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

ESTUDOS EPIDEMIOLÓGICOS, GENÉTICOS E AVALIAÇÃO DE VACINAS PARA PESTIVÍRUS DE RUMINANTES

SIMONE SILVEIRA

Porto Alegre
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Tese apresentada como requisito parcial para obtenção de grau de Doutora em Ciências Veterinárias na área de Medicina Veterinária, subárea Medicina Veterinária Preventiva, especialidade Virologia.

Orientador: CLÁUDIO WAGECK CANAL

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Simone Silveira

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Aprovado em: 22 de fevereiro de 2019

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RESUMO

Para manter a competitividade da bovinocultura brasileira no mercado internacional é fundamental o monitoramento da sanidade animal. Como a Febre Aftosa está erradicada com ou sem vacina em muitos países, os pestivírus de ruminantes têm sido reconhecidos como os vírus que ainda causam mais perdas econômicas em todo mundo. A infecção pode ser desde subclínica até fatal, acometendo o trato respiratório, gastrointestinal ou reprodutivo. As quatro espécies virais principais que causam estas infecções pertencem à família *Flaviviridae,* gênero *Pestivirus,* e são comumente chamadas de: vírus da diarreia viral bovina tipo 1 (BVDV-1), tipo 2 (BVDV-2), vírus da doença da fronteira (BDV) e HoBi-like pestivírus, respectivamente. Cada uma destas espécies pode ainda ser dividida em vários subgenótipos ou grupos genéticos. Essa grande diversidade genética e antigênica dos pestivírus de ruminantes, aliada ao grande número de espécies suscetíveis implica em desafios para o desenvolvimento e aplicação de testes de diagnóstico precisos e de vacinas eficazes. Deste modo, a presente tese objetivou realizar estudos epidemiológicos, genéticos e avaliação de vacinas contra pestivírus de ruminantes. No Capítulo 1, a diversidade genética de pestivírus foi investigada em ruminantes do Rio Grande do Norte e Maranhão através de RT-PCR do soro de animais amostrados para monitoria da Febre Aftosa. O HoBi-like pestivírus foi a única espécie detectada em bovinos, mas nenhum pestivírus foi detectado em pequenos ruminantes. No Capítulo 2, cinco HoBi-like pestivírus brasileiros tiveram o seu genoma completo sequenciado. Análises filogenéticas, genéticas e evolutivas foram realizadas de todos os genomas de HoBi-like pestivírus disponível no GenBank. Essas análises revelaram que o gene da glicoproteína E2 é o mais variável e o que mais sofre seleção positiva, informações estas que podem ser usadas para o desenvolvimento racional de novas vacinas. Os resultados deste estudo também sugerem que o HoBi-like pestivírus pode ter se originado na Ásia e, posteriormente, tenha sido introduzido no Brasil e, deste, foi levado para a Europa. No Capítulo 3, a prevalência de animais soropositivos para pestivírus foi determinada em ovinos do estado do Wyoming, Estados Unidos. Um total de 5,6% dos animais foram considerados positivos, e a maioria destes foram provavelmente infectados pelo BVDV-1. O Capítulo 4 teve como objetivo avaliar a competição viral de cepas dos principais subgenótipos (BVDV-1a, BVDV-1b e BVDV-2a) através de uma técnica *in vitro* denominada de PrimeFlow RNA assay. Cepas de BVDV-2a excluíram cepas de BVDV-1a e BVDV-1b em coinfecções. Estes resultados foram semelhantes aos observados com as mesmas cepas em um estudo prévio *in vivo,* concluindo-se que a técnica utilizada permite, por exemplo, avaliar a competição entre cepas antes destas serem incluídas juntas em vacinas polivalentes, além de auxiliar na elucidação do porquê há cepas que ocorrem com mais frequência a campo. O Capítulo 5 teve como objetivo avaliar a resposta humoral induzida por vacinas de replicon (Vrep) contendo uma cepa de BVDV-1a, BVDV-1b ou BVDV-2a em cada vacina frente a 26 cepas pertencentes a sete subgenótipos de BVDV. As três Vreps induziram anticorpos neutralizantes contra todas as cepas, contudo, a BVDV-1b Vrep foi a vacina que induziu os maiores títulos de anticorpos. Os resultados destes 5 capítulos acrescentam conhecimentos sobre a

diversidade, evolução, biologia e resposta a vacinas de pestivírus de ruminantes. Estes conhecimentos serão importantes para aumentar a nossa habilidade de detectar e controlar esses vírus, por meio de testes de diagnóstico e vacinas mais eficazes utilizadas em programas de controle e erradicação. Portanto, os resultados obtidos servirão de base para adoção de medidas de controle mais racionais e efetivas contra estes vírus que causam um grande impacto econômico na bovinocultura brasileira e mundial.

Palavras-chave: BVDV, HoBi-like, bovinos, ovinos.

ABSTRACT

Animal health monitoring is essential to maintain the competitiveness of Brazilian cattle in the international market. Since the Foot-and-mouth Disease is eradicated with or without vaccination in many countries, ruminant pestiviruses have been recognized as the viruses that still cause the most economic losses worldwide. The infection could be subclinical or even fatal, affecting the respiratory, gastrointestinal and reproductive tract. The four major viral species that cause this infection belong to the family Flaviviridae, pestivirus genus, which are formerly known as: bovine viral diarrhea virus type 1 (BVDV-1), type 2 (BVDV-2), border disease virus (BDV) and HoBi-like pestivirus, respectively. Besides, each species can be further segregated in several subgenotypes or genetic groups. This great genetic and antigenic variability of the ruminant pestiviruses, alongside with the broad range of susceptible host species, implies challenges for the development and application of accurate diagnostic tests and effective vaccines. Thus, the current thesis aimed to perform epidemiologic, genetic studies and evaluation of vaccines against ruminant pestivirus. In the first study, the genetic diversity of pestivirus was investigated in ruminants from Rio Grande do Norte e Maranhão States. All isolates were classified as HoBi-like pestivirus. All small ruminant samples tested negative. In the second study, genome of five Brazilian HoBi-like pestiviruses were sequenced. Beyond that, phylogenetic, genetic and evolutive analyses were performed based on all HoBi-like pestivirus, whose full-length genome sequences were available in a public database. The surface glycoprotein E2 encodes the most variable gene which also shows the greater number of sites under positive selection, data useful for the rational development of new vaccines. Phylogenetic inference suggests the HoBi-like pestivirus may have originated in Asia, specifically in India, and after it was introduced in Brazil and later spread to Europe. In the third study, the pestivirus antibody prevalence was assessed in sheep from Wyoming State, United States. The overall seroprevalence was 5.6%. It was suggested that sheep flocks were likely exposed to BVDV-1. The fourth study aimed to evaluate the competitive fitness of BVDV-1a, BVDV-1b and BVDV-2a strains in vitro using the PrimeFlow RNA assay. The BVDV-2a strains eliminated the BVDV-1a and BVDV-1b strains in coinfections. These results were similar to those observed with the same strains in a previous in vivo study. Then, this technique allows, for example, the evaluation of the competition between strains before they are included together in vaccines. In addition to this technique may help elucidate why there are virus strains that occur more frequently in the field. The fifth study assessed the humoral immune response induced by virus replicon (Vrep) vaccines based on BVDV-1a, 1b and BVDV-2a strains against 26 representative BVDV strains of seven subgenotypes. The three Vrep vaccines induced neutralizing antibodies against all strains. However, the BVDV-1b-Vrep vaccinated animals had higher titers than those vaccinated with BVDV-1a and BVDV-2a Vrep and similar to those elicited by a modified-live virus (MLV) vaccine. The results presented herein add knowledge on diversity, evolution, biology and response to ruminant pestivirus vaccines. This knowledge will be important to increase our ability to detect and control these viruses, through more accurate and effective diagnostic tests and vaccines

used in control and eradication programs. Therefore, the results obtained will serve as a basis for the adoption of more rational and effective control measures against these viruses that cause a great economic impact on Brazilian and worldwide cattle.

Keywords: *BVDV, HoBi-like, cattle, sheep*

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APV:	Vírus Pronghorn
APPV:	Vírus atipíco de suínos
5'UTR:	Região 5' não traduzida
BD:	Doença da fronteira
BDV:	Vírus da doença da fronteira
BVDV:	Vírus da diarreia viral bovina
BVDV-1:	Vírus da diarreia viral bovina tipo 1
BVDV-2:	Vírus da diarreia viral bovina tipo 2
cp:	Citopático
CSFV:	Vírus da peste suína clássica
DM:	Doença das mucosas
ELISA:	Ensaio imunoenzimático
EUA:	Estados Unidos da América
GPV:	Pestivírus de girafa
HS:	Síndrome hemorrágica aguda
ICTV:	Comitê Internacional de Taxonomia Viral
IHC:	Imunohistoquímica
IRES:	Sítio interno de entrada ribossomal
kb:	Kilobases
KV:	Vacina com vírus inativado
MA:	Maranhão
MLV:	Vacina com vírus vivo modificado
NADC-USDA:	*National Animal Disease Center – United States Department of Agriculture*
ncp:	Não-citopático
nm:	Nanômetros
N^{pro}:	Autoprotease N terminal
NrPV:	Pestivírus de ratos
ORF:	Fase de leitura aberta
PB:	Paraíba
PCR:	Reação em cadeia da polimerase

PI: Persistentemente infectado

RaPestV: Pestivírus de morcego

RNA: Ácido ribonucleico

RT-PCR: Transcrição reversa seguida da reação em cadeia da polimerase

RT-qPCR: Transcrição reversa seguida da reação em cadeia da polimerase quantitativa

SFB: Soro fetal bovino

SN: Soroneutralização

UTR: Região não traduzida

SUMÁRIO

1. INTRODUÇÃO

As infecções em ruminantes causadas por pestivírus resultam em grandes perdas econômicas em todo mundo. Estas podem variar de subclínicas até fatais e podem envolver sinais clínicos respiratórios, reprodutivos e digestivos (MACLACHLAN; DUBOVI, 2011). As quatro espécies virais principais que causam estas infecções pertencem à família *Flaviviridae,* gênero *Pestivirus,* são: *Pestivirus A*, *Pestivirus B, Pestivirus D* e *Pestivirus H*. No entanto, elas são comumente chamadas de: vírus da diarreia viral bovina tipo 1 (BVDV-1) e tipo 2 (BVDV-2), vírus da doença da fronteira (BDV) e HoBi-like pestivírus, respectivamente (SMITH et al., 2017).

A bovinocultura é um dos destaques do agronegócio brasileiro no cenário mundial. Uma vez que o País tem o maior rebanho bovino comercial do mundo, com mais de 232 milhões de animais, é o maior exportador de carne bovina e o quarto de leite. Ademais, o Brasil tem o 22° maior rebanho de caprinos do mundo e o 18º maior de ovinos (MARTINS et al., 2016; USDA-FAS, 2018). Para manter e melhorar esta competitividade no mercado internacional é fundamental o monitoramento da sanidade animal e a aplicação de programas sanitários adequados e eficientes, especialmente em relação às doenças virais que causam grande impacto na produtividade a exemplo daquelas causadas por pestivírus em ruminantes. Apesar desses vírus serem detectados há décadas no Brasil, as informações acerca da diversidade viral são provenientes principalmente do sul do País e de rebanhos bovinos (SILVEIRA et al., 2017; WEBER et al., 2014a). Deste modo, mais conhecimentos precisam ser obtidos sobre quais pestivírus circulam em diferentes regiões brasileiras e não apenas em bovinos, como também nos demais ruminantes suscetíveis a infecção. Isso se faz necessário para determinar possíveis fontes de infecção, para informar programas de controle e para o desenvolvimento de testes de diagnóstico e vacinas (BOOTH et al., 2013; LUZZAGO et al., 2014; YILMAZ et al., 2012). O Brasil ainda não possui nenhum programa nacional ou regional de controle e erradicação de pestivírus de ruminantes. Além disso, agravando o quadro, a vacinação é incipiente e realizada de maneira desigual nas diferentes regiões e sistemas de produção (WEBER et al., 2014b).

Ao contrário do Brasil, os Estados Unidos (EUA) possuem um conhecimento mais aprofundado sobre quais pestivírus circulam em bovinos no país, além do engajamento ser maior no controle de BVDV. O país é um dos líderes em produção animal, têm o

quarto maior rebanho mundial de bovinos com 94 milhões de cabeças, é o maior produtor de carne bovina e o quarto maior exportador da mesma. Apesar de não ter grande destaque no cenário internacional de criação de ovinos, a ovinocultura é praticada quase que de forma homogênea em todo o território nacional (USDA-FAS, 2018; USDA-NASS, 2018).

Entretanto, este país também enfrenta desafios e alguns dos mais urgentes para serem resolvidos são: explorar a diversidade de pestivírus em outros hospedeiros, como em ovinos e animais silvestres e desenvolver vacinas mais eficientes. Essas necessidades ocorrem devido a possibilidade de transmissão viral interespécie, o que pode ter um grande impacto para a saúde animal e controle de BVDV, e pela falha das vacinas em eliminar o BVDV dos rebanhos, o que é causado em parte pela ampla diversidade genética e antigênica dos isolados e a presença dos animais persistentemente infectados. Consequentemente, vacinas que forneçam uma proteção mais ampla, que sejam eficientes contra uma maior diversidade de tipos de pestivírus, que evitem a infecção fetal e que sejam seguras são altamente requeridas (RIDPATH et al., 2009; WORKMAN et al., 2016).

Além do conhecimento sobre quais pestivírus circulam em um determinado país ou região e em quais frequências, entender sobre a biologia viral é imprescindível. Como exemplos pode-se citar o estudo do *fitness* viral de diferentes isolados, como eles se relacionam em coinfecções, o porquê e como eles evoluem e se dispersam. Esses discernimentos podem ajudar a explicar variações na predominância e na localização geográfica dos pestivírus detectados, mudanças na patogênese e resposta imune. O objetivo final desses estudos sobre os pestivírus convergem em aumentar a nossa habilidade de detectar e controlar esses vírus (PETERHANS; SCHWEIZER, 2014; RIDPATH, 2010).

A partir dos problemas e questionamentos acima abordados, o presente doutorado objetivou realizar estudos epidemiológicos, genéticos e evolutivos de pestivírus de ruminantes, com foco naqueles detectados no Brasil e nos Estados Unidos, além de desenvolver e avaliar vacinas para pestivírus. Deste modo a tese será introduzida por um referencial teórico, e posteriormente, apresentada na forma de cinco artigos científicos.

2. REVISÃO BIBLIOGRÁFICA

2.1 Classificação e história dos pestivírus de ruminantes

O gênero *Pestivirus* pertence à família *Flaviviridae* e é composto por onze espécies virais reconhecidas pelo Comitê Internacional de Taxonomia Viral (ICTV) (Tabela 1). Seis espécies têm sido detectadas em ruminantes e são comumente nomeadas como BVDV-1, BVDV-2, BDV, pestivírus de Girafa, HoBi-like pestivírus, Aydin-like pestivírus e APV (SIMMONDS et al., 2011). A figura 1 representa a relação filogenética entre as espécies de Pestivírus.

Tabela 1 – Espécies de pestivírus segundo o ICTV, seguidos da nomenclatura usual, hospedeiro e doença que causam.

Espécie	Nomenclatura usual	Hospedeiro	Doença	Primeira Referência
*Pestivirus A**	BVDV-1 (vírus da diarreia viral bovina tipo 1)	Bovinos, ovinos, outros ruminantes, suínos	Diarreia viral bovina / doença das mucosas (BVD/DM)	OLAFSON; MacCALLUM; FOX, 1946.
*Pestivirus B**	BVDV-2 (vírus da diarreia viral bovina tipo 2)	Bovinos, ovinos, outros ruminantes, suínos	BVD/DM	CORAPI; FRENCH; DUBOVI, 1989
Pestivirus C	CSFV (vírus da peste suína clássica)	Suínos	Peste suína clássica	SALMON,1885
*Pestivirus D**	BDV (vírus da doença da fronteira)	Ovinos, outros ruminantes, suínos	Doença da fronteira	HUGHES; KERSHAW; SHAW, 1959
Pestivirus E	APV (Vírus Pronghorn)	Antílope	Desconhecida	VILCEK et al., 2005a
Pestivirus F	Bungowannah	Suínos	Miocardite	KIRKLAND et al., 2007
*Pestivirus G**	GPV (Pestivírus de girafa)	Girafas, bovinos	DM-like (girafa) / desconhecida (bovina)	AVALOS-RAMIREZ et al., 2001a
*Pestivirus H**	HoBi-like pestivírus	Bovinos, ovinos, búfalos	BVD/DM	SCHIRRMEIER et al., 2004
*Pestivirus I**	Aydin-like pestivírus	Ovinos, caprinos	Aborto, malformações	OGUZOGLU et al., 2009

Pestivirus J	Pestivirus de rato	Ratos	Desconhecida	FIRTH et al., 2014
Pestivirus K	APPV (Pestivírus atípico suíno)	Suínos	Tremor congênito	HAUSE; HAUSE, 2015

* Detectado em ruminantes de interesse econômico (bovinos, pequenos ruminantes).

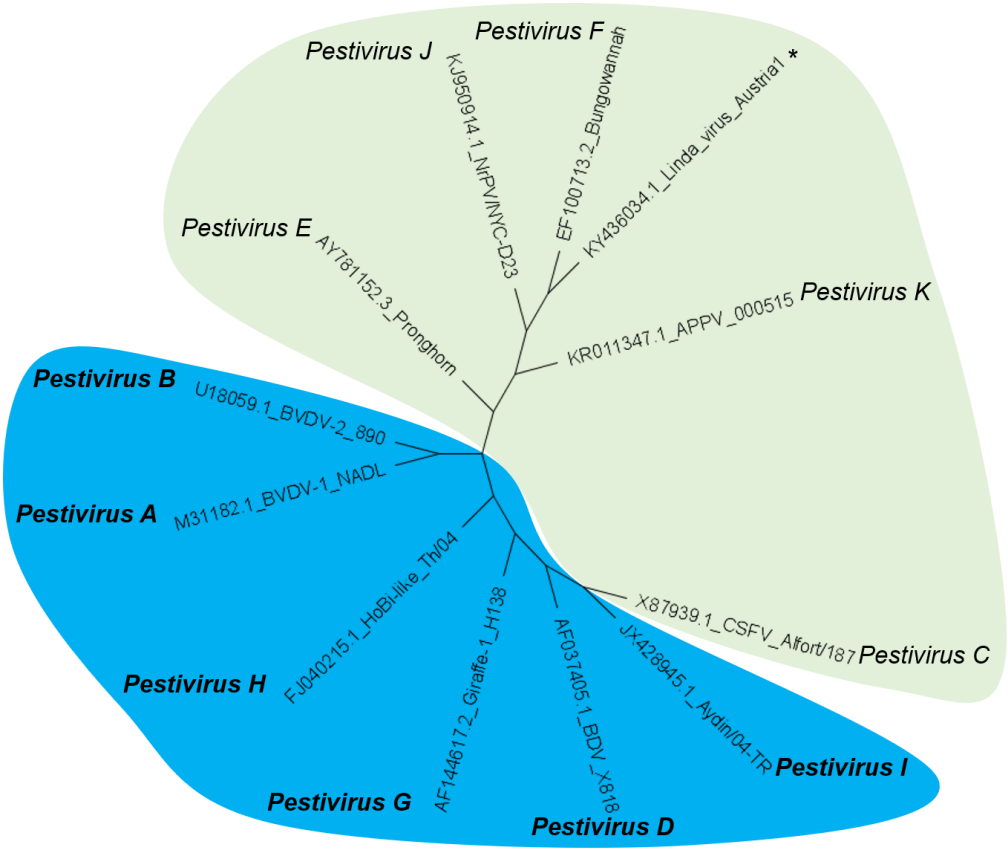

Figura 1: Árvore filogenética baseada na sequência completa do genoma das cepas protótipos das espécies de pestivírus (10102nt). As espécies que já foram detectadas em ruminantes estão em negrito. * Pestivírus ainda não classificado como espécie. O software MEGA 10 foi usado para a inferência filogenética de acordo com o método de Maximum Likelihood.

O primeiro pestivírus de ruminante que foi associado a doença foi relatado em 1946, quando uma nova doença transmissível em bovinos foi descrita nos Estados Unidos. Esta doença foi caracterizada por leucopenia, pirexia, depressão, diarreia, anorexia, lesões gastrointestinais e hemorragias (OLAFSON; MacCALLUM; FOX, 1946). O agente etiológico foi isolado em 1957 e nomeado como BVDV e a doença como BVD (LEE; GILLESPIE, 1957). Uma doença similar causada também pelo BVDV foi observada no Canadá, porém, esta se apresentava com mais severidade e maior taxa de mortalidade e foi denominada de doença das mucosas (CHILDS, 1946; RAMSEY; CHIVERS, 1953).

