Alegre, BR-RS, 2021.

1. BVDV. 2. APPV. 3. filogenia. 4. soroneutralização. 5. Análise de Componentes Principais. I. Canal, Cláudio Wageck, orient. II. Weber, Matheus Nunes, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

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Pestivírus em suínos e bovinos: uma abordagem sorológica, molecular e evolutiva

Aprovada em 25 de março de 2021

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AGRADECIMENTOS

Gostaria de agradecer imensamente aos meus pais Silvana e Moacir por toda dedicação e apoio durante todos os anos de graduação e pós-graduação; sem eles eu não teria liberdade de me dedicar ao mestrado e doutorado. Agradeço também à minha irmã Júlia pela ajuda e apoio sempre que precisei; e ao Timo por me fazer companhia durante todos estes anos.

Agradeço muito ao Professor Cláudio Canal, do Laboratório de Virologia Veterinária da FaVet, por abrir as portas para o aprendizado, tanto na pesquisa como no crescimento pessoal, e por ter me concedido a oportunidade de conviver com grandes mestres e colegas, e tê-los transformado acima de tudo em grandes amigos com os quais convivi todos os dias e levarei para sempre comigo.

Estes foram anos de muito estudo, muito aprendizado, muitas experiências vibrantes e desafiadoras, muitas pessoas especiais que me tornaram quem sou hoje...a tudo e a todos, muito obrigada!

RESUMO

O gênero *Pestivirus*, pertencente à família *Flaviviridae*, compreende vírus de genoma RNA classificados em 11 espécies que, na sua grande maioria, estão associadas a infecções de animais ungulados. No Brasil, assim como em vários países, os pestivírus podem afetar a atividade pecuária e gerar prejuízos econômicos, como infecção por *Pestivirus C* (vírus da peste suína clássica, CSFV) e *K* (pestivírus atípico porcino) em suínos e por *Pestivirus A, B* (vírus da diarreia viral bovina 1, 2, BVDV 1 e 2) e *H* (pestivírus *hobi-like*) em bovinos. Com o objetivo de contribuir com mais informações acerca da epidemiologia e diversidade dos pestivírus no Brasil e da caracterização antigênica entre cepas de pestivírus de bovinos para avaliar a eficiência de vacinas, o presente trabalho será apresentado sob a forma de 5 capítulos. O objetivo e resultados alcançados nos trabalhos foram, respectivamente: 1) a caracterização de pestivírus detectados em suínos de criação de subsistência no Rio Grande do Sul, através de soroneutralização para detecção de anticorpos contra BVDV 1 e 2, além de RT-PCR para detecção de pestivírus seguida de sequenciamento e análise filogenética. Como resultado, 28 (4,4%) das amostras foram positivas na sorologia e duas amostras foram positivas na RT-PCR e classificadas como BVDV-1d e BVDV-2ª, demonstrando o risco destes vírus serem fonte de contaminação para a indústria suinícola e prejudicarem o diagnóstico da Peste Suína Clássica ; 2) a primeira descrição de *Pestivirus K* no Brasil, através de análise de amostras de leitões nascidos com tremores congênitos em duas granjas do Sul do país por RT-PCR. Como resultado, foi realizada a primeira detecção e caracterização genética deste vírus na América do Sul, o que propiciará o estudo de sua patogenia e epidemiologia; 3 e 4) o uso da Análise de Componentes Principais (PCA) como ferramenta estatística para caracterização da resposta imune de cepas de campo e vacinais de BVDV-1 e 2 através de soroneutralização. Os resultados demonstraram que a PCA gerou gráficos de fácil visualização e interpretação de diferenças e semelhanças entre agrupamentos antigênicos e que não houve um mesmo padrão de antigenicidade entre isolados pertencentes a um mesmo subgenótipo, conhecimento este que será utilizado para a seleção de cepas para compor vacinas mais eficientes na proteção contra o BVDV; e 5) a caracterização de pestivírus detectados em amostras de bovinos recebidas no Laboratório de Virologia Veterinária-UFRGS entre 2016-2018, bem como inferência temporal (através da ferramenta relógio molecular) dos subgenótipos de pestivírus de bovinos já escritos no Brasil. Como resultado, foram descritas amostras de subgenótipos BVDV-1a, 1b e 2b (mais prevalentes no País) e 1d e 1e (subgenótipos já descritos, porém menos frequentes), e análise temporal da

filodinâmica destas cepas e outras representando os subgenótipos já descritos no país pode ser inferida e explicada pela história da pecuária bovina no país. Os resultados aqui apresentados somam novos conhecimentos acerca dos pestivírus e servirão de base para o estabelecimento de planos de controle e erradicação das doenças causadas por pestivírus em suínos e bovinos.

Palavras-chave: pestivírus, vacina, BVDV, APPV, filogenia, Análise de Componentes Principais, relógio molecular.

ABSTRACT

The Pestivirus genus, belonging to the family *Flaviviridae*, comprises RNA genome viruses classified into 11 species, mostly associated to ungulate hosts infections. In Brazil, as in many other countries, pestiviruses can affect livestock causing economic losses, such as Pestivirus C (classical swine fever virus, CSFV) and K (atypical porcine pestivirus) in swine and Pestivirus A, B (bovine viral diarrhea virus 1, 2- BVDV 1 and 2) and H (Hobi-like pestivirus- HoBiPeV) in cattle. The aim of this thesis is contributing to the epidemiology and diversity of pestiviruses in Brazil, and the antigenic characterization among bovine pestivirus strains to evaluate the efficiency of vaccines. The present work is presented in 5 chapters. The aim and results achieved in each chapter were, respectively:1) characterization of pestiviruses detected in subsistence swine (backyard pigs) in Rio Grande do Sul state, through detection of antibodies against BVDV 1 and 2, in addition to RT-PCR for pestivirus detection followed by sequencing and phylogenetic analysis. As result, 28 (4.4%) samples were positive in serology and two samples were positive in RT-PCR and classified as BVDV-1d and BVDV-2a subgenotypes, demonstrating the risk of ruminant pestiviruses as source of contamination for the swine industry and impair the diagnosis of Classical Swine Fever; 2) the first description of *Pestivirus K* in Brazil, through the analysis of samples from piglets born with congenital tremors in two farms in South states by RT-PCR. As result, this is the first detection and genetic characterization of this virus in South America, creating a stepstone for the study of its pathogenesis and epidemiology in the country; 3 and 4) the use of Principal Component Analysis (PCA) as a statistical tool for characterizing the antigenic relationship of field and vaccine strains of BVDV-1 and 2 through serum neutralization. The results showed that PCA generates easy visualization and interpretation graphics showing differences and similarities between antigenic groups, and that there was not a same pattern of antigenicity between isolates belonging to the same subgenotype; this knowledge can be used for the selection of strains to produce more efficient vaccines in protecting against BVDV; and 5) genetic characterization of pestiviruses detected in bovine samples received at the Veterinary Virology Laboratory-UFRGS between 2016-2018, as well as a temporal inference (through the molecular clock tool) of the emergence of bovine pestivirus subgenotypes in Brazil. As result, positive samples detected in the laboratory were classified as subgenotypes BVDV-1a, 1b and 2b (most prevalent in the country) and 1d and 1e (subgenotypes already described, but less frequent), and a phylodynamic temporal analysis of these other strains representing the subgenotypes described in the country could be inferred and

explained by the history of cattle introduction and ranching in Brazil. The results presented here add new knowledge about pestiviruses and can be used in the establishment of control and eradication programs for diseases caused by pestiviruses in swine and cattle.

Keywords: pestivirus, vaccine, BVDV, APPV, Principal Component Analysis, molecular clock.

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LISTA DE ABREVIAÇÕES

BVDV	Vírus da Diarreia Viral Bovina (*Bovine Viral Diarrhea Virus*)
PCA	Análise de Componentes Principais (*Principal Component Analysis*)
APPV	Pestivírus atípico porcino (*Atypical Porcine Pestivirus*)
ICTV	Comitê Internacional de Taxonomia Viral (*International Comitee on Taxonomy of Viruses*)
CSFV	Vírus Da Peste Suína Clássica (*Classical Swine Fever Virus*)
OIE	Organização Mundial de Saúde Animal
N^{pro}	proteína N-terminal
cp	citopático
ncp	não citopático
BDV	Vírus da Doença da Fronteira (*Border Disease Virus*)
5'UTR	5′ não traduzida (5' *untranslated*)
ORF	fase aberta de leitura (*open reading frame*)
C	proteína do capsídeo
p7	proteína 7
DM	doença das mucosas
PI	persistentemente infectado
HoBiPeV	*HoBi-like* vírus
SN	soroneutralização

SUMÁRIO

1 INTRODUÇÃO

O Brasil tem papel importante na produção animal e agrícola mundial. Além de ser significante produtor e exportador mundial de soja, cana de açúcar, café, e de o agribusiness ser responsável por grande fatia da geração de empregos no país, grande destaque é dado à produção de proteína animal (FAPDA-FAO, 2014). O Brasil é o quarto maior exportador de carne suína e tem tido a liderança em exportação mundial de carne de frango e bovina por muitos anos (USDA, 2021).

A carne suína é a carne mais consumida no mundo e a suinocultura é um setor que tem apresentado crescimento nos últimos anos. Com uma produção de 3,76 milhões de toneladas em 2016, o Brasil é o quarto maior exportador mundial em produção e exportação de carne suína (EMBRAPA SUÍNOS E AVES, 2019). Já em relação à carne bovina, o Brasil possui o maior rebanho comercial e é o maior exportador mundial (USDA, 2021).

Apesar de serem ambas atividades econômicas significativas, o modelo de criação suína e bovina são distintos com relação a desafios microbiológicos. O crescimento da produção suína levou à adoção de sistemas com alta densidade de animais nas instalações e avanços no controle e prevenção de doenças; porém a produção de suínos ainda enfrenta perdas por doenças infecciosas transmissíveis e emergentes, provenientes de dentro e fora das fronteiras (BARCELLOS *et al*, 2008). Já para o rebanho bovino, quase toda a produção animal provém de animais criados a pasto (FERRAZ; DE FELÍCIO, 2010), portanto com densidade animal muito menor do que na criação de suínos. Ainda assim, a bovinocultura enfrenta desafios microbiológicos devido à parcial adoção de medidas de biossegurança como sistema aberto na aquisição de animais e inseminação com sêmen não controlado ou monta natural (DE OLIVEIRA *et al*, 2015; FERNANDES *et al*, 2016).

Os pestivírus são vírus que ameaçam e causam prejuízos para ambos os rebanhos no Brasil. A peste suína clássica é uma doença infecciosa viral causada pelo *Pestivirus C*, anteriormente classificado como vírus da peste suína clássica (CSFV), um agente viral de grande importância para a suinocultura no mundo todo, podendo afetar a economia dos países de forma impactante. É uma doença de notificação obrigatória na Organização Mundial de Saúde Animal (OIE), causando sanções econômicas importantes quando presente no rebanho devido ao impedimento das exportações de carne e subprodutos (OIE, 2021). O Pestivírus Atípico Porcino (APPV) já foi

descrito nos principais países com suinocultura desenvolvida e causa perdas reprodutivas e de desempenho, principalmente em leitões que podem nascer com síndrome de tremores congênitos (POSTEL *et al*, 2017; YUAN *et al*, 2017). Os bovinos são hospedeiros afetados pelos *Pestivirus A, B* e *H*, e apesar de não ser uma doença de notificação obrigatória, estes pestivírus estão presente em grande parte dos rebanhos bovinos e geram prejuízos econômicos mesmo não sendo diagnosticados ou gerando sintomas leves a moderados (PINIOR *et al*, 2017).

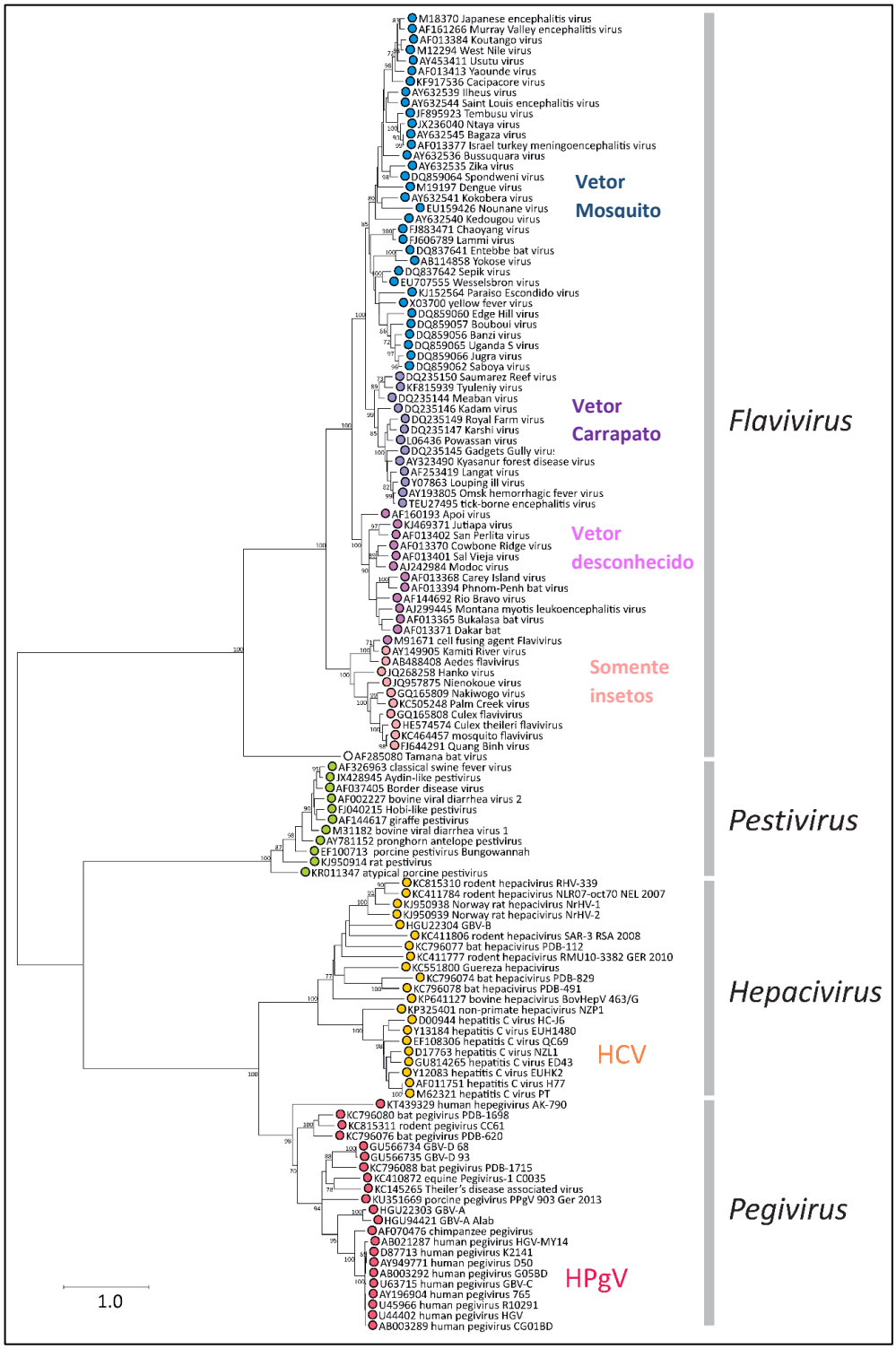
Obter informações constantes sobre a presença dos pestivírus e o potencial impacto destes na suinocultura e bovinocultura brasileira é essencial para o melhoramento destas atividades tão importantes na economia. Desta forma, o objetivo da presente tese de doutorado foi verificar a presença, distribuição, epidemiologia molecular e diversidade genética e antigênica das infecções causadas por pestivírus em suínos e bovinos, visando conhecimento que possa ser utilizado para benefício sanitário e econômico deste setor produtivo.

2 REVISÃO BIBLIOGRÁFICA

2.1 Família *Flaviviridae*

A família *Flaviviridae*, da qual fazem parte inúmeros vírus de importância na saúde humana e animal, é composta por quatro gêneros: *Flavivirus*, *Hepacivirus*, *Pegivirus* e *Pestivirus* (Figura 1). Os vírus do gênero *Flavivirus* são transmitidos principalmente por artrópodes (arbovírus), contando com 53 espécies, dentre as quais estão patógenos muito importantes para a saúde humana como o *Vírus da dengue*, *Vírus da febre amarela*, *Vírus da encefalite japonesa*, *Vírus do Oeste do Nilo* e *Vírus Zika*. O gênero *Hepacivirus* é composto por 14 espécies, dentre as quais o mais antigo e importante é o *Hepacivirus C* (*Vírus da hepatite C)*, que infecta humanos. As onze espécies descritas no gênero *Pegivirus* estão relacionadas a diversos hospedeiros, sendo que correlações entre estes vírus e algum tipo de patogenia vem aos poucos sendo relatada (SIMMONDS *et al*, 2017). Por fim, o gênero *Pestivirus* abriga onze espécies (*Pestivirus A-K*) (SMITH *et al*, 2017) sendo que a maioria destes tem importante relevância e impacto na saúde animal.

Figura 1. Família *Flaviviridae*. Árvore filogenética de sequência de aminoácidos da região NS5B utilizando cepas referência para cada um dos quatro gêneros.



Adaptado de: SIMMONDS *et al* (2017).

2.2 **Gênero *Pestivirus***

Uma característica única do gênero *Pestivirus* é a presença de duas proteínas exclusivas, a E RNAse (Erns) e a proteína N-terminal (Npro), não sendo encontradas proteínas homólogas nem nos gêneros mais próximos, como *Flavivirus* e *Hepacivirus* (NEILL, 2013). Além disso, os isolados também podem ser classificados em biotipos citopático (cp) ou não-citopático (ncp), de acordo com a capacidade de produzir efeito citopático em cultivo celular (POCOCK *et al*, 1987). Já para a demarcação de espécies dentro do gênero, são consideradas como espécies diferentes as que apresentam no mínimo 25% de divergência de nucleotídeos no genoma completo. Podem auxiliar nesta demarcação entre espécies outros dois critérios: diferença de pelo menos 10 vezes no título de neutralização em testes de neutralização cruzada com soros imunes policlonais e gama de hospedeiros que podem ser infectados (SMITH *et al*, 2017).

2.2.1 Taxonomia

O gênero *Pestivirus* era oficialmente composto, até o ano de 2017, por quatro espécies reconhecidas: BVDV-1, BVDV-2, CSFV e Vírus da Doença da Fronteira (BDV). Em 2018, a nomenclatura das espécies foi modificada, e outras possíveis novas espécies já descritas foram adicionadas oficialmente ao gênero, de acordo com o ICTV (SMITH *et al*, 2017) (Tabela 1). Desta maneira, BVDV -1, BVDV-2, CSFV e BDV passaram a ser *Pestivirus A*, *B*, *C* e *D*, respectivamente. As possíveis novas espécies adicionadas ao gênero *Pestivirus* foram: *Pestivirus E* (VILCEK *et al*, 2005), *Pestivirus F* (KIRKLAND *et al*, 2007), *Pestivirus G* (AVALOS-RAMIREZ *et al*, 2001), *Pestivirus H* (SCHIRRMEIER *et al*, 2004), *Pestivirus I* (OGUZOGLU *et al*, 2009), *Pestivirus J* (FIRTH *et al*, 2014) e *Pestivirus K* (HAUSE *et al*, 2015) (Tabela 1; Figura 2). Esta nova classificação foi feita com base na sequência de nucleotídeos do genoma completo, portanto duas novas prováveis espécies, um pestivírus descrito em morcego (WU *et al*, 2012) e pestivírus descritos em pequenos ruminantes na Tunísia (THABTI *et al*, 2005) não foram oficialmente reconhecidos como pertencentes ao gênero *Pestivirus* devido aos genomas estarem somente parcialmente sequenciados.

Tabela 1. Espécies do gênero *Pestivirus* de acordo com nova classificação designada pelo ICTV.