Já o BDV foi descrito pela primeira vez em 1959 causando doença em ovinos na fronteira entre a Inglaterra e o País de Gales. Essa enfermidade ficou conhecida como a

doença da fronteira (BD) e era caracterizada pelas perdas reprodutivas e pós-natais em ovinos e cordeiros apresentando tremores e cobertura escassa e anormal de lã (HUGHES; KERSHAW; SHAW, 1959).

No Quênia, em 1967, um terceiro pestivírus foi isolado de girafa (GPV) (AVALOS-RAMIREZ et al., 2001b). No entanto, após 1967, só houve mais uma detecção desse vírus, que ocorreu na década de 90 a partir de um cultivo celular bovino inoculado com *Theileria spp*., também proveniente do Quênia (SCHWEIZER; PETERHANS, 2014).

Um pouco mais tarde, em 1973, decidiu-se denominar como pestivírus um grupo de vírus geneticamente e antigenicamente similares, composto pelas espécies: BVDV, CSFV e BDV (HORZINEK, 1973). Mais tarde, a análise filogenética de isolados identificados como BVDV, revelou tratar-se de duas espécies, BVDV-1 e BVDV-2 (RIDPATH; BOLIN; DUBOVI, 1994). A espécie BVDV-2 foi inicialmente identificada a partir do final da década de 80 na América do Norte, em surtos de BVD aguda e hemorrágica (CARMAN et al., 1981; CORAPI; FRENCH; DUBOVI, 1989).

Após os anos 2000, outros três pestivírus foram identificados em bovinos e em pequenos ruminantes (HoBi-like, Aydin, pestivírus da Tunísia) e um pestivírus em ruminantes silvestres (APV). Em 2004, o HoBi-like vírus foi relatado pela primeira vez, decorrente da sua detecção em um lote brasileiro de soro fetal bovino (SFB) comercializado na Alemanha (SCHIRRMEIER et al., 2004). No ano seguinte descobriu-se um isolado mais antigo de HoBi-like vírus, detectado em uma amostra que havia sido coletada no final dos anos 90 de um búfalo doente no Brasil (STALDER et al., 2005). Subsequentemente, esse vírus tem sido detectado tanto como contaminante de SFB, quanto causando doença similar a BVD em bovinos na Ásia (STAHL et al., 2007), Europa (DECARO et al., 2011) e no Brasil (CORTEZ et al., 2006); e mais recentemente em ovinos (SHI et al., 2016).

Em 2005, o vírus Pronghorn (APV) foi detectado em um antílope Pronghorn cego nos Estados Unidos (VILCEK et al., 2005c). Por mais de uma década, não houve detecções do APV. Contudo, em um estudo de amostras coletadas de animais silvestres entre 2011 e 2014, provenientes dos Estados Unidos, esse pestivírus foi detectado em veado-mula, carneiro-selvagem e em cabra-das-rochosas (artigo ainda não publicado). No entanto, não se sabe ainda se o APV pode infectar espécies domésticas e qual o real impacto em animais silvestres (RIDPATH, 2015).

Ainda em 2005, um pestivírus atípico, ainda não classificado como espécie, foi identificado como contaminante de vacinas e também em ovinos na Tunísia; associado a surtos similares a doença da fronteira (THABTI et al., 2005). O mesmo vírus foi detectado posteriormente causando doença em ovinos na França e Itália, e em caprinos na Itália (CIULLI et al., 2017; DUBOIS et al., 2008; MARTIN et al., 2015). Mais tarde na Turquia, outro pestivírus, chamado de Aydin, foi detectado em pequenos ruminantes domésticos, tanto associado a sinais clínico, como em animais aparentemente saudáveis (OGUZOGLU et al., 2009).

Além dos pestivírus citados que emergiram em ruminantes na última década, outros emergiram em hospedeiros como os suínos (HAUSE; HAUSE, 2015; KIRKLAND et al., 2007). Estas descobertas foram possibilitadas pelo advento da melhoria das técnicas moleculares, do sequenciamento de alta performance e de estudos de metagenoma. Assim, em 2017 o ICTV passou a reconhecer mais espécies de pestivírus, aumentando o número de quatro para onze e os nomeando de Pestivírus A-K (SIMMONDS et al., 2011). Porém, alguns pestivírus permanecem não oficializados como espécies, a exemplo do Linda vírus, detectado em suínos (LAMP et al., 2017), do RaPestV em morcegos (WU et al., 2012) e o pestivírus da Tunísia em ruminantes (THABTI et al., 2005), como previamente mencionado.

Com a constante emergência de novos pestivírus, sua conhecida ampla gama de hospedeiros e a capacidade dos pestivírus de cruzar barreiras interespécie, há grande preocupação sobre o impacto desses vírus para a saúde animal e consequente perdas econômicas. Dentre todos os pestivírus identificados em ruminantes, quatro espécies se destacam por apresentarem uma maior distribuição mundial e pelo maior número de pesquisas relacionadas, são elas: BVDV-1, BVDV-2, BDV e HoBi-like vírus. Diferentemente, os demais pestivírus de ruminantes (APV, GPV, Aydin ou atípicos da Tunísia) possuem um número limitado de detecções.

2.2 Caracterização, Genoma e Replicação Dos Pestivírus

Os vírus do gênero *Pestivirus* são esféricos, pequenos, medem entre 40 e 60 nm de diâmetro, possuem nucleocapsídeo icosaédrico e envelope (FIGURA 2) (MACLACHLAN; DUBOVI, 2011; SIMMONDS et al., 2011). O genoma consiste de uma fita linear de RNA de sentido positivo com aproximadamente 12,3 kilobases (kb) de

comprimento e que possui nas extremidades 5’ e 3’ duas regiões não traduzidas (UTR). Esta molécula de RNA codifica somente uma única fase aberta de leitura (ORF) que é traduzida em uma longa poliproteína com aproximadamente quatro mil aminoácidos, que é clivada em 12 proteínas individuais à medida que é traduzida: a autoprotease N terminal ou proteína N (N^{pro}), proteína do capsídeo (C), glicoproteínas do envelope (E^{rns}, E1, E2) e as proteínas não-estruturais (p7, NS2, NS3, NS4A, NS4B, NS5A e NS5B) (FIGURA 3) (SIMMONDS et al., 2011).

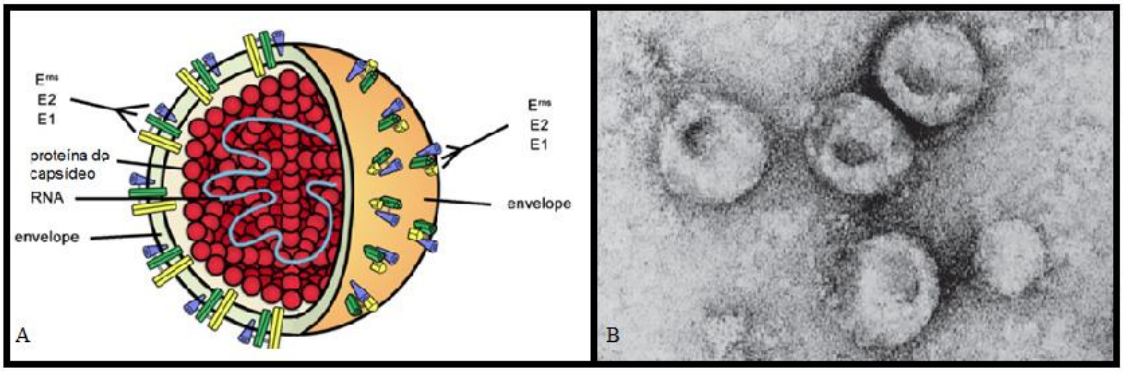

Figura 2. Morfologia e estrutura dos vírus do gênero *Pestivírus*. A) Ilustração esquemática de um vírion com seus principais componentes; B) Foto de microscopia eletrônica de vírions do vírus da diarreia viral bovina. Adaptado: BEER et al., 2007; SIMMONDS et al., 2011.

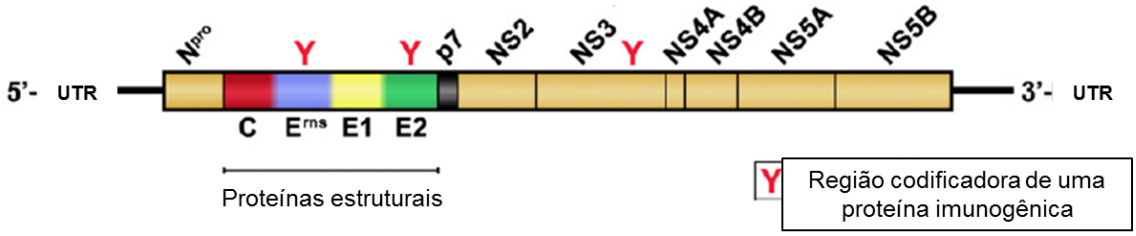

Figura 3. Representação esquemática da estrutura e organização genômica dos pestivírus. Adaptado de BEER et al. (2007).

As UTRs se dobram e formam estruturas secundárias que interagem com proteínas celulares e virais. As UTRs têm função relacionada à regulação da replicação e transcrição do RNA, como também na tradução da ORF. A 5’UTR possui um sítio interno de entrada ribossomal (IRES) que é reconhecido por proteínas celulares que dirigem os ribossomos a se ligar nesta região e iniciar a tradução da poliproteína (NEILL, 2013). Esta região é a mais utilizada para detecção e caracterização pois ela apresenta regiões altamente conservadas que são favoráveis à amplificação por PCR (RIDPATH et al., 2010).

Dentre as proteínas processadas, a N^{pro} é uma proteína autoproteolítica que é responsável por sua liberação da poliproteína. A sua função é relacionada ao bloqueio da produção de interferons. Esta proteína é exclusiva dos pestivírus, altamente conservada e

frequentemente usada em análises filogenéticas (NEILL, 2013; RIDPATH, 2010; WISKERCHEN; BELZER; COLLETT, 1991). A proteína do capsídeo (C) reveste o RNA genômico, para protegê-lo dentro da partícula viral (MURRAY; MARCOTRIGIANO; RICE, 2008). A E^{rns} possui atividade de RNase, sendo importante na depressão do sistema imune do hospedeiro. Esta proteína é secretada na forma solúvel pelas células infectadas e anticorpos gerados contra esta podem ser neutralizantes (NEILL, 2013; RIDPATH, 2010).

Pouco é conhecido sobre a função da E1, no entanto é sabido, que ela precisa formar um heterodímero com a E2 para que o vírus seja infeccioso (NEILL, 2013). A glicoproteína E2 é a região genética e antigênica mais variável dos pestivírus, possuindo os principais epítopos antigênicos neutralizantes, assim sendo o maior alvo da resposta imune humoral protetora. Muitos anticorpos produzidos contra E2 são neutralizantes e efetivamente inibem a infecção. Ademais, a E2 media a ligação ao receptor celular, o CD46, portanto, é o maior determinante do tropismo celular (DEREGT et al., 1998; DONIS; CORAPI; DUBOVI, 1988; LIANG et al., 2003; PATON; LOWINGS; BARRETT, 1992; RONECKER et al., 2008).

Por fim, a proteína p7 é necessária para a patogenicidade viral e importante na montagem e egresso do vírion, facilitando a migração do vírus de célula a célula (GLADUE et al., 2012). As proteínas NS2 e NS3 podem ser encontradas individualizadas ou como um único polipeptídeo. Elas são necessárias para a replicação e viabilidade viral, mas também exercem função na clivagem da poliproteína viral (LACKNER et al., 2004; LI et al., 2013; TAUTZ et al., 1997; WARRENER; COLLETT, 1995). A proteína NS4a é um co-fator da NS3. As proteínas NS4b, NS5a e NS5b são componentes do complexo de replicação viral. Além disso, a NS4b é envolvida no rearranjo da membrana celular na célula infectada; NS5a na tradução das proteínas e montagem do vírion através da interação com proteínas virais e do hospedeiro (LING et al., 2018); e a NS5b tem função de RNA polimerase e também está envolvida com a montagem do vírion (ANSARI et al., 2004; WEISKIRCHER et al., 2009; ZHONG; GUTSHALL; DELVECCHIO, 1998).

A infecção da célula inicia após a ligação do heterodímero E1-E2 aos receptores presentes na superfície celular. O vírus é então internalizado por endocitose dependente de clatrina, desencapsidado e o RNA é liberado no citoplasma, onde ocorrem a transcrição e a tradução. Na replicação, ocorre a síntese de um RNA complementar de sentido negativo que serve como um molde para a síntese do RNA genômico. Este RNA de

sentido positivo é traduzido em uma poliproteína que é auto clivada e forma as proteínas estruturais e não-estruturais. A partícula é montada e maturada no retículo endoplasmático e no complexo de Golgi e, por fim, o vírion é liberado via exocitose (MACLACHLAN; DUBOVI, 2011; NEILL, 2013; SIMMONDS et al., 2011).

2.3 Evolução

Como os pestivírus são vírus de RNA de fita simples com uma RNA polimerase que não possui capacidade de correção, os nucleotídeos que são incorporados erroneamente introduzem mutações. Essas, junto com os eventos de recombinação homóloga e heteróloga do genoma viral levam a alta variabilidade genética e mudanças associadas ao tropismo, gama de hospedeiros, patôgenese e virulência dos pestivírus (DOMINGO, 1997; NAGAI et al., 2004; PETERHANS et al., 2010).

As inúmeras mutações que surgem durante a replicação viral produzem uma população viral no hospedeiro, denominada quasispécie, que consiste em um conjunto de vírus com genomas similares, que interagem cooperativamente em nível funcional e coletivamente contribuem para as características da população (DOMINGO; SHELDON; PERALES, 2012; LAURING; FRYDMAN; ANDINO, 2013). A evolução de quasispécies é influenciada pelas altas taxas de mutação durante a replicação viral. Quasispécies são fontes de adaptabilidade viral, porque elas constituem-se em repositórios dinâmicos de variantes virais genotípicas e fenotípicas que surgem espontaneamente ou em resposta a pressões seletivas internas ou externais, tais como a resposta imune inata e adaptativa (DOMINGO; SHELDON; PERALES, 2012; SCHWEIZER; PETERHANS, 2014).

Quasispécies já foram descritas em animais infectados com BVDV e HoBi-like vírus, nos quais a variação da dominância e composição viral foi observada ao longo do tempo, de hospedeiro para hospedeiro e entre diferentes órgãos de um mesmo animal (COLLINS; DESPORT; BROWNLIE, 1999; DOW et al., 2015; WEBER; JONES; ZANDOMENI, 2002; WEBER et al., 2016a, 2017).

Fenômenos evolutivos como as quasispécies levam ao surgimento de diferentes grupos genéticos, espécies, genótipos e subgenótipos. Esta diversidade genética pode ser benéfica para a sobrevivência viral, contudo, pode causar dificuldades na detecção e diferenciação destes vírus (RIDPATH, 2003). Além disso, discrepâncias observadas em

análises filogenéticas de pestivírus sugerem que a ocorrência de recombinação seja um fenômeno frequente (LIU et al., 2010; WEBER et al., 2015). Eventos de recombinação homóloga já foram evidenciados *in silico* entre subgenótipos de BVDV-1 e BVDV-2. Estes eventos podem desempenhar um importante papel na evolução e diversificação viral, no entanto podem representar um desafio para estudos filogenéticos e taxonômicos (HE et al., 2007; JONES; WEBER, 2004). Entretanto, para ocorrer recombinação, duas variantes virais precisam infectar a mesma célula simultaneamente. Além disto, para o vírus recombinante se tornar a variante predominante, ele precisa estar viável e apresentar vantagens em relação às cepas parentais para poder competir com elas e se sobrepor (JONES; WEBER, 2004). Apesar da baixa probabilidade de ocorrer recombinação homóloga, ela pode ser facilitada devido a endemicidade do BVDV em muitos países e pela presença de animais persistentemente infectados (KŐVÁGÓ et al., 2016).

A recombinação também pode levar ao surgimento de vírus citopáticos (cp), que são aqueles que causam efeito citopático em cultivo celular. Enquanto que os vírus cp são raros, os vírus não-citopáticos (ncp) predominam na natureza. Os vírus cp surgem de cepas ncp através de vários eventos genéticos, além da recombinação já citada, têm-se inserção de sequências celulares, duplicações, deleções, mutações pontuais e rearranjos. Esses eventos genéticos resultam na clivagem da proteína NS2/3 gerando a NS2 e NS3 individualizadas. Essa superinfecção com uma cepa cp que é homóloga a cepa ncp, com a qual os animais foram primeiramente infectados, origina a doença das mucosas, uma forma fatal da infecção por pestivírus em ruminantes (MACLACHLAN; DUBOVI, 2011; NEILL, 2013; SIMMONDS et al., 2011).

Assim a diversidade viral é decorrente de mutações e recombinações no genoma dos pestivírus e, juntamente com a evolução viral, são influenciadas e dirigidas por múltiplos fatores relacionados ao hospedeiro, ao tipo de infecção e como resposta ao sistema imune ou às vacinas (CHERNICK et al., 2018; DOW et al., 2015; KUCA et al., 2018; NEILL et al., 2012; WEBER et al., 2016a). Consequentemente, a variabilidade genética é diferente ao longo do genoma e proteínas estruturais tendem a mudar mais do que as não-estruturais (CHERNICK et al., 2018; NEILL et al., 2012), o que ocorre principalmente com o gene que codifica a E2, cujas mudanças têm sido associadas a mudança de hospedeiro e ao escape do sistema imune (KUCA, et al., 2018; NEILL et al., 2015, WEBER et al., 2017).

2.4 Diversidade Genética e Diversidade Antigênica

Os pestivírus apresentam uma considerável diversidade genética (NEILL, 2013). Dentre os principais pestivírus que infectam ruminantes (BVDV-1, BVDV-2, BDV, HoBi-like), 21 subgenótipos de BVDV-1 já foram descritos, (1a a 1u), quatro subgenótipos de BVDV-2 (2a a 2d) (YEŞILBAĞ; ALPAY; BECHER, 2017), oito genótipos de BDV (1 a 8), (ARNAL et al., 2004; BECHER et al., 2003; DUBOIS et al., 2008; GIAMMARIOLI et al., 2011; PELETTO et al., 2016; VILČEK et al., 1997) e quatro grupos genéticos de HoBi-like vírus (3a a 3d) (GIAMMARIOLI et al., 2015a).

As análises filogenéticas constituim-se em ferramentas úteis para estudar a diversidade genética e desvendar as histórias evolutivas dos microorganismos. No caso dos pestivírus as análises filogenéticas têm sido embasada principalmente na comparação de sequências de nucleotídeos da região 5'UTR, N^{pro} e E2 (BOOTH et al., 2013; LIU et al., 2010; OĞUZOĞLU et al., 2012; PECORA et al., 2014; TAJIMA et al., 2001; VILČEK et al., 2001). O conhecimento gerado sobre a diversidade de pestivírus é importante para determinar relações evolutivas entre cepas, possíveis fontes de infecção, para informar programas de controle, ou ainda para o desenvolvimento de testes de diagnóstico e vacinas mais eficientes (BOOTH et al., 2013; GIAMMARIOLI et al., 2015b; YILMAZ et al., 2012).

Os pestivírus são antigenicamente relacionados e apresentam reatividade cruzada em teste de soro-neutralização (SN) (SIMMONDS et al., 2011). Contudo, quanto maior a diferença genética entre os pestivírus, menor é a reatividade cruzada. Essa diversidade antigênica tem sido observada, através testes de SN usando antissoro ou painéis de anticorpos monoclonais, entre as espécies de pestivírus (BVDV-1, BVDV-2, BDV, HoBi-like), entre os subgenótipos e genótipos, e inclusive entre cepas de um mesmo grupo genético. Além disso, foi verificado que o BVDV-1 e 2 são mais parecidos antigenicamente entre si do que com HoBi-like pestivírus e BDV (BACHOFEN et al., 2008; BAUERMANN; FLORES; RIDPATH, 2012; BECHER et al., 2003; BEHERA et al., 2011; GIAMMARIOLI et al., 2011; MINAMI et al., 2011; NAGAI et al., 2001; PECORA et al., 2014; PIZARRO-LUCERO et al., 2006; RIDPATH et al., 2010). O significado prático destas diferenças antigênicas já foi evidenciado, por exemplo, por falhas vacinais em animais imunizados com vacinas contendo cepas de BVDV-1 e que não foram eficazes em evitar a infecção experimental com cepas de BVDV-2 (RIDPATH;

BOLIN; DUBOVI, 1994). Em outro estudo, uma vacina contendo BVDV-1a induziu baixo nível de anticorpos contra BVDV-1b (FULTON et al., 2003).