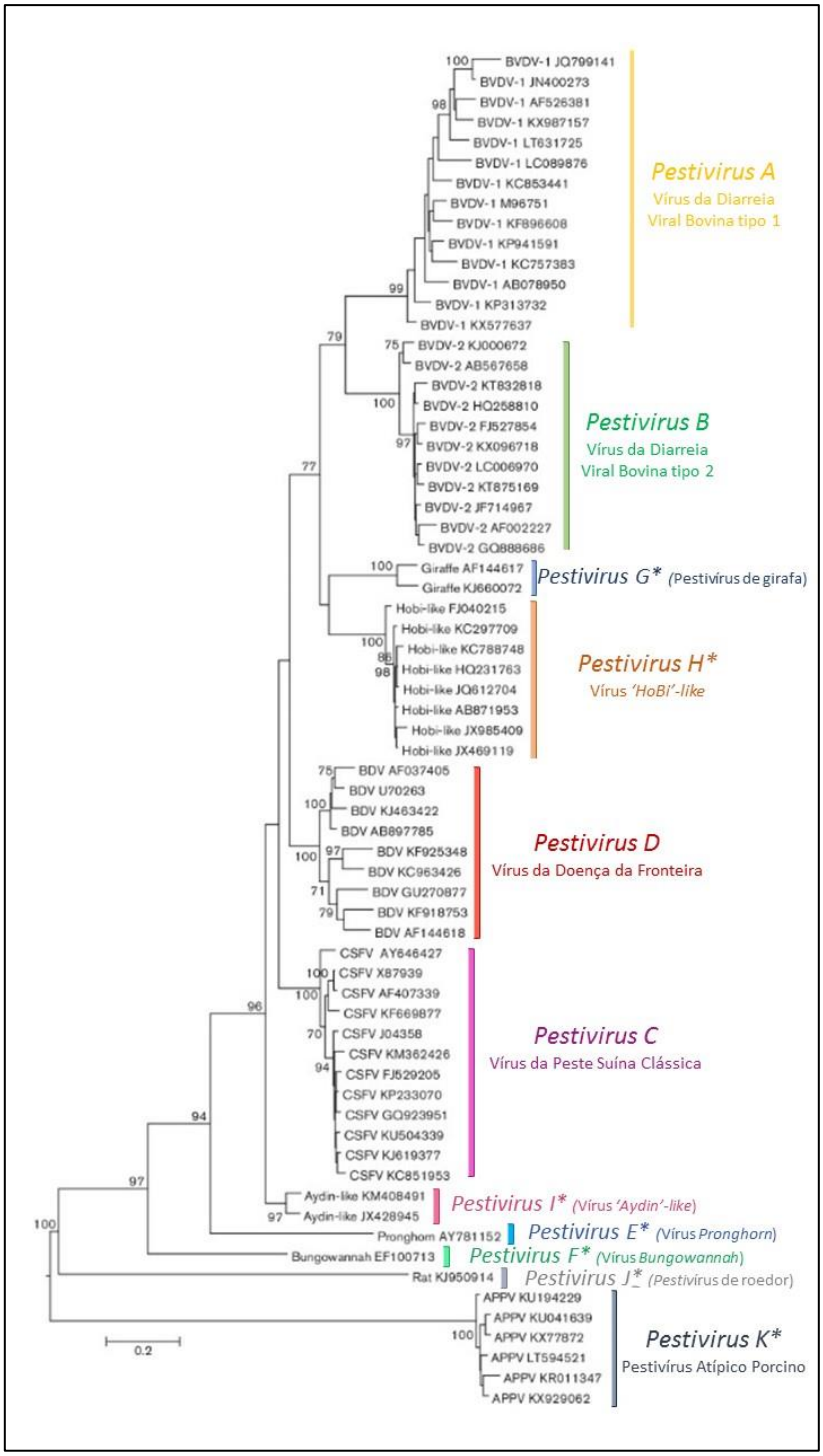
Espécie	Denominação anterior	Cepa referência	Número de acesso GenBank
Pestivirus A	Vírus da diarreia viral bovina 1	NADL	M31182
Pestivirus B	Vírus da diarreia viral bovina 2	890	U18059
Pestivirus C	Peste suína clássica	Alfort 187	X87939
Pestivirus D	Vírus da doença da fronteira	X818	AF037405
Pestivirus E	Pestivírus Pronghorn		AY781152
Pestivirus F	Pestivírus porcino	Bungowannah	EF100713
Pestivirus G	Pestivírus de girafa	H138	AF144617
Pestivirus H	Pestivírus 'HoBi-*like*'	Th/04_KhonKaen	FJ040215
Pestivirus I	Pestivírus 'Aydin-*like*'	04-TR	JX428945
Pestivirus J	Pestivírus de rato	NrPV/NYC-D23	KJ950914
Pestivirus K	Pestivírus atípico porcino	515	KR011347

Adaptada de: SMITH *et al*, 2017.

Há importantes variações genéticas e antigênicas até mesmo dentro das espécies de pestivírus. A classificação de subgrupos dentro das espécies, apesar de não reconhecida pelo ICTV, é amplamente utilizada e feita de acordo com análises filogenéticas da sequência de nucleotídeos de regiões como a 5′ não traduzida (5'UTR), Npro e E2, através dos métodos Maximum Likehood e análise Bayesiana (LIU *et al*, 2009). Com base em diversos estudos de filogenia, o *Pestivirus A*, por exemplo, atualmente pode ser dividido em pelo menos 21 subgenótipos nomeados por ordem alfabética (1a a 1q) (DENG *et al*, 2012; VILCEK *et al*, 2001). O *Pestivirus B* é dividido em três

subgenótipos (2a a 2c) (FLORES *et al*, 2002b; TAJIMA *et al*, 2001), o *Pestivirus D* em sete genótipos (BDV-1 a BDV-7) (BECHER *et al*, 2003; GIAMMARIOLI *et al*, 2011; KAWANISHI *et al*, 2014) e o *Pestivirus C* em três genogrupos (1, 2 e 3) que se dividem em vários subgenótipos (LOWINGS *et al*, 1996; PATON *et al*, 2000; POSTEL *et al*, 2013). Algumas das novas espécies incluídas no gênero também demonstram grande diversidade genética, como é o caso do *Pestivirus H* ('HoBi'-like), com 5 subgenótipos propostos de acordo com a origem geográfica das cepas (MISHRA *et al*, 2014, KALAIYARASU *et al*, 2021) e do *Pestivirus K*, que apesar de descrito pela primeira vez em 2015, já tem relatada grande diversidade entre isolados (BEER *et al*, 2016). Estas divisões em subgrupos dentro das espécies devem-se a grandes variações genéticas entre as cepas, e a maior implicância prática destas variações é que as variações genéticas podem gerar baixa reatividade sorológica cruzada, levando a falhas vacinais e perda da habilidade de detecção dos testes de diagnóstico (BACHOFEN *et al* 2008; FULTON *et al* 2003; RIDPATH 2003).

Figura 2. Gênero Pestivirus. Árvore filogenética construída a partir de sequência de aminoácidos da região NS5B de representantes de todas as espécies do gênero. O asterisco identifica as espécies recentemente reconhecidas como pertencentes ao gênero.

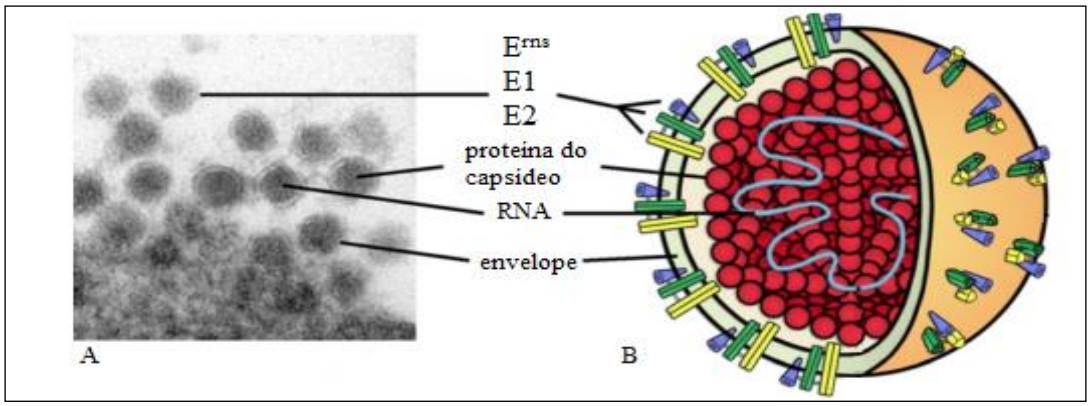


Adaptado de: SMITH *et al* (2017)

2.2.2 Vírion

Os vírions do gênero Pestivirus possuem 40–60 nm de diâmetro e têm formato esférico devido ao envelope composto de membranas da célula hospedeira (SIMMONDS *et al*, 2017). O capsídeo pode apresentar formato icosaédrico (Figura 3), porém sugere-se que a estrutura é polimórfica e sem formato definido (RIEDEL *et al*, 2017).

Figura 3. Descrição esquemática da estrutura de um vírion do gênero Pestivirus. A) Foto de microscopia eletrônica de vírions do Pestivirus C. B) Ilustração esquemática do vírion.

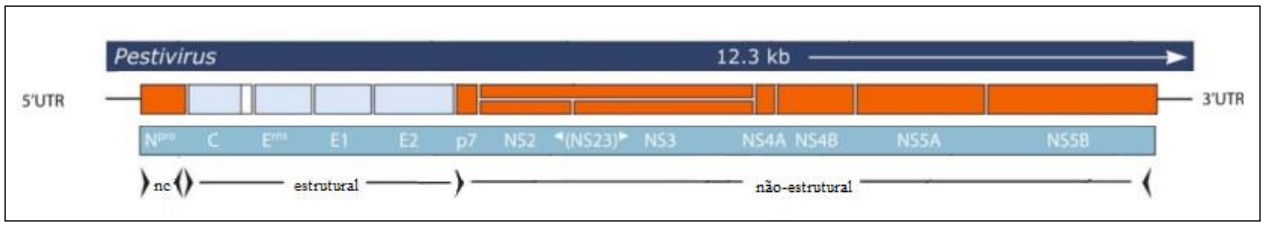


Adaptada de: TAUTZ et al (2015).

2.2.3 Genoma

Os pestivírus são vírus envelopados que têm o genoma constituído por uma fita simples de RNA de polaridade positiva de 11,5 a 13 kilobases, possuindo duas regiões não traduzidas nas extremidades 5’ e 3’. O genoma tem uma única fase aberta de leitura (ORF), que é traduzida em uma longa poliproteína de aproximadamente quatro mil aminoácidos. Esta poliproteína é posteriormente clivada em onze a doze proteínas, sendo quatro estruturais e o restante não estruturais (BROCK; DENG; RIBLET, 1992; COLLETT *et al*, 1988; KIRKLAND *et al*, 2015). A poliproteína é clivada nas proteínas individuais à medida que é traduzida: Npro, proteína do capsídeo (C), glicoproteínas do envelope (Erns, E1 e E2), proteína 7 (p7) e as proteínas não-estruturais NS2, NS3, NS4A, NS4B, NS5A e NS5B (Figura 4) (SIMMONDS *et al*, 2017).

Figura 4. Esquema de organização do genoma de pestivírus. O genoma é codificado em uma única poliproteína, posteriormente clivada em proteínas estruturais e não estruturais.



Adaptada de PETERHANS *et al* (2010).

Algumas regiões genômicas são utilizadas na caracterização genética e filogenia devido a propriedades específicas citadas a seguir.

2.2.3.1 Região 5’ UTR

O genoma dos membros da família *Flaviviridae* não possui estrutura *cap* na região 5’do genoma (BROCK; DENG; RIBLET, 1992), que tem função de interação com o ribossomo e sinalização de início da tradução. A estratégia para garantir a tradução do RNA utilizando a maquinaria celular envolve a ligação dos ribossomos em uma estrutura secundária e terciária formada pelo RNA nesta região, denominada sítio interno de entrada no ribossomo (IRES). Neste sítio é sinalizada e iniciada a tradução (POOLE *et al*, 1995) e, por ser altamente conservada entre os pestivírus, a sequência de nucleotídeos é utilizada em análises filogenéticas (THURNER *et al*, 2004).

2.2.3.2 Npro

A primeira proteína viral traduzida é a proteína não estrutural Npro, que possui atividade autoproteolítica, sendo responsável pela própria clivagem da poliproteína (STARK *et al*, 1993). A Npro só é encontrada nos pestivírus e não possui homologia com nenhuma outra protease (RAWLINGS; BARRETT; BATEMAN, 2012) e, por interferir na via do Interferon tipo I, já foi associada à habilidade dos pestivírus de gerar infecções persistentes de fetos (CHARLESTON *et al*, 2001).

2.2.3.3 Glicoproteínas do envelope E1 e E2

A E1 e E2 são proteínas integrais da membrana e fortemente inseridas a ela. A proteína E1 forma heterodímeros com a proteína E2 (THIEL *et al*, 1991), sendo que estes heterodímeros são necessários para a entrada do vírus na célula. Com base na proteína dos flavivírus, supõe-se que a função da E1 nos pestivírus é ser chaperona da proteína E2, que teria função de ligação a receptores celulares (LI *et al*, 2008). Embora seja uma glicoproteína do envelope, animais infectados não produzem anticorpos contra E1 e esta não é alvo importante de linfócitos T (KIMMAN *et al*, 1993).

A proteína E2 possui características que a tornam capaz de realizar fusão de membranas, sendo indicada como uma das responsáveis pela entrada do vírus na célula (FERNÁNDEZ-SAINZ *et al*, 2014) e determinante do tropismo celular (LIANG *et al*, 2003). O receptor celular mais descrito e tido como principal receptor do Pestivirus A é o CD46 bovino, uma proteína de membrana reguladora da atividade do complemento. Porém é suposto que os pestivírus necessitem de outros cofatores para entrada na célula, como receptores de heparan-sulfato (DRAGER; BEER; BLOME, 2015). A E2 é a glicoproteína que contém grande parte dos determinantes antigênicos e é alvo da maioria da resposta humoral protetora, com a maioria dos epítopos localizados na porção N-terminal (DEREGT *et al*, 1998).

2.2.3.4 Proteínas não estruturais NS2 e NS3

A proteína NS2 induz estresse no retículo endoplasmático da célula hospedeira e desempenha um papel importante na patogênese e virulência (GUO *et al*, 2011). Ela é encontrada fusionada com a NS3 em células infectadas com cepas ncp, porém é encontrada na forma individualizada em grande quantidade em infecção por cepas cp. A clivagem entre NS2 e 3 é processada pela própria NS2, havendo a necessidade de interação com uma chaperona celular denominada Jiv (proteína do domínio J interagindo com a proteína viral) (LACKNER; THIEL; TAUTZ, 2006). Acredita-se, portanto, que este controle da atividade de protease e clivagem entre NS2 e NS3 através da limitação na quantidade de Jiv celular pode ser um importante fator regulatório do vírus (RINCK *et al*, 2001), já que a habilidade de estabelecer infecções persistentes depois de exposição intrauterina é restrita ao biotipo ncp, onde estas duas proteínas permanecem

na forma não clivada. Quando este fator regulatório é perdido, como em Jiv celular expressada em excesso ou quando um fragmento desta chaperona é inserido no genoma de cepas de pestivírus, ocorre a indução da clivagem entre NS2–3 em alta eficiência. Este e outros casos de inserções no genoma de cepas cp, principalmente entre NS2 e NS3, são capazes de induzir um quadro fatal denominado Doença das Mucosas (DM) em bovinos persistentemente infectados (PETERHANS *et al*, 2010).

A clivagem das outras proteínas não estruturais ocorre em quatro sítios de clivagem (NS3/NS4A, NS4A/NS4B, NS4B/NS5A, e NS5A/NS5B) através de uma serino protease cujo sítio catalítico está na NS3. Essa protease necessita da proteína NS4A como cofator para exercer sua atividade de clivagem (BAZAN; FLETTERICKT, 1988). A NS3 também possui um domínio helicase que funciona no desenrolamento de estruturas secundárias na 3' UTR ou em regiões de codificação, permitindo a iniciação ou facilitando a atividade da RNA polimerase viral (proteína NS5B), caracterizada como uma RNA polimerase dependente de RNA (COLLETT, 1992). Apesar de não se ter muito conhecimento sobre elas, as proteínas NS4B e NS5A são necessárias para a replicação do genoma (SHENG *et al*, 2012).

2.2.4 Biotipos

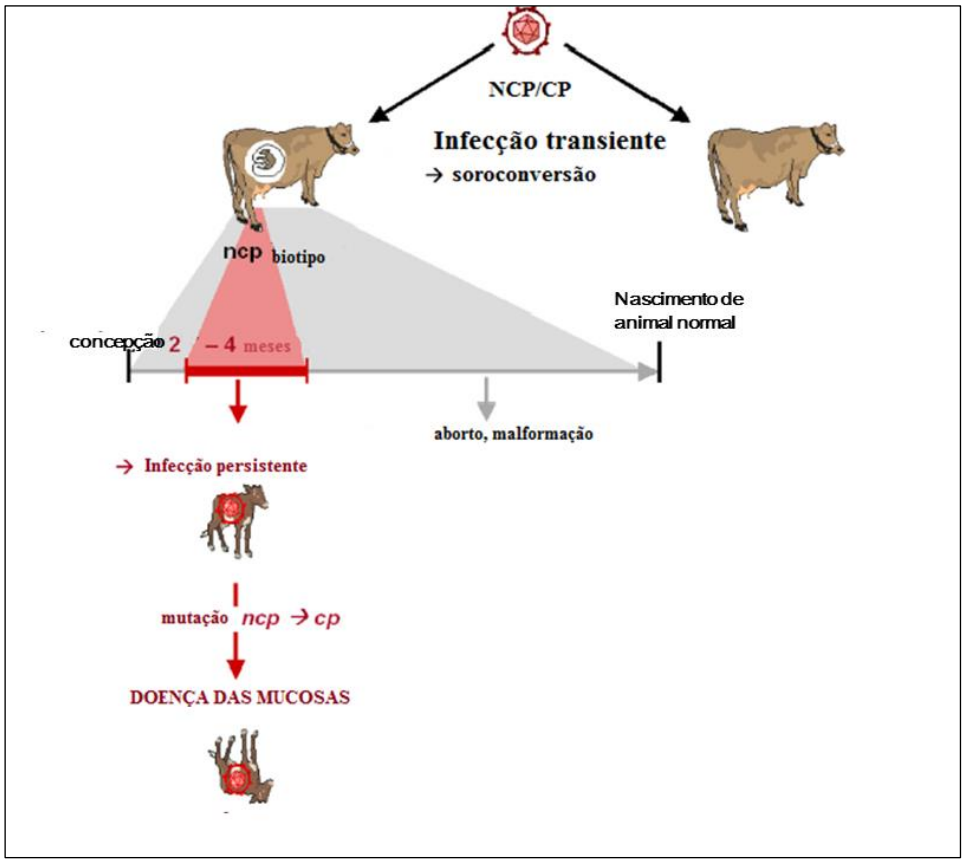
Os pestivírus podem apresentar dois biotipos: o biotipo ncp, que não causa destruição celular, e o biotipo cp, que causa a aparição de efeito característico nas células em cultivo. O biotipo ncp é o mais encontrado na natureza, sendo o único biótipo capaz de gerar animais persistentemente infectados (PI) quando ocorre infecção intra-uterina. Nos isolados caracterizados como ncp, as proteínas virais NS2 e NS3 encontram-se em sua maioria na forma fusionada NS2-3; já o biotipo cp é originário de mutações, deleções e rearranjos genéticos que afetam a proteína viral NS2-3, que passa a ser clivada sob a forma de duas proteínas individuais (POCOCK *et al*, 1987). Várias mudanças no genoma já foram relatadas como formas de geração da NS2 e NS3 individuais: inserções de sequências celulares do hospedeiro, como a sequência Jiv (BECHER; TAUTZ, 2011), duplicações de sequências do próprio genoma (MEYERS *et al*, 1992; TAUTZ *et al*, 1996), deleções de parte do genoma (TAUTZ *et al*, 1994), mutações pontuais (KUMMERER; MEYERS, 2000) ou rearranjos dentro do genoma (MEYERS *et al*, 1992).

2.2.5 Animais PI

A infecção de fêmeas prenhes suscetíveis por alguns pestivírus cursa com transmissão transplacentária do vírus. As consequências desta infecção para o concepto dependem da idade gestacional em que ocorre a infecção e da cepa do vírus: no início da gestação podem ocorrer perdas embrionárias e fetais, nascimento de filhotes fracos e inviáveis ou o nascimento de animais PI. A infecção no final da gestação frequentemente leva ao nascimento de animais normais, livres do vírus (MACLACHLAN; DUBOVI, 2011). Se a infecção transplacentária ocorrer antes do desenvolvimento do sistema imune do feto, o vírus não é reconhecido como antígeno e o animal gera imunotolerância à cepa infectante, se tornando um animal PI. Somente cepas ncp são capazes de estabelecer e manter infecção persistente (PETERHANS *et al*, 2010) e o animal PI excretará altos títulos do vírus em todas as secreções corporais por toda a vida, constituindo-se em um reservatório e fonte de disseminação viral entre os animais. Já foi descrita a geração de animais PI em infecções transplacentárias por *Pestivirus A* (DEREGT; LOEWEN, 1995), *B* (RIDPATH; BOLIN; DUBOVI, 1994), *C* (MOENNIG; FLOEGEL-NIESMANN; GREISER-WILKE, 2003), *D* (NETTLETON *et al*, 1998) e *H* (DECARO *et al*, 2014).