O mesmo foi verificado em um estudo com uma vacina de BDV, a qual induziu uma resposta humoral fraca e uma proteção parcial contra infecção por BVDV (VANTSIS et al., 1980). Também já foi relatado que vacinas com BVDV-1 e BVDV-2 induziram um título baixo de anticorpos neutralizantes contra o HoBi-like pestivírus, o que pode não conferir a proteção necessária contra infecção por esse pestivírus (BAUERMANN et al., 2013a; DECARO et al., 2013; DIAS et al., 2017). Este questionamento também foi abordado após um estudo *in vivo* que verificou que a imunidade desenvolvida por uma vaca após a gestação de progênie persistentemente infectada (PI) por BVDV não foi suficiente para prevenir uma subsequente infecção fetal causada por HoBi-like pestivírus (BAUERMANN; FALKENBERG; RIDPATH, 2017).

2.5 Epidemiologia dos Pestivírus de Ruminantes

2.5.1 Hospedeiros

O BVDV e o HoBi-like vírus tem como hospedeiro natural os bovinos e o BDV os ovinos (BAUERMANN et al., 2013b; GRONDAHL et al., 2003). Embora a classificação clássica dos pestivírus se referia à espécie hospedeira de origem e a doença que estes ocasionavam, vários estudos têm comprovado que os pestivírus não são estritamente espécie-específicos e infectam, além da espécie de origem, outros animais domésticos e silvestres (SAKODA et al., 1999; THABTI et al., 2005; VILCEK et al., 2005b). O BVDV-1, BVDV-2 e BDV têm sido detectados em mais de 50 espécies da ordem Artiodáctila, classe Mammalia (BECHER et al., 1997, 1999; GRONDAHL et al., 2003; NETTLETON, 1990; PASSLER; WALZ, 2009; TERPSTRA; WENSVOORT, 1988; THABTI et al., 2005; VAN CAMPEN et al., 2001; VILČEK et al., 1997; VILČEK; BELÁK, 1996; VILČEK; NETTLETON, 2006). O HoBi-like vírus além de ter sido detectado em bovinos, também já foi identificado em búfalos e pequenos ruminantes (SHI et al., 2016; STALDER et al., 2005).

Infecções com pestivirus em várias espécies animais pode ocorrer amplamente na natureza, com estes os vírus podem disseminar-se entre animais de vida livre e animais de criação, o que tem grande importância epidemiológica (VILČEK; BELÁK, 1996; VILČEK; NETTLETON, 2006). Por exemplo, a infecção de hospedeiros que não sejam

bovinos (ex: animais silvestres) com o potencial de atuarem como reservatórios do vírus, pode ser um obstáculo para o sucesso na implantação de estratégias de controle de BVDV em bovinos (BRODERSEN, 2014; PASSLER; WALZ, 2009).

2.5.2 Distribuição Viral

BVDV é uma dos agentes infecciosos mais disseminados em bovinos mundialmente, com uma alta prevalência em quase todas as populações estudadas. Notavelmente, o BVDV pode ser um dos vírus que habita animais de vida terrestre mais amplamente distribuídos, juntamente com o BDV (SCHWEIZER; PETERHANS, 2014; YEŞILBAĞ; ALPAY; BECHER, 2017).

Estudos têm mostrado que o BVDV-1 é mais frequente no mundo do que o BVDV-2, e que vários subgenótipos predominam em diferentes países. O subgenótipo com o maior número de deteções no mundo é o BVDV-1b, seguido de BVDV-1a, BVDV-1c e BVDV-2a. Enquanto o BVDV-1b predomina na Europa, América e Ásia, o BVDV-1c predomina na Oceania. Em relação a África, poucos artigos têm sido publicados sobre a diversidade de BVDV nesse continente. Alguns países europeus e a China possuem uma grande diversidade genética de BVDV, com subgenótipos que circulam exclusivamente em animais desses países. Ao contrário, a Austrália, África e América possuem uma variabilidade limitada de BVDV, com exceção do Brasil. Por mais que alguns estudos possam não refletir a distribuição precisa de subgenótipos nos países e continentes, eles fornecem uma estimativa da frequência dos vários subgenótipos e de quais estão circulando (YESILBAG et al., 2017). Esse desconhecimento e imprecisão sobre a diversidade genética de BVDV acontece devido ao número limitado de pesquisas em alguns países e regiões e também ao tipo de metodologia empregada nesses estudos.

Há menos informações sobre o BDV que sobre o BVDV, pois ele é menos detectado e estudado. A maioria dos trabalhos baseiam-se em sorologia, mostrando que as taxas de soroprevalência de BDV em ovinos variam entre 0,5-50% entre países e entre regiões (EVANS et al., 2018; FERNÁNDEZ-AGUILAR et al., 2016; KRAMETER^FRÖTSCHER et al., 2007; OIE, 2017; O'NEILL; O'CONNOR; O'REILLY, 2004; PANIAGUA et al., 2016). Porém, em vários estudos, observou-se que os ovinos e caprinos possuíam títulos maiores de anticorpos e/ou em uma maior frequência contra BVDV do que contra o BDV (BRAUN et al., 2013; CZOPOWIZC et

al., 2011; GRAHAM et al., 2001; KRAMETER^FRÖTSCHER et al., 2007; PRATELLI et al., 2001; SCHIEFER et al., 2006; SCHLEINER et al., 2006). O BDV tem sido identificado majoritariamente na Europa, principalmente em ovinos, mas também em camurça (*Rupricapra rupicapra*) e em caprinos (ARNAL et al., 2004; CARUSO et al., 2017; DE MIA et al., 2005; DUBOIS et al., 2008; GIAMMARIOLI et al., 2011; STALDER et al., 2017).

Poucas detecções do BDV têm ocorrido fora do continente europeu (SULLIVAN; CHANG; AKKINA, 1997; GÓMEZ-ROMERO et al., 2018), ou seja, a maioria dos isolados de BDV foram identificados em áreas onde os pequenos ruminantes eram criados isolados de outras espécies. Em regiões onde há um contato próximo de pequenos ruminantes e bovinos, o BVDV-1, BVDV-2 e também o HoBi-like pestivírus têm sido isolados de pequenos ruminantes (ABDEL-LATIF et al., 2013; BEHERA et al., 2011; FROETSCHER et al., 2006; HAN et al., 2016; MISHRA et al., 2012; OIE, 2017; SHI et al., 2016). Enquanto que os ovinos parecem ser frequentemente infectados com pestivírus que comumente circulam em bovinos, os bovinos não parecem muito propensos a infecção por BDV (RUSSELL et al., 2017).

O HoBi-like pestivírus tem sido identificado principalmente em bovinos, mas também em pequenos ruminantes e como contaminantes de SFB, provenientes do Brasil, Itália, Tailândia, Bangladesh, Austrália, Peru e da Índia (BERGNER et al., 2018; CORTEZ et al., 2006; DECARO et al., 2011; HAIDER et al., 2014; LIU et al., 2009; MAO et al., 2012; MISHRA et al., 2014; STAHL et al., 2007; WEBER et al., 2016b). A distribuição dos 4 grupos genéticos (3a-3d) de HoBi-like pestivírus se dá conforme diferenças geográficas. O grupo 3a consiste em todas as cepas isoladas na Europa, na América do Sul e como contaminantes de SFB. O grupo 3b é formado por vírus detectados em Bangladesh e os grupos 3c e 3d são de cepas que circulam na Índia (GIAMMARIOLI et al., 2015a).

2.5.3 Transmissão

A transmissão viral pode ocorrer horizontalmente, por contato direto ou indireto, ou ainda verticalmente. A rota mais comum de transmissão é o contato direto entre animais, principalmente entre PIs e animais suscetíveis, via oro-nasal. Entretanto, várias maneiras de transmissão indireta têm sido demonstradas: agulhas, luvas para palpação retal, vacinas contaminadas, soro fetal bovino, semen, transferência de embriões e

inseminação artificial (GIVENS et al., 2003; HOUE, 1999; LINDBERG et al., 2006; MACLACHLAN; DUBOVI, 2011). A introdução do vírus em um rebanho e a prevalência da infecção são associadas com determinantes epidemiológicos como: densidade animal, comércio de animais, práticas de pastagem que permitem contato com outras hospedeiros suscetíveis, a exemplo dos animais silvestres (ARNAL et al., 2004; CARUSO et al., 2016; HOUE, 1999; LANYON; REICHEL, 2014; LINDBERG; HOUE, 2005; NETLETTON, WILLOUGHBY, 2007).

Porém, a forma mais eficiente de introdução e disseminação viral no rebanho ocorre pela compra de animais PI ou fêmeas gestando fetos PI, coloquialmente conhecidas como “vacas de Tróia”. Touros também podem desempenhar um papel importante de disseminação viral, pois podem apresentar uma infecção prolongada no aparelho reprodutor e disseminar o vírus através do sêmen por meses (GIVENS et al., 2003; NEWCOMER et al., 2014).

2.6 Patogenia e Sinais Clínicos dos Pestivírus em Ruminantes

A infecção por pestivírus em ruminantes apresenta-se com uma ampla gama de apresentações clínicas, que não são patognomônicas e podem ocorrer de forma atípica (BIANCHI et al., 2017; FULTON et al., 2017; LANYON et al., 2014). Embora o termo “diarreia” componha o nome da doença em bovinos, sinais respiratórios e reprodutivos são os mais comumente relatados (RIDPATH, 2010). A apresentação clínica e patológica varia dependendo da cepa do vírus, espécie do hospedeiro, estado imunológico, presença de infecções concomitantes por outros patógenos, idade e a situação reprodutiva do hospedeiro (MACLACHLAN; DUBOVI, 2011; RIDPATH, 2010).

Entretanto, a sintomatologia das infecções causadas por pestivírus em pequenos ruminantes e bovinos é semelhante. Por exemplo, a apresentação clínica desenvolvida em bovinos pela infecção pelo HoBi-like pestivírus é indistinguível da infecção causada por BVDV (BAUERMANN et al., 2013b; BIANCHI et al., 2011; CORTEZ et al., 2006; DECARO et al., 2011, 2012a; OIE, 2016; OIE, 2017; RIDPATH et al., 2013). Os mesmos sinais clínicos causados por BDV podem ser causados por BVDV em ovinos, o que acontece quando há um contato próximo de bovinos e ovinos (OIE, 2017). Porém, há algumas peculiaridades da infecção em cada espécie animal. Como exemplo, em ovinos, a maior evidência que há pestivírus no rebanho são os sinais clínicos decorrentes de

infecção de uma fêmea prenhe, como morte fetal e aborto (OIE, 2017). A infecção causada por pestivírus em ruminantes pode ser dividida em quatro categorias: infecção de animais não-prenhes, infecção de fêmeas prenhes, infecção persistente e doença das mucosas (MACLACHLAN; DUBOVI, 2011).

2.6.1 Infecção Aguda de Animais Não-prenhes

A infecção aguda acomete animais de todas as idades, porém, é mais comum em animais jovens em rebanhos com infecção endêmica. Ela acontece quando um animal soronegativo e imunocompetente é exposto ao vírus (NEWCOMER; GIVENS, 2013). Esta forma da doença é uma das manifestações mais importantes da infecção por pestivírus (BRODERSEN, 2014) e é caracterizada por alta morbidade e baixa mortalidade (OIE, 2016).

A viremia inicia de forma transitória aos três dias após a infecção e dura até 14 dias, até que a imunidade se desenvolva. Após a soroconversão, os animais ficam protegidos contra uma reinfecção pelo mesmo vírus (HOUE; LINDBERG; MOENNIG, 2006; LANYON et al., 2014; PETERHANS et al., 2010). O período de incubação dura de cinco a sete dias, e a maioria das infecções são subclínicas. Quando presente, os sinais clínicos são brandos, os animais podem apresentar febre, leucopenia, diarreia, secreção nasal e ocular, erosões ou úlceras no focinho e na mucosa oral, depressão, inapetência e diminuição na produção de leite. Se o animal afetado não tiver nenhuma infecção oportunista, ele tende a se recuperar completamente em três semanas (HOUE; LINDBERG; MOENNIG, 2006; LANYON et al., 2014; MACLACHLAN; DUBOVI, 2011; NEWCOMER; GIVENS, 2013). Fêmeas podem ter infertilidade, enquanto que touros podem apresentar diminuição na fertilidade e infecção prolongada nos testículos, assim, eles podem disseminar o vírus por meses pelo sêmen (BRODERSEN, 2014; OIE, 2016).

Cepas ncp altamente virulentas podem ainda induzir úlceras disseminadas na orofaringe, laringe e esôfago, como também pode provocar severa trombocitopenia, o que leva a hemorragia extensa e altas taxas de mortalidade em animais suscetíveis (BRODERSEN, 2014; MACLACHLAN; DUBOVI, 2011; NEWCOMER; GIVENS, 2013; FULTON et al., 2017).

Em surtos com essas características foi detectado pela primeira vez o BVDV-2a, causando uma síndrome hemorrágica aguda (HS) em várias propriedades na década de

90 na América do Norte (CARMAN et al., 1981; PELLERIN et al., 1994; RIDPATH; BOLIN; DUBOVI, 2014). Cepas de BDV altamente virulentas também já foram identificadas causando surtos em ovinos jovens, com mortalidade próxima a 50% (OIE, 2017).

O vírus induz uma variedade de efeitos no sistema imune e consequentemente imunodepressão, o que exacerba as infecções secundárias bacterianas e/ou virais, e aumenta a severidade da doença e a mortalidade (BRODERSEN, 2014; MACLACHLAN; DUBOVI, 2011; NEWCOMER; GIVENS, 2013; RIDPATH, 2010). Assim, o BVDV está frequentemente associado ao complexo respiratório bovino (BRD), uma doença multifatorial que pode ser causada por diversos vírus e bactérias. O BRD é a doença que causa maior prejuízo econômico para a bovinocultura dos Estados Unidos e cuja incidência tem aumentado na última década (MOSIER, 2015; USDA, 2011).

A excreção do vírus ocorre por vários fluídos corporais, como: secreção nasal e ocular, urina, leite, sêmen, saliva e fluídos fetais (LANYON et al., 2014), além de haver distribuição altamente variável do antígeno nos tecidos e uma acentuada depleção dos folículos linfoides em vários tecidos (BRODERSEN, 2014).

2.6.2 Infecção de Fêmeas Prenhes

Enquanto que a infecção da fêmea prenhe geralmente cursa de maneira subclínica ou branda, como uma infecção transitória, as consequências para o feto são mais sérias (OIE, 2017). A disseminação transplacentária ocorre após a infecção de uma fêmea prenhe e suas consequências são dependentes da virulência da cepa, do tempo gestacional e da raça, no caso em ovinos. Tanto em bovinos (FIGURA 4) quanto em ovinos (FIGURA 5), como resultados da infecção pode-se observar: reduzidas taxas de concepção, morte fetal ou embrionária, aborto, problemas congênitos, infecção persistente ou infecção inaparente com presença de resposta imune (BRODERSEN, 2014; LANYON et al., 2014; MACLACHLAN; DUBOVI, 2011; OIE, 2016; RIDPATH, 2010).

Em bovinos, enquanto o aborto pode acontecer em quase toda a extensão da gestação, os defeitos congênitos, por exemplo, tendem a acontecer no final da organogênese fetal, entre o 3ª e o 5ª mês de gestação (FIGURA 4). Os defeitos congênitos geralmente são observados no sistema nervoso central e os mais comuns são: a hipoplasia cereberal, hidrocefalia, bragnatismo e catarata ocular. Se a infecção ocorre após esse

período e após o desenvolvimento da imunocompetência, o feto desenvolve uma resposta imune protetiva e geralmente não apresenta nenhum sinal clínico (GROOMS, 2004).

Em ovinos, a morte fetal, por exemplo, pode ocorrer em qualquer estágio da gestação, mas é mais comum no início. Antes do feto desenvolver o sistema imunológico (60-85 dias da gestação), a mortalidade varia entre 50-75%, dependendo da cepa. No período dos 60-85 dias da gestação, as consequências da infecção são imprevisíveis, os animais podem nascer soropositivos e sem vírus, ou alguns são soronegativos e virêmicos. Se há sobrevivência fetal após infecção, os cordeiros geralmente nascem fracos e menores, muitas vezes incapazes de se levantar. Eles têm uma suscetibilidade aumentada a outras doenças, tendem a crescer devagar e sob condições normais a campo, muitos morrem perto da época de desmame (CAMPBELL et al., 1995; NETLETTON; WILLOUGHBY, 2007; OIE,2017).

Já em caprinos a infecção por pestivírus é menos comum, sendo o aborto o principal sinal clínico e as demais consequências da infecção em fêmeas prenhes são semelhantes ao que se observa em ovinos. No entanto, a mortalidade fetal tende a ser maior, variando de 80-100%. Consequentemente, caprinos PIs são raros e quando presentes apresentam uma baixa taxa de sobrevivência (DEPNER et al., 1991; GIAMMARIOLI et al., 2011; LAMM; BROADDUS; HOLYOAK, 2009; OIE,2017; PASSLER et al., 2014).

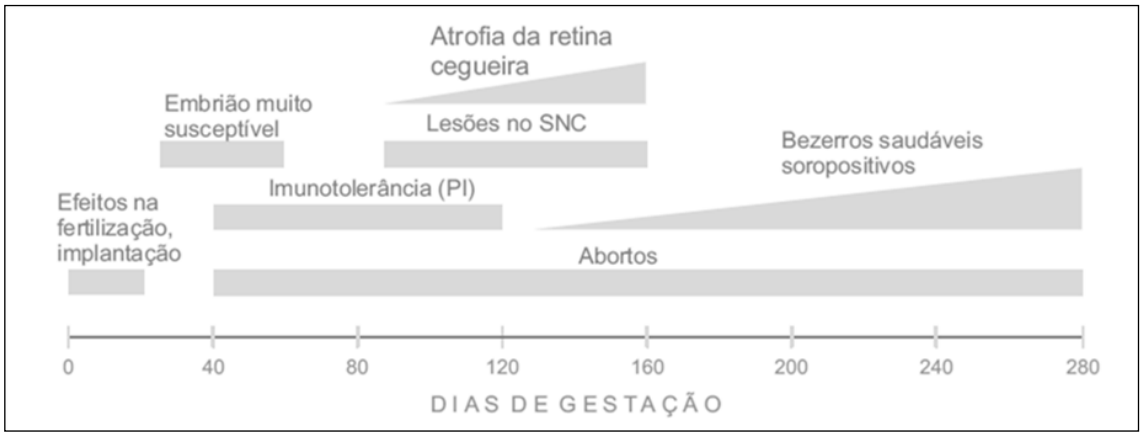

Figura 4. Representação esquemática das consequências da infecção de fêmeas bovinas prenhes de acordo com o estágio de gestação. Fonte: Ridpath et al. (2012).

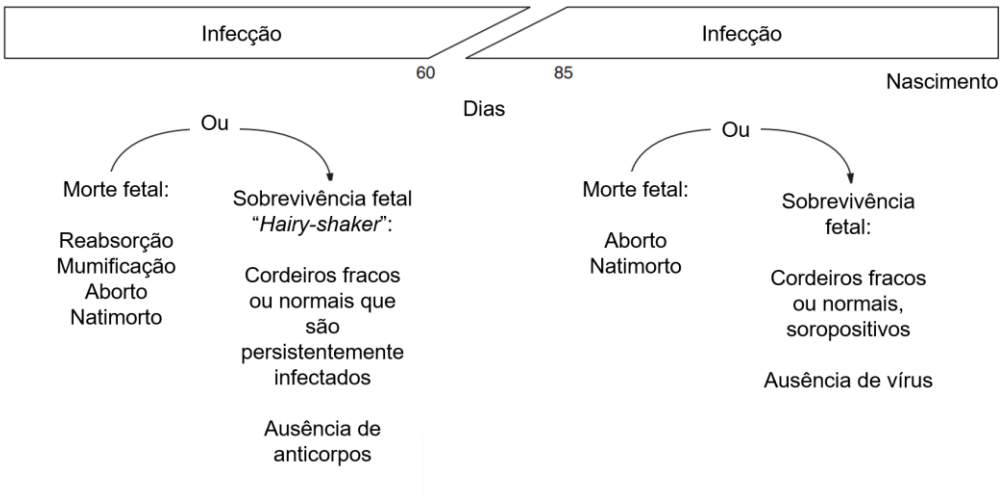

Figura 5. Possíveis consequências da infecção por pestivírus em ovinos. Antes e depois do feto desenvolver competência imunológica entre aproximadamente 60 e 85 dias da gestação. Adaptado: Netletton, Willoughby (2007).

2.6.3 Infecção Persistente

A infecção persistente ocorre tanto em bovinos, quanto em pequenos ruminantes (Figura 4, Figura 5). Porém, somente cepas ncp estabelecem este tipo de infecção, que ocorre entre os 30 e 125 dias de gestação em bovinos, ou antes dos 60 dias em ovinos, ou entre os dias 17-38 em caprinos. Além disto, fêmeas prenhes que são PI também darão origem a animais PI e no caso de carneiros PIs, a qualidade do sêmen e a fertilidade são reduzidas (BACHOFEN et al., 2013; LANYON et al., 2014; NETTLETON, 2013; OIE, 2017; PASSLER et al., 2014; PETERHANS et al., 2010; RIDPATH, 2010; SCHWEIZER; PETERHANS, 2014).

O sistema imune está imaturo no feto durante os períodos mencionados, assim o animal não consegue montar uma resposta imune adequada contra o vírus, tornando-se imunotolerante a este. Estes animais permanecem soronegativos e disseminam grande quantidade de vírus em todas as secreções e excreções por toda a vida, e eficientemente transmitem o vírus para animais suscetíveis no rebanho (LANYON et al., 2014; MACLACHLAN; DUBOVI, 2011; PETERHANS et al., 2010; RIDPATH, 2010). Desta maneira, os PIs são o principal reservatório e fonte de transmissão do vírus. O PI desempenha um papel substancialmente maior na transmissão viral que um animal transitoriamente infectado. Isto porque o vírus é excretado em títulos inferiores em animais transitoriamente infectados e somente por alguns dias (LINDBERG; HOUE, 2005)HOUE, 1999; RIDPATH; BAUERMANN; FLORES; 2012).

As quatro espécies de pestivírus (BVDV-1, BVDV-2, BDV, HoBi-like) causam

animais PIs. Em uma população infectada, em média de 1 a 2% dos bovinos são PIs. Enquanto que essa porcentagem é menor em pequenos ruminantes, há relatos de frequência de 0,3-0,6% em ovinos e 0,08% em caprinos (HOUE, 1999; KRAMETTER-FROETSCHER et al., 2006; VALDAZO-GONZÁLEZ; ÁLVAREZ-MARTÍNEZ; GREISER-WILKE, 2006; WEBER et al., 2016).

Os sinais clínicos de um animal PI são muito variáveis. Os animais PIs podem parecer clinicamente normais, ou possuírem retardo no crescimento, diminuição no ganho de peso anemia, refugagem, bem como, podem apresentar conformação anormal do corpo e defeitos no sistema nervoso central, como tremores e cegueira (HOUE; LINDBERG; MOENNIG, 2006; LANYON et al., 2014; MACLACHLAN; DUBOVI, 2011; NEWCOMER; GIVENS, 2013; PETERHANS et al., 2010; OIE, 2016; SCHWEIZER; PETERHANS, 2014).

Em ovinos e caprinos, as mudanças patológicas mais marcantes são no sistema nervoso central e na pele. Mudanças na pelagem são frequentemente aparentes, e acontecem no pescoço e nas costas, como cobertura escassa e anormal de lã, pelos eriçados e mudança na pigmentação. Devido a isso a doença é comumente chamada de *"hairy shaker"*. Os sinais neurológicos são a característica mais marcante da doença e podem ser desde tremores até problemas de locomoção e malformações esqueléticas. Os tremores podem variar de praticamente indetectáveis para contrações rítmicas violentas. (CAMPBELL et al., 1995; MAO et al., 2013; NETLETTON, WILLOUGHBY, 2007; OIE,2017).

Dado que os pestivírus replicam-se em quase todos os tecidos dos animais PIs, há uma ampla distribuição do antígeno viral, incluindo nos linfonodos, epitélio do trato gastrointestinal, pulmões, timo, cérebro, pele e nas glândulas salivares e uma depleção localmente restrita ou moderada dos tecidos linfoides (BRODERSEN, 2014; LANYON et al., 2014).

A taxa de mortalidade para os PIs no primeiro ano de vida é maior que 50%, pois, devido uma imunidade deficiente, eles são suscetíveis a infecções secundárias e morrem de doenças como pneumonia e enterite. Isto, combinado com a doença das mucosas (discutidas posteriormente), leva a diminuição do tempo de sobrevivência para cerca de 30% para bovinos com mais de dois anos de idade (LANYON et al., 2014; MACLACHLAN; DUBOVI, 2011).

2.6.4 Doença das Mucosas

A doença das mucosas (DM) acomete apenas animais PIs. Assim, ela tem baixa morbidade, podendo-se manifestar na forma de surto acometendo todos os PIs de um rebanho, mas é invariavelmente fatal (BIANCHI et al., 2017; DARWEESH et al., 2015; METCALFE; YARNALL, 2017). A DM é mais bem documentada em bovinos infectados por BVDV. Contudo, por exemplo, ela também tem sido descrita acometendo ovinos infectados com BDV e em bovinos por HoBi-like pestivírus (DECARO et al., 2014; NETLETTON; WILLOUGHBY, 2007, MONIES; PATON; VILCEK, 2004). A DM ocorre quando os dois biótipos estão presentes: o vírus ncp com qual o animal originalmente foi infectado persistentemente *in utero*, e a cepa cp homóloga genética e antigenicamente à ncp, sendo estas duas cepas chamadas de "par viral" (FIGURA 6). A cepa cp surge da cepa ncp, através de mutações, incluindo recombinação, inserções de sequências celulares, duplicações, deleções e rearranjos (BRODERSEN, 2014; MACLACHLAN; DUBOVI, 2011; PETERHANS et al., 2010). Com menos frequência, pode ocorrer uma superinfecção com uma cepa cp proveniente de uma fonte externa, homóloga à cepa ncp, proveniente de vacinas ou de animais PIs (LANYON et al., 2014; MACLACHLAN; DUBOVI, 2011; SCHWIZER; PETERHANS, 2014).

As características clínicas da DM são as mesmas que as de uma infecção aguda de animais não prenhes, no entanto, com maior gravidade. A DM é rara, tem início súbito e pode se estender por várias semanas ou meses, de forma crônica com sinais clínicos recorrentes. Os animais podem ter febre, anorexia, salivação, diarreia aquosa, secreção nasal, desidratação, definhamento e por fim morte (HOUE; LINDBERG; MOENNIG, 2006; MACLACHLAN; DUBOVI, 2011; OIE, 2016; SCHWEIZER; PETERHANS, 2014). A forma aguda é caracterizada por erosões e úlceras na mucosa oral, nasal, esôfago, intestino delgado e nas placas de Peyer. Por outro lado, na forma crônica, os animais tipicamente apresentam úlceras na pele (MACLACHLAN; DUBOVI, 2011; NEWCOMER; GIVENS, 2013; BIANCHI et al., 2017).

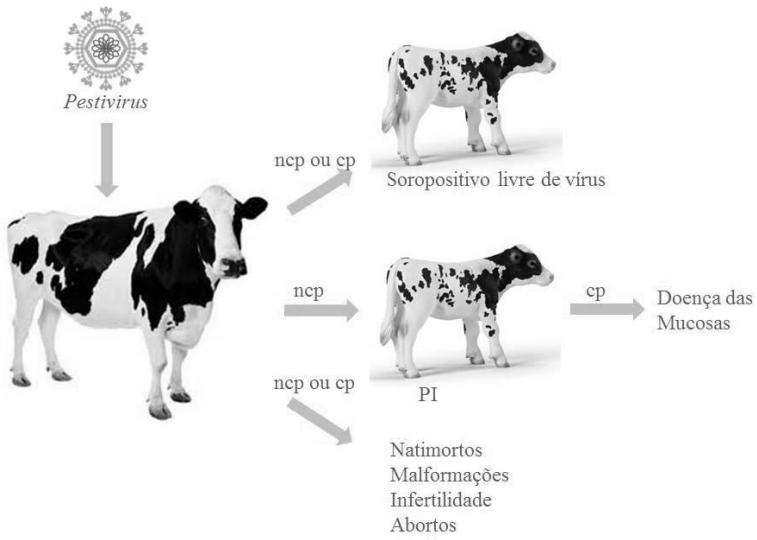

Figura 6. Consequências da infecção de fêmeas bovinas prenhes de acordo com o biótipo do vírus. Adaptado de Ridpath et al. (2012).

Na DM é observada uma ampla distribuição do antígeno viral, incluindo nas glândulas salivares, ocorrendo severa depleção de todos os tecidos linfoides e lesões graves no trato gastrointestinal (BRODERSEN, 2014). Esta forma da infecção não é transmissível, pois, somente animais PI a desenvolvem, e estes possuem os dois biótipos cp e ncp envolvidos na patogênese (SCHWEIZER; PETERHANS, 2014). A DM representa um exemplo raro onde a evolução viral associada com a mudança de biótipo leva a morte do animal afetado e, por fim, leva a extinção do vírus cp que causou a doença (PETERHANS et al., 2010). A citopatologia *in vitro* não se correlaciona com a virulência *in vivo*, ou seja, as cepas cp não são mais patogênicas do que as ncp, tanto que as formas clínicas mais severas da forma aguda da infecção são associadas com cepas ncp, e somente estas podem gerar animais persistentemente infectados (NEILL,2013; RIDPATH, 2010).

2.7 Diagnóstico dos Pestivírus de Ruminantes

O diagnóstico presuntivo de uma infecção causada por pestivírus em ruminantes deve ser feito com base na história clínica, nos sinais clínicos e nas lesões microscópicas e macroscópicas (MACLACHLAN; DUBOVI, 2011). No entanto, como a infecção é clinicamente diversa e não apresenta sinais clínicos patognomônicos, um diagnóstico definitivo é somente obtido por testes laboratoriais (NEWCOMER; GIVENS, 2013; OIE,

2016; SANDVIK, 1999). Os testes laboratoriais são baseados no isolamento viral em cultivo celular, detecção do antígeno viral (imunofluorescência, imunohistoquímica, ELISA), detecção do RNA viral (RT-PCR, RT-qPCR) e detecção de anticorpos por sorologia (ELISA, soroneutralização) (NEWCOMER; GIVENS, 2013; MACLACHLAN; DUBOVI, 2011). A coleta de amostras biológicas irá depender da sintomatologia observada e da condição do animal. O soro pode ser utilizado tanto para a detecção de anticorpo, quanto de antígeno. Para detecção viral, fragmento de diversos tecidos também podem ser usados, como de: orelha, tireoide, cérebro, rim, baço, linfonodos, intestino e sêmen (OIE, 2015; OIE, 2017).

No entanto, a alta diversidade genética dos pestivírus causa problemas relativos à detecção e diferenciação destes vírus (RIDPATH, 2003). Desta maneira, é necessário avaliação dos testes de diagnóstico a fim de certificar que eles detectam a ampla gama de diversidade antigênica e genética encontrada entre os pestivírus, bem como a realização de pesquisa de rotina nos rebanhos bovinos e nas demais espécies de hospedeiros, com o objetivo de detectar novas variantes virais que possam não estar sendo detectadas com os testes de diagnóstico tradicionais (DUBOVI, 2013b; OIE, 2015).

Assim, não há teste capaz de detectar todos os pestivírus em todos os hospedeiros. Devido à maior demanda, a maioria dos testes de diagnóstico comerciais e *in house* para pestivírus, são aqueles desenvolvidos e validados para BVDV em bovinos. No entanto, alguns testes específicos estão disponíveis para BDV e HoBi-like pestivírus, ou para BVDV em pequenos ruminantes (CHU et al., 1987; DECARO et al., 2012b; DUBEY et al., 2014; EVANS; LANYON; REICHEL, 2017; LIU et al., 2008). Como os ovinos podem ser infectados com BVDV, é preferível usar testes de diagnóstico “panpestivírus” capazes de detectar tanto BDV quanto BVDV nesses hospedeiros (OIE, 2017).

A detecção rápida e sensível de pestivírus é um aspecto relevante no contexto de diagnóstico para programas de controle e erradicação, que têm sido focados na identificação de animais PIs. Para isso, além dos testes convencionais para diagnóstico rápido (RT-PCR, ELISA), outros testes têm sido elaborados para serem simples e apresentarem um bom custo-benefício, a exemplo de um tipo de amplificação isotermal para detecção de RNA de BVDV, sem necessidade de enzima e com uso de nanopartículas de ouro (ASKARAVI et al., 2017; GHASEMI MONJEZI et al., 2016).

Além da escolha da amostra biológica a ser coletada, do conhecimento de qual ou quais pestivírus podem ter causado a doença, a escolha e interpretação dos testes de

diagnóstico irão depender do tipo de infecção. Deve-se avaliar se a infecção é aguda (transitória) ou persistente, pois, em uma infecção aguda o vírus só é detectável por alguns dias, já na infecção persistente, por toda a vida do animal. Entretanto, após uma infecção aguda é possível detectar anticorpos, enquanto na infecção persistente os animais serão soronegativos (OIE, 2015; OIE, 2017). Como os animais PIs possuem alta carga viral, os testes de diagnóstico empregados para detectar estes alcançam excelente sensibilidade e especificidade (LANYON et al., 2014). Recomenda-se que para a identificação de animais PIs, haja coleta de sangue de todo o rebanho. Os animais precisam ser soronegativos e apresentarem dois testes com resultados positivos para detecção do antígeno viral, de amostras coletadas com no mínimo três semanas de diferença (LIU et al., 2008; OIE, 2015).

2.7.1 Isolamento Viral

O isolamento viral é historicamente considerado a prova ouro para o diagnóstico de pestivírus, porém, é caro e laborioso, assim outras técnicas têm sido mais utilizadas, pela facilidade e pelo baixo custo. No entanto, o isolamento viral é o único que consegue diferenciar vírus não viáveis de vírus biologicamente ativos (BRODERSEN, 2014; NEWCOMER; GIVENS, 2013; RIDPATH, 2010).

Há muitas variações da técnica e todas devem ser otimizadas para obter a máxima sensibilidade na detecção. O vírus pode ser isolado em vários tipos celulares e a partir de secreção nasal, sangue, soro, sêmen, *pool* de leite e tecidos de animais afetados, como fragmento de orelha ou tecidos de fetos abortados (LANYON et al., 2014; NEWCOMER; GIVENS, 2013; MACLACHLAN; DUBOVI, 2011; OIE, 2015; OIE, 2017).

Para o diagnóstico de uma infecção persistente através do isolamento viral, adota-se como critério que as amostras biológicas (ex: soro) sejam coletadas com no mínimo três semanas de diferença, sem soroconversão (OIE, 2015; SANDVIK, 1999). Em relação ao diagnóstico de DM, a confirmação deve ser realizada pelo isolamento do vírus cp (OIE, 2015).

Um problema enfrentado no isolamento viral em cultivo celular é a contaminação com pestivírus ncp, geralmente provenientes do soro fetal bovino utilizado como um suplemento nos meios de cultivo celular. O monitoramento, a fim de assegurar que isto não aconteça, é uma medida essencial para o controle de qualidade no diagnóstico de pestivírus. Além disto, o soro fetal bovino precisa ser livre de anticorpos neutralizantes

contra os pestivírus (OIE, 2015; SANDVIK, 1999). Como as células infectadas por vírus ncp não podem ser distinguidas do controle de células não infectadas, é necessário o uso de imunofluorescência ou imunoperoxidase (HOUE; LINDBERG; MOENNING, 2006; OIE, 2015; SANDVIK, 1999).

2.7.2 Imunohistoquímica

A imunohistoquímica (IHC) fornece um diagnóstico específico, sensível e robusto para detecção de pestivírus (OIE, 2015; LANYON et al., 2014; SANDVIK, 1999). Para animais PIs, praticamente todos os tecidos podem ser usados, especialmente, os linfonodos, glândulas tireoides, pele, cérebro, abomaso e placenta (OIE, 2015). Nestes animais, o antígeno viral está presente na pele em altos títulos, assim a IHC tem desempenhado um importante papel em programas de controle e erradicação, que visam identificar animais PIs através do uso da técnica a partir de fragmentos de orelha (DUBOVI, 2013). Desta maneira, por exemplo, o teste tem alcançado sensibilidade de 100% inclusive para detecção de PI infectado com vírus HoBi-like pestivírus (BAUERMANN et al., 2014).

2.7.3 RT-PCR e RT-qPCR

A presença de RNA viral de pestivírus pode ser detectada por RT-PCR ou RT-PCR em tempo real (RT-qPCR). Essas técnicas são rápidas e oferecem alta sensibilidade, o que é adequado para amostras biológicas com baixa quantidade viral, como amostras provenientes de: tanques de leite, amostras de *pool* de soro ou provenientes de uma infecção aguda. Além de ser úteis para avaliar contaminações por pestivírus em soro fetal bovino e em vacinas. Porém, essas técnicas podem ser empregadas a partir de qualquer tipo de amostra, como fragmentos de tecidos, secreção nasal, pelo, sêmen e embriões (LANYON et al., 2014; NEWCOMER et al., 2013; OIE, 2016; SINGH et al., 2011; ZOCCOLA et al., 2017).

Testes de RT-PCR usando primers específicos para a região 5’UTR são os mais empregados, por esta região ser bastante conservada (OIE, 2015; SANDVIK, 1999). RT-PCR a partir de tanque de leite pode ser útil para um teste inicial, a fim de indicar se há algum animal PI ou transitoriamente infectado entre as vacas lactantes (HOUE; LINDBERG; MOENNING, 2006; LANYON et al., 2014). Esta técnica é sensível o suficiente para detectar uma vaca PI lactante em um grupo de 132 animais (LANYON et

al., 2014; OIE, 2015). O mesmo princípio pode ser aplicado para *pools* de soro e para *pools* de fragmento de orelha, que se constituem em técnicas rápidas e econômicas (LANYON et al., 2014; WEINSTOCK; BHUDEVI; CASTRO, 2001).

A identificação de um PI pode ser feita através da detecção do RNA viral no sangue através de RT-qPCR. No entanto, na presença de resultado positivo, uma nova coleta deve ser realizada com no mínimo três semanas de diferença em relação a primeira amostra, e esta precisa ser positiva para confirmar o estado de PI (NEWCOMER; GIVENS, 2013; OIE, 2015). Para facilitar o diagnóstico, testes de RT-qPCR têm sido desenhados a fim de diferenciar infecções agudas de persistentes, com base na carga viral presente na amostra (LANYON et al., 2014). Além disto, testes de RT-qPCR também têm sido desenvolvidos com a capacidade de diferenciar BVDV-1 e 2 (BAXI et al., 2006; BHUDEVI; WEINSTOCK, 2001; LETELLIER; KERKHOFS, 2003), ou para apenas detectar HoBi-like pestivírus (LIU et al., 2008). Recentemente, kits comerciais de RT-qPCR têm sido validados para detectar RNA de BVDV em amostras de fragmento de orelha, sangue, plasma, soro e leite. Alguns destes kits já apresentaram uma ótima sensibilidade (100%) para detecção de HoBi-like pestivírus em fragmentos de orelha (BAUERMANN et al., 2014).

Entretanto, um problema tem sido enfrentado no uso de RT-PCR e RT-qPCR, pois, vários testes desenhados para detectar BVDV falham em detectar e diferenciar HoBi-like pestivírus ou possuem sensibilidade diminuída comparada a detecção de BVDV. Isto ocorre devido a variações na sequência na região do primer e/ou da sonda (BAUERMANN et al., 2014; LIU et al., 2008; SCHIRRMEIER et al., 2004). Assim, há necessidade de vigilância contínua e de desenvolvimento de novos testes para detectar cepas divergentes, como por exemplo, para identificação dos HoBi-like pestivírus (DUBOVI, 2013).

Considerando que os ovinos podem ser infectados tanto com BDV quanto com BVDV, uma RT-PCR panpestivírus reativa deve ser usada. Como o BDV apresenta oito grupos genéticos, precisa ser conhecida a capacidade da RT-PCR empregada em detectar esses genótipos (OIE, 2017).

2.7.4 Testes Sorológicos

As técnicas mais usadas para a detecção de anticorpos contra pestivírus são a SN e o ELISA (HOUE; LINDBERG; MOENNING, 2006; OIE, 2015). A SN é um teste

sensível, específico, adequado para quantificação de anticorpos por titulação e é reconhecido como teste de referência para sorologia de BVDV e BDV. Devido à sua alta especificidade e as diferenças antigênicas entre os pestivírus, é essencial usar um vírus-teste similar aos vírus de campo (HOUE; LINDBERG; MOENNING, 2006; OIE, 2015; SANDVIK, 1999).