Em infecção de vacas prenhas por *Pestivirus A* e *B*, pode ocorrer a geração de bezerros PI se houver transmissão transplacentária dos 40 aos 120 dias de gestação (PETERHANS *et al*, 2010) (Figura 5). Estes animais que desenvolvem imunotolerância ao vírus jamais conseguem erradicá-lo do organismo e, geralmente, possuem desenvolvimento retardado e morrem no primeiro ano de vida devido a infecções secundárias causadas pela imunodepressão gerada. Alguns destes animais PI podem desenvolver o quadro clínico DM (MACLACHLAN; DUBOVI, 2011), que está associado ao surgimento do biotipo cp da cepa de BVDV a qual o animal PI é imunotolerante (BACHOFEN *et al*, 2010). O *Pestivirus H* possui características em comum com os *Pestivirus A* e *B*, tendo como principal hospedeiro o bovino e cursando com sintomatologia clínica semelhante (DECARO *et al*, 2011; STAHL *et al*, 2007): surtos de aborto e malformações (Bianchi *et al* 2011; Decaro *et al* 2012), geração de animais PI e quadro semelhante à DM (BIANCHI *et al* 2016; DECARO *et al* 2014; DECARO *et al* 2012; WEBER *et al* 2016).

Figura 5. Consequências de infecção persistente pelo BVDV. A infecção de um animal soronegativo conduz a uma infecção transitória sem sintomatologia clínica. Em contraste, a infecção com cepas ncp em fêmeas soronegativas entre o segundo e quarto mês de gestação pode levar a um feto PI. Se o vírus ncp muta para biotipo cp ou se o animal é infectado com um vírus cp antigenicamente semelhante, o bezerro PI desenvolverá a letal DM.



Adaptado de: PETERHANS *et al* (2010).

Infecções por *Pestivirus C* em porcas com 50 a 70 dias de gestação podem levar ao nascimento de leitões PI, que geralmente apresentam crescimento inferior, refugo e tremores congênitos; alguns leitões PI são clinicamente normais e podem sobreviver por vários meses (MOENNIG; FLOEGEL-NIESMANN; GREISER-WILKE, 2003). Para *Pestivirus D*, que tem como hospedeiro principal o ovino, a infecção de fêmeas prenhes até 80 dias de gestação pode gerar filhotes PI e estes são cordeiros que nascem com pouca chance de sobrevivência por causa da imunodepressão. Estes animais nascem pequenos e fracos, não conseguem manter-se em pé, podem ter sinais neurológicos como tremores e anormalidades na cor e textura da lã. Alguns animais PI podem se tornar adultos e excretar o vírus por anos (NETTLETON *et al*, 1998).

2.3 Pestivírus em Suínos

Suínos são hospedeiros importantes de alguns pestivírus, sendo hospedeiros naturais do *Pestivirus C, Pestivirus F* e *Pestivirus K* e ocasionais dos *Pestivirus A, B* e *D*. O principal pestivírus isolado de suínos é o *Pestivirus C*, considerado o agente infeccioso viral mais importante na suinocultura no mundo, podendo afetar a economia de forma tão impactante que está na lista de doenças de notificação obrigatória da OIE (OIE, 2021). Outros pestivírus que podem afetar a suinocultura têm sido relatados com certa frequência e são os relacionados a hospedeiros ruminantes (TERPSTRA; WENSVOORT, 1991; VILCEK; BELÁK, 1996) e o APPV (HAUSE *et al*, 2015).

2.3.1 *Pestivirus C*

O *Pestivirus C* causa doença grave e contagiosa denominada Peste Suína Clássica, afetando tanto suídeos domésticos como silvestres (MOENNIG, 2000). Por ser de difícil controle em altas densidades populacionais de suínos, está na lista de doenças de notificação obrigatória da OIE (OIE, 2021). Os primeiros surtos descritos ocorreram na França em 1822 e nos Estados Unidos em 1833, e a doença acabou tornando-se endêmica nos séculos XIX e XX, com distribuição mundial, embora atualmente alguns países já tenham erradicado a doença (EDWARDS *et al*, 2000).

A gravidade dos sinais clínicos depende principalmente da idade do animal e virulência do isolado. Em suínos adultos, a doença é geralmente leve ou sub-clínica, porém o quadro grave da doença é caracterizado por febre alta, lesões hemorrágicas, diarreia, imunodepressão com infecções secundárias e alta mortalidade (MOENNIG; FLOEGEL-NIESMANN; GREISER-WILKE, 2003). A via de transmissão é a oro-nasal, sendo as tonsilas o órgão de entrada; posteriormente o vírus é drenado para linfonodos, medula óssea e tecido linfóide do trato digestivo, atinge a corrente sanguínea em altos títulos e se instala no fígado, pâncreas e rins. O vírus pode atravessar a barreira transplacentária em fêmeas gestantes e, dependendo da fase gestacional, pode gerar desde perdas gestacionais ao nascimento de leitões PI (RIDPATH & FLORES, 2007).

A maioria dos países com produção de suínos já tem tomado medidas de controle e erradicação da doença, e esta é considerada erradicada na América do Norte, Austrália e Nova

Zelândia, Europa, Chile e Paraguai; partes da Colômbia e Brasil também são consideradas livres do vírus (OIE, 2020). No Brasil, a região com a maior produção na suinocultura industrial é a região Sul, onde não são identificados casos desde 1988 (EDWARDS *et al*, 2000). Em 1992 foi implantado o Programa Nacional de Controle e Erradicação da Peste Suína Clássica, e a monitoria e vigilância continuam atualmente com o Plano de Contingência para peste suína clássica.

2.3.2 *Pestivirus A* e *B*

A prevalência de infecção por *Pestivirus A* e *B* em rebanhos suínos tem crescido substancialmente nos últimos anos, levantando a suspeita de que estes pestivírus de ruminantes atualmente podem estar causando alguma perda econômica na indústria da suinocultura (TAO *et al*, 2013). Acredita-se que *Pestivirus A* e *B* já tenham sido isolados de suínos no passado, e erroneamente identificados como *Pestivirus C* utilizando anticorpos policlonais, já que os pestivírus apresentam alguma reatividade sorológica cruzada entre si e a diferenciação por antigenicidade pode ser complicada (DEKKER; WENSVOORT & TERPSTRA, 1995).

Na década de 1960, já havia sido provado que antígenos de *Pestivirus A* e *C* possuíam alguma reatividade cruzada (WENSVOORT *et al* 1989), porém somente em 1973 vírions de *Pestivirus A* foram recuperados de porcos doentes naturalmente infectados (FERNELIUS *et al*, 1973). A partir deste estudo, casos de suínos com sinais clínicos consistentes com Peste Suína Clássica foram diagnosticados como infecção por *Pestivirus A* (TERPSTRA & WENSVOORT, 1991) e testes antigênicos foram desenvolvidos para diferenciar as espécies de pestivírus em suínos. Durante um surto de Peste Suína Clássica na Holanda em 1997/1998, 26,5% das amostras positivas para *Pestivirus C* através de ELISA, analisadas posteriormente por outros métodos, demonstraram ser positivas para outros pestivírus que não o *Pestivirus C* (PASSLER & WALZ, 2010).

Infecção de suínos por pestivírus relacionados a ruminantes geralmente cursa sem sinais clínicos, permitindo ao vírus se disseminar no rebanho sem detecção. Estudos de *Pestivirus A* em suínos não prenhes raramente relataram sinais clínicos apesar da viremia e soroconversão (WALZ *et al*, 1999). Porém em surtos onde os animais afetados eram mais novos, a infecção cursou com anemia, pelagem áspera, crescimento retardado, tremores, conjuntivite, diarreia, poliartrite, petéquias na pele e cianose de orelha. A infecção de fêmeas gestantes pode gerar problemas reprodutivos (TERPSTRA& WENSVOORT, 1988).

As maiores fontes de *Pestivirus A* e *B* para infecção de suínos são os bovinos, sendo que fatores de risco para a soropositividade de suínos são a presença de gado na mesma fazenda, alta densidade de pequenos ruminantes próximos ao rebanho, vacinas contaminadas e a idade do animal (LOEFFEN *et al*, 2009; WENSVOORT; TERPSTRA, 1988). Alguns estudos sorológicos de prevalência têm sido realizados para detecção de pestivírus de bovinos em rebanhos suínos ao longo do tempo, e o decréscimo na soroprevalência em rebanhos tecnificados pode ser explicado pela especialização na suinocultura, com diminuição no contato com outras espécies e o aumento no controle de qualidade da produção de vacinas (BOLIN; MATTHEWS; RIDPATH, 1991).

Os *Pestivirus A* e *B*, assim como *Pestivirus C*, podem infectar javalis, e tanto em estudos sorológicos (SEDLAK; BARTOVA; MACHOVA, 2008; VILCEK; NETTLETON, 2006; ZUPANCIĆ *et al*, 2002), quanto em detecção direta por RT-PCR (Weber *et al* 2016), a presença deste vírus corrobora com a hipótese de que os javalis podem exercer algum papel na transmissão destes pestivírus (ZUPANCIĆ *et al*, 2002).

2.3.3 *Pestivirus F*

Este pestivírus foi identificado em suínos em um surto único ocorrido em 2003 na Austrália. O surto foi caracterizado por mortalidade neonatal e fetos mumificados, sendo que o achado patológico mais marcante nos leitões foi miocardite não-supurativa multifocal; a busca pelo possível agente causal resultou na identificação de um pestivírus que ainda não havia sido descrito (KIRKLAND *et al*, 2007). A apresentação clínica teve semelhanças com infecção uterina causada por outros pestivírus, cursando com mortalidade em leitões de três a quatro semanas e aumento no número de natimortos em fêmeas afetadas. As perdas de leitões ultrapassaram 50% em algumas granjas e este pestivírus não foi detectado por métodos diagnósticos que eram considerados como adequados para todos os membros do gênero Pestivirus.

O *Pestivirus F* possui baixa identidade nucleotídica quando comparado aos outros pestivírus já descritos, além de limitada reatividade sorológica cruzada com os outros membros do gênero (KIRKLAND *et al*, 2015). Apesar de ser divergente, as características únicas do gênero *Pestivirus* estão presentes nesta espécie (RICHTER *et al*, 2014). Ainda não foram relatados casos desta doença fora da Austrália.

2.3.4 *Pestivirus K*

Com o auxílio das novas técnicas de sequenciamento de alta eficiência, nos últimos anos tem sido possível a descrição de pestivírus muito divergentes antes não detectados. Uma possível nova espécie de pestivírus detectada em morcegos *Rhinolophus affinis* (WU *et al*, 2012), a espécie *Pestivirus J* detectada em roedores *Rattus norvegicus* (FIRTH *et al*, 2014) e a espécie *Pestivirus K* foram descritas através destes novos métodos. O *Pestivirus K* foi identificado em amostras de soro de suínos destinada inicialmente ao sequenciamento do vírus da Síndrome Respiratória e Reprodutiva Suína em um banco de soros suínos nos Estados Unidos. Um contig de 11.276 pares de bases, codificando uma poliproteína de 3.635 aminoácidos, teve identidade de 40% com a poliproteína dos pestivírus já conhecidos. Apesar da poliproteína ter 250 aminoácidos a menos que a poliproteína da maioria dos pestivírus e a proteína Npro não ter homologia com nenhuma sequência nos bancos de dados, características conservadas do gênero foram encontradas neste genoma. Estas características conservadas sugeriram que, apesar de muito divergente, esta seria uma possível nova espécie do gênero e foi levantada a hipótese de que este agente poderia causar alguma síndrome ou doença nos suínos (HAUSE *et al*, 2015).

Após a descrição deste possível pestivírus, estudos foram realizados para compreender se este agente geraria algum tipo de sintomatologia. Uma síndrome de tremores congênitos em leitões, descrita há quase cem anos, ainda possuía causa infecciosa, provavelmente viral, desconhecida. Com o uso de sequenciamento de alta eficiência, este vírus foi detectado tanto em leitões que apresentavam esta síndrome de tremores congênitos quanto em adultos assintomáticos, mas estava ausente em leitões assintomáticos, traçando forte correlação entre a síndrome de tremores congênitos sem etiologia definida e este novo pestivírus (ARRUDA *et al*, 2016; POSTEL *et al*, 2016).

A inoculação do vírus em fêmeas suínas gestantes soronegativas resultou na geração de leitegadas com tremores congênitos e infectados com o *Pestivirus K*, confirmando que a via transplacentária, uma característica dos pestivírus, é a via pela qual os leitões têm o sistema nervoso infectado (ARRUDA *et al*, 2016; DE GROOF *et al*, 2016), e que alguns animais continuam a excretar o vírus até a idade de abate, sendo estes a possível fonte de contaminação para as fêmeas primíparas soronegativas introduzidas nas granjas (DE GROOF *et al*, 2016).

Através de imunofluorescência e imunohistoquímica, a localização do vírus no sistema nervoso central de leitões afetados foi identificada como sendo a camada granular interna do cerebelo, explicando porque os leitões com tremores conseguem se recuperar após algumas semanas, inclusive com completa remissão dos tremores (POSTEL *et al*, 2016). A rota de infecção fecal-oral foi confirmada devido à presença do vírus nas glândulas salivares, pâncreas, duodeno e cólon (DE GROOF *et al*, 2016; POSTEL *et al*, 2016; SCHWARZ *et al*, 2017), porém a infecção via inseminação artificial também foi sugerida, já que o vírus também foi detectado em sêmen (GATTO *et al*, 2017). O principal achado histopatológico em leitões afetados por tremores foi desmielinização leve da matéria branca do cérebro e da medula espinhal, com quantidade moderada de vacúolos (POSTEL *et al*, 2016; SCHWARZ *et al*, 2017).

A grande diversidade genética do *Pestivirus K*, a relevante porcentagem de suínos positivos tanto em RT-PCR quanto em sorologia, e sua presença em amostras de 1997 (MUÑOZ-GONZÁLEZ *et al*, 2017) sugerem que o vírus circula há muito tempo nos rebanhos suínos, corroborando relatos desta síndrome há quase cem anos. O *Pestivirus K* já foi descrito nos EUA (ARRUDA *et al*, 2016; HAUSE *et al*, 2015), Canadá (DESSUREAULT *et al*, 2018), Áustria (SCHWARZ *et al*, 2017), Alemanha (BEER *et al*, 2016; POSTEL *et al*, 2016; POSTEL *et al*, 2017), Inglaterra, Itália, Sérvia, Suíça (POSTEL *et al*, 2017), Holanda (DE GROOF *et al*, 2016), Espanha (MUÑOZ-GONZÁLEZ *et al*, 2017), China e Taiwan (POSTEL *et al*, 2017; YUAN *et al*, 2017; ZHANG *et al*, 2017), Coréia do Sul (CHOE *et al*, 2020) e até 2018 não havia sido descrito no Brasil (MOSENA *et al*, 2018; POSSATTI *et al*, 2018). Após o primeiro relato (descrito nesta tese), outros estudos revelaram a presença do vírus em rebanhos no país (GATTO; SONÁLIO & DE OLIVEIRA, 2019).

2.4 Pestivírus em bovinos

2.4.1 *Pestivirus A* e *B*

O gênero *Pestivirus* contém três espécies virais de importância para a indústria pecuária bovina: BVDV-1, BVDV-2 e HoBiPeV, nomeados oficialmente como *Pestivirus A*, *B* e *H*, respectivamente (SMITH *et al*, 2017). O BVDV está presente em todos os países onde existe algum nível de atividade pecuária e causa prejuízos econômicos diretos e indiretos para a criação de

bovinos (ROBERT *et al*, 2004; RODNING *et al*, 2012; RILEY *et al*, 2019). Os primeiros casos descritos de diarreia viral bovina eram de uma doença com sintomas severos, porém a maioria das infecções causam sintomatologia leve a branda de curta duração. Porém alguns surtos graves relacionados à infecção aguda por BVDV, especialmente BVDV-2, tem sido relatados (RIDPATH *et al*, 2006).

O BVDV foi segregado em duas espécies distintas pois possuem alta variabilidade genômica e antigênica. Enquanto o quadro de sintomas é o mesmo entre infecções por BVDV-1 e BVDV-2, há diferenças biológicas significantes entre as duas espécies (RIDPATH, 2003), como a reatividade sorológica cruzada entre BVDV-1 e BVDV-2 que é geralmente baixa, apresentando implicações importantes no diagnóstico e eficácia das vacinas (RIDPATH; FLORES, 2007).

As duas espécies levam, na maioria, a infecções com sinais clínicos leves ou inaparentes. Em animais não prenhes, sinais brandos como febre curta, sinais respiratórios, gastroentéricos e leucopenia podem estar presentes, e decréscimo na produção de leite e ganho de peso podem ser observados em gado leiteiro e de corte (RICHTER *et al*, 2017). Os anticorpos adquiridos após uma infecção aguda protegem o animal da reinfecção homóloga pela vida toda (PETERHANS *et al*, 2010).

A infecção pelo BVDV em fêmeas prenhes suscetíveis é a chave na sobrevivência do vírus no rebanho, devido à habilidade de transmissão transplacentária do vírus. As consequências desta infecção para o feto dependem principalmente da idade gestacional em que ocorre a infecção. Infecções no início da gestação geram reabsorção embrionária e retorno ao estro. Em idade gestacional mais avançada podem ocorrer abortos, mumificação fetal, natimortos, bezerros com malformações, fracos e inviáveis, ou o nascimento de animais PI. Ao terço final da gestação os bezerros já possuem sistema imune ativo e nascem normais, soropositivos e livres do vírus (RIDPATH; FLORES, 2007).

Os animais PI podem apresentar características como crescimento retardado e malformações congênitas, ou podem ter aparência normal (BACHOFEN *et al*, 2010). Estes animais replicam e excretam o vírus em altos títulos em todas as secreções durante toda a vida, constituindo-se no principal reservatório de disseminação viral entre os animais (ARENHART *et al* 2009). Apesar de sobreviverem por poucos meses, alguns animais PI podem sobreviver por anos, inclusive se tornando reprodutores, e transmitir o vírus pelo sêmen no caso de machos e gerar bezerros PI no caso de fêmeas (RIDPATH; FLORES, 2007). Os animais PI podem desenvolver o quadro clínico

fatal DM, geralmente associado ao surgimento do biótipo citopático da cepa de BVDV a qual o animal PI é imunotolerante (BACHOFEN *et al*, 2010).

Pequenos ruminantes domésticos e silvestres bi ungulados são suscetíveis aos pestivírus, já que BVDV já foi detectado em ovelhas, cabras (KRAMETTER-FROETSCHER *et al*, 2010) e camelos (GAO *et al*, 2013), e animais silvestres como cervos, alpacas (PASSLER; WALZ, 2010) e antílopes. Biungulados não-ruminantes podem também ser hospedeiros acidentais, já que suínos e javalis (TAO *et al*, 2013; WEBER *et al*, 2016) podem ser infectados e manifestarem sintomatologia.

Em estudo global com escala temporal, a prevalência mundial de animais PI em rebanhos decaiu de 1,85% em 1980 para 0,36% em 2016. Já a prevalência de animais soropositivos se manteve estável durante este período na maioria das regiões do globo, com prevalência de 46,23% a nível individual e 66,08% a nível de rebanhos soropositivos em 2016 (SCHARNBÖCK *et al*, 2018). Isto demonstra que os pestivirus ainda tem grande circulação em rebanhos bovinos. Os perfis de subgenótipos prevalentes se mostrou muito dependente da região geográfica, sendo os subgenótipos BVDV-1b e 1a os mais presentes no rebanho mundial. BVDV-1b é também o subgenótipo mais prevalente no continente americano, asiático e europeu, porém a maioria dos isolados australianos pertencem ao subgenótipo BVDV-1c. Muitos dos subgenótipos menos frequentes estão limitados à Europa e Ásia (subgenótipos 1e a 1t) (YESILBAG; ALPAY; BECHER, 2017). BVDV-2 tem alta frequência em alguns países do continente americano, porém a espécie já foi descrita em vários países europeus. O subgenótipo 2a é o mais frequente no rebanho mundial, porém em alguns países o subgenótipo 2b é o mais presente.

No Brasil, estudos retrospectivos mostraram prevalências de BVDV-1 de 53,9% a 54,4%, e os subgenótipos mais comuns são 1a (33,9% a 35,9%) e 1b (16,3% a 10,1%), e os subgenótipos 1d, 1e e 1i tiveram poucos relatos. Isolados de BVDV-2 tiveram de 25,7% a 33,7% de frequência, com a maioria (>84%) pertencendo ao subgenótipo 2b. Relatos de isolados de HoBiPev também se tornaram mais comuns no Brasil, com prevalências de 12,4% a 19,9% dos pestivirus detectados dependendo da região do país (FLORES *et al*, 2018; SILVEIRA *et al*, 2018). Na região Nordeste este foi o pestivírus mais detectado em amostragem representativa do rebanho, mostrando que, apesar de ser o pestivírus de bovino mais recente em termos de descrição, já circula nos rebanhos brasileiros (SILVEIRA *et al*, 2018).