A SN pode ser biologicamente mais relevante que o ELISA, porque ela pode avaliar o título de anticorpos em relação as variações que existe entre os isolados de pestivírus. Além do mais, ela pode ser empregada em amostras de diferentes hospedeiros, não apenas de bovinos (DUBOVI, 2013). Porém, a SN é trabalhosa, cara, precisa de cultivo celular e como há poucas cepas cp, uma imunocitoquímica precisa ser realizada para a leitura dos resultados. Além do mais, os resultados podem variar entre laboratórios devido ao uso de diferentes cepas ou tipos celulares (DUBOVI, 2013; HOUE; LINDBERG; MOENNING, 2006; LANYON et al., 2014).

Há muitos testes de ELISA desenvolvidos *in house* ou na forma de kits comerciais para detecção de anticorpos em soro, leite individual ou tanque de leite, contra BVDV e BDV. No entanto, esses ELISAs não são capazes de distinguir anticorpos contra essas espécies de pestivírus. Eles são desenhados, geralmente, para detecção de anticorpos contra a proteína NS23 e possuem como vantagens a facilidade de triagem de muitas amostras, alta sensibilidade e especificidade, rapidez e custo menor em relação à SN disponíveis (CHU et al., 1987; DUBOVI, 2013; HOUE; LINDBERG; MOENNING, 2006; LANYON et al., 2014; NOGAROL et al., 2017; OIE, 2015; SANDVIK, 1999).

Os testes sorológicos podem ser usados, por exemplo, para investigar o estado de exposição de um rebanho ou de um animal, o estágio da infecção no rebanho e para avaliar a eficácia de vacinas. No entanto, testes sorológicos são limitados quando a vacinação é praticada, devido à dificuldade de diferenciar animais infectados de animais vacinados (DUBOVI, 2013; NEWCOMER; GIVENS, 2013; SANDVIK, 1999). Além disto, na interpretação de resultados negativos deve-se levar em conta o estado de imunotolerância de animais PI (MACLACHLAN; DUBOVI, 2011). Pois, como estes animais não desenvolvem resposta imune contra o vírus que lhe está infectando, os testes sorológicos não conseguem identificá-los (NEWCOMER; GIVENS, 2013).

Para rebanhos leiteiros não vacinados, o teste em tanque de leite para anticorpos é uma ferramenta útil e mais barata que permite uma triagem inicial para classificação do estado do rebanho (HOUE; LINDBERG; MOENNING, 2006; LANYON et al., 2014;

OIE, 2015; SANDVIK, 1999). Também é possível testar *pools* de soro por ELISA para detecção de anticorpos, o que permite estimar a soroprevalência dos animais do *pool* (LANYON et al., 2014).

2.7.5 Testes de ELISA para Detecção de Antígeno

Os testes de ELISA para detecção de antígeno geralmente se baseiam na detecção da proteína NS2/3 ou E^{rns} em tecidos, como sangue e, principalmente, fragmento de orelha (HOUE; LINDBERG; MOENNING, 2006; OIE, 2015; QUINET et al., 2016; SANDVIK, 1999; OIE, 2017), o que tem possibilitado um teste economicamente viável para os programas de controle e erradicação de BVDV (DUBOVI, 2013; LANYON et al., 2014). Estes testes de ELISA apresentam sensibilidade similar ao isolamento viral, porém, são mais efetivos na presença de anticorpos do colostro do que o isolamento viral. Mesmo assim, podem gerar resultados falso-negativos em ovinos virêmicos menores de 2 meses (DUBOVI, 2013; NEWCOMER; GIVENS, 2013; OIE, 2015; OIE, 2017). Geralmente, os testes de ELISA são mais sensíveis para detectar animais PIs do que os animais transitoriamente infectados. Não estão disponíveis kits comerciais para BDV, somente para BVDV (DUBOVI, 2013; OIE, 2015; OIE, 2017).

2.8 Controle e Profilaxia

Há uma significativa perda econômica causada pelo BVDV em bovinos, devido a sua ampla distribuição pelo mundo, a apresentação clínica variada, a imunossupressão que predispõe o animal a outras doenças e principalmente devido as perdas reprodutivas. Estima-se que o custo de um animal infectado varia em média de U$175-200, sendo maior em vacas leiteiras do que em gado de corte. A partir disso, vários estudos têm provado que é mais vantajoso economicamente gastar para controlar e erradicar o BVDV que pagar os gastos oriundos da infecção (CHAROENLARP et al., 2018; LAUREYNS, 2017; RICHTER et al., 2017).

Consequentemente, programas de erradicação de BVDV sem vacinação começaram nos países da Escandinávia na década de 90. O sucesso alcançado por esses programas tem levado ao desenvolvimento de programas nacionais e regionais de controle e erradicação em vários outros países, como: Áustria, Alemanha, Itália, Holanda, Suíça, Eslovênia, França, Escócia, Bélgica, Irlanda, Austrália e Nova Zelândia (HOUE;

LINDBERG; MOENING, 2006; LANYON et al., 2014; LINDBERG; ALENIUS, 1999; NETTLETON, 2013; QUINET et al., 2016; REICHEL; LANYON; HILL, 2018; SANDVIK, 2004).

Estes programas são baseados na remoção dos animais PIs, com ou sem vacinação. Para a identificação dos animais PIs a estratégia mais utilizada é o teste de todos os animais da propriedade, utilizando ELISA para detecção de antígeno a partir de um fragmento de orelha do animal. Após a identificação e remoção dos PIs, em países onde não há o uso de vacinas, o segundo estágio do programa consiste no monitoramento dos rebanhos quanto a presença de anticorpos, o que é verificado através da aplicação de um teste de ELISA a partir de amostras de soro ou leite (QUINET et al., 2016; THOMANN et al., 2017; THULKE et al., 2018).

Além desse monitoramento contínuo dos rebanhos livres para rápida detecção de uma possível reinfecção, os programas têm focado em medidas de biossegurança para prevenir a introdução viral. Estas medidas agem especialmente sobre o comércio animal para prevenir a comercialização de animais PIs, fêmeas portando um feto PI e mesmo de animais transitoriamente infectados (ALPAY; TOKER; YEŞILBAĞ, 2018; LANYON et al., 2014; REARDON et al., 2018; SCHWEIZER; PETERHANS, 2014).

Depois dos animais PIs terem sido eliminados, o uso da vacina é decidido conforme a situação epidemiológica, o que irá variar conforme a prevalência do BVDV, densidade animal, características do comércio, contato com populações silvestres, variação das cepas circulantes e tipo de produção. Em propriedades com alta densidade animal, comércio intensivo e alta prevalência de BVDV, a vacinação precisa ser empregada. Em propriedades com baixa prevalência, a vacinação não é obrigatória, já que o risco de reintrodução pode ser muito pequeno (NEWCOMER, 2013). Portanto, um programa pode ter duas fases, primeiro, eliminação dos PIs, seguida de vacinação e erradicação. Este tipo de programa, por exemplo, foi adotado em estados da Alemanha que apresentavam alta prevalência da infecção e ele minimiza o risco de reintrodução em rebanhos livres do vírus (WERNIKE et al., 2017).

Apesar de que as vacinas vêm sendo usadas por mais de 50 anos, tanto de forma sistemática em países com programas de controle quanto nos demais países, elas não têm se mostrado efetivas na eliminação do BVDV nos rebanhos, o que ocorre principalmente devida a grande diversidade genética e antigênica observada nas cepas de BVDV, pela falta de uma proteção fetal completa e pela falha em remover animais PIs. Assim, para

alcançar uma melhor proteção vacinal, as vacinas necessitam refletir as cepas prevalentes ou tipos antigênicos presentes no país do seu uso, pois, a proteção contra cepas homólogas a da vacina geralmente é superior que contra cepas heterólogas. Portanto, as pesquisas que visam conhecer quais pestivírus circulam em um país são fundamentais (BRODERSEN, 2014; MAHONY et al., 2005; NEWCOMER; GIVENS, 2013; RIDPATH et al., 2010). Desta maneira, não há nenhuma vacina considerada 100% eficaz. No entanto, a escolha da vacina e o protocolo vacinal ajudam a maximizar essa eficácia. Há muitas vacinas comercialmente disponíveis, geralmente elas estão em combinação com outras vacinas virais ou bacterianas. Elas podem conter: vírus vivos atenuados (MLV) ou inativados (KV), cepas cp ou ncp, de BVDV-1a e/ou de BVDV-2a. Apesar de o BVDV-1b ser o subgenótipo prevalente no mundo, poucas vacinas contêm cepas dele na composição. Até o momento, não há nenhuma vacina disponível para o HoBi-like pestivírus (BAUERMANN et al., 2013a; NEWCOMER et al., 2017).

Recomenda-se o uso de duas doses de MLV. Se uma KV for usada, o recomendado é que seja realizada uma segunda dose com MLV (HOUE; LINDBERG; MOENING, 2006). As MLVs geralmente induzem uma resposta imune mais rápida, mais ampla e duradoura que as KV, além de fornecerem uma proteção fetal mais robusta. Isso porque elas induzem títulos maiores de anticorpos neutralizantes e uma resposta imune celular mais potente (DOWNEY-SLINKER et al., 2016; PLATT et al., 2017; WALZ et al., 2018). Contudo, muitas vezes a KV é administrada por ela ser mais segura do que as MLVs, por não serem nem imunossupressores nem patogênicas. Isto porque, historicamente, aconselhou-se a não administração de MLV a fêmeas prenhes, devido ao risco de infecção transplacentária, além de haver o risco de indução da DM em animais PI. Porém, atualmente há vacinas MLV aprovadas para a vacinação de fêmeas prenhes, mas sob algumas condições (KALAYCIOGLU; RUSSELL; HOWARD, 2012; NEWCOMER; CHAMORRO; WALZ, 2017).

Atualmente, o principal foco no desenvolvimento de vacinas é melhorar a eficácia na proteção fetal, basicamente para prevenir o nascimento de PIs, e ampliar a proteção heteróloga. Portanto, vacinas mais avançadas, empregando novas tecnologias e novos adjuvantes vem sendo desenvolvidas e testadas experimentalmente. Como exemplo têm-se as vacinas de DNA, de nanopartículas ou recombinantes de subunidade, que geralmente tem como antígeno a E2 de BVDV, que é carreada e/ou expressa em vetores como alfavírus, baculovírus ou adenovírus (CAI et al., 2018; CANTÚ-MARTÍNEZ et al.,

2013; CHUNG et al., 2018; CIBULSKI et al., 2018; EL-ALTAR et al., 2015; LOKHANDWALA et al., 2017; LOY et al., 2013; MODY et al., 2014; PECORA et al., 2015).

Para o sucesso no controle e erradicação de pestivírus, além das ações quanto a eliminação dos PIs, das medidas de biossegurança e das vacinações, faz-se necessária a educação dos veterinários e proprietários e a pesquisa contínua, a fim de se fazerem reavaliações do programa, por exemplo. Uma vez que há risco de emergência de novos pestivírus, de vírus que mudaram de hospedeiros ou que aumentaram a sua virulência. Recordando que os pestivírus não são espécie-específicos, assim eles podem infectar tanto animais de produção quanto animais silvestres, de vida livre ou de cativeiro. Consequentemente, a transmissão viral interespécie pode ocorrer e deste modo os animais silvestres, ou mesmo os pequenos ruminantes, podem ter um impacto em programas de controle de BVDV em rebanhos bovinos, podendo atuar como vetores para reintrodução de pestivírus já erradicados, além da possibilidade de disseminarem espécies virais consideradas atípicas para os bovinos, como o BDV (MARTIN et al., 2015; RIDPATH, 2015; RIDPATH, PASSLER, 2016; KAISER et al., 2017).

Como exemplo de emergência de uma espécie de pestivírus, tem-se o vírus HoBi-like. Vírus que tem sido detectado na última década em vários países e que é um risco para programas de controle e erradicação de BVDV, já que tantos animais soronegativos quanto os soropositivos para BVDV são suscetíveis a infecção por HoBi-like. Como resultado, ele pode causar grandes prejuízos econômicos, comprometendo programas de controle, e assim podendo até se tornar restritivo para o comércio internacional de animais e produtos com países que são livres deste pestivírus (BAUERMANN et al., 2013b, 2013a; BAUERMANN; FALKENBERG; RIDPATH, 2017).

Diferentemente da preocupação que se tem com o BVDV em bovinos, o controle de pestivírus em ovinos, mais precisamente do BDV, é menos realizado e depende da extensão da infecção no rebanho. Surtos esporádicos podem ser controlados pela remoção dos animais suspeitos em introduzir o vírus no rebanho. No entanto, para que não ocorra essa introdução várias medidas são aconselhadas. Idealmente, todos os animais comprados devem ser testados e todas fêmeas compradas devem ser acasaladas e mantidas separadas do rebanho, até o período de parição. Devido ao risco de infecção em ovinos por BVDV, é essencial que principalmente as ovelhas prenhes sejam mantidas distante dos bovinos. Devido à baixa demanda, poucas vacinas estão disponíveis contra

BDV para ovinos. A vacina comercial mais empregada é composta por cepas de BDV e BVDV-1 inativadas e recomenda-se que ela seja administrada em fêmeas antes da época reprodutiva para prevenção da infecção transplacentária (SANDVIK et al., 2014; OIE, 2017).

2.9 Situação dos Pestivírus de Ruminantes no Brasil

A infecção pelo BVDV tem sido descrita desde a década de 60 no Brasil (CORREA; NETO; BARROS, 1968). Atualmente o BVDV está amplamente distribuído pelos rebanhos bovinos brasileiros, com 22-67,3% dos animais e 43-95% dos rebanhos soropositivos (ALMEIDA et al., 2013; CANAL et al., 1998; CHAVES et al., 2012; FERNANDES et al., 2016; QUINCOZES et al., 2007; SOUSA et al., 2013; THOMPSON et al., 2006).

O Brasil apresenta uma ampla e única diversidade genética de pestivírus, com diferenças regionais marcantes. Desta forma, BVDV-1a, 1b, 1d, 1e, 1i, 2a, 2b, 2c e o HoBi-like pestivírus têm sido identificados no país, principalmente em bovinos, mas também em soros fetais bovinos e em búfalos (1b, HoBi-like) (ALVES et al., 2016; BIANCHI et al., 2011; CORTEZ et al., 2006; FLORES et al., 2002; MONTEIRO et al., 2018; MÓSENA et al., 2017; OTONEL et al., 2014; PAIXAO et al., 2018; STALDER et al., 2005; WEBER et al., 2014a, 2016b).

Apesar de que ainda não tenha sido detectado nenhum pestivírus em pequenos ruminantes no país, trabalhos sorológicos mostram que eles circulam nesses hospedeiros, mas em baixa prevalência (GAETA et al., 2016; LÚCIDI et al., 2016; SILVA et al., 2014).

A maioria dos estudos sobre pestivírus concentra-se na Região Sul e consistem de isolados virais detectados em animais com sinais clínicos ou de rebanhos com histórico sugestivo de infecção por pestivírus (BIANCHI et al., 2011; OTONEL et al., 2014; SILVEIRA et al., 2017). Poucos estudos tiveram como objetivo a vigilância para a identificação dos pestivírus circulantes em uma população e a determinação da frequência de infecções ativas (SILVEIRA et al., 2018a; WEBER et al., 2014a).

Em amostras coletadas majoritariamente no Sul observou-se uma proporção de BVDV-2 semelhante à de BVDV-1, com frequências variando de 40-57,5% para BVDV-1 e 33,7-45% para BVDV-2 (BIANCHI et al., 2011; SILVEIRA et al., 2017; WEBER et

al., 2014c). Estas frequências de BVDV-2 são similares às descritas no Chile (PIZARRO-LUCERO et al., 2006) e na Coréia do Sul (OEM et al., 2009), porém, superiores que aquelas relatadas nos demais países (BOOTH et al., 2013; KUTA et al., 2013; LUZZAGO et al., 2014; MAHONY et al., 2005; MISHRA et al., 2014; PECORA et al., 2014; RIDPATH et al., 2010; ULARAMU et al., 2013; YILMAZ et al., 2012; ZHANG et al., 2014).

Diferentemente da realidade do Sul, no Nordeste (MA, PB) um trabalho recente revelou uma predominância do HoBi-like pestivírus, situação que só é observada de forma semelhante em uma região da Índia (MISHRA et al., 2014; SILVEIRA et al., 2018a). O Brasil além de ser o lugar de origem dos primeiros isolados de HoBi-like, é o País com o maior número de detecções do mesmo (CORTEZ et al., 2006; CRUZ et al., 2018; MONTEIRO et al., 2018; SILVEIRA et al., 2017; WEBER et al., 2016b). Ademais, dos três casos de síndrome similares a DM causada por HoBi-like pestivírus que ocorreram até hoje no mundo, dois ocorreram no Brasil e são os mais antigos. Um ocorreu no estado do Mato Grosso (CRUZ et al., 2018) e outro na Paraíba (WEBER et al., 2014b).

Apesar da ampla distribuição dos pestivírus em bovinos pelo País, há uma carência de pesquisa em algumas regiões. O conhecimento sobre a realidade dos pestivírus de ruminantes em todo o território brasileiro é necessário para o desenvolvimento e aprimoramento de métodos de detecção e controle e para servir de base para um futuro programa nacional. Atualmente, não há um programa de controle nacional e estima-se que apenas uma pequena porcentagem do rebanho seja vacinada.

Além disto, no Brasil a maioria das vacinas licenciadas são inativadas e estas contêm cepas estrangeiras somente de BVDV-1 ou de BVDV-1 e de BVDV-2. No entanto, questiona-se sobre a eficácia destas vacinas, devido à alta variabilidade antigênica entre os pestivírus e ao uso majoritário de vacinas inativadas, apesar do conhecimento de que as vacinas atenuadas são mais eficientes (FLORES et al., 2005; NEWCOMER; GIVENS, 2012; WEBER et al., 2014a).

Assim, oito vacinas comerciais usadas no Brasil foram avaliadas quanto à imunogenicidade. Não foram detectados anticorpos contra BVDV-1 e BVDV-2 em pelo menos quatro e seis vacinas, respectivamente. Além disso, apenas uma vacina induziu resposta em 100% dos animais em títulos moderados a altos, tanto para o BVDV-1 quanto para o BVDV-2 (ANZILIERO et al., 2015). Além da eficiência das vacinas disponíveis no Brasil ser questionável, elas não têm cepa de HoBi-like em sua composição. Em um

estudo experimental, ovinos vacinados com vacinas inativadas de BVDV apresentaram baixo ou indetectável nível de anticorpos neutralizantes contra o HoBi-like, indicando que provavelmente eles são suscetíveis a infecção por esse vírus. Desta maneira estes resultados reforçam a necessidade de revisão na formulação das vacinas para pestivírus no Brasil, com a inclusão de cepas brasileiras de BVDV-1, BVDV-2 e do HoBi-like vírus (ANZILIERO et al., 2015; DIAS et al., 2017).

2.10 Situação dos Pestivírus de Ruminantes nos Estados Unidos

O BVDV-1 tem sido detectado desde a década de 40 em bovinos nos EUA (OLAFSON; MacCALLUM; FOX, 1946), enquanto o BVDV-2 começou a ser detectado apenas no final da década de 80 (CORAPI; FRENCH; DUBOVI, 1989). Assim, as vacinas que já eram muito utilizadas até então começaram a ter em sua composição além do BVDV-1a o BVDV-2a (RIDPATH; FULTON, 2009).

Posteriormente, o BVDV-1a que era o subgenótipo predominante seguido do BVDV-2a e BVDV-1b, passou a ser identificado com menor frequência, até que nas últimas duas décadas houve uma mudança dessa predominância, quando o BVDV-1b passou a ser majoritariamente o subgenótipo mais detectado (FULTON et al., 2002; RIDPATH et al., 2011; WORKMAN et al., 2016). Além dos três subgenótipos mencionados, o BVDV-2b e BVDV-2c tiveram detecções esporádicas no país (FULTON et al., 2009; JENCKEL et al., 2014; WORKMAN et al., 2016). O vírus HoBi-like, até o momento, não foi detectado em nenhum hospedeiro nos EUA (BAUERMANN et al., 2014; BAUERMANN; RIDPATH; DARGATZ, 2017).

O BVDV é amplamente disseminado pelos rebanhos dos EUA (BAUERMANN; RIDPATH; DARGATZ, 2017; WITTUM et al., 2001). No entanto, não há programa nacional de controle de BVDV, como ocorre em outros países, somente programas regionais voluntários onde o principal foco é a detecção e remoção de bovinos PIs. Esses programas são impulsionados pela agregação de valor comercial nos bovinos que não são PIs e que possuem anticorpos anti-BVDV. Porém, como apenas alguns produtores adotam essa medida, não há redução efetiva da prevalência de BVDV, porque o vírus pode ser reintroduzido nos rebanhos (RIDPATH; BOLIN; DUBOVI; 1994; VAN CAMPEN, 2010).