2.4.2 *Pestivirus H*

A espécie nomeada *Pestivirus H*, referida como ‘HoBi’-like vírus, é o mais recente dos pestivírus de ruminantes descritos, sendo primeiramente detectada por pesquisadores da Alemanha em 2004. Este novo pestivírus, primeiramente chamado pestivírus atípico, foi isolado de um lote de soro fetal bovino importado do Brasil (SCHIRRMEIER *et al*, 2004). A partir deste relato, pesquisadores em muitos países identificaram este pestivírus primeiramente em cultivo celular, porém descrições de infecção natural de bovinos e búfalos por esta espécie começaram a ser comuns na Ásia (STAHL *et al*, 2007), Itália (DECARO *et al*, 2012) e Brasil (BIANCHI *et al*, 2011; SILVEIRA *et al*, 2018; MONTEIRO *et al*, 2019). Apesar de sua semelhança nos sinais clínicos com BVDV-1 e 2, esta espécie é geneticamente e antigenicamente distinta (BAUERMANN; FLORES & RIDPATH, 2012). Infecções naturais e experimentais geram a mesma sintomatologia, com cepas ncp e cp identificadas e quadro semelhante à doença das mucosas e geração de animais PI (DECARO *et al*, 2011; DECARO *et al*, 2012; BIANCHI *et al*, 2011; WEBER *et al*, 2016). A prevalência em rebanhos brasileiros do HoBiPev é de 12,4% a 19,9%, dependendo da região do país, porém em estudo de prevalência na região Nordeste este foi o pestivírus mais detectado (SILVEIRA *et al*, 2018). Isso mostra que, apesar de ser o pestivírus de bovino mais recente em termos de descrição, já circula nos rebanhos brasileiros e esta frequência significativa tem chamado a atenção para a indústria vacinal, já que as vacinas contra BVDV disponíveis no mercado não possuem HoPiPev em sua formulação (BAUERMANN; FLORES; RIDPATH, 2012).

3 CAPÍTULOS

O objetivo geral da tese foi caracterizar pestivírus em bovinos e suínos, especialmente pestivírus de ruminantes (*Pestivirus A*, *B* e *H*) e o recém descrito *Pestivirus K*. Além de caracterização epidemiológica e genética, o uso de PCA como método estatístico na caracterização antigênica de isolados e cepas vacinais de diferentes subgenótipos de BVDV-1 e 2 foi descrito em estudo realizado no Laboratório de BVDV no NADC (*National Animal Disease Center*, USDA-Ames, Iowa, EUA) sob co-orientação da Dra. Shollie Falkenberg.

3.1 Capítulo 1: Detecção de pestivírus e anticorpos contra *Pestivirus A* e *B* em suínos de subsistência do Rio Grande do Sul

O presente manuscrito intitulado "*Survey for ruminant pestiviruses in backyard pig from Southern Brazil*" foi aceito no periódico *Journal Of Veterinary Diagnostic Investigation* e publicado em 2020 no volume 32(1), páginas 136–141, sob DOI: 10.1177/1040638719896303. O artigo é de acesso aberto e será apresentado a seguir tal qual foi publicado.

Brief Communication

Journal of Veterinary Diagnostic Investigation
2020, Vol. 32(1) 136–141

DOI: 10.1177/1040638719896303
jvdi.sagepub.com

Survey for pestiviruses in backyard pigs in southern Brazil

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Abstract. The *Pestivirus* genus comprises species that affect animal health and productivity worldwide. Members of the *Suidae* family are hosts for classical swine fever virus (CSFV), an important pathogen tracked by the World Organization for Animal Health (OIE). However, swine are also susceptible to other pestivirus species that can result in disease or compromise CSFV detection. We searched for pestivirus infection in swine sera collected from 320 backyard pig herds in southern Brazil. We used reverse-transcription PCR primers for Bungowannah virus; atypical porcine pestivirus (APPV); and a panpestivirus pair that detects bovine viral diarrhea virus (BVDV)-1, -2, and HoBi-like pestivirus (HoBiPeV), border disease virus (BDV), and CSFV. Two samples were positive using the panpestivirus primer pair and were classified as BVDV-1d and -2a, respectively. Serum samples were tested for virus neutralization against BVDV-1a, -1b, and -2 strains, resulting in 28 (4.4%) positive samples. Of those, 16 samples had the highest titers against BVDV-1a (2), BVDV-1b (5), and BVDV-2 (9). Our results indicate that Bungowannah virus, APPV, CSFV, BDV, and HoBiPeV have not been circulating in these specific backyard swine populations. However, ruminant pestiviruses were detected and must be considered in future pestivirus control programs conducted in Brazil.

Key words: antibody; Brazil; BVDV; detection; pestivirus; swine.

The *Pestivirus* genus (family *Flaviviridae*) was previously composed of 4 recognized species named *Bovine viral diarrhea virus 1* (BVDV-1), BVDV-2, *Border disease virus* (BDV), and *Classical swine fever virus* (CSFV). A new taxonomy and species nomenclature for the genus was adopted in 2018. Now, BVDV-1 has been named *Pestivirus A*, BVDV-2 is *Pestivirus B*, CSFV is *Pestivirus C*, and BDV is *Pestivirus D*. As previously described, 7 putative pestivirus species—from *Pestivirus E* to *Pestivirus K*—were recognized as official members of the genus, including Bungowannah virus and atypical porcine pestivirus (APPV), which are known to infect and cause disease in pigs.[15]

Pestiviruses have positive single-stranded RNA genomes of ~12.3 kb, with a single open reading frame (ORF) translated into 12 viral polypeptides flanked by untranslated regions (UTRs) at the 5′- and 3′-ends. Conserved genomic regions, especially 5′-UTR, have been used for genotyping pestivirus species and variants through phylogenetic analysis, which has resulted in at least 21 subtypes for BVDV-1 (1a–1u) and 3 for BVDV-2 (2a–2c).[3]

The pestivirus species were initially named according to their host, with pigs as hosts for CSFV, sheep for BDV, and cattle for BVDV-1 and -2, although alternative hosts have been described over time. CSFV is considered one of the most important swine viral pathogens, which has led to the official eradication of this disease in many countries. BVDV has spread worldwide and is considered an important pathogen for the cattle population, having economic impacts, especially as a result of reproductive losses and the consequent birth of persistently infected calves. Given this fact, some countries have included BVDV in official eradication programs in cattle.

Some pestiviruses, such as BVDV, can infect unusual hosts sporadically; BVDV has been found in several members of the order Artiodactyla, such as sheep, goats, deer, bison, swine, and wild boars.[15] BVDV infection in swine can be asymptomatic, which allows propagation of the virus in the herd. In some cases, BVDV infection can cause signs similar to those of CSFV infection; antigenic and structural similarities among pestiviruses can also lead to misleading detection of CSFV.[14]

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Other pestiviruses, such as Bungowannah virus and APPV, are apparently restricted to infecting only *Suidae* members.[7,8] Bungowannah virus was described and detected in only Australia, causing myocarditis in piglets[8]; APPV was recently associated with congenital tremors in piglets worldwide, including in Brazil.[11]

To assess information about several pestivirus species infections in pigs, we tested sera from backyard pig herds for pestivirus genomes and BVDV antibodies. These samples composed the target population for the CSFV Surveillance Program given that backyard pig farms typically lack biosecurity measures. Samples were analyzed by reverse-transcription PCR (RT-PCR) for CSFV, APPV, Bungowannah virus, and ruminant pestiviruses (BVDV-1, BVDV-2, HoBiPeV, and BDV). Additionally, we used virus neutralization (VN) to quantify the amount of antibody reaction to such infections.

Brazil is considered largely as a CSFV-free zone by the World Organization for Animal Health (OIE), resulting from established eradication and control programs. Despite the large volume of pork meat production in southern Brazil, more than half of the swine farms in Rio Grande do Sul State are backyard herds, characterized as having a small number of animals that are slaughtered and consumed mostly on the farm. This type of herd is characterized by lack of biosecurity measures, and hence these animals were an important target population for the CSFV Surveillance Program. The 741 swine sera from backyard pigs used in our study were collected in 2014 during CSFV surveillance tests in the state of Rio Grande do Sul, southern Brazil. The Official Veterinary Office (Secretaria Estadual de Agricultura, Pecuária e Irrigação, SEAPI-RS) randomly collected blood samples from 320 backyard farms selected from a source population while setting predefined risk criteria for CSFV, such as proximity to waste dumps and the practice of feeding food waste to pigs, to increase the effectiveness of the surveillance system. The samples were collected in 202 counties and comprised sera from male and female animals 6–72 mo old. All samples tested negative in CSFV serology assays performed at the official laboratory in Brazil.

Total RNA was isolated (TRIzol LS reagent; Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Complementary DNA was synthesized (GoScript reverse transcriptase; Promega, Madison, WI), and PCR was performed (GoTaq; Promega). For ruminant pestivirus and CSFV detection, PCR was carried out using primers named PanPesti F and PanPesti R, which amplified a 118-bp fragment of the conserved 5′-UTR genomic region of ruminant pestivirus species.[18] Additionally, the samples were assayed for Bungowannah virus and APPV using the primer pairs UTR_Left and UTR_Right[1] and Pesti-11453-F and PestiV NS5-R,[2] respectively. PCR products were subjected to electrophoresis in 2% agarose gels and visualized under ultraviolet illumination.

Samples that were found to be positive in the ruminant pestivirus RT-PCR assay were further tested with the primers 324 and 326,[17] resulting in a longer amplification product of 288 bp that was sequenced and used in phylogenetic analysis. Amplification products were purified (PureLink PCR purification kit; Thermo Fisher Scientific). Both DNA strands were sequenced (ABI PRISM 3100 genetic analyzer; BigDye Terminator v.3.1 cycle sequencing kit; Thermo Fisher Scientific).

The sequences were assembled (Geneious v.9.1.5; Biomatters Limited, Auckland, New Zealand) and analyzed (nucleotide BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi). For phylogenetic analysis, 27 reference sequences were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/genbank/), and multiple alignments were performed (ClustalW; available in MEGA v.6.06, https://www.megasoftware.net/). A phylogenetic tree based on the 5′-UTR nucleotide sequences was constructed with MEGA 6 using the neighbor-joining method and the Kimura-2 substitution model.

The serum samples were also tested for the presence of antibodies by VN, using strains of the pestivirus species that were detected by RT-PCR and sequencing. Pestivirus strains used in the comparative VN assay included cytopathic (cp) BVDV-1a Oregon C24V, BVDV-1b Osloss, and BVDV-2a VS-253. BVDV strains were propagated and titered in Madin–Darby bovine kidney (MDBK) cells. MDBK cells used during the study were grown in Dulbecco modified eagle medium (DMEM; Thermo Fisher Scientific) supplemented with antibiotics at a final concentration of 100 units/mL penicillin, 100 µg/mL streptomycin, and 5% equine serum. MDBK cells were previously found to be free of pestivirus RNA by RT-PCR.

Serum aliquots were placed in a water bath at 60°C for 60 min for complement system deactivation. A first screening by VN was performed against the reference strains (BVDV-1a, -1b, and -2), as described in the OIE Manual of Standards for Diagnostic Tests and Vaccines.[20] The samples were initially tested in triplicate at a dilution of 1:8 given the rare infection of swine by ruminant pestiviruses and the low titers produced against these infections. Seropositive samples at a 1:8 dilution were retested using 2-fold serial dilutions in DMEM from 1:8 to 1:1,024; 100 tissue culture infectious doses ($TCID_{50}$) of virus were added to each well. Plates containing serum dilutions and virus were incubated for 100 min at 37°C in 5% CO_2. Following incubation, a 50 µL/well cell suspension containing 10^5 cells/mL was added. The plates were incubated for another 96 h at 37°C in 5% CO_2. Sera of animals that previously tested negative by VN for BVDV-1 and -2 were used as negative controls. Samples that neutralized the virus at a dilution of 1:1,024 were further tested in dilutions from 1:512 to 1:32,768. Plates were observed using an optical microscope, searching for characteristic cytopathic effects.

In the survey of pestivirus genomes through RT-PCR, we tested all 741 samples. No sample was found to be positive

for Bungowannah virus or APPV using specific RT-PCRs. Two serum samples (0.3%) were positive in the ruminant pestivirus RT-PCR with either the PanPesti[18] and 324/32634[17] primers. These samples were named SUI 1 and SUI 2, and the 5′-UTR partial sequences obtained from DNA sequencing of the amplification products using the 324/326 primers were deposited in GenBank as accessions MK334041 and MK334042, respectively.

In a nucleotide BLAST search, the SUI 1 nucleotide sequence was found to have the highest similarity (~98%) to isolates LV/C3P/13 (KP715116) and LF80/11 (JX122862), both BVDV-1d samples from Brazil. In the phylogenetic tree produced based on 5′-UTR nucleotide sequences, the strain SUI 1 and those cited above with the highest nucleotide identity were clustered with the reference strain (BJ1308) for BVDV-1d, along with other Brazilian strains that were classified as the same subtype. These nucleotide sequences were grouped into a BVDV-1d strain branch within the phylogenetic tree with a bootstrap value of 100% (Fig. 1).

For the SUI 2 sample, the nucleotide sequence was found to be 94% identical to that of BVDV-2 strains from Argentina (isolates 106 and 76/08; GenBank accessions JX848364 and MF120586, respectively). Strain SUI 2 and other strains previously described as BVDV-2a as well as the reference strain for the subtype known as New York93 were grouped to form a branch composed of BVDV-2a strains with a bootstrap of 89% (Fig. 1).

The 741 swine sera tested by VN resulted in 102 (13.7%) samples with toxic effects on the cell culture that were excluded from the VN results. From the 639 nontoxic serum samples, antibodies against the ruminant pestiviruses were detected in 27 samples (4.2%) from 18 households (5.6%).

Despite great genetic and antigenic diversity within the pestiviruses, there is serologic cross-reaction over an extended range between species and even subtypes. To accurately detect antibody titers, OIE establishes a difference in titer by >4-fold to define a strain for which the antibodies have a specific neutralization reaction greater than the cross-neutralization reaction.[20] Comparative neutralization of the 27 VN-positive samples from among the 639 tested samples with the BVDV-1a, -1b, and -2b strains revealed the following: 3 of 27 (11.1%) had significantly higher titers against BVDV-1a than against the other subtypes, 9 of 27 (33.3%) had higher titers against BVDV-1b, and 9 of 27 (33.3%) had higher titers against BVDV-2 (Fig. 2). Six samples had a higher titer against 1 of the strains than against the other 2 strains (but <4-fold increase). Of these 6 samples, 2 had higher titers against BVDV-1a (7.4%), 4 against BVDV-1b (14.8%), and 1 against BVDV-2 (3.7%). One sample had the same titer for BVDV-1a and BVDV-2 (3.7%; Supplementary Table 1).

Studies involving the detection of pestiviruses other than CSFV in pigs are scarce. We tested 741 backyard swine sera for pestiviruses using RT-PCR and sequencing, followed by VN. We found no evidence of Bungowannah virus and APPV genomes. Apparently, Bungowannah virus infection is restricted to Australia.[8] Bungowannah virus was not observed in one study in the United States,[1] in accord with our results. We also searched for APPV without success, although it has been detected previously in southern Brazil.[11] We speculate that the age of the sampled animals contributed to the negative results, given that APPV is associated with congenital tremors in newborn piglets.[7]

We found 2 (0.3%) pig samples to be positive for a ruminant pestivirus genome. The occurrence of positive samples in our study was lower than in previous studies given differences in sampling. Our study was conducted by random sampling representative of the backyard swine population in the State, in which clinical appearance data were not collected, whereas most surveys for these pestiviruses in swine were performed in clinically affected animals, which may have overestimated the data. A study with 511 samples from sick pigs in China detected 26.8% BVDV RT-PCR positivity,[4] suggesting its widespread circulation in the swine herds in that country. Although previously detected in a wild boar from southern Brazil,[19] a search for the BVDV genome in domestic pigs through RT-PCR has not been conducted in South America, which suggests a lack of studies on pestiviruses other than CSFV in *Suidae* hosts in this region.

In phylogenetic analysis, the samples that we sequenced were classified as BVDV-1d and BVDV-2a. BVDV-1d is frequently reported in cattle in Brazil,[18] which could raise suspicions about the cattle origin of the SUI 1 strain given that backyard pigs usually come in contact or are raised with other animal species. We detected one BVDV-2a sample, although 2a strains are rarely reported in Brazil,[5] but BVDV-2b is frequently found in cattle in southern Brazil.[18] BVDV-2a was reported in cattle from Argentina,[12] and the sequence that we obtained had a high degree of identity with those samples.

The BVDV seroprevalence observed herein was 4.2% at the individual level and 5.6% at the herd level, similar to that in other studies performed in commercial farm pigs in Brazil, ranging from 2.4–4.7% at the animal level.[6] In Europe, commercial farm pigs had a lower BVDV seroprevalence at the animal level using VN, especially in Norway (2.2%) and The Netherlands (2.5%).[10] Higher BVDV seroprevalences in pigs were found in Asian countries, such as in South Korea,[9] with a seroprevalence of 5.3%. Regarding Chinese swine herds,[16] the epidemic status of BVDV infection was a serious issue, linking low seroprevalence rates to high biosecurity in farms and abolished mixed farming practices.

BVDV-infected cattle are speculated to be the main source of BVDV infection in swine given increased seroprevalence in pigs when cattle are reared on the same farm[10]; close contact with cattle farms or cattle in the same facilities were identified as risk factors for BVDV infection in swine. Rio Grande do Sul, southern Brazil, contains >13 million cattle, according to the State Veterinary Office (SEAPA-RS), and serologic studies performed on cattle from this region found

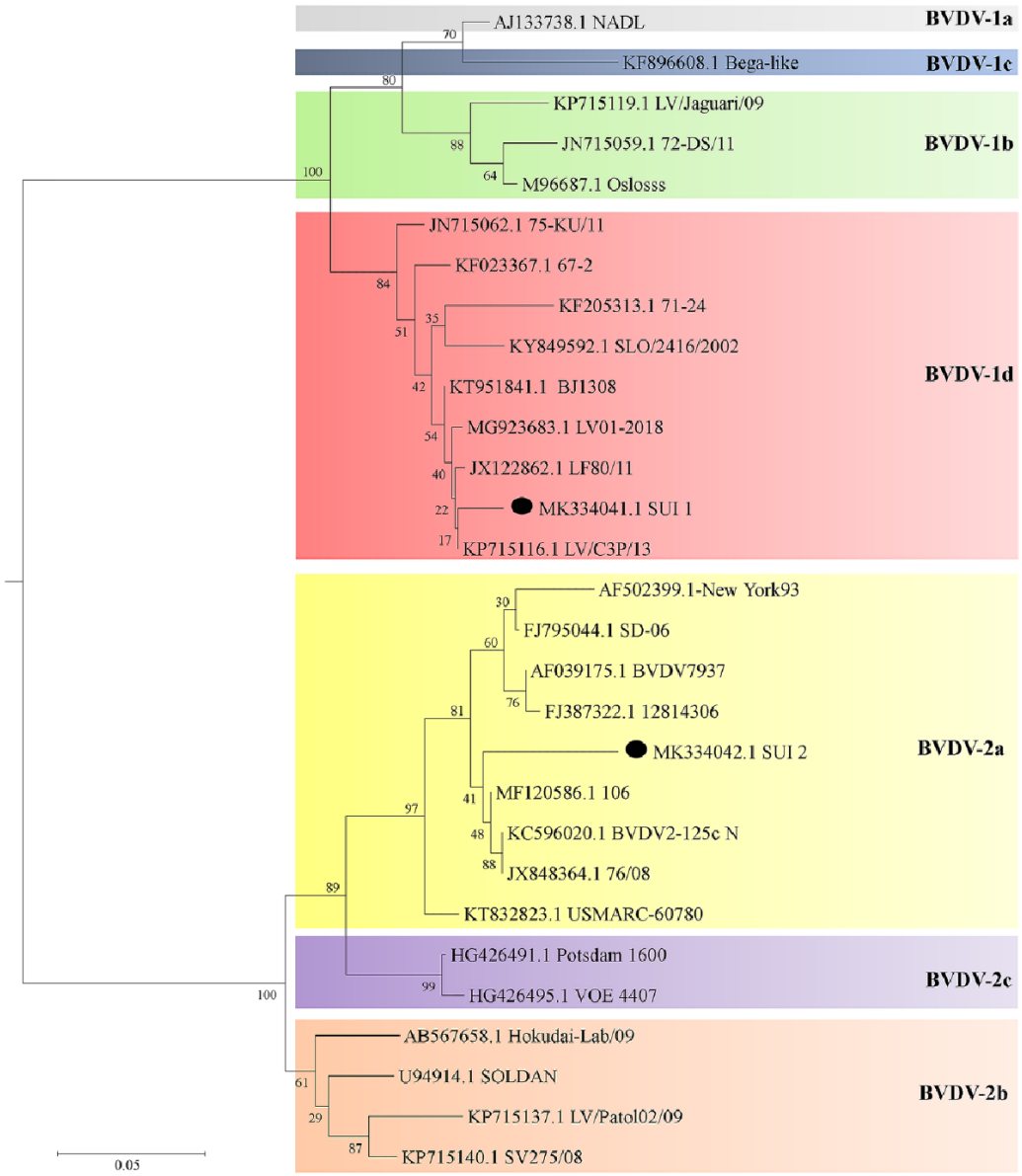

Figure 1. Phylogenetic tree based on the 5′-UTR nucleotide sequence. Neighbor-joining linear tree based on the partial 5′-UTR nucleotide sequence of representative strains for some BVDV-1 and BVDV-2 subtypes. For phylogenetic tree construction, representative sequences were used from GenBank. The samples from our study, SUI 1 and SUI 2, are marked with a black dot. Kimura-2 model with gamma-distributed rate variation and 1,000-bootstrap repetitions were used as parameters to build the tree.