Diferentemente dessas tentativas regionais de controle de BVDV, a vacinação é praticada em todo o território nacional. Estima-se que 68-87% dos bovinos são imunizados com vacinas, tanto KV, quanto MLV, contendo BVDV-1a e/ou BVDV-2a (BAUERMANN; RIDPATH; DARGATZ, 2017; NEGRÓN et al., 2011; WALZ et al., 2017). Contudo, por mais que o BVDV-1b seja o subgenótipo mais prevalente, ele não está incluso na maioria das vacinas comerciais (RIDPATH et al., 2011).

Consequentemente, nessa necessidade intrínseca de se ter vacinas mais eficientes, a inclusão de cepa de BVDV-1b é recomendada. Outras necessidades e desafios são enfrentados no controle de pestivírus nos EUA. Essencialmente, a vigilância precisa ser contínua para identificar quais pestivírus estão infectando os bovinos, a qual é realizada por uma rede de laboratórios em todo o país (DRISKELL; RIDPATH, 2006; RIDPATH, FULTON, 2009). Também se faz necessário conhecer sobre quais pestivírus estão circulando em outros ruminantes, tanto domésticos como os ovinos, quanto em silvestres, como os cervídeos, devido a possibilidade de transmissão interespécie e o impacto que isso pode ter para a saúde animal e o controle de BVDV (RIDPATH, FULTON, 2009), pois em muitas regiões dos EUA, por exemplo, bovinos e ovinos tem contato próximo. Ao contrário do BDV que foi poucas vezes detectado em ovinos no país (NEIL, RIDPATH, 2014; SULLIVAN; CHANG; AKKINA, 1997), o BVDV parece estar mais presente nesse hospedeiro (AMES et al., 1982; BRAKO et al., 1984; GOYAL et al., 1988; SILVEIRA et al., 2018b) o que demonstra que de fato bovinos e ovinos podem transmitir pestivírus entre si. Apesar disso, o conhecimento sobre a realidade dos pestivírus em ovinos no EUA é limitado e não há relato de uso de vacinas.

3. OBJETIVOS

3.1 Gerais

Realizar estudos epidemiológicos, genéticos e avaliar vacinas para pestivírus de ruminantes.

3.2 Específicos

- Investigar a diversidade genética de pestivírus detectados em ruminantes da região nordeste do Brasi.
- Sequenciar o genoma completo de HoBi-like pestivírus brasileiros.
- Realizar análises filogenéticas, genéticas e evolutivas de todos os HoBi-like pestivírus com genoma completo disponível no GenBank.
- Determinar a soroprevalência de anticorpos anti-pestivírus em ovinos domésticos do estado do Wyoming, Estados Unidos.
- Avaliar o *fitness v*iral *in vitro* de isolados de BVDV-1a, BVDV-1b e BVDV-2a através da técnica de PrimeFlow RNA.
- Avaliar a resposta humoral induzida por vacinas de replicon baseadas no gene E2 de BVDV-1a, BVDV-1b e BVDV-2a contra 26 cepas pertencentes a sete subgenótipos de BVDV; comparar esses resultados com a resposta humoral induzida por uma vacina de vírus vivo modificado, contendo cepas de BVDV-1a e BVDV-2a.

4. CAPÍTULOS

4.1 Capítulo 1: HoBi-like is the most prevalent ruminant pestivirus in Northeastern Brazil

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ORIGINAL ARTICLE

WILEY Transboundary and Emerging Diseases

HoBi-like is the most prevalent ruminant pestivirus in Northeastern Brazil

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Summary

The ruminant pestiviral species BVDV-1, BVDV-2 and BDV, along with the putative species HoBi-like, may cause substantial economic losses in cattle, sheep and goats. Brazil's large size, variable biomes and wide range of ruminant animal production within different geographic regions suggest that the presence and prevalence of ruminant pestivirus may differ by regions within Brazil. This study investigated the genetic diversity of ruminant pestiviruses and determined the frequency of active infections within two states of the Northeast Region of Brazil, Maranhão and Rio Grande do Norte. Serum samples from 16,621 cattle and 2,672 small ruminants from 569 different herds residing in this region were tested by RT-PCR followed by DNA sequencing. Seventeen positive cattle were detected (0.1%) from fifteen different herds (2.64%). All isolates were classified as HoBi-like pestiviruses based on phylogenetic analysis. All small ruminant samples tested negative. The findings presented herein suggest that the Northeast Region of Brazil has a uniquely high prevalence of HoBi-like viruses. The increasing reports of HoBi-like viruses detected in cattle in the field suggest that natural infection with these viruses may be more widespread than previously thought. The identification of HoBi-like viruses as the most prevalent type of ruminant pestivirus circulating in the Northeast Region of Brazil indicates the need for both continued monitoring and determination of the extent of economic losses associated with HoBi-like virus infections. In addition, it must be taken into account in the choice of diagnostic tests and in vaccine formulations.

KEYWORDS
active infection, cattle, HoBi-like, pestivirus, phylogeny, small ruminant

1 | INTRODUCTION

Ruminant pestivirus infections cause substantial economic losses to the livestock industry worldwide (Houe, 1999). Clinical manifestations range from mild to severe and may affect the respiratory, digestive and/or reproductive system (MacLachlan & Dubovi, 2011). The causative agent can be one of the three recognized species within the family *Flaviviridae*, genus *Pestivirus*: *Bovine Viral Diarrhea Virus type 1* (BVDV-1), BVDV-2, *Border Disease Virus* (BDV) (Simmonds et al., 2011) or one of the emerging putative pestivirus species. In particular, a putative species referred to as HoBi-like or BVDV-3 causes clinical signs similar to those caused by BVDV-1 and 2 infections in cattle and small ruminants (Decaro et al., 2011, 2014; Liu, Xia, Wahlberg, Belák, & Baule, 2009; Shi et al., 2016; Weber et al., 2016).

Pestiviruses are single-stranded, positive-sense RNA viruses that contain a genome of approximately 12.3 kb with a unique open reading frame that is flanked by a 5′ and 3′ untranslated region (UTR) (Simmonds et al., 2011).The 5′UTR is conserved among pestiviruses and is frequently used as the basis for diagnostic tests. The 5′UTR and the N-terminal autoprotease (N^{pro}) coding regions are frequently used for phylogenetic analysis (Booth, Thomas, El-Attar, Gunn, & Brownlie, 2013; Nagai et al., 2008; Vilcek et al., 2001).

Understanding the genetic diversity of pestiviruses in a country is essential to the development of regional control programmes (Dubovi, 2013; Fulton, 2015). Thus far, BVDV-1 is the most prevalent pestivirus species identified worldwide; however, the proportion of bovine pestivirus species varies by geographic region (Abe et al., 2016; Booth et al., 2013; Giammarioli, Ceglie *et al.*, 2015; Jones, Zandomeni, & Weber, 2001; Maya et al., 2016; Nagai et al., 2004; Weber et al., 2014; Workman et al., 2016). Brazil has several distinct geographic regions and a large agroindustry based on cattle production (IBGE, Instituto Brasileiro de Geografia e Estatística, 2016; USDA – FAS, United States Department of Agriculture, Foreign Agricultural Service, 2016). The type of industry and ecosystems differ widely between regions. While pestiviruses are widespread in Brazil, the type and prevalence of bovine pestivirus species within specific geographic regions are largely unknown. The goal of this study was to investigate the genetic diversity of bovine pestiviruses and determine the frequency of active pestivirus infections in ruminants from Brazilian Northeastern region.

2 | MATERIALS AND METHODS

2.1 | Study area, target population and sample size

This study was performed in the states of Maranhão (MA) and Rio Grande do Norte (RN), Northeast Brazil (Figure 1a, b). The sera analysed in this study were collected as part of the biannual surveillance study performed in Brazil in 2012–2013 to expand the *Foot-and-mouth disease* (FMD)-free zone using vaccination. Samples were stored at −20°C until analysis in 2016. The target population included all farms enrolled in the national FMDV control and eradication programme of these states. Samples were randomly drawn from farms located in counties with high cattle movement as defined by the State Veterinary Office data on livestock movement. Each state was subjected to a two-stage, sampling scheme, which considered farms as primary units and cattle up to 24 months of age as secondary units. Immunization of the target population with BVDV vaccines is unknown. A total of 11,264 cattle were sampled from 394 MA herds. In RN, 5,357 cattle from 175 herds and 2,672 small ruminants from 89 herds were sampled. The selected MA herds were predominantly beef cattle. Approximately 70% of the MA production units consisted of up to 500 cattle, whereas 30% consisted of small ruminants. Approximately 42% of the RN herds have dairy and beef cattle breeding together, and ~82% of the RN herds has up to 500 cattle. Small ruminants are present in ~58% of the selected RN herds.

2.2 | Sample preparation, RNA isolation and RT-PCR

A total of 19,293 serum samples were initially tested for pestivirus infection in 429 pools of up to 45 samples. Samples from the positive pools were then individually tested. This methodology of the pool testing was validated in a previous article (Weber et al., 2014). Total RNA was isolated using Quick-Zol® (Ludwig Biotec, Porto Alegre, Brazil) according to the manufacturer's instructions. cDNA synthesis and PCR were performed using a GoScript™ Reverse Transcription System and GoTaq® G2 Hot Start Polymerase (Promega, Madison, USA). The PCR amplification resulted in a product of 118 bp of 5′UTR (Weber et al., 2014).

2.3 | DNA sequencing and phylogenetic reconstruction

Partial sequences of the 5′UTR, N^{pro} and E2 coding regions were amplified by RT-PCR using the protocols described in Table 1. PCR amplification products were purified using a Wizard® SV Gel and PCR Clean-up System (Promega) according to the manufacturer's instructions. Both strands were sequenced using a Big Dye Terminator cycle sequencing kit, version 3.1, on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Multiple alignments of sequences generated in this study and representative strains within genus *Pestivirus* (whose accession numbers are shown in Table S1) were performed using the MUSCLE algorithm (Edgar, 2004). Phylogenetic reconstructions were performed applying the Maximum Likelihood method (Guindon & Gascuel, 2003) with Kimura 2-parameter substitution model (Kimura, 1980). All evolutionary analyses were conducted using the Molecular Evolutionary Genetics Analysis software package 7 (MEGA7) (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) with 1,000 bootstrap replicates each. HoBi-like genetic groups were defined according to Giammarioli, Ridpath et al. (2015).

3 | RESULTS

In this work, a total of 19,293 serum samples were tested by RT-PCR assay. Of these, 17 (0.09%) samples tested positive for pestivirus, where 0.09% (10/11,264) and 0.13% (7/5,357) of the samples correspond to cattle from MA and RN states, respectively. None of the small ruminant samples tested positive. At the herd level, nine of 394 MA herds (2.28%) and six of 175 RN herds (3.43%) had active infections, that means at least one RT-PCR positive animal (persistently or acutely infected). Thirteen of the positive herds had only one positive animal, whereas two herds (herds #125 and #168) had two positive animals. Figure 1 shows the location of the herds sampled and, of these, which had positive animals.

Sixteen partial 5′UTR sequences, 14N^{pro} sequences and 13 E2 sequences were obtained from the 17 positive samples. These sequences were successfully amplified using the following primers: forward and reverse for 5′UTR (Deregt et al., 2006); TF3 and TR3

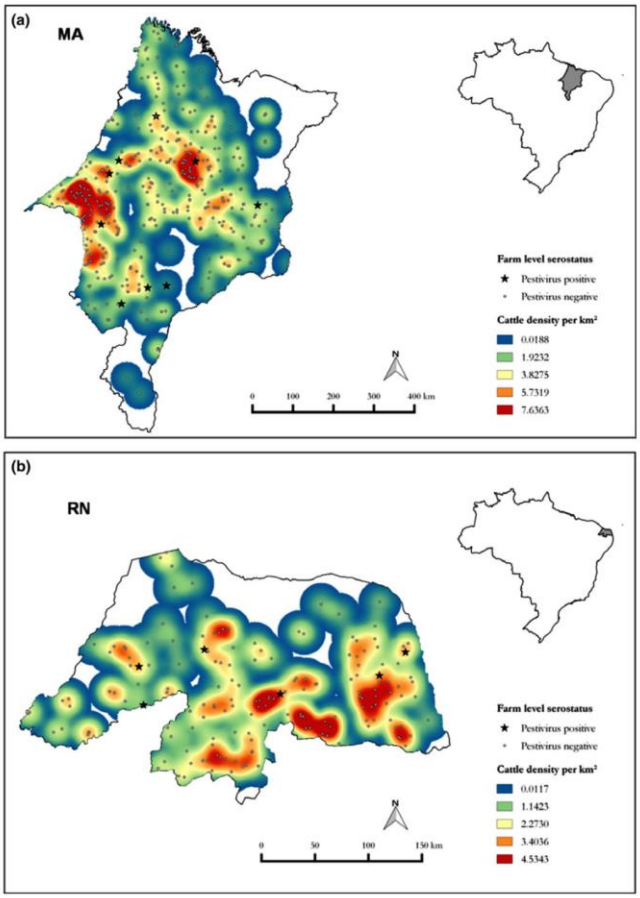

FIGURE 1 Spatial distribution of herds infected by ruminant pestiviruses in the states of Maranhão (MA) (a) and Rio Grande do Norte (RN) (b), Northeast Brazil. Cattle density was calculated assuming a radius of 40 km (MA) and 20 km (RN).

for N^{pro} (Liu, Kampa, Belák, & Baule, 2009); SF3 and SR3 for E2 (Liu, Xia et al., 2009).

Upon nucleotide BLAST analysis (https://blast.ncbi.nlm.nih.gov/), all sequences obtained in this study were found to share a high degree of identity with HoBi-like strains ranging from 84% to 100%, 79% to 99% and 89% to 98% in the 5′UTR, N^{pro} and E2 coding regions, respectively. The nucleotide similarity among the isolates generated herein ranged from 93.5% to 100% in 5′UTR, 92.2% to 100% in N^{pro} and 92.2% to 99% in E2 sequences.

Two herds, in particular, presented two positive animals. In herd #125, the sequences obtained for the RT-PCR-positive calves presented nucleotide identity of 97.5% in the 5′UTR and 93.5% in the E2 region. On the other hand, the sequences of the two animals from herd #168 shared 100% of identity in the 5′UTR, N^{pro} and E2 regions.

The phylogenetic tree obtained for the 5′UTR (Figure 2a) showed three well-separated clusters corresponding to HoBi-like viruses genetic groups a, c and d. All of the sequences obtained in this work were grouped into the genetic group "a" cluster. This cluster presented three branches corresponding to Th/04KhonKaen (GenBank accession number NC012812.1) detected in Thailand (Ståhl et al., 2007); HN1507 (GenBank accession number KU563155.1) detected in China (Shi et al., 2016); and the last branch contained strains detected in South America, Europe and China, as well as all the sequences reported herein. In general, the sequences obtained in this study were more closely related to previously reported strains than to each other. The N^{pro} phylogenetic tree (Figure 2b) revealed two well-separated clusters, HoBi-like viruses genetic groups a and c. The strains characterized in this study were grouped into genetic group a. To avoid loss of information due to

TABLE 1 Primers used for RT-PCR and DNA sequencing

Genomic region	Primers	References	Pestivirus species[a]
5′UTR	UTR-DL1F, UTR-DL4R	Refaat, Salem, Gafer, & Dardiri, (2010)	BVDV-1, BVDV-2
	Forward, reverse	Deregt et al., (2006)	BVDV-1, BVDV-2, BDV
	324, 326	Vilcek et al., (1994)	BVDV-1, BVDV-2, BDV
N^{pro}	TF3, TR3	Liu, Kampa et al., (2009)	HoBi-like pestivirus
	LV Pesti F, LV Pesti R	Weber et al., (2014)	BVDV-2
	BD1, BD3	Vilcek et al., (2001)	BVDV-1
	BD1, BD2	Vilcek et al., (1997)	BVDV-1, BVDV-2, BDV
E2	SF3, SR3	Liu, Xia et al., (2009)	HoBi-like pestivirus
	E2F, E2R	Tajima et al., (2001)	BVDV-1, BVDV-2

[a]Ruminant pestivirus species detected according to primer design.

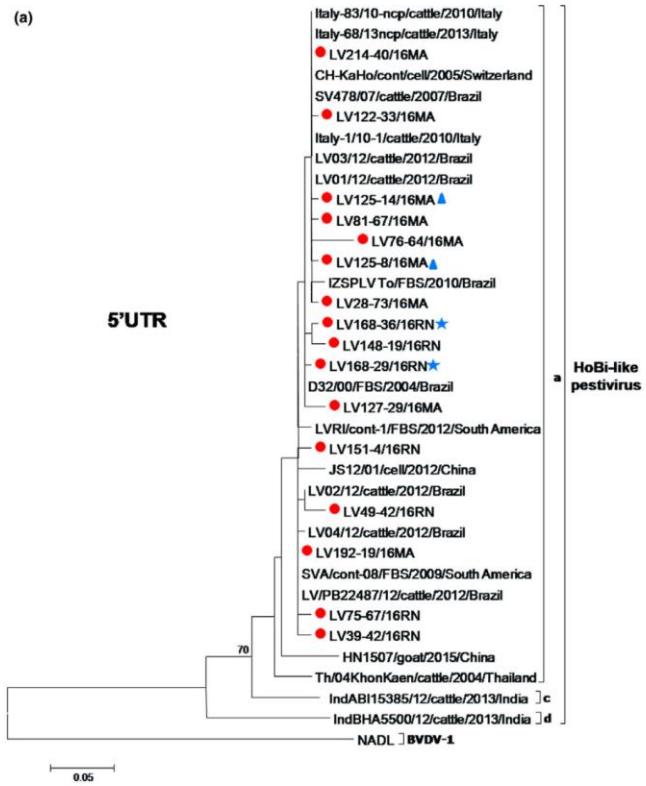

FIGURE 2 Phylogenetic tree based on the 5′UTR—198 bp (a), N^{pro}-432 bp (b) and E2—320 bp (c) sequences of reference strains and Brazilian pestivirus isolates. MEGA 7 was used for phylogeny inference according to the maximum likelihood algorithm. Bootstrap analyses that were supported by >70% of 1,000 replicates are indicated in nodes. Brazilian pestivirus isolates are highlighted with a symbol (●). HoBi-like virus sequences from the same herds are marked with a symbol (triangle and star). The GenBank accession numbers are listed in Table S1. The bar represents genetic distance.

FIGURE 2 (continued)

the shorter available sequences, strains from genetic group b were not included in the phylogenetic reconstruction of N^{pro}. Lastly, the E2 phylogenetic tree (Figure 2c) presented two clusters, with one containing the Th/04KhonKaen sequence and the other containing all of the remaining HoBi-like sequences, including those generated in this study.

4 | DISCUSSION

In the present study, the frequency of active infections caused by ruminant pestiviruses and the genetic diversity of these pestiviruses were determined in ruminants from Northeastern Brazil. Frequencies of 0.09% and 0.13% of pestivirus infections in cattle were obtained by RT-PCR followed by DNA sequencing. The frequencies of active infections reported in this study were similar to a previous study conducted in Southern Brazil that found 0.36% pestivirus-positive calves (Weber et al., 2014); however, the infection frequencies reported in the present study were lower than the 2.8% frequency reported in Belgium (Hanon et al., 2014). None of the small ruminant samples tested positive for pestivirus infection despite reports of natural infection of small ruminants with the bovine pestiviruses: BVDV-1, BVDV-2 and HoBi-like viruses (Han et al., 2016; Kim et al., 2006; Shi et al., 2016; Vilcek, Nettleton, Paton, & Bélak, 1997).