BVDV seroprevalences of 56–68%.[13] Backyard or noncommercial farms are frequently found to have ≥2 livestock species reared in the same property, which was true in the farms in our study as 96.6% of the farms were identified as raising pigs and cattle. Among the 18 swine herds with BVDV-positive serologic results, in 10 of them, the pigs and cattle had nose-to-nose contact, whereas only one farm did not rear any ruminant species. Although our aim was strictly to determine the frequency of pestivirus genomes and antibodies, sampling was performed according to predefined risk criteria for CSFV infection. Hence, it is not possible to define the risk factors related to our study.

Epidemiologic investigation of pestiviruses in heterologous host species is important to understand their impact on

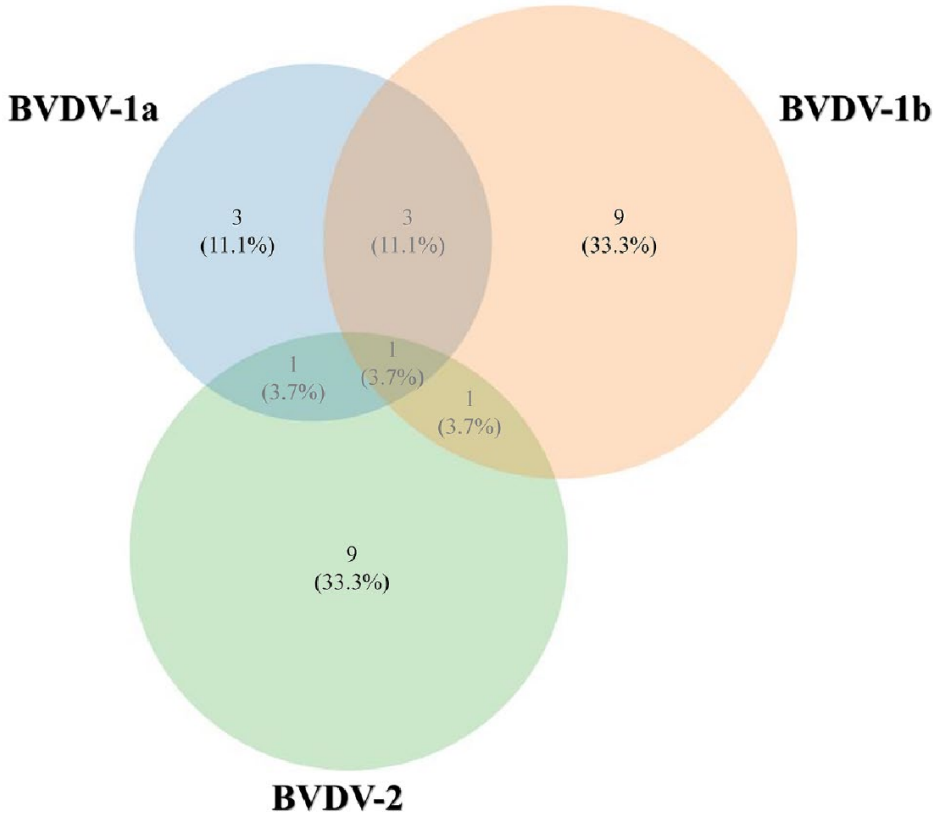

Figure 2. Virus neutralization (VN) results. Antibody titers were determined in swine serum samples tested in the VN assay against BVDV-1a, -1b, and -2 strains. During cross-neutralization between 2 or 3 reference strains, highest titers (>4-fold) are considered as a significant neutralization reaction against the particular strain (outer circles); for cross-neutralization results with highest titer against 1 strain (but <4-fold), the strain for which the neutralization reaction occurred could not be determined (inner circles).

health, potential virus reservoirs in nature, and laboratory testing. Thus, our results reinforce the notion that Bungowannah virus, APPV, CSFV, BDV, and HoBiPeV have not been circulating in this Brazilian backyard swine population. However, there is a need to generate epidemiologic studies for pestiviruses, especially BVDV, in heterologous host species for future control programs.

Declaration of confliction interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

Our study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior–Brasil (CAPES)–Finance Code 001, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and PROPESQ/UFRGS.

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Supplementary material

Supplementary material for this article is available online.

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JVDI: Supplementary material

Mosena ACS, et al. Survey for pestiviruses in backyard pigs in southern Brazil

Supplementary Table 1. Antibody titers determined in the virus neutralization test against BVDV-1a, -1b, and -2 in swine serum tested in our study.

Sample identification	Anti–BVDV-1a titer	Anti–BVDV-1b titer	Anti–BVDV-2 titer
052/03	512	<8	<8
052/04	256	<8	<8
122/03	16	<8	<8
252/09	16	8	<8
062/01	<8	256	<8
259/04	<8	256	<8
076/01	<8	256	16
135/03	<8	128	8
135/01	64	512	32
002/08	<8	32	<8
051/03	<8	32	<8
232/03	<8	32	<8
180/08	<8	16	<8
135/05	<8	32	16
252/08	1,024	2,048	<8
217/06	32	64	16
008/01	<8	16	512
153/02	16	8	1,024
181/01	8	64	1,024
181/02	8	16	1,024
222/04	16	16	1,024
154/01	<8	<8	128
154/05	<8	<8	32
154/02	<8	64	512
154/03	16	16	128
233/06	256	<8	1,024
180/05	128	64	128

The antibody titers are expressed as the reciprocal value of the maximum dilution of the sample in which neutralizing antibodies were detected (<8 means that these samples tested negative in the start dilution of the assay, which was 1:8). When cross-neutralization occurs between 2 or 3 reference strains, higher titers (>4-fold) are shaded in dark gray; high titer (but <4-fold) are shaded in light gray.

3.2 Capítulo 2: Detecção do *Pestivirus K* no Brasil e sua diversidade genética

O experimento com o objetivo de detectar o *Pestivirus K* pela primeira vez no Brasil já foi concluído e o artigo científico "*Presence of atypical porcine pestivirus (APPV) in Brazilian pigs*" foi aceito e publicado em 8 de novembro de 2017 no periódico *Transboundary and Emerging Diseases*. O artigo é de acesso restrito e por razões de direitos autorais não será incluído neste documento, podendo ser encontrado em https://doi.org/10.1111/tbed.12753. O resumo disponibilizado encontra-se abaixo:

Summary

Recently, a putative new pestivirus species, provisionally named as Atypical Porcine Pestivirus (APPV), was associated with the congenital tremor in piglets in North America and consequently in Europe and Asia. The present research aimed to describe the detection and characterization of APPV employing NS5B gene partial sequencing, gross pathology and histologic examination of piglets displaying congenital tremor from two different farms of Southern Brazil. No gross lesions were observed, and the histological findings revealed moderate vacuolization of the white matter of the cerebellum. RT-PCR followed by DNA sequencing and a phylogenetic analysis confirmed the presence of APPV in samples from the two farms, which the samples were distinct in nature. Phylogenetic reconstruction reinforced the high genetic variability within the APPVs previously reported. This is the first report of APPV in South America suggesting that this new group of viruses may be widespread in swine herds in other countries as it is in Brazil.

3.3 Capítulo 3: Uso da PCA na caracterização antigênica de cepas de BVDV-1 e 2

A caracterização antigênica de pestivírus é difícil devido à grande diversidade antigênica entre isolados e ao fato de haver neutralização cruzada em diferentes níveis entre espécies. O método tradicional para avaliar as relações antigênicas entre isolados é a soroneutralização (SN), porém os resultados podem ser de difícil interpretação quando múltiplos isolados são avaliados devido ao grande volume de dados e neutralização cruzada. O objetivo dos dois capítulos a seguir foi aplicar o método estatístico PCA para visualização e interpretação dos resultados de SN e relações antigênicas entre cepas utilizadas em vacinas comerciais e vários isolados de campo geneticamente diversos, além das relações antigênicas entre cepas pertencentes a um mesmo subgenótipo. O estudo foi realizado no *BVDV Laboratory* do NADC (*National Animal Disease Center*) em Ames, Iowa- EUA, durante o período de dezembro de 2018 a junho de 2019 sob co-orientaçao da Dra. Shollie Falkenberg. Dois artigos sobre este tema foram redigidos e publicados. O primeiro artigo descreve pela primeira vez o uso de PCA na visualização de relações antigênicas entre cepas vacinais e isolados de campo através de resultados de SN. Este método já foi utilizado para caracterização antigênica de vírus altamente diversos genética e antigenicamente como o vírus HIV. Este artigo foi publicado no periódico *Vaccine*, volume 38, número 36, páginas 5764-5772 em 10 de agosto de 2020, sob o título "*Multivariate analysis as a method to evaluate antigenic relationships between BVDV vaccine and field strains*". O artigo é de acesso restrito e, portanto, não será disponibilizado neste documento, podendo ser encontrado em https://doi.org/10.1016/j.vaccine.2020.07.010. O resumo disponibilizado encontra-se abaixo:

Abstract

Bovine viral diarrhea virus (BVDV) is comprised of two species, BVDV-1 and BVDV-2, but given the genetic diversity among pestiviruses, at least 21 subgenotypes are described for BVDV-1 and 4 for BVDV-2. Genetic characterization can be achieved through complete or partial sequencing and phylogeny, but antigenic characterization can be difficult to determine due to the antigenic diversity and cross-neutralization that exists among isolates. The traditional method for evaluating antigenic relationships between pestivirus isolates is the virus neutralization (VN) assay, but interpretation of the data to determine antigenic difference can be unclear. Data from this study utilized a multivariate analysis for visualization of VN results to analyze the antigenic relationships

between vaccine strains and multiple field isolates. Polyclonal sera were generated against 6 BVDV strains currently contained in vaccine formulations, and each serum was used in VN's to measure the neutralizing antibody titers against 15 BVDV field isolates characterized as prevalent and divergent subgenotypes in the USA. Principal component analysis (PCA) were performed on the VN assay datasets, and results were interpreted from PCA clustering within the PCA dendrogram and scatter plot. The results demonstrated clustering patterns among isolates suggestive of antigenic differences. While expected, the BVDV-1 and BVDV-2 isolates did not cluster together and had the greatest spatial distribution. In addition, other BVDV isolates had distinct spatial patterns suggesting antigenically divergent isolates. This analysis provides an alternative and more efficient means to analyze large VN datasets to visualize antigenic relationships between pestivirus isolates. This analysis could be beneficial for vaccine development and evaluation of efficacy, since most vaccines cannot fully protect animals from the broad range diversity of BVDV viruses.

3.4 Capítulo 4: Análise de relações antigênicas entre de cepas de BVDV geneticamente similares ou divergentes

Após a publicação aceita para validação do uso de PCA para interpretação de dados de relação antigênica entre isolados de BVDV, o segundo artigo visou analisar a relação antigênica entre cepas vacinais e isolados divergentes classificados em diferentes subgenótipos. O objetivo foi verificar se há relação entre classificação genética e relação antigênica, ou seja, se isolados próximos geneticamente (pertencentes ao mesmo subgenótipo) tem uma maior proximidade antigênica. Este método pode auxiliar no melhoramento de vacinas contra BVDV, já que atualmente as cepas utilizadas para produção das vacinas são pertencentes somente aos subgenótipos BVDV-1a e BVDV-2a. Este artigo foi publicado no periódico *Journal of Virological Methods*, volume 299, número 36, em 25 de outubro de 2021, sob o título "*Use of multivariate analysis to evaluate antigenic relationships between US BVDV vaccine strains and non-US genetically divergent isolates*". O artigo é de acesso aberto e será disponibilizado a seguir no formato que foi aceito para publicação.

Journal of Virological Methods 299 (2022) 114328

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Use of multivariate analysis to evaluate antigenic relationships between US BVDV vaccine strains and non-US genetically divergent isolates

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ARTICLE INFO

Keywords:
Bovine viral diarrhea virus
Antigenic characterization
Cross neutralization
Principal component analysis
Virus neutralization

ABSTRACT

Bovine viral diarrhea virus (BVDV) comprises two species, BVDV-1 and BVDV-2. But given the genetic diversity among pestiviruses, at least 22 subgenotypes are described for BVDV-1 and 3-4 for BVDV-2. Genetic characterization is generally accomplished through complete or partial sequencing and phylogeny, but it is not a reliable method to define antigenic relationships. The traditional method for evaluating antigenic relationships between pestivirus isolates is the virus neutralization (VN) assay, but interpretation of the data to define antigenic relatedness can be difficult to discern for BVDV isolates within the same BVDV species. Data from this study utilized a multivariate analysis for visualization of VN results to analyze the antigenic relationships between US vaccine strains and field isolates from Switzerland, Italy, Brazil, and the UK. Polyclonal sera were generated against six BVDV strains currently contained in vaccine formulations, and each serum was used in VNs to measure the titers against seven vaccine strains (including the six homologous strains) and 23 BVDV field isolates. Principal component analysis (PCA) was performed using VN titers, and results were interpreted from PCA clustering within the PCA dendrogram and scatter plot. The results demonstrated clustering patterns among various isolates suggesting antigenic relatedness. As expected, the BVDV-1 and BVDV-2 isolates did not cluster together and had the greatest spatial distribution. Notably, a number of clusters representing antigenically related BVDV-1 subgroups contain isolates of different subgenotypes. The multivariate analysis may be a method to better characterize antigenic relationships among BVDV isolates that belong to the same BVDV species and do not have distinct antigenic differences. This might be an invaluable tool to ameliorate the composition of current vaccines, which might well be important for the success of any BVDV control program that includes vaccination in its scheme.

1. Introduction

Three out of 11 currently accepted viral species from the genus Pestivirus, within the family Flaviviridae, exist in most cattle-producing countries worldwide (Yeşilbağ et al., 2017). The BVDV-1, BVDV-2 and HoBi-like pestivirus (HoBiPeV) species represent important economic impact for the cattle industry, mainly due to reproductive losses on all pregnancy stages and development of persistently infected (PI) calves upon in utero infection. Such PI animals mount no immune response against the infecting virus and constantly secrete high amounts of infectious virus particles and maintain the infection cycle within the herd (Richter et al., 2017; Pinior et al., 2019; Rodning et al., 2012; Basqueira et al., 2020).

Pestiviruses can be classified into two biotypes, noncytopathic (ncp)

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https://doi.org/10.1016/j.jviromet.2021.114328
Received 30 June 2021; Received in revised form 27 September 2021; Accepted 8 October 2021
Available online 25 October 2021

and cytopathic (cp), according to their activity in cell culture, where cp viruses kill infected cells and ncp viruses do not. In addition, pestivirus species (Smith et al., 2017) possess a high genetic diversity due to several mechanisms, such as high rate of mutations during replication, a feature common to RNA viruses (Becher and Tautz, 2011; Jones and Weber, 2004). The broad genetic diversity within species, especially BVDV-1 and 2, led to the necessity of a further classification of isolates into subgenotypes. Based on phylogenetic analysis of genomic regions, BVDV-1 can be currently classified into at least 22 subgenotypes (1a to 1 u) and BVDV-2 into 3–4 subgenotypes (2a to 2c, and potentially 2d). The most prevalent subgenotypes worldwide are 1a, 1b and 2a, and most of the less prevalent subgenotypes are restricted to European, Asian and South American countries (Yeşilbağ et al., 2017; Silveira et al., 2017).

Several in vitro studies have attempted to link genetic to antigenic characterization, in an attempt to establish if isolates of the same subgenotype would present similar antigenic characteristics, but divergent results were found (Minami et al., 2011; Ridpath et al., 2010; Pecora et al., 2014). Antigenic differences not only between species (Bauermann et al., 2012; Ridpath et al., 1994), but also between subgenotypes and even between isolates of the same subgenotype were described (Minami et al., 2011; Ridpath et al., 2010; Ridpath, 2003; Bachofen et al., 2008). However, the practical consequences of the antigenic diversity between subgenotypes are still not know (Ridpath et al., 1994; Bachofen et al., 2008; Ridpath, 2005; Sozzi et al., 2020a). While the clear difference in levels of cross-neutralization between BVDV-1, 2 and HoBiPeV neutralizing antibodies have been widely described, identifying antigenically divergent or similar isolates within the same species and subgenotype by the use of VN assay can be challenging. The broad diversity of isolates, varying levels of cross-reactivity between multiple isolates and interpretation of a great amount of data are barriers to a clear interpretation of VN assay results. Typically, serological relatedness is expressed as coefficient of antigenic similarity (R) between strains using VN titers. There are a limited number of studies that have evaluated the serological relationships that exist among isolates from different BVDV subgenotypes (Minami et al., 2011; Ridpath et al., 2010; Bachofen et al., 2008; Becher et al., 2003; Couvreur et al., 2002) and these studies have aimed at evaluating the antigenic variability not only between BVDV species but within BVDV subgenotypes. Recently, a multivariate analysis called Principal Component Analysis (PCA) was for the first time applied to BVDV antigenic characterization (Mosena et al., 2020), and antigenic clusters and differences could be identified. This contrasts with previous reports (Couvreur et al., 2002) where BVDV-1 isolates were homogeneous since BVDV-1 isolates could not be subdivided into smaller serotypes based on neutralization patterns and R value calculations. While smaller serological subgroups could not be determined, some of the VN titers among BVDV-1 strains and antisera were rather low (>20) and these VN titers were as low as the VN titers observed between BVDV-1 and BVDV-2 strains and their corresponding antisera (Couvreur et al., 2002).

Given that most current commercially available BVDV vaccines contain BVDV-1a and/or 2a strains, there is concern about the level of cross protection that can be attained against other BVDV species and subgenotypes. Although, studies have demonstrated that BVDV-1a vaccinated cattle were proven to be fully or in part protected against BVDV-1b infection (Brock and Cortese, 2001; Xue et al., 2010). While these vaccination/challenge studies support the thinking that current vaccines confer protection against other subgenotypes, PI surveillance studies would suggest a lack of protection as the most frequent subgenotype detected in BVDV PI calves in US is BVDV-1b (Fulton et al., 2009; Workman et al., 2016). In addition, recently other genetically diverse isolates (1c, 1i, 2b and 2c subgenotypes) were recently identified for the first time (Neill et al., 2019a; Neill et al., 2019b) in the US, increasing the genetic diversity of BVDV in this country, raising more concerns about the level of cross protection associated with current vaccines. Given the increased genetic diversity within the US and genetic diversity that is observed globally, a better understanding is necessary to determine the level of protection that can be attained with current BVDV vaccines against other pestivirus species and subgenotypes. This information is imperative with regard to development of new vaccines or vaccination strategies that can elicit great cross protection against genetically and antigenically diverse isolates. Historical data would suggest that the breadth of the genetic and antigenic diversity will continue to increase. Therefore, a better understanding of antigenic relationships is required to inform potential intervention strategies such as vaccination as part of control programs moving forward.

The aim of this study was to use the PCA approach for interpretation of VN data from genetically distinct BVDV-1 and 2 field isolates from several subgenotypes and distinct geographical origins, and to describe the cross neutralizing patterns of serum raised against 1a and 2a strains used in current vaccine formulations in neutralizing these genetically divergent BVDV field isolates.

2. Material and methods

2.1. Viruses

Twenty-three field isolates, twenty-two field isolates representing BVDV-1 subgenotypes (1a, d, e, f, g, h, i and k) along with one HoBiPeV isolate (HoBi/D32), and seven US based vaccine strains (3 BVDV-1a and 4 BVDV-2a) were selected for this study (Table 1). The field isolates were selected in an attempt to represent a broad genetic diversity within the BVDV-1 species in contrast to strains used in most BVDV vaccines, which are 1a and 2a strains of US origin. In the USA, the majority of field isolates are classified as 1a, 1b and BVDV-2a, with few descriptions of BVDV-2b and recent descriptions of BVDV-1c, BVDV-1i and BVDV-2c subgenotypes (Neill et al., 2019a; Neill et al., 2019b). Non-US isolates provided greater genetic diversity, as selection of non-US field samples for antigenic characterization included 22 cp and ncp BVDV-1 field isolates from Italy (1e, 1f, 1g and 1k subgenotypes), Switzerland (1e, 1h and 1k), United Kingdom (1a, 1d, 1e and 1i), and one HoBi-like pestivirus isolate from Brazil (Table 1). The seven BVDV vaccine strains (3 BVDV-1a and 4 BVDV-2a) chosen for the current study are routinely used in BVDV modified-live vaccine formulations and also in killed vaccines, although a couple of strains contained in some killed vaccines were not represented in this study. Six out of the seven vaccine strains were used to generate antiserum that was subsequently used in VN assays.

The seven vaccine strains and 23 field isolates were propagated in Madin-Darby bovine kidney cells (MDBK) the laboratory according to standard protocol. Each strains and isolate aliquot was used to inoculated a flask of MDBK cells with 75 % confluence layer for 1 h, followed by cell layer wash to remove the inoculum and addition of fresh Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % of fetal bovine serum (FBS) for incubation at 37 °C in a 5% CO2 atmosphere for 4–5 days. Cytopathic effect was observed in MDBK cells inoculated with cp strains and field isolates. Viruses were titered through serial 10-fold dilutions with replicates of five wells per dilution in bovine turbinate (BT) cells. Cytopathic effect was observed in wells inoculated with cp strains and field isolates, and for ncp isolates the cell layer was fixed and stained according to standard immunoperoxidase staining protocol using the E2 protein-specific monoclonal antibody N2 and horseradish peroxidase-conjugated protein G (Bolin and Ridpath, 1995). Titration 96-well plates were briefly rinsed with distilled water and cells fixed in 60/40 PBS-BSA/acetone at room temperature and dried for 1 h at 37C°. Cells were then incubated with E2 protein-specific monoclonal antibody N2 diluted in PBST for 1 h. After application of this primary antibody, wells were rinsed twice with PBS wash buffer in between steps. Cell layers were then incubated with 1:60 dilution of goat antiserum to mouse gamma globulin (IgG) (Cappel, catalog number 55455) in PBSTN binding buffer for 1 h. Cells were then incubated with rec-Protein G-Peroxidase Conjugate (ZYMED, catalog number 10-1223) diluted in

Table 1
Strains and isolates used in this study with referred ID, genetic classification, country of origin and GenBank accession number.

	Country	Species/ Subgenotype	Isolate ID / Virus ID	Complete Genome Genbank Acession no.	Biotype
Field isolates	Italy	1e	D7219_1e / MA/101/05	MW054940	ncp
		1f	D14688 _1f / LA/230/14	MW054933	cp
			D18648_1f / LA/87,88,90/05	MW054934	ncp
		1 g	D23284_1 g / UM/111/06	MW054936	ncp
		1k	D18892_1k / TO/197/11	MW054935	ncp
			D59460_1k / SA/159/09	MW054937	ncp
	Swiss	1e	CH_Maria_1e	MW655625	ncp
			S03_1175_1e	MW655631	ncp
			Carlito_1e	KP313732	ncp
			R2000_95_1e	MW655627	ncp
		1 h	CH_04_01b_1 h	MW655625	ncp
			R3572/90_1 h	MW655629	ncp
			SM09_20_1 h	MW655632	ncp
		1k	CH_Suwa_1k	AY894998	ncp
			R3230/95_1k	MW655628	ncp
			R5013/96_1k	MW655630	ncp
	UK	1a	62_2_1a	MW250798	ncp
			63_1_1a	MW250799	ncp
		1d	67_1_1d	MW250800	ncp
		1e	68_1_1e	MW250802	ncp
		1i	58_2_1i	MW250797	ncp
			69_1_1i	MW250803	ncp
	Brazil	HoBiPeV	HoBi/D32	AB871953	ncp
Vaccine strains	USA	1a	C24V _1a	AF091605	cp
			Singer _1a	DQ088995	cp
			NADL _1a	M31182	cp
		2a	125c_2a	MH806434	cp
			296c_2a	MH806436	cp
			5912c_2a	MH231129	cp
	Canada	2a	53637c_2a	MH231127	cp

PBSTN binding buffer for 1 h at room temperature. Staining was developed with AEC substrate prepared in acetate buffer with hydrogen peroxide until red color appeared and wells were rinse with tap water. All cell lines, medium and fetal bovine serum were tested free of pestivirus antigen and antibodies (Ridpath et al., 1994).

2.2. Genetic characterization

All isolates and vaccine strains used in this study are available in GenBank and specific details can be found in Table 1. Isolates previously described but that had no 5′UTR or E2 sequence available at GenBank (all European field isolates with exception of Carlito_1e (Stalder et al., 2015) and CH_Suwa_1k (Bachofen et al., 2008)) had the whole genome sequenced according to previously described protocol (Neill et al., 2019b) and submitted to Genbank (Table 1). Simultaneous multiple whole genomes sequencing was obtained through cDNA synthesis of viral RNA with primers composed of 20 bases of known sequence with 8 random bases at the 3′-end, so that resultant cDNAs could be barcode identified and amplified by primer-specific PCR and sequenced on the Ion Torrent PGM platform. Virus genomes were assembled by both de novo and reference-assisted assembly methods. Nevertheless, phylogenetic analysis for subgenotype classification was performed using the 5′UTR sequences of all isolates and strains, as 5′UTR phylogeny is commonly accepted and used for subgenotype classification (Bauermann et al. (2012); Ridpath et al. (1994)). All viruses were confirmed to be classified as same subgenotype as literature. A more detailed phylogenetic analysis of the E2 amino acid sequence of the same strains and isolates was obtained so the genetic relationship between viruses could be compared to the antigenic characterization. A neutralizing humoral immune response in pestiviruses infections is mostly directed against the highly immunogenic E2 protein. To determine amino acid differences in the major neutralizing glycoprotein E2 (Fulton et al., 1995; Deregt et al., 1998), MEGA6 software tools Clustal W alignment and UPGMA method were used to obtain the phylogenetic tree with branch support estimated using 1000 bootstrap replicates and Poisson correction method used to calculate evolutionary distances.

2.3. Antisera

From the seven selected vaccine strains, specific antiserum was generated against six strains (C24V_1a, Singer_1a, NADL_1a, 125_2a, 296_2a and 53637_2a). The same antisera were used in a previous study where PCA was applied in VN assay results interpretation (Mosena et al., 2020). Briefly, antisera were generated by intranasal instillation (2.5 ML/nostril, 1 × 106 TCID50/ML) of each viral preparation in BVDV antigen/antibody-free colostrum deprived calves according to

previously described protocol (Neill et al., 2019a). Each calf received a subcutaneous booster injection of 2 ML of virus after 21–28 days, and at approximately 48 days a sample of blood was drawn by jugular puncture and serum was prepared and stored at −20C for use in VN assays. All animals were handled in accordance with the Animal Welfare Act Amendments (7 U.S. Code §2131 to §2156) and all study procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the National Animal Disease Center (protocol #ARS-2017–673). The vaccine strain 5912_2a was not included in the antiserum production.

2.4. VN assays

VN assays were performed according to previously described BVDV VN protocol (Bolin and Ridpath, 1995) and as previously described utilizing the PCA analysis (Mosena et al., 2020), where each of the six antisera were tested against the seven vaccine strains (six of them used as inoculum for the antisera production, called homologous strains) and the 23 genetically divergent field isolates from different subgenotypes and geographic origin (Table 1). In 96-well plates, 50 u L of serial two-fold dilutions of one antiserum was incubated with 50 TCID50 of virus per well (replicates of five wells per dilution)for one hour, and then $2 \times 10^{\wedge}4$ bovine turbinate cells were inoculated per well. Four days after inoculation, cp effect was observed on each well for cp strains, while growth of ncp viruses was tested using monoclonal antibody N2 (E2 protein-specific) and horseradish peroxidase-conjugated protein G as described in Viruses methods section and according to previously described (Bolin et al., 1991). Neutralization titers of each antiserum against each of the strains and isolates were calculated using the Spearman-Kärber method (Sozzi et al., 2020b). The same methodology was performed for each of the six antisera against the 30 strains and isolates.

2.5. VN titers distribution and PCA

The VN titers were calculated, transformed into log2 values and used to generate distribution of the data represented by box and whisker plots. These values were then used to conduct PCA using the prcomp function in R. The first and second principle component (PC) were then used to generate the cluster analysis dendrogram by hierarchical cluster analysis with an unweighted pair group mean arithmetic (UPGMA) method using the function hclust in R. The relative positions of the subgenotypes were drawn by ggplot with the first PC (PC1) representing the x and second PC (PC2) representing the y axis. All the analyses were conducted by R (version 3.6.1).

3. Results

3.1. E2 amino acid phylogeny

The phylogenetic tree generated with E2 amino acid sequences of the 30 strains and isolates showed that the HoBi/D32 isolate was the most divergent of the ruminant pestiviruses, presenting a low amino acid identity to BVDV-1 and 2 species. Within the BVDV species branch, two main clusters grouped all BVDV-1 and 2 strains and isolates according to the previous classification described in literature (Fig. 1). The criteria for clusters analysis was established as 9 amino acids substitution per 100 residues (Fig. 1), in order to keep vaccine strains classified as 1a subgenotype in the same cluster, since these strains have been extensively characterized for many years.

In the BVDV-1 branch, 1a vaccine strains were located in two different clades. One of the clades included the strain C24V_1a and two UK 1a field isolates, and the other clade contained the strains NADL_1a and Singer_1a (Fig. 1). 1i isolates (58_2_1i and 69_1_1i) were clustered together, while isolates belonging to the 1e subgenotype were separated into two clusters, where two Swiss 1e isolates generated one cluster (CH_Maria_1e and Carlito_1e) and the other Swiss isolates along with UK isolate 68_1_1 and Italian D7219_1e formed an individual cluster (Fig. 1). One clade included all 1k isolates, and the same happened to 1 h and 1f clade. Subgenotypes that were represented by one isolate (67_1_1d and D23284_1 g) generated single isolate clusters (Fig. 1).

The BVDV-2 vaccine strains clustered into one clade, consisting of one clade with 296_2a, 5912_2a and 125_2a strains grouped together while vaccine strain 53637c_2a was divergent from others and was placed in a separated branch (Fig. 1).

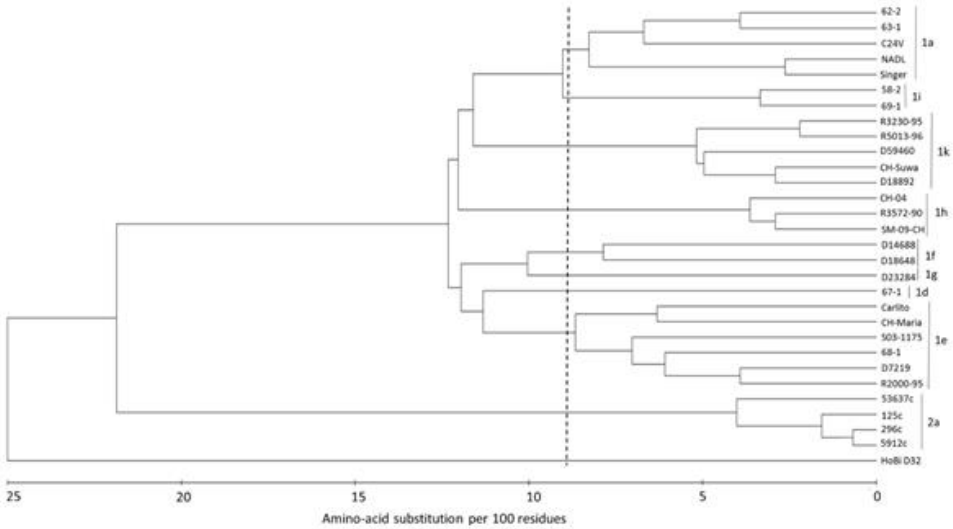

Fig. 1. Phylogenetic analysis of the E2 coding sequence of BVDV. Phylogenetic analysis of the E2 sequences of 30 BVDV strains and isolates (5 BVDV-1a, 1 BVDV-1d, 6 BVDV-1e, 2 BVDV-1f, 1 BVDV-1g, 3 BVDV-1h, 2 BVDV-1i, 5 BVDV-1k, 4 BVDV-2a, and 1 HoBi) to determine amino acid differences within the E2 protein among strains for the major neutralizing protein.

3.2. VN titers distribution

The distribution of VN titers for the six antisera against the BVDV isolates (Supplementary Table 1) were plotted using box and whisker plot (Fig. 2). VN titers (log2) were distributed along the X axis for each antiserum in Y axis. In Fig. 2, the mean was denoted by bar, median titers were denoted by a dot, and boxes extended from the 25th to the 75th percentile with whiskers indicating the minimum and maximum titer (Fig. 2). The maximum titer in all antisera was against the strain which the antisera was specifically generated. Antisera generated against Singer_1a and 53637_2a had the highest mean neutralizing titers and, in a comparison of the means of the neutralizing titers, antisera against BVDV-1 vaccine strains had the highest mean titers (Fig. 2A), as among the isolates there were no BVDV-2 isolates.

3.3. Antigenic clusters through PCA

As previously described (Mosena et al., 2020), results from the PCA yielded two different illustrations of the data, both a PCA cluster dendrogram (Fig. 3A-5A) and scatter plot (Fig. 3B-5B). The PCA cluster dendrogram combines the variation from both PC1 and PC2 into one value to cluster the strains and isolates into antigenically similar groups as determined by VN titers and this is accomplished by hierarchical cluster analysis with an unweighted pair group mean arithmetic (UPGMA) method using the function hclust in R.Whereas the two-dimensional PCA scatter plot represents two axes, the PC1 associated with the X axis and PC2 associated with the Y axis, and the relative positions of the subgenotypes were drawn by ggplot in R. The percent variability represented by PC1 and PC2 is denoted on each axis. These plots allowed the identification of viruses that cluster together in the PCA dendrogram and subsequent categorization of these isolates to groups within the PCA scatter plot. Collectively, by superimposing the PCA cluster dendrogram groups on to the scatter plots the spatial orientation of isolates and potential antigenic groups could be assessed. The antigenic groups are denoted by Roman numerals (I, II, III, etc.) and color shading was utilized to better characterize antigenic groups within each respective Figure. A height of 1 within the PCA cluster dendrogram was used as the minimum cutoff value to characterize strains and isolates that cluster together into the antigenic groups based on VN results and this criterion was used to superimposed on the PCA scatter plot.

The dataset (Figs. 3, 4 and 5) was analyzed using both BVDV-1a and BVDV-2a antisera titers (Fig. 3), only BVDV-1a antisera titers (Fig. 4) and only BVDV-2a antisera titers (Fig. 5). A height of 1 within the PCA cluster dendrogram was used as the criteria to characterize strains and isolates into like groups (Figs. 3, 4 and 5A). The PCA two-dimensional approach generated a scatter plot where the contribution of each PC value can be visualized. When viruses that clustered into like groups in the dendrogram were identified within the PCA scatter plot, the spatial orientation of antigenically related groups could be visualized, as the PC1 and PC2 contribution to the variability of the data (Figs. 3, 4 and 5B).

To identify groups with similar neutralization patterns, initial antigenic comparisons using the PCA were obtained by evaluating VN titers using the six vaccine strains antisera against a total of 30 viruses presented here that included 7 vaccine strains and 23 non-US origins genetically divergent BVDV field isolates belonging to subgenotypes not typically observed in the US (Fig. 3). Two main branches can be observed in the PCA cluster dendrogram, with BVDV-1 and -2 viruses forming the two major branches. Five clusters formed by more than one virus were identified within the BVDV-1 main cluster. Singer_1a, NADL_1a, and C24V_1a vaccine strains formed one cluster (VI), but no field isolates clustered with the vaccine strains (Fig. 3A). The remaining four clusters within the BVDV-1 main cluster were comprised of field isolates apart from Italian D23284_1 g and D14688_1f isolates forming individual branches (Fig. 3A). Cluster V only contained BVDV-1i isolates (Fig. 3A). Most BVDV-2 strains and isolates were grouped into one cluster (I), while viruses 5912_2a and HoBi/D32 formed individual branches within the BVDV-2 main cluster (Fig. 3A).

The PCA scatter plot generated a two-dimensional view of the plot and demonstrated the spatial position of the strains and isolates in relation to each other, as well as the position in the PC1 and PC2 axis (Fig. 3B). The PC1 represented by the X axis in the PCA scatter plot was representative of 73.07 % of the variability (Fig. 3B). While a different representation of the data, the two main branches observed in the PCA dendrogram illustrate similar categorization as the PC1 axis largely grouped isolates into BVDV-1 and BVDV-2 species. Thus, BVDV-2 strains were plotted in the left PC1 quadrant, very distant from BVDV-1 groups in the right quadrant (Fig. 3B). Isolates that formed individual clusters in the PCA cluster dendrogram (Fig. 3A) were observed in the scatter plot as having no proximity to the multiple isolates/strains groups (Fig. 3B). In addition, PC2 represented by the Y axis accounted for 18.07 % of the variability (Fig. 3B). For the cluster of viruses containing the vaccine strains, cluster I for the BVDV-2 strains and cluster VI for the BVDV-1 strains, the VN titers are higher than the other cluster of viruses, which would be expected given that the homologous titers, in general, are greater than heterologous titers among other viruses. This is best

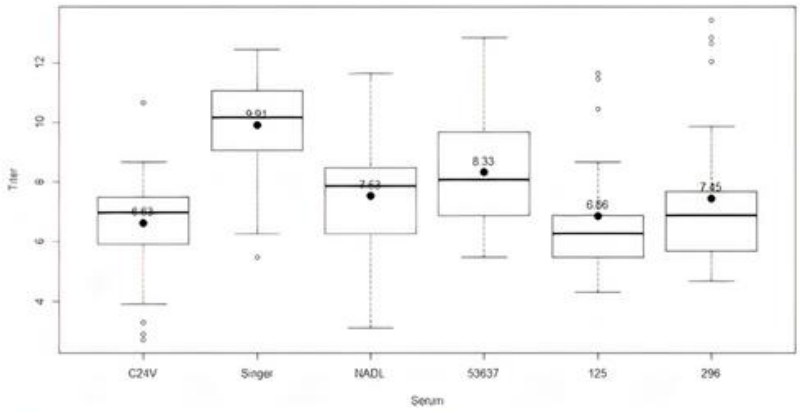

Fig. 2. Box plot for neutralizing antibody titers using 30 BVDV isolates (5 BVDV-1a, 1 BVDV-1d, 6 BVDV-1e, 2 BVDV-1f, 1 BVDV-1g, 3 BVDV-1h, 2 BVDV-1i, 5 BVDV-1k, 4 BVDV-2a, and 1 HoBi) against antisera generated against three BVDV-1a vaccine strains (C24V, Singer, and NADL) and three BVDV-2a strains (53637c, 125c, and 296c).

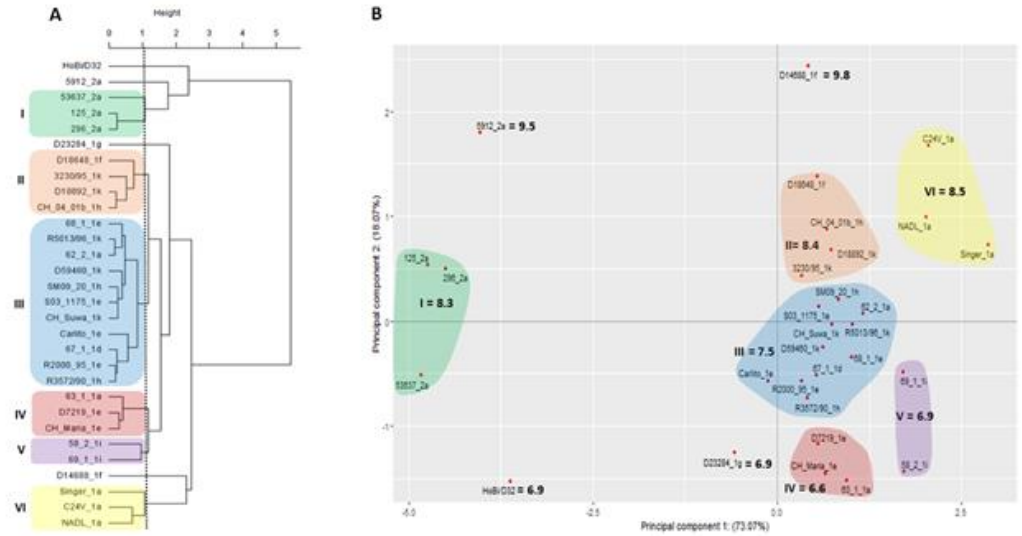

Fig. 3. Methods to evaluate similar antigenic clustering using 30 BVDV strains (5 BVDV-1a, 1 BVDV-1d, 6 BVDV-1e, 2 BVDV-1f, 1 BVDV-1g, 3 BVDV-1h, 2 BVDV-1i, 5 BVDV-1k, 4 BVDV-2a, and 1 HoBi) against antisera generated against three BVDV-1a vaccine strains (C24V, Singer, and NADL) and three BVDV-2a strains (53637c, 125c, and 296c). (A) Cluster analysis dendrogram using Ward's method combing the variation from both principal component 1 and 2 to cluster strains into like groups. (B) Principal component scatter plot displaying independent contribution of the first two principal components accounting for the largest variation in the samples. The mean neutralizing titer of the antigenic cluster is demonstrated along with the cluster identification based on dendrogram A.