The genetic diversity of ruminant pestivirus species was verified by sequencing the 5′UTR, N^{pro} and E2 coding regions. Following detection by RT-PCR using a panpestivirus protocol (Weber et al., 2014), sequences were submitted to protocols specific for either BVDV-1, 2 or HoBi-like viruses (Table 1). At least one of these regions was sequenced from each isolate, and all strains generated herein were classified as HoBi-like viruses (Figure 2). The predominance of HoBi-like viruses observed in the present study contrasted with previous reports from Brazil (Silveira et al., 2017), South America (Jones et al., 2001; Maya et al., 2016), North America (Workman et al., 2016), Europe (Giammarioli, Ceglie *et al*., 2015; Tajima et al., 2001), Asia (Nagai et al., 2001; Zhang et al., 2014) and Australia (Ridpath, Fulton, Kirkland, & Neill, 2010), with BVDV-1 being the most prevalent pestivirus detected. A similar study was conducted in Southern Brazil, where BVDV-1 and BVDV-2 were detected; however, HoBi-like viruses were not present (Weber et al., 2014). It is important to highlight that a recent survey performed in India also

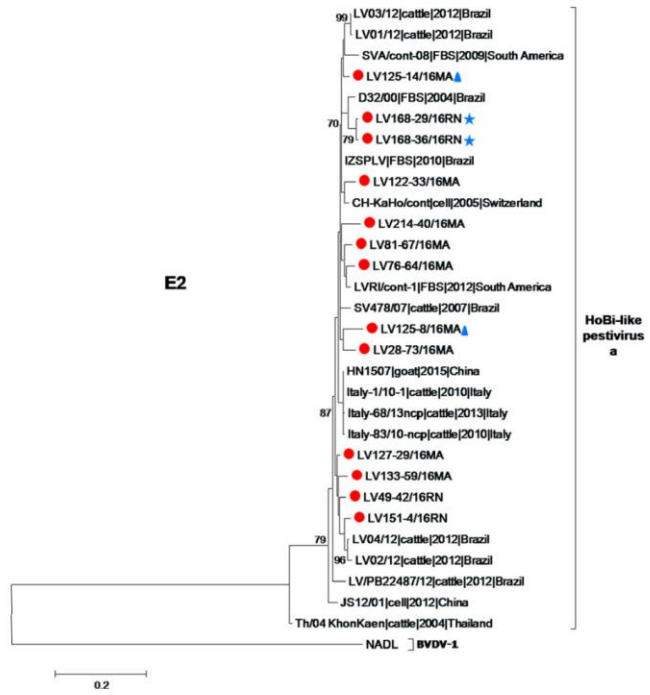

FIGURE 2 (continued)

found HoBi-like viruses as the most common bovine pestivirus species present in that subcontinent (Mishra et al., 2014).

The HoBi-like sequences analysed in the present study were also classified into genetic groups as defined by Giammarioli, Ridpath et al. (2015). All of the Brazilian strains were grouped into subtype "a," and only the strains reported in India were grouped into subtypes c and d (Figure 1a). Additional studies are required to determine the clinical and/or antigenic implications of these different genetic groups. Studies suggested that, even though HoBi-like viruses have a common ancestor, their evolution was related to geographic location (Giammarioli, Ridpath et al., 2015; Mishra et al., 2014).

Understanding regional genetic diversity of ruminant pestiviruses is important for establishing appropriate vaccination protocols that should include the viral variants present in each region (Mahony et al., 2005). It is important to highlight that cross-neutralization between different pestivirus species is low (Bauermann, Flores, & Ridpath, 2012; Bauermann et al., 2013; Pizarro-Lucero, Celedón, Aguilera, & Calisto, 2006; Ridpath et al., 2010); moreover, previous exposure to BVDV-1 and 2 may not protect against HoBi-like infection (Bauermann, Falkenberg, & Ridpath, 2016). The results presented in this study reinforce the need for vaccines against HoBi-like viruses, especially in regions where this pestivirus species is prevalent. The possibility of dissemination of HoBi-like viruses by biological products is a troubling threat, considering that approximately 40% of all HoBi-like pestiviruses identified in the world are from FBS or contaminated cell cultures (Giammarioli, Ridpath et al., 2015; Liu, Xia, Baule, & Belák, 2009; Mao, Li, Zhang, Yang, & Jiang, 2012; Pelleto et al., 2012; Schirrmeier, Strebelow, Depner, Hoffmann, & Beer, 2004; Silveira et al., 2017; Stalder et al., 2005; Xia, Vijayaraghavan, Belák, & Liu, 2011; Xia et al., 2012). Furthermore, the increasing worldwide identifications of this pestivirus species represent an emerging problem for countries with control and eradication programmes for ruminant pestiviruses.

In conclusion, the present study reported a HoBi-like frequency that was similar to the frequency reported in India (Mishra et al., 2014), but higher than those reported in other regions worldwide. This study extends the epidemiological understanding of HoBi-like viruses and suggests Northeastern Brazil as a possible original source of this pestivirus species. Considering the growing evidence for the occurrence of HoBi-like pestiviruses in geographically distant cattle populations, these emerging viruses present considerable risk to cattle health and management as well as to BVD control programmes. The detection of HoBi-like viruses in Northeastern Brazilian cattle emphasizes the need for continued herd monitoring and further

investigations into determining the extent to which HoBi-like virus infections cause economic losses in cattle production.

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CONFLICT OF INTEREST

None declared.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Table S1 – Pestivirus species and GenBank accession numbers of the pestivirus sequences that were analyzed in the present study and representative strains.

		Accession number		
Strain	Pestivirus species	5'UTR	N^{pro}	E2
Representative strains				
IZSPLV To	HoBi-like pestivirus	HM151361.1	HM151362.1	HM151363.2
D32/00	HoBi-like pestivirus	AB871953.1	AH013732.2	AY604725.1
SV478/07	HoBi-like pestivirus	KP715142	KP743038	KP743116
LV01/12	HoBi-like pestivirus	KC465388	KC465392	KC465396
LV02/12	HoBi-like pestivirus	KC465389	KC465393	KC465397
LV03/12	HoBi-like pestivirus	KC465390.1	KC465394.1	KC465398.1
LV04/12	HoBi-like pestivirus	KC465391	KC465395	KC465399
LV/PB22487/12	HoBi-like pestivirus	KP715144	KP743039	KP743115
Brzbuf9	HoBi-like pestivirus	-	AY895010.1	-
CH-KaHo/cont	HoBi-like pestivirus	JX985409.1	AY895011.1	JX985409.1
Italy-83/10-ncp	HoBi-like pestivirus	JQ612704.1	JQ612704.1	JQ612704.1
Italy-68/13ncp	HoBi-like pestivirus	KJ627179.1	KJ627179.1	KJ627179.1
Italy-1/10-1	HoBi-like pestivirus	HQ231763.1	HQ231763.1	HQ231763.1
JS12/01	HoBi-like pestivirus	JX469119.1	JX469119.1	JX469119.1
SVA/cont-08	HoBi-like pestivirus	FJ232692.1	FJ232693.1	FJ232694.1
LVRI/cont-1	HoBi-like pestivirus	KC297709.1	KC297709.1	KC297709.1
HN1507	HoBi-like pestivirus	KU563155.1	KU563155.1	KU563155.1
Th/04 KhonKaen	HoBi-like pestivirus	NC012812.1	NC012812.1	NC012812.1
IndBHA5500/12	HoBi-like pestivirus	KM201302.1	-	-
IndABI15385/12	HoBi-like pestivirus	KM201313.1	KM261877.1	-
NADL	BVDV-1	AJ133738.1	AJ133738.1	AJ133738.1
Study				
LV28-73/16MA	HoBi-like pestivirus	KY864929	KY864950	KY864965
LV39-42/16RN	HoBi-like pestivirus	KY864936	-	-
LV49-42/16RN	HoBi-like pestivirus	KY864926	KY864951	KY864954
LV75-67/16RN	HoBi-like pestivirus	KY864934	-	-
LV76-64/16MA	HoBi-like pestivirus	KY864924	KY864952	KY864955
LV81-67/16MA	HoBi-like pestivirus	KY864930	KY864953	KY864956
LV122-33/16MA	HoBi-like pestivirus	KY864931	KY864940	KY864957
LV125-8/16MA	HoBi-like pestivirus	KY864927	-	KY864958
LV125-14/16MA	HoBi-like pestivirus	KY864932	KY864941	KY864959
LV127-29/16MA	HoBi-like pestivirus	KY864925	KY864942	KY864960
LV133-59/16MA	HoBi-like pestivirus	-	KY864943	KY864961
LV148-19/16RN	HoBi-like pestivirus	KY864928	KY864944	-
LV151-4/16RN	HoBi-like pestivirus	KY864935	KY864945	KY864962
LV168-29/16RN	HoBi-like pestivirus	KY864939	KY864946	KY864963
LV168-36/16RN	HoBi-like pestivirus	KY864938	KY864947	KY864964
LV192-19/16MA	HoBi-like pestivirus	KY864937	KY864948	-
LV214-40/16MA	HoBi-like pestivirus	KY864933	KY864949	KY864966

"-" absent

4.2 Capítulo 2: Phylogenetic and evolutionary analysis of HoBi-like pestivirus: Insights into the origin and dispersal

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Phylogenetic and evolutionary analysis of HoBi-like pestivirus: Insights into origin and dispersal

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Abstract

The HoBi-like pestivirus (HoBiPeV), currently classified as *Pestivirus H* species, is a pathogen associated with a broad spectrum of clinical manifestations in ruminants, particularly in cattle. Since HoBiPeV complete genome sequencing data is scarce, in the present study we described five nearly complete new Brazilian HoBiPeV genomes and further perform a more complete genetic and evolutionary characterization with all additional genome sequences available in the GenBank database. Entropy and selection pressure analysis showed the E2 gene, a surface glycoprotein, is the most variable gene, which also displays the greatest number of sites under positive selection. Phylogenetic and Bayesian inference based on complete genome and N^{pro} gene, respectively, from all HoBiPeV sequences available so far, confirms the existence of three main clades (a, b, and c). The abovementioned analysis suggest that this pestivirus species probably emerged in Asia and spread to different regions including Brazil, where only strains belonging to specific genetic group "a" have been found. The hypothesis of the HoBiPeV introduction in Brazil (between 1890 and 1962), formulated based on Bayesian inference, coincides with a period of intensive importation of water buffalo (*Bubalus arnee*) and indicine cattle (*Bos taurus indicus*) from Asia to Brazil, suggesting that this could be the origin of the current Brazilian HoBiPeV genetic group "a".

Keywords: Pestivirus, selection pressure, entropy, phylogeny, virus evolution

Running title: HoBiPeV: Insights into origin and dispersal

1 Introduction

Pestiviruses are positive-sense single-stranded RNA viruses that belong to the genus *Pestivirus*, family *Flaviviridae*. The HoBi-like virus (HoBiPeV), or bovine viral diarrhea virus-3 (BVDV-3) and currently named as *Pestivirus H*, is an emerging cattle pathogen (Shi et al., 2016; Silveira et al., 2018). The viral genome is around 12.3 Kb long and contains a unique open reading frame (ORF) that is flanked by two untranslated regions (UTRs) of roughly 600 nucleotides at total. The ORF encodes a polyprotein of ~3900aa or ~11.7 Kb nucleotides long, that is processed into four structural proteins (C-E^{rns}-E1-E2) and seven non-structural proteins (N^{pro}-p7-NS2/NS3-NS4A-NS4B-NS5A-NS5B) (Tautz et al., 2015; Smith et al., 2017).

Initially, HoBiPeV was identified in Brazilian fetal bovine serum samples exported to Germany (Schirrmeier et al., 2004). Nonetheless, it was discovered that the virus had been circulating in Brazil for a longer time than previously estimated. In 1996, it was isolated from Brazilian water buffaloes (Stalder et al., 2005). Subsequently, the virus was detected in cattle, small ruminants, and fetal bovine sera from South America (Weber et al., 2016; Cruz et al., 2018; Silveira et al., 2018), Asia (Ståhl et al., 2007; Shi et al., 2016) and Italy (Decaro et al., 2012). This growing number of HoBiPeV detections, especially after the identification of the highly divergent Asian strains (Mishra et al., 2014), allowed a proposed classification into four distinct genetic groups, starting from BVDV-3a to -3d (Giammarioli et al., 2015), here named as groups "a"-"d".

The clinical signs caused by HoBiPeV infection are similar to those caused by other ruminant pestiviruses, such as the BVDV-1 and BVDV-2. This includes respiratory disease, reproductive failure, acute gastroenteritis, and mucosal disease-like syndrome (Decaro et al., 2012; Weber et al., 2016). However, despite similarities in clinical presentation with other ruminant pestiviruses, the HoBiPeV has genetic and antigenic differences that may impact both vaccine protection and the efficiency of diagnostic tools (Mósena et al., 2017; Riitho et al., 2018). Currently, there are no vaccines available to prevent infections caused by HoBiPeV in cattle. Hence, this virus may present an emerging threat to countries with BVDV-1/BVDV-2 eradication programs and to countries where this virus is endemic (Bauermann et al., 2017).

Despite the increasing number of studies detecting the infections caused by HoBiPeV, the understanding of its origin, dispersion, and genetic variability is still limited, especially due to low number of complete genome sequences and diversity on geographical origin strains. Thus, the present study describes the nearly complete genome sequence of five new Brazilian HoBiPeV and,

along with all available complete genome sequences, performed a broader phylogenetic and selection pressure analysis, alongside temporal dispersion to increase the extant knowledge on HoBiPeV and attempt to elucidate its origin and diversification. This study demonstrates the importance of increase the number of complete genome sequences available in public databases to enable more complete analysis on this species.

2 Material and methods

2.1. HoBiPeV genome sequencing

Five serum samples positive by RT-PCR for HoBiPeV, named LV168-29/16RN, LV168-36/16RN, LV125-8/16MA, LV127-29/16MA, and LV03-12, were selected to obtain the complete genome sequences in this study. These strains were recovered from cattle in the Brazilian Northeast, region of country with the highest number of HoBiPeV identified so far (Weber et al., 2016; Silveira et al., 2018). Briefly, serum samples harboring HoBiPeV were centrifuged at 3,000 x g for 15 minutes to remove small debris. The total RNA was isolated using a TRIzol LS reagent (Ambion, USA) according to the manufacturer's instructions. A whole transcriptome amplification kit (WTA2, Sigma Aldrich, US) was utilized to convert the viral RNA into dsDNA. Illumina libraries were prepared using a Nextera XT DNA Sample Preparation kit (Illumina, US) and sequenced on an Illumina MiSeq platform, with a paired-end run of 2 x 150 nucleotides (nt).

The quality of the reads was assessed by FastQC and trimmed with the Geneious software (v. 11.1). The reads were assembled into contigs using SPAdes 3.10 and imported into the Geneious software (v. 11.1), subsequently being pre-processed and compared to sequences in the GenBank nucleotide and protein databases using BLASTn and BLASTx, respectively. The genome organization and annotation were predicted based on previous annotated full-length HoBiPeV sequences available in the GenBank database, using the Geneious software.

2.2. Complete genomics analysis

All HoBiPeV complete genomes available on the GenBank database until 13 April 2019 (Table S1) were aligned and compared with the five nearly complete sequences obtained in the present study. Multiple sequence alignments were carried out by employing the MUSCLE software. The host DNA sequence insertions were removed from the cytopathogenic strain sequences.

The dataset was checked for the presence of the recombinant sequences prior to any analysis, using the GARD under the Datamonkey web application (http://www.datamonkey.org/) and the Recombination Detection Program 4 (RDP4) software. Different detection methods (RDP, Genecov, Bootscan, Maxchi, Chimaera, Siscan, 3Seq) were implemented in the RDP4, with a P-value adjusted to 0.01. If the sequence presented a signal of the recombination events, supported by at least four methods, they were excluded from the analysis. The final dataset, comprising 16 nearly complete genome sequences, was submitted for phylogenetic reconstruction as well as entropy and selection pressure analysis.

2.2.1. Phylogenetic analysis

Phylogenetic reconstruction was performed by applying the Maximum Likelihood method, using the best-fit substitution model for the dataset, a general time-reversible with a gamma distribution and invariant sites (GTR+G+I). All phylogenetic analysis were conducted using the Molecular Evolutionary Genetics Analysis software package 7 (MEGA7), with 1000 bootstrap replications.

2.2.2. Entropy and selection pressure analysis

The variation in the amino acid composition of the polyprotein in the alignment dataset was analyzed by the Shannon entropy plot, as per the Los Alamos National Laboratory HIV sequence database website (https://www.hiv.lanl.gov/content/sequence/ENTROPY/entropy_one.html). The selection pressure acting on individual codons of the ORF was investigated by employing five algorithms: single-likelihood ancestor counting (SLAC), fixed effects likelihood (FEL), mixed effects model of evolution (MEME), along with the fast-unbiased Bayesian approximation (FUBAR), available on DataMonkey, and HyPhy, available on the MEGA7 software.

The strength of the selection pressure was determined on the basis of the ratio (□) of non-synonymous (d*N*) to synonymous (d*S*) substitutions per site. When □ > 1 with $p < 0.1$ or posterior probability ≥ 0.9 (FUBAR) was an indicative the action of positive selection. If the amino acid site met the criteria mentioned above through at least two algorithms, it was considered to be evolving under a positive selection.

The E2 amino acid sequence of the Brazilian HoBiPeV strain SV478/07 (GenBank accession number KY767958) was retrieved from the GenBank database and used as a target for

homology modelling, using the SWISS-MODEL server. The best homology models were selected according to the GMQE (Global Model Quality Estimation) and QMEAN statistical parameters. The overall stereochemical quality, including the backbone torsional angles, was checked through the Ramachandran plot, according to PROCHECK (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/). Lastly, the sites under positive selective pressure were mapped onto the E2 model using the UCSF Chimera v 1.13.

2.3. Time to the most recent common ancestor (tMRCA) analysis

A dataset of all the available 23 HoBiPeV N^{pro} gene sequences from GenBank in 13 April 2019 (Table S1) was aligned and used to infer a maximum likelihood (ML) genealogy in PhyML. The SH-aLRT method was utilized to gather support for branching in the tree topology. The resultant phylogeny was evaluated in TempEst (http://beast.community/tempest) to assess the temporal signal. The dataset was then used to estimate the rate of molecular evolution and the tMRCA, using a Bayesian MCMC method implemented in BEAST v1.8.3.

The Bayesian analysis assumed an uncorrelated log-normal relaxed molecular clock and a non-parametric Bayesian Skyline Plot as a model for the demographic history. The MCMC chain was run for 2 x 10^9 chain steps, and the convergence was evaluated in a TRACER v1.6. A Maximum clade credibility (MCC) tree was summarized using a TreeAnnotator v1.8.3 and visualized with FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). Uncertainties in the parameter estimates were reflected in the 95% highest probability density (HPD) values.

3 Results

3.1. Sequence analysis

The nearly complete genome sequences of five Brazilian HoBiPeV strains were obtained (Table 1). All genomes displayed a classic virus genome organization (Tautz et al., 2015), comprising 12,032–12,295 nt. The complete sequence of the single ORF (11,700 nt long) was determined, encoding a polyprotein of 3,899 amino acids. The UTRs sequences were partially determined, whose length ranged from 332-595 nt at total. Neither viral genomic rearrangements nor host genomic insertions were observed in the five genomes.

The nearly complete Brazilian strain genomes were compared with all 11 complete HoBiPeV sequences available in the GenBank in 13 February 2019 (Table S1). The pairwise

comparisons revealed that the nucleotide identity among the complete genome sequences ranged between 90%–99.5% and the similarity between the amino acid sequences were 93.2%–99.3%.

3.2. Entropy and selection pressure analysis

The polyprotein amino acid sequence of the five Brazilian strains from this study and 11 from the literature were aligned and their variability was estimated using the Shannon entropy. A high entropy value indicates a higher variability at a given position, while values close to zero denote amino acid conservation. These current analysis revealed an entropy range between 0–1.180 (Fig. 1A) throughout the genome. The whole ORF showed 15% of the positions presenting amino acid variability, with the Shannon entropy ≥ 0.234. E2 was the most variable gene, presenting 25.2% of the sites under variation. This gene encodes a surface glycoprotein that is the main immunoprotective protein of viruses, and it displays receptor-binding activity. The E2 is divided into four antigenic domains, ranging from A to D (El Omari et al., 2013). The domain A (Fig. 1B) presented the highest frequency of amino acid sites under variation (34.5%) as well as the highest values of Shannon entropy. It was followed by domain B (27.6%), domain C (24.5%), and domain D (13.1%). After the E2, the genes that showed the greatest amino acid variability within their genomes were NS5A (21.1%) and N^{pro} (20.2%).

Amino acid substitutions were also evaluated to elucidate the nature of the selective pressure. A total of 18 sites were identified in terms of positive selection (9 sites by at least two methods, 6 sites by 3 methods, and 3 sites by 4 methods), corroborating the results of the Shannon entropy. The E2 region was found to present the highest number of sites (11/373) under positive selection (Table 2). These sites were mainly concentrated in domains A (n= 5) and C (n= 3) of E2 gene (Fig. 2). On the other hand, both N^{pro} and N5A had only one amino acid under each positive site, while NS5B and NS23 had two and three, respectively (Table 2).

3.3. Phylogenetic analysis and time to the most recent common ancestor (tMRCA) analysis

A phylogenetic tree using all nearly complete genome sequences was reconstructed under a maximum likelihood approach (Fig. 3). All HoBiPeV clustered in genetic group "a", including the Brazilian strains described in this study, which were grouped within the group "a" into three separated clades. Then, strains LV125-8/16MA and LV03/12 shared a direct common ancestor as well as Strains LV168-36/16RN and LV168/29/16RN, while strain LV127-29/16MA was in an exclusive clade.