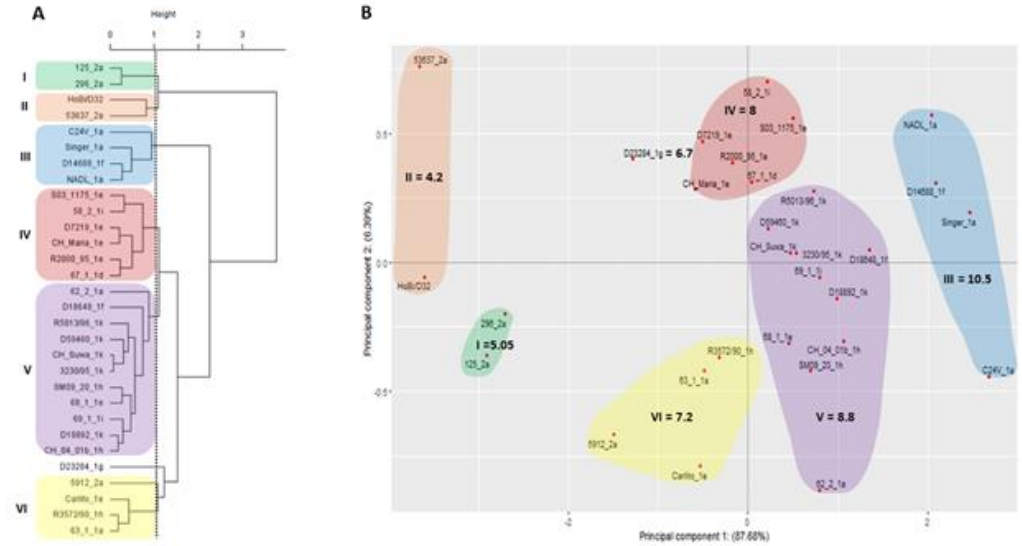

Fig. 4. Methods to evaluate similar antigenic clustering using 30 BVDV strains (5 BVDV-1a, 1 BVDV-1d, 6 BVDV-1e, 2 BVDV-1f, 1 BVDV-1g, 3 BVDV-1h, 2 BVDV-1i, 5 BVDV-1k, 4 BVDV-2a, and 1 HoBi) and antisera generated against three BVDV-1a vaccine strains (C24V, NADL, and Singer). (A) Cluster analysis dendrogram using Ward's method combing the variation from both principal component 1 and 2 to cluster strains into like groups. (B) Principal component scatter plot displaying independent contribution of the first two principal components accounting for the largest variation in the samples. The mean neutralizing titer of the antigenic cluster is demonstrated along with the cluster identification based on dendrogram A.

demonstrated by clusters III, IV, and V that have lower VN titers as they are on the opposing side of the Y axis and are more spatially distant from the cluster containing the BVDV-1 vaccine strains (Fig. 3B).

Interestingly, one vaccine strain (5912_2a) and field isolate (D14688_1f) that lacked proximity to the clusters had high VN titers, which were greater than average VN titers for the vaccine virus clusters for each respective BVDV species (Fig. 3B). Although two of the isolates (HoBi/D32 and D23284_1 g) with distinct spatial proximity did not have the lowest VN titers, the titers in general were lower than the majority of viral clusters (Fig. 3B). The same was observed for cluster V,

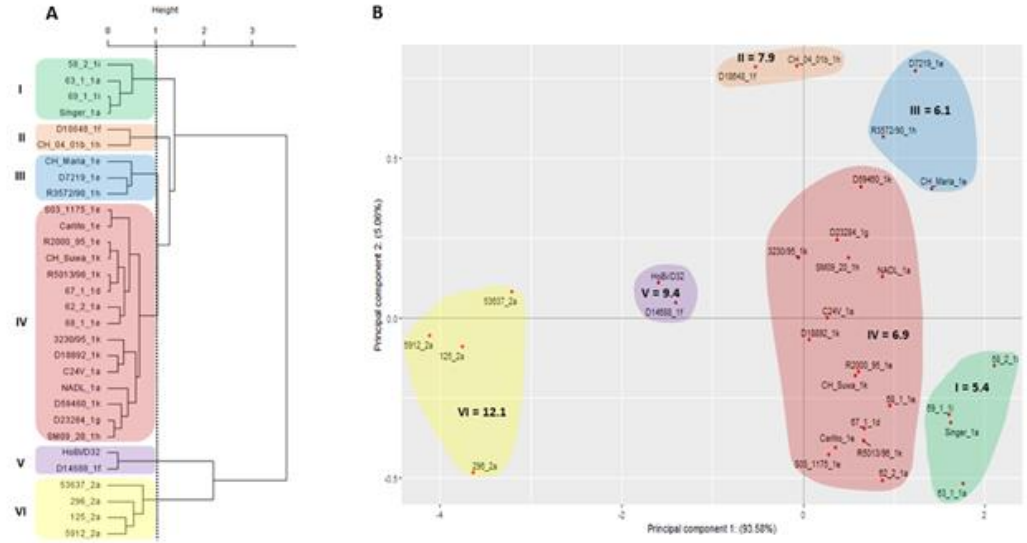

Fig. 5. Methods to evaluate similar antigenic clustering using 30 BVDV strains (5 BVDV-1a, 1 BVDV-1d, 6 BVDV-1e, 2 BVDV-1f, 1 BVDV-1g, 3 BVDV-1h, 2 BVDV-1i, 5 BVDV-1k, 4 BVDV-2a, and 1 HoBi) and three BVDV-2a antisera generated against vaccine strains (296c 53637c, and 125c). (A) Cluster analysis dendrogram using Ward's method combing the variation from both principal component 1 and 2 to cluster strains into like groups. (B) Principal component scatter plot displaying independent contribution of the first two principal components accounting for the largest variation in the samples. The mean neutralizing titer of the antigenic cluster is demonstrated along with the cluster identification based on dendrogram A.

representative of the BVDV-1i isolates, and cluster IV, comprised of BVDV-1e and -1a isolates, that were observed to have some of the lowest titers (Fig. 3B).

To better understand the contribution of each BVDV species-specific antisera to the cross-neutralization patterns associated with the same 30 virus dataset, antigenic comparisons were made using only the BVDV-1 vaccine strains specific-antisera (C24V_1a, NADL_1a and Singer_1a antisera; Fig. 4) as well as only using the BVDV-2 vaccine strains specific-antisera (125_2a, 296_2a and 53637_2a; Fig. 5). These comparisons were evaluated because of the percent variability that was accounted for by PC1 and PC2 for each antisera group (Figs. 4 and 5).

PCA using only the VN data for the BVDV-1 vaccine strains specific-antisera yielded similar trends as observed when using all six vaccine strains antisera, but specific VN titer patterns could be observed. Notable differences observed for the BVDV-1 specific-antisera were larger clusters containing BVDV-1 field isolates and the presence of only one individual branch within the PCA dendrogram. In the dendrogram (Fig. 4A), strain 5912_2a grouped in a cluster with other BVDV-1 isolates (VI). BVDV-2 vaccine strains were separated in two clusters, with cluster I with vaccine strains 125_2a and 296_2a and cluster II with HoBi/D32 clustered along with vaccine strain 53637_2a (Fig. 4A). Cluster 2 was the most spatially divergent group in scatter plot (Fig. 4B).

Viruses that formed individual branches (Fig. 3A) when using all six vaccine strains antisera for comparisons were all contained in clusters with other viruses (Fig. 4A), except for D23284-1g cluster (Fig. 4A), which also formed a single cluster when the six antisera titers were analyzed (Fig. 3A). Vaccine BVDV-1 strains formed one cluster like in the PCA with all the antisera titers (Fig. 3A), but the field isolate D14688-1f, which was previously divergent as a single cluster (Fig. 3A) clustered within the vaccine cluster (III, Fig. 4A). This is suggestive that this isolate is very antigenically similar to the vaccine strains.

It should also be noted that most of the BVDV-1 isolates were observed on the same side of the PC1 axis as the BVDV-1a vaccine strains in the PCA dendrogram, but isolates D7219_1e, R200_95_1e, CH_Maria_1e (IV), Carlito_1e, R3572/90_1 h, 63_1_1a (VI) and D23284_1g were observed on the adjacent side of the PC1 axis in the PCA scatterplot (Fig. 4B). Specifically, these results are noteworthy as they demonstrate the majority of BVDV-1e isolates may be more antigenically different than BVDV-1a vaccine strains as well as the observation that also one BVDV-1a isolate, namely 63_1_1a may also be antigenically divergent albeit is a BVDV-1a isolate and genetically similar to the BVDV-1a vaccine strains.

PCA using only the VN data for the BVDV-2 vaccine strains specific-antisera demonstrated further antigenic differences as specific VN titer patterns could be observed (Fig. 5), similar to observations associated with using only BVDV-1 vaccine strains specific-antisera. Since no non-US BVDV-2 field isolates were included in the study, the dendrogram build with only BVDV-2 antisera titers showed the four BVDV-2a vaccine strains as having a similar behavior in neutralization and belonging to one antigenic cluster (VI, Fig. 5A and 5B). Interestingly, isolates D14688-1f and HoBi/D32 clustered together and average VN titers for these two isolates tended to be higher than other BVDV-1 isolates and strains (Fig. 5), and D14688_1f was not similar to any other isolate when the six antisera were analyzed (Fig. 3A). When using only BVDV-2 antisera results, as for when analyzing only BVDV1 antisera, the highest VN titers among BVDV-1 isolates were observed against isolate D14688_1f. BVDV-1i isolates were grouped into one cluster when all six antisera results were analyzed (Fig. 3A) and a similar trend was observed when using the BVDV-2 specific-antisera (Fig. 5A and 5B). Two BVDV-1i isolates along with two BVDV-1a isolates/strains formed one cluster (I; Fig. 5) and this cluster also had the lowest VN titers as compared to the other BVDV-1 isolates/strains.

4. Discussion

More than a growing number of species, the description of new subgenotypes within BVDV species has been growing expansively in the last years, with at least 22 subgenotypes described for BVDV-1, 3–4 for BVDV-2 and initial subgenotype classification in the HoBiPeV species (Yeşilbağ et al., 2017). In the USA the previously described subgenotypes were 1a, 1b and 2a, but in the last two years 1i, 2b and 2c isolates were firstly detected (Neill et al., 2019a; Neill et al., 2019b).

Commercial vaccines available in the country contain well known 1a and 2a strains in their composition, but cross protection is not well defined within the diverse species.

Finding patterns or relationships between subgenotypes and antigenic properties could help in establishing an antigenic classification for isolates, since genetic classification is easier than antigenic characterization through VN titers. VN assay is the gold standard test for determining BVDV antigenic properties, but interpretation of data can be difficult when multiple isolates are analyzed. The antigenic variability measured by neutralization in VN assays results is mostly due to antibodies against the E2 protein, which is an envelope glycoprotein that is highly variable, immunogenic and induces the production of most virus-neutralizing antibodies (Deregt et al., 1998). However, as observed, E2 gene or amino acid phylogeny is not able to describe antigenicity of isolates, since E2 epitopes that are potential targets for neutralizing antibodies are conformational epitopes subjected to post translational processes, as glycosylation (Li et al., 2013).

Additionally, serological relatedness is typically expressed as coefficient of antigenic similarity (R) using VN titers (Bachofen et al., 2008). The coefficient of antigenic similarity (R) is calculated based on A antiserum titer against B virus strain and B antiserum titer against A virus strain and it generates a scale in which a result closest to 100 means less antigenic differences in between the two viral strains. Thus, as viral titers, this calculation provides a value between the specific viral strain and the corresponding antisera and does not allow for comparisons of antigenic relationships among multiple isolates.

In a previous study (Mosena et al., 2020), a new method for interpretation of VN results used PCA to generate antigenic clustering plots. This method allowed visualization of antigenically similar and divergent BVDV isolates when analyzing multiple isolates against multiple antisera. Thus, the relationship between isolates could be clearly interpreted. In this study, we used the same PCA analysis to antigenically characterize vaccine strains and several field isolates from a variety of subgenotypes and different geographic origins. Some of the Swiss isolates (CH_04_01b_1 h and CH_Maria_1e) used in the current study were previously used for antigenic comparisons against BVDV-1a antisera (Bachofen et al., 2008). R values < 25 are generally considered to indicate significant antigenic differences and R values were previously reported to be > 25 for the CH_04_01b_1 h isolate against BVDV-1a antisera (Bachofen et al., 2008). Therefore, at least one of the previously reported R values for the CH_Maria_1e isolate was > 25 which would suggest these isolates were antigenically similar to the BVDV-1a antisera (Bachofen et al., 2008). Interestingly, in the current PCA, the CH_Maria_1e and CH_04_01b_1 h isolates clustered in separate clusters from each other as well as separate clusters from the BVDV-1a vaccine strains, and this was regardless of antisera used for comparisons and is suggestive of antigenic differences. The Italian isolate D7219_1e was also evaluated previously in a cross neutralization study, where sera generated against vaccines containing only 1a or 1b strain in its composition were tested through VN assay for cross neutralization potential against 1a, 1b and 1e isolates (Sozzi et al., 2020b). No vaccine antisera could generate cross neutralization titers higher than 1/10 for D7219_1e (Sozzi et al., 2020b). In the current PCA, this field isolate did not cluster with 1a and 1b isolates other than 63_1_1a when BVDV-1 antisera were used.

Antigenic relatedness could be observed in the antigenic PCA plots presented in this study. Given that more isolates from several subgenotypes were used for the PCA as compared to the previous study that evaluated only isolates of US origin (Mosena et al., 2020), more marked trends associated with antigenic differences could be more clearly discerned. Since no specific pattern was associated with antigenic relatedness, it is rather dependent on the isolate than the subgenotype, i.e., isolates of the same subgenotype were observed to cluster in various BVDV-1 PCA groups. Dendrogram and scatter plots did highlight that some isolates are antigenically divergent from most isolates described in this study. Isolate D14688_1f and D18648_1f were each positioned in different clusters thus showing different antigenic relationships for each of the PCA scenarios and as well as different antigenic relationship between the two BVDV-1f isolates. This points that some isolates are antigenically very divergent from members of the same species and subgenotype.

However, some subgenotypes presented relationships that remained similar in all the plots regardless of the antisera used for comparisons. Similarly, antigenic patterns could be observed for the BVDV-1i subgenotype. In two out of three scenarios the two 1i isolates (UK isolates 58_2_1i and 69_1_1i) clustered together, sometimes with isolates of other subgenotypes isolates inside the same cluster. This was not observed with other subgenotypes included in the study, where some isolates from the same subgenotype would be clustered separately. Although only two BVDV_1i isolates were included, it should be further analyzed as there may be some feature that this subgenotype might provide to investigate specific antigenic determinants linked to patterns of neutralizing antibodies.

In addition to the illustration that antigenic relationships between isolates could not be linked to subgenotype classification, some of our results highlight previous concerns raised about current vaccines efficacies in protecting against the broad diversity of BVDV. Even BVDV-1a, the most frequent subgenotype worldwide (Yeşilbağ et al., 2017), presented in this study isolates that were antigenically divergent from each other and vaccine strains. It raises questions on the cross protection of 1a vaccine strains against other subgenotypes. Antigenic characterization of pestiviruses has been challenging since the standard VN titer results can be hard to visualize and interpret due to differences in comparison between different antisera and the number of isolates and antisera used. PCA was already shown to be a statistical method that can turn VN titers into an easy and clear interpretation of antigenic relationships between multiple isolates using multiple antisera and can be a tool to identify antigenic patterns. This could become a useful resource to design vaccine with improved efficacy against a broader diversity of BVDV field isolates. In order to turn VN results with PCA interpretation into a tool for the vaccine industry, vaccination and challenge studies are necessary to understand if and how antibody neutralization patterns in vitro are related to cross protection or failure in vivo. Furthermore, VN titers are a measure only for the humoral immune response and provide an understanding of how efficiently the virus is neutralized by antibodies. VN titers do not account for the cell mediated immune response, which is also related to protection observed during in vivo vaccination studies (Ridpath, 2013; Chase, 2013; Platt et al., 2009). Although it is important for immune protection, there is still neither consensus on the role and mechanisms of cell mediated immune responses in BVDV infection, nor does an established method exist for its measurement (Becher et al., 2003; Chase, 2013). Nevertheless, characterization of isolates regardless of their type of immune response will allow for a more directed analysis of the genome and its relationship to immunological and antigenic differences that may exist. We believe the results presented in this study could further lead to a deeper genetic comparison among antigenically similar and dissimilar isolates to determine if there are molecular genetic signatures that could be related to antigenic similarity or divergence between isolates. This might be a tool to improve the composition of current vaccines and broaden its cross protection against diverse pestiviruses, which is important for the success of BVDV control programs.

Funding

CWC and ACSM were supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) - Finance Code 001, Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS), and Pró-reitoria de Pesquisa da Universidade Federal do Rio Grande do Sul (Propesq/UFRGS).

This research was conducted at a USDA research facility and all

funding was provided through internal USDA research dollars.

Author contributions

Conceived and designed the experiment: ACM, SMF, JDN.
Performed the experiment: ACM, SMF, RPD, JDN.
Analyzed the data: ACM, HM, EC.
Contributed reagents/materials/analysis tools: SMF, RPD, RB, GMD, MS, CWC, JDN.
Wrote the paper: ACM and SMF.
Reviewed the paper: all authors.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

The authors would like to thank Kathryn McMullen, Patricia Federico and Renae Lesan for their excellent technical assistance and Brian Conrad, Jeremy Spieker, Parker Ness, Dalene Whitney and Chase Conis for assistance with animal studies. Mention of trade name, proprietary product, or specified equipment does not constitute a guarantee or warranty by the USDA and does not imply approval to the exclusion of other products that may be suitable. USDA is an Equal Opportunity Employer. This is a short text to acknowledge the contributions of specific colleagues, institutions, or agencies that aided the efforts of the authors.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2021.114328.

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Supplementary Table 1. Neutralization titers (in log2) of the six antisera against field isolates and vaccine strains used in this study. Dark grey color represents the homologous titers of each specific antiserum against the homologous vaccine strain.

			Virus isolate	Antisera 1a			Antisera 2a		
	Country	Species/ Subgenotype	Isolate ID	C24V	Singer	NADL	125	296	53637
Field isolates	Italy	1e	D7219	5.68	9	7.87	4.28	5.68	8.07
		1f	D14688	8.67	11.66	10.46	8.67	9.06	10.06
			D18648	7.47	11.46	9.46	6.27	8.27	10.06
		1g	D23284	5.9	7.87	6.27	6.07	6.87	8.27
		1k	D18892	7.67	11.06	8.47	6.67	7.67	8.07
			D59460	6.67	10.06	8.07	5.68	6.27	8.27
	Swiss	1e	CH-Maria	6.67	8.67	6.87	5.08	4.68	7.47
			S03-1175	6.87	9.86	9.06	6.87	7.47	7.27
			Carlito	6.27	10.26	5.68	7.47	6.27	7.47
			R2000-95	6.47	9.26	7.87	6.07	7.07	7.27
		1h	CH-04-01b	7.47	11.46	8.47	6.07	7.07	9.66
			R3572/90	5.88	10.26	6.87	5.32	5.67	8.27
			SM09-20	7.47	11.06	7.67	6.67	5.68	8.27
		1k	CH-Suwa	6.87	10.46	8.27	6.67	6.27	7.47
			R3230/95	7.07	10.46	8.27	6.67	7.47	8.67
			R5013/96	8.27	9.86	8.27	6.27	7.07	6.87
	UK	1a	62-2	7.27	11.86	7.27	6.27	6.67	6.47
			63-1	6.27	9.86	6.27	5.32	5.32	5.47
		1d	67-1	7.27	9.26	7.67	6.07	7.27	6.87
		1e	68-1	7.67	10.46	7.27	6.27	5.88	6.87
		1i	58-2	8.07	8.67	8.07	4.48	4.68	5.68
			69-1	7.27	10.86	8.47	5.48	5.08	6.07
	Brazil	HoBiPeV	HoBi/D32	3.27	6.26	3.08	8.47	9.86	10
Vaccine strains	USA	1a	C24V	10.66	12.45	9.26	6.67	6.87	8.07
			Singer	8.47	12.45	11.06	5.08	5.68	5.88
			NADL	7.07	12.05	11.66	5.47	6.47	7.47
		2a	125c	2.88	7.87	4.08	11.46	12.65	12.45
			296c	3.88	7.47	4.08	11.66	12.85	11.65
			5912c	4.68	9.46	5.08	11.65	13.45	12.85
	Canada	2a	53637c	2.68	5.47	4.48	10.46	12.05	12.05

3.5 Capítulo 5: Caracterização antigênica de cepas de BVDV detectadas na Região Sul e análise temporal de subgenótipos detectados no Brasil

O conhecimento das espécies e subgenótipos de BVDV circulantes em uma região é importante para o desenvolvimento de ferramentas diagnósticas, métodos de controle, desenvolvimento de vacinas, e é essencial para estudos de epidemiologia molecular e filodinâmica. A análise do tempo do ancestral comum mais recente (tMRCA) pode ser usada para inferir a epidemiologia viral, flutuações históricas na dinâmica populacional e dispersão espacial. O objetivo deste capítulo foi, além de caracterizar os subgenótipos de BVDV mais diagnosticados no Laboratório de Virologia Veterinária-UFRGS no período 2016-2018, inferir relações filogenéticas entre os subgenótipos descritos no Brasil através de escala temporal utilizando a ferramenta do relógio molecular. O artigo foi submetido para publicação durante a escrita desta tese e teve o aceite final para publicação com o título "*Temporal analysis of bovine pestivirus diversity in Brazil*" no *Brazilian Journal of Microbiology* com a identificação DOI 10.1007/s42770-022-00735-z no formato de acesso restrito, portanto não será disponibilizado nesta tese. O resumo apresentado a seguir está disponível *on line*.

TEMPORAL ANALYSIS OF BOVINE PESTIVIRUSES DIVERSITY IN BRAZIL

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ABSTRACT

In this study, phylogenetic and evolutionary analysis of cattle pestiviruses (BVDV-1, 2 and HoBiPeV) originated in Brazil were used to investigate the temporal diversification of subgenotypes in the country. Inferred dated phylogeny and time of the most recent common ancestor (tMRCA) demonstrated that some BVDV subgenotypes (1a, 1b, 1d, 1e and 2b) and HoBi-like sequences grouped according to region of origin, and the diversification of subgenotypes appears to have occurred around the introduction of, firstly *Bos Taurus*, and then *Bos Indicus,* and expansion to form adapted Brazilian breeds. The present results help to elucidate the temporal facts that led to diversification of ruminant pestiviruses in cattle in Brazil.

4 CONCLUSÃO

Os trabalhos que compõem a presente tese de doutorado geraram as seguintes conclusões:

A) BVDV-1 e 2 circulam em suínos de criação de subsistência;

B) A investigação de pestivirus em suínos é importante para a percepção do impacto destes hospedeiros como potenciais reservatórios e diferencial em testes laboratoriais (de Peste Suína Clássica por exemplo);

C) O *Pestivirus K*, que afeta leitões, está presente no Brasil especialmente devido à intensiva e significante produção de suínos no país;

D) O uso do método estatístico Análise de Componentes Principais (ACP) para análise de multivariáveis mostrou-se uma ferramenta promissora para visualizar relações antigênicas entre pestivírus;

E) Além disso, o ACP mostrou de forma clara que isolados de BVDV de mesmo perfil genético (subgenótipo) não possuem a mesma relação de proximidade antigênica;

F) A ACP é um método poderoso para a escolha de cepas vacinais que gerem resposta imune mais específica contra as cepas de campo de BVDV;

G) A frequência de subgenótipos de BVDV dentre amostras do Rio Grande do Sul não mudou nos últimos anos, com BVDV-1a, 2b, 1b, 1d e 1e sendo relatados;

H) Análise temporal de espécies de BVDV presentes no Brasil pode sugerir que a alta diversidade de pestivírus bovino no país se formou a partir da introdução de espécies e raças de bovinos, o aumento da população bovina e as migrações de gado ligadas a fatores econômicos.

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ANEXO A- Artigo "*A new highly divergent copiparvovirus in sheep"*

O presente artigo foi publicado pela doutoranda como primeira autora e não faz parte da Tese. Ele descreve o uso de sequenciamento de alta eficiência na análise de amostras de um surto de aborto em uma propriedade de ovinos em que a etiologia não foi identificada. O genoma de um parvovírus, divergente dos demais, foi identificado e caracterizado. Este artigo foi aceito para publicação em 08 de março de 2021 no periódico *Archives of Virology* sob o título "*A new highly divergent copiparvovirus in sheep*", DOI: 10.1007/s00705-021-05020-2, como "*Annotated sequence record*". O artigo é de acesso restrito e, portanto, não será incluído neste documento, podendo ser acessado em https://doi.org/10.1007/s00705-021-05020-2. O resumo disponibilizado encontra-se abaixo:

Abstract

The subfamily Parvovirinae within the family Parvoviridae consists of viruses that can infect a wide range of vertebrate hosts and cause effects ranging from severe disease to asymptomatic infection. In the present study, high-throughput sequencing (HTS) was utilized to analyze samples obtained from an abortion outbreak in a sheep flock to identify a putative viral etiology. A highly divergent nearly complete parvovirid genome sequence, approximately 4.9 kb in length, was determined. The nonstructural protein (NS1) amino acid (aa) sequence of this virus shared less than 30% identity with those of other copiparvoviruses and less than 22% identity with those of members of other genera in the subfamily Parvovirinae. Phylogenetically, this virus, which we have provisionally named "sheep copiparvovirus 1", formed a cluster with copiparvovirus sequences and should be classified as a member of a new species in the genus Copiparvovirus.

ANEXO B- Trajetória Acadêmica da Doutoranda

A doutoranda iniciou a prática acadêmica na pesquisa no quarto semestre do curso de graduação da Faculdade de Veterinária-UFRGS no ano de 2011 no Laboratório de Virologia Veterinária, seguindo com a trajetória acadêmica ate o fim do doutorado, em 2021.

- O primeiro contato com a pesquisa foi como estagiária voluntária e posterior como bolsista de Iniciação Científica no Laboratório de Virologia Veterinária sob supervisão do professor Cláudio Canal. Foram 7 semestres de Iniciação Científica continua no Laboratório (de 2011 ate 2014) até a graduação da aluna. Durante o período de bolsa PIBIC como Iniciação Científica, a aluna participou de 3 Salões de Iniciação Científica da UFRGS e do Congresso Brasileiro de Virologia:

-XXIV Salão de Iniciação Científica UFRGS (2012), com o trabalho "Detecção de Parvovírus e Anelovírus em pulmão de javalis em cativeiro";

-XXV Salão de Iniciação Científica UFRGS (2013), com o trabalho "Prevalência de bovinos persistentemente infectados pelo vírus da diarréia viral bovina no estado do Rio Grande do Sul";

-XXVI Salão De Iniciação Científica UFRGS (2014), com o trabalho "Detecção e de terminação das espécies de parvovírus em gatos";

-**Mósena, A.C.S.**; Chiappetta, C. M.; Silva, M.S.; Weber, M. N.; Budaszewski, R.F.; Streck, A. F.; Canal, C.W. Detection and characterization of parvoviruses in stool samples from diseased and healthy cats. In: **XXV Brazilian Congress of Virology - IX Mercosul Meeting of Virology**, 2014, Ribeirão Preto. Virus Reviews and Research, 2014. v.19. p.40 - 40

Durante o período de Iniciação Científica também auxiliou na pesquisa de diversos alunos de graduação e pós graduação, sendo co-autora em diversos artigos e resumos de congresso:

- Weber, M. N.; **Mósena, A. C. S**.; Simões, S. V. D.; Almeida, L. L.; Pessoa, C. R. M.; Budaszewski, R. F.; Silva, T. R.; Ridpath, J. F.; Riet-Correa, F.; Driemeier, D.; Canal, C. W. Clinical Presentation Resembling Mucosal Disease Associated with -HoBi'-like Pestivirus in a Field Outbreak. TRANSBOUNDARY AND EMERGING DISEASES: v.61, p.n/a - n/a, 2014

- Weber, M.N.; Silveira, S.; Machado, G.; Groff, F.H.S.; **Mósena, A.C.S.**; Budaszewski, R.F.; Dupont, P.M.; Corbellini, L.G.; Canal, C.W. High frequency of bovine viral diarrhea virus type 2 in Southern Brazil. VIRUS RESEARCH: v.191, p.117 - 124, 2014

-Weber, M.N.; Galuppo, A.G.; Budaszewski, R.F.; Corbellini, A.O.; **Mósena, A.C.S.**; Pinto, L.D.; Marques, L.S.; Rodrigues, J.L.; Canal, C.W. Evaluation of prenucleic acid extraction for increasing sensitivity of detection of virus in bovine follicular fluid pools. THERIOGENOLOGY: v.79, p.980 - 985, 2013

-Weber, M.N.; Silveira, S.; **Mósena, A.C.S**.; Silva, M.S.; Flores, E. F.; Canal, C. W. Genetic diversity of ruminant pestiviruses in Brazil In: Joint U.S. BVDV/ESVV Pestivirus Symposium Pestiviruses: Old Enemies, New Challenges, 2014, Kansas City. Pestiviruses: Old Enemies, New Challenges. 2014. p.46 - 46

-Weber, M.N.; Silveira, S.; Machado, G.; Groff, F.; **Mósena, A.C.S.**; Silva, M.S.; Budaszewski, R.F.; Dupont, P.; Corbellini, A.O.; Canal, C. W. High frequency of bovine viral diarrhea virus 2 in Rio Grande do Sul, Brazil. In: XXV Brazilian Congress of Virology - IX Mercosul Meeting of Virology, 2014, Ribeirão Preto. Virus Reviews and Research, 2014. v.19. p.39 - 39

-Budaszewski, R.F.; Weber, M. N.; **Mósena, A.C.S.**; Silveira, S.; Dupont, P.; Borchardt, A.; Streck, A. F.; Canal, C.W. Identification of potential multi-recombinant strains of canine distemper virus In: XXV Brazilian Congress of Virology - IX Mercosul Meeting of Virology, 2014, Ribeirão Preto. Virus Reviews and Research, 2014. v.9. p.41 - 41

-Silva, M.S.; **Mósena, A. C. S.**; Weber, M.N.; Silveira, S.; Dupont, P.; Torikashvili, M.; Budaszewski, R.F.; Streck, A. F.; Canal, C.W. Pestivirus contamination in cell culture. In: XXV Brazilian Congress of Virology - IX Mercosul Meeting of Virology, 2014, Ribeirão Preto. Virus Reviews and Research, 2014. v.19. p.40 - 40

-Silveira, S.; Weber, M.N.; **Mósena, A.C.S.**; Silva, M.S.; Streck, A. F.; Cruz, R. A. S.; Pescador, C.A; Canal, C. W. Phylogenetic Analysis of HoBi-like Viruses from Brazil between 2007 and 2014 In: Joint U.S. BVDV/ESVV Pestivirus Symposium Pestiviruses: Old Enemies, New Challenges, 2014, Kansas City. Pestiviruses: Old Enemies, New Challenges, 2014. p.30 - 30

-Weber, M. N.; Galuppo, A. G.; Budaszewski, R. F.; Corbellini, A.; **Mósena, A. C. S.**; Pinto, L. D.; Rodrigues, J. L.; Canal, C. W. An optimized protocol for screening virus in bovine follicular fluid In: XXIII Encontro Nacional de Virologia - VII Encontro de Virologia do Mercosul, 2012, Foz do Iguaçu. Virus Reviews and Research, 2012. v.17. p.96 - 96

-Pinto, L. D.; Streck, A. F.; Budaszewski, R. F.; Souza, C. K.; Antunes, J. R.; **Mósena, A.C.S.**; Canal, C. W. Coinfection of canine parvovirus with other gastroenteritis viruses In: XIV International Parvovirus Workshop, 2012, Ithaca NY. Proceedings of XIV International Parvovirus Workshop, 2012

-Silva, T. R.; **Mósena, A.C.S.**; Weber, M. N.; Budaszewski, R. F.; Pinto, L. D.; Souza, C. K.; Riet-Correa, F; Almeida, L. L.; Driemeier, D.; Canal, C. W. Detecção e caracterização filogenética de pestivírus atípicos de bovinos In: XXIV Salão de Iniciação Científica UFRGS, 2012, Porto Alegre. Resumos do XXIV Salão de Iniciação Científica da UFRGS. , 2012.

-Weber, M. N.; Galuppo, A. G.; Budaszewski, R. F.; Corbellini, A.; Marques, L. S; **Mósena, A.C.S.**; Egocheaga, R.M.F; Rodrigues, J. L.; Canal, C. W. Detection of bovine viral diarrhea virus and bovine herpesvirus in follicular fluid by RT-PCR and PCR In: XXII Encontro Nacional de Virologia - VI Encontro de Virologia do Mercosul, 2011, Atibaia. Virus Reviews and Research, 2011. v.16. p.66 – 66

- Após graduação em Medicina Veterinária, a trajetória acadêmica continou com o ingresso da aluna no mestrado do Progama de Pós Graduação em Ciências Veterinárias no período de 2015 a 2017, com o professor Cláudio Canal como orientador. Deste período, fizeram parte da dissertação os seguintes artigos:

- **Mósena, Ana Cristina S.**; Cibulski, Samuel P.; Weber, Matheus N.; Silveira, Simone; Silva, Mariana S.; Mayer, Fabiana Q.; Roehe, Paulo M.; Canal, Cláudio W. Genomic and antigenic relationships between two -HoBi'-like strains and other members of the Pestivirus genus. ARCHIVES OF VIROLOGY, v.jul, p.1 - , 2017

- **Mósena, Ana Cristina S.**; Weber, Matheus N.; Cibulski, Samuel P.; Silveira, Simone; Silva, Mariana S.; Mayer, Fabiana Q.; Canal, Cláudio W. Genomic characterization of a bovine viral diarrhea virus subtype 1i in Brazil. ARCHIVES OF VIROLOGY, v.(), p.1 - 5, 2016

Além de co autoria em artigos e congresso:

- Weber, Matheus Nunes; Streck, André Felipe; Silveira, Simone; **Mósena, Ana Cristina Sbaraini**; Da Silva, Mariana Soares; Canal, Cláudio Wageck. Homologous recombination in pestiviruses: Identification of three putative novel events between different subtypes/genogroups. INFECTION GENETICS AND EVOLUTION, v.30, p.219 - 224, 2015.

- Weber, M. N.; Pino, E.H.M.; Souza, Carine Kunzler De; **Mósena, A. C. S.**; Sato, J.P.H.; Barcellos, David E.S.N.; Canal, C. W. First Evidence of Bovine Viral Diarrhea Virus Infection in Wild Boars. ACTA SCIENTIAE VETERINARIAE (Online), v.44, p.1398 - , 2016.

- Silveira, Simone; Weber, Matheus Nunes; **Mósena, Ana Cristina Sbaraini**; Da Silva, Mariana Soares; Streck, André Felipe; Pescador, C.A; Flores, E. F.; Weiblen, R.; Driemeier, D.; Ridpath, J. F.; Canal, Cláudio Wageck. Unique combination of BVDV-1, BVDV-2 and Hobi-like pestiviruses present in Brazil. In: XXVI Brazilian Congress of Virology, X Mercosur Meeting of Virology, 2015., 2015. v.20. p.41 – 41

- Em seguimento ao mestrado, os 4 anos de doutorado geraram significativa produção científica, além de crescimento pessoal e acadêmico como bolsista de doutorado sanduíche (PDSE) por 6 meses no NADC-USDA (National Animal Disease Center-United States Department of Agriculture) em Ames, Iowa- EUA, sob supervisão da Dr[a] Shollie Falkenberg. Os resultados do doutorado foram os artigos apresentados nesta tese, alem de participação em congressos:

- **Mosena, Ana Cristina S.**; Falkenberg, Shollie M.; Ma, Hao; Casas, Eduardo; Dassanayake, Rohana P.; Walz, Paul H.; Canal, Cláudio W.; Neill, John D. Multivariate analysis as a method to evaluate antigenic relationships between BVDV vaccine and field strains. VACCINE, v.I, p.1 - , 2020

- **Mosena, Ana Cristina S.**; Falkenberg, Shollie M.; Ma, Hao; Casas, Eduardo; Dassanayake, Rohana P.; Booth, Richard; De Mia, Gian Mario; Schweizer, Matthias; Canal, Cláudio W.; Neill, John D. Use of multivariate analysis to evaluate antigenic relationships between US BVDV vaccine strains and non-US genetically divergent isolates. JOURNAL OF VIROLOGICAL METHODS, v.299, p.114328 -, 2022

- **Mósena, A. C. S.**; Weber, M. N.; Da Cruz, R. A. S.; Cibulski, S. P.; Da Silva, M. S.; Puhl, D. E.; Hammerschmitt, M. E.; Takeuti, K. L.; Driemeier, D.; De Barcellos, D. E. S. N.; Canal, C. W. Presence of atypical porcine pestivirus (APPV) in Brazilian pigs. TRANSBOUNDARY AND EMERGING DISEASES, v.65, p.22 - 26, 2018

- **Mosena, Ana C. S.**; Weber, Matheus N.; Cibulski, Samuel P.; Silva, Mariana S.; Paim, Willian P.; Silva, Gustavo S.; Medeiros, Antônio A.; Viana, Nathália A.; Baumbach, Letícia F.; Puhl, Daniela E.; Silveira, Simone; Corbellini, Luis G.; Canal, Cláudio W. Survey for pestiviruses in backyard pigs in southern Brazil. JOURNAL OF VETERINARY DIAGNOSTIC INVESTIGATION, v.32, p. 141, 2020

- **Mosena, Ana C. S**.; Da Silva, Mariana S.; Lorenzett, Marina P.; Cibulski, Samuel P.; Weber, Matheus N.; Budaszewski, Renata F.; Mayer, Fabiana Q.; Henker, Luan C.; De Cecco, Bianca S.; Pavarini, Saulo P.; Driemeier, David; Canal, Cláudio W. A new highly divergent copiparvovirus in sheep. ARCHIVES OF VIROLOGY, v.166, p.1517 - 1520, 2021

- **Moséna, Ana Cristina Sbaraini**; Paim, Willian P.; Da Silva, Mariana S.; Weber, Matheus N.; Cibulski, Samuel P.; Puhl, Daniela E.; Baumbach, Letícia F.; Olegário, Juliana C.; Silveira, Simone; Canal, Cláudio W. Detection and genetic characterization of bovine pestivirus between 2016 and 2018 In: **XXIX Brazilian Congress of Virology & XIII Mercosur Meeting of Virology**, 2018, Gramado, RS- Brasil

-**Mosena, A. C. S**; Baumbach, L; Puhl, D. E; Silva, G; Medeiros, A. A; Corbellini, L. G; Canal, C. W. Pesquisa por pestivírus de ruminantes em suínos de subsistência no Estado do Rio Grande do Sul In: **3º Encontro Nacional de Epidemiologia Veterinária**, 2018, Porto Alegre, RS-Brasil. https://doi.org/10.22456/1679-9216.88655

ANEXO C- Produções científicas em co-autoria no doutorado:

- Bianchi, M. V.; Konradt, G.; De Souza, S. O.; Bassuino, D. M.; Silveira, S.; **Mósena, A. C. S.**; Canal, C. W.; Pavarini, S. P.; Driemeier, D. Natural Outbreak of BVDV-1d-Induced Mucosal Disease Lacking Intestinal Lesions. VETERINARY PATHOLOGY, v.54, p.242 - 248, 2017.

-Silveira, S.; Weber, M. N.; **Mósena, A. C. S.**; Da Silva, M. S.; Streck, A. F.; Pescador, C. A.; Flores, E. F.; Weiblen, R.; Driemeier, D.; Ridpath, J. F.; Canal, C. W. Genetic Diversity of Brazilian Bovine Pestiviruses Detected Between 1995 and 2014. TRANSBOUNDARY AND EMERGING DISEASES, v.64, p.613 - 623, 2017.

-Silveira, S.; Baumbach, L. F.; Weber, M. N.; **Mósena, A. C. S.**; Da Silva, M. S.; Cibulski, S. P.; Borba, M. R.; Maia, R. D.; Coimbra, V. C. S.; De Moraes, G. M.; Ridpath, J. F.; Canal, C. W. HoBi-like is the most prevalent ruminant pestivirus in Northeastern Brazil. TRANSBOUNDARY AND EMERGING DISEASES, v.jul, p.1 -, 2017.