After verifying that the whole-genome dataset gave an anomalous temporal signal, we tested three different gene alignments: N^{pro}, E2, and NS5A. These genes encode proteins essential to host immune response escape (N^{pro}, E2), cellular tropism and host range (E2), and virus replication (NS5A). Besides, both genes showed the greatest amino acid variability within their genome.

Notwithstanding, only N^{pro} had a significant correlation between sampling date and evolutionary rate, which enabled the inference of the evolutionary rate under a Bayesian approach. A mean evolutionary rate of 5.51×10^{-5} substitution/site/year was estimated by using a relaxed molecular clock parameter. The MCC tree (Fig. 4) included 23 HoBiPeV N^{pro} sequences (encompassing the five genomes recovered in this study) and corroborated the results of the whole-genome ML phylogeny (Fig. 3). Three well-supported clades were clearly observed, corresponding to the genetic groups "a", "c", and "d". All the sequences isolated in Brazil belonged to group "a". Unfortunately, no complete N^{pro} sequence of the genetic group "b" was available in GenBank, interfering on the analysis performance.

For both the main clades and the 95% HPD, the tMRCA was inferred; the first evolutionary event for HoBiPeV took place when the genetic group "c" and "d" diverged and evolved independently, most likely in the 16th (1566, 95% HPD 1001–1901) and 18th (1724, 95% HPD 1345–1933) centuries. The diversification of group "a" started around 1856 (95% HPD 1656–1969), when two sub-clades arose. One of these clades was represented by only one strain (Th/04), detected in Thailand. The second group encompasses 20 HoBiPeV strains and includes all Brazilian strains, in addition to European and Asian sequences. This cluster can be dated back to 1936 (95% HPD 1840–1987).

4 Discussion

Even though HoBiPeV have been detected since 2004, its identification became more common only after 2011, mainly from Brazilian samples. Thereby, this study described five nearly complete Brazilian HoBiPeV genomes sequences from a Brazilian region (northeast) which shows an abnormally high prevalence of HoBiPeV (Silveira et al., 2018). Besides, this study also performed a more complete genetic and evolutionary characterization of all HoBiPeV genome sequences available in the GenBank, complementing previous characterizations performed with a limited set of available sequences and restricted genomic regions.

Herein, the E2 was the most variable gene and bearer of most sites evolving under a diversifying (positive) selection, reinforcing previous studies results on HoBiPeV. The positive sites observed in this study lies mainly in DA followed by DC, which corroborates with previous analysis based on the E2 of the HoBiPeV (Weber et al., 2017), BVDV-1 and BVDV-2 (Tang and Zhang, 2007; Lang et al., 2014; Chernick et al., 2018), and are a consequence of the DA being the target of most neutralizing antibodies. Some non-structural genes (NS5A, p7, N^{pro}) also showed high amino acid variability at some sites and need to be further characterized alongside the role of these proteins.

Since the origin and divergence of HoBiPeV is still a matter of speculation, a Bayesian molecular clock was carried out to increase information and infer hypothesis about this issue. The N^{pro} was chosen for these analysis due to its suitable variability and presence of temporal signal. The NS5A gene seems to be less variable than required for the Bayesian analysis, and E2 seems highly variable with respect to performing well in reconstructing past events. Therefore, among the candidate genes, N^{pro} was the only which allowed some acceptable molecular clock analysis.

Phylogenetic reconstruction by N^{pro} suggests a relatively recent origin for the HoBiPeV, despite the high confidence interval (1566, 95% HPD 1001–1901). The presence of Indian sequences rooting the tree may suggest an Asian origin of these viruses, and apparently genetic group "a" (that includes the Brazilian isolates) emerged more than a century after the initial putative origin of the virus (1856, HPDs 1656–1969). Although the high confidence interval could limit the inference on time scale, our study result corroborates with a previous study (Liu et al., 2009), where the genetic group "a" also underwent a spread starting around 1880 (HPDs 1651–1993). Bayesian analysis from this previous study was performed only with sequences of the three HoBi-like strains that were available at that time, while the current study uses a broader set of sequences along with the five new Brazilian genomes.

Despite the uncertainty of the Bayesian analysis, the presented dates coincide with an intense importation of water buffaloes (*Bubalus arnee)* and indicine cattle (*Bos taurus indicus*) from Asia to Brazil in the period between 1890–1962. These events were responsible for the increase in livestock in the Brazilian northeast, the virus' main entry region in this country (Ajmone-Marsan et al., 2010; Cabrera, 1986). Coincidentally, The Brazilian northeast has a high prevalence of HoBiPeV (Silveira et al., 2018), similar to India (Mishra et al., 2014), a scenario that

is not observed in any other region in the world. This strengthens the hypothesis on a possible origin of the HoBiPeV in Asia and not in Brazil, as initially proposed (Bauermann et al., 2013).

Brazilian viruses belong to the genetic group "a", along with European and a few Asian viruses (Figure 3, Figure 4). Brazil seems to have an important role on the HoBiPeV description in other countries. In 2004 the HoBiPeV was first identified in Europe, in FBS imported from Brazil (Schirrmeier et al., 2004), and to date, more than one third of all HoBiPeV identified in the world have come from FBS, labeled as having a Brazilian or (broadly) South American origin (Bauermann et al., 2013; Silveira et al., 2017; Jardim et al., 2018; Monteiro et al., 2018). This fact could be explained by an increase of the Brazilian bovine population in the 1970's (ABIEC, 2016) and a increasing exportation of Brazilian bovine products to several countries.

Unfortunately, the scarce number of sequences prevent deep phylogenetic conclusions and may interfere with the confidence intervals for the age of the common ancestors. Besides, only the genetic group "a" viruses have their complete genome sequence available in the Genbank database. We encourage additional data and research in order to obtain more HoBiPeV sequences from different countries and geographic regions. Therefore, more studies will confirm, elucidate or reject the hypothesis raised in our study about the origin and dissemination of the HoBiPeV.

We observed that HoBiPeV probably emerged in Asia, thereafter spreading to different regions around the world. This virus was probably introduced in Brazil several times after the 1900's, coinciding with an intensive importation of water buffalo and humped cattle (*Bos taurus indicus*) from Asia. Besides, sites under positive pressure were identified mainly in the E2 gene, encoding the most immunogenic pestivirus protein. Thus, this study expands the limited knowledge on the HoBiPeV diversification and suggests an evolutionary history hypothesis based on a more complete set of genetic sequences.

Conflict of Interest

None declared.

Acknowledgements

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Ethical Statement

Ethical approval was not required for this study. Biological samples used to RNA extraction followed by DNA sequencing were collected in previous studies (Weber et al., 2016; Silveira et al., 2018) and the present study involved only *in silico* analysis.

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Figures

Fig. 1. Global amino acid variability of the HoBiPeV polyprotein (A) and the E2 glycoprotein (B). Variability is represented by the Shannon entropy plot. Analysis were performed using the Shannon Entropy-One from NCBI HIV sequence Database tool. Highest entropy value shows the maximum variability at that particular position. The genomic regions and their percentage of amino acid variability are shown in the figure bottom (A). The four E2 domains, DA, DB, DC, and DD, along with the transmembrane domain (TM), are shown in the figure bottom (B).

Fig. 2. Structural model of the HoBiPeV E2 protein. The model was predicted by homology based on BVDV-1 E2 crystal, as described in the Methods sections. Structural domains are represented on monomer. Domain 'A' starts from the N terminus, colored in yellow (DA), green (DB), blue (DC), and magenta (DD). Amino acids under positive selection are colored red.

Fig. 3. Phylogenetic tree based on full-length sequences (12,074 nt) of the HoBiPeV, genetic group a. MEGA 7 was used for phylogeny inference under a maximum likelihood approach. Bootstrap analysis supported by > 70% of 1000 replicates are indicated in nodes. The GenBank accession numbers are listed in Table S1 (available in the online version of this article). The branch lengths are drawn to scale, with the bar at the bottom indicating the nucleotide substitutions per site. Sequence name: strain/host/country/collection date. The HoBiPeV sequenced in this study are highlighted with a green dot (●).

Fig. 4. Bayesian phylogenetic tree of 23 HoBiPeV N^{pro} sequences (504 nt). The phylogeny was estimated using the Bayesian MCMC approach under a log-normal relaxed molecular clock model and a non-parametric Bayesian Skyline Plot (as a model to determine demographic history). The grey horizontal bars denote the 95% HPD credible region used to determine the age of each node (see the timescale below the phylogeny). The GenBank accession numbers are listed in Supplementary Table 1. Sequence names include information about strain/country/collection date. Genetic groups "a", "c", and "d" are denoted by different colors. The HoBiPeV strains isolated in this study are highlighted with a symbol (o).

Tables

Table 1. Summary of sequencing data for five HoBiPeV nearly complete genome sequences obtained in this study.

Table 2. Positively selected sites in HoBiPeV polyprotein ORF (3,889 codons). Sites positive by at least two methods are included in the list.

Supplementary material

Table S1 – Accession numbers, reference, country of origin and year of the HoBiPeV included in the data set.

Table 1. Summary of sequencing data for five HoBiPeV nearly complete genome sequences obtained in this study.

Strain	Genome lenght (nt)	Accession number	5'UTR (nt)	3'UTR (nt)	GC content
LV168-29/16RN	12,184	MH410813	335	149	46.2%
LV125-8/16MA	12,156	MH410814	254	202	46.3%
LV127-29/16MA	12,096	MH410815	346	49	46.5%
LV168-36/16RN	12,295	MH410812	392	203	46.2%
LV03-12	12,032	MH410816	278	54	46.3%

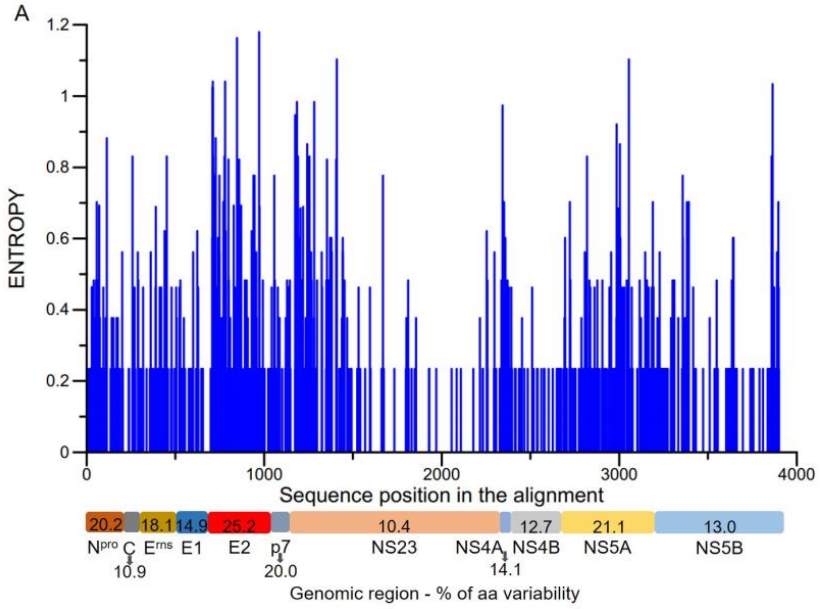
Table 2. Positively selected sites in HoBiPeV polyprotein ORF (3,889 codons). Sites positiv
by at least two methods are included in the list.

	aa position	aa†	FEL‡	MEME‡	SLAC‡	FUBAR§
N^{pro}	114	V	✓	✓	✓	✓
E2	710	N	✓	✓		✓
	711	L	✓	✓	✓	✓
	712	L	✓	✓		✓
	722	Y	✓	✓		
	748	R	✓	✓		
	780	L	✓	✓		✓
	847	T	✓	✓	✓	✓
	870	E/K	✓	✓		✓
	946	K	✓	✓		
	957	L	✓	✓		
	974	T	✓	✓		
NS2-3	1204	T	✓	✓		
	1292	L	✓	✓		
	1353	G	✓	✓		
NS5A	2995	K	✓	✓		✓
NS5B	3357	A	✓	✓	✓	
	3381	V	✓	✓		

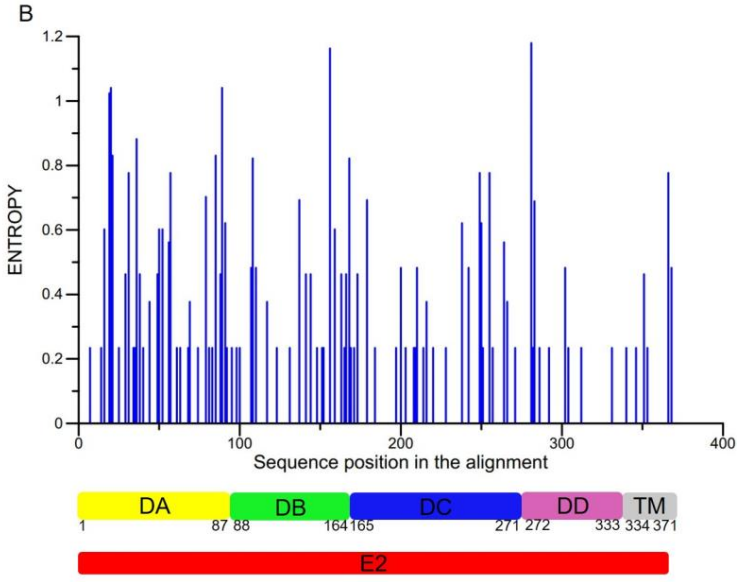
† aa more frequent.

‡ Criteria to consider sites with significant evidence of positive selection: p-value <0.1 in SLAC (single-likelihood ancestor counting), FEL (fixed effects likelihood), MEME (mixed effects model of evolution).

§ Posterior probability ≥0.9 in FUBAR (fast unbiased Bayesian approximation).



tbed_13520_f1a.tiff



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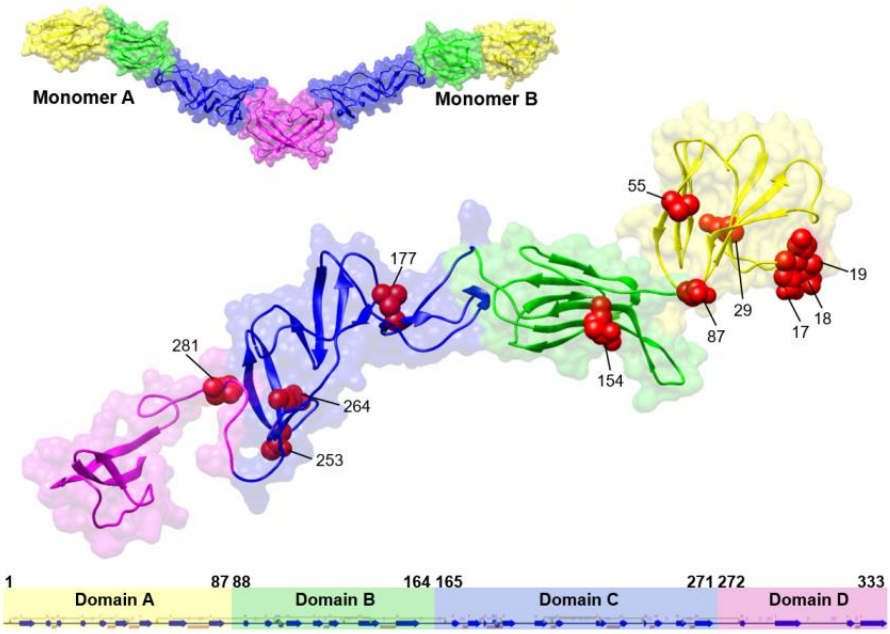

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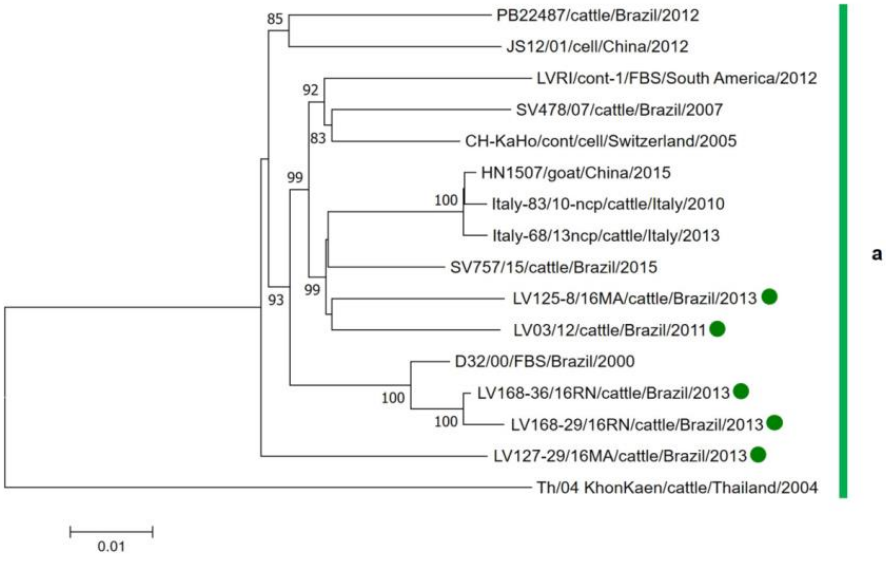
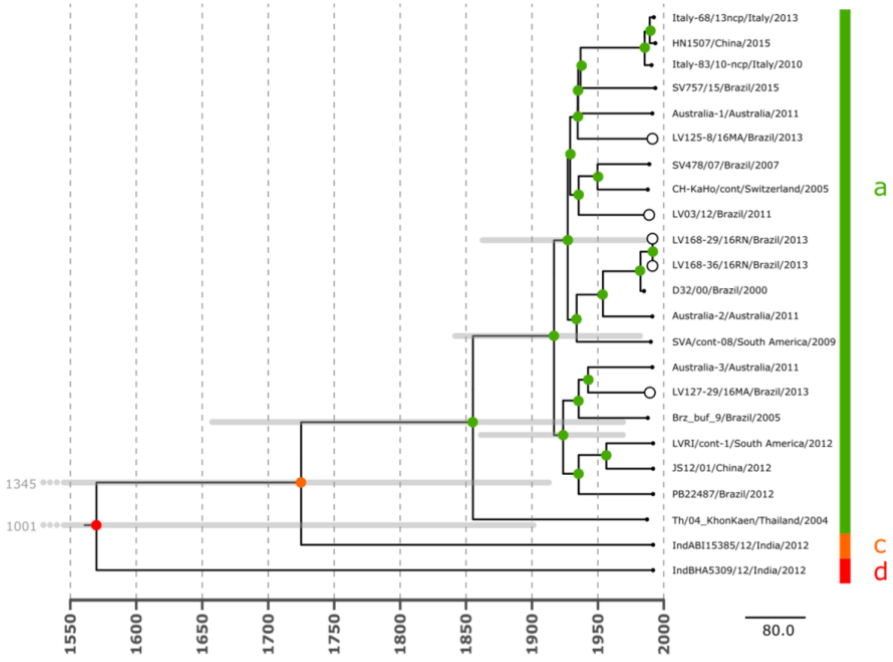


tbed_13520_f3.tif



tbed_13520_f4.tif

Supplementary material

Table S1 – Accession numbers, reference, country of origin and year of the HoBiPeV included in the data set.

HoBiPeV	Accession No.	Country	Year†	Reference
Whole genome				
Italy 83/10	JQ612704	Italy	2010	Decaro et al., 2012
Italy 68/13	KJ627179	Italy	2013	Decaro et al., 2014
JS12/01	JX469119	China	2012	Mao et al., 2012
Th/04	NC012812.1	Thailand	2004	Stahl et al., 2007
CH-KaHo/cont	JX985409	Switzerland	2005	Stalder et al., 2005
LVRI/cont-1	KC297709	South America	2012	Gao et al., 2016
PB22487	KY762287	Brazil	2012	Mósena et al., 2017
SV478/07	KY767958	Brazil	2007	Mósena et al., 2017
SV757/15	KY683847	Brazil	2015	Cortez et al., 2017
HN1507	KU563155	China	2015	Shi et al., 2016
D32/00	AB871953	Brazil	2000	Schirrmeier et al., 2004
LV03-12	MH410816	Brazil	2011	Weber et al., 2016
LV125-8/16MA	MH410814	Brazil	2013	Silveira et al., 2018
LV127-29/16MA	MH410815	Brazil	2013	Silveira et al., 2018
LV168-29/16RN	MH410813	Brazil	2013	Silveira et al., 2018
LV168-36/16RN	MH410812	Brazil	2013	Silveira et al., 2018
N^{pro}				
Australia-3	HQ403056.1	Australia	2011	Xia et al., 2013
Australia-2	HQ403055.1	Australia	2011	Xia et al., 2013
Australia-1	HQ403054.1	Australia	2011	Xia et al., 2013
IndBHA5309/12	KM261864.1	India	2012	Mishra et al., 2014
IndABI15385/12	KM261877.1	India	2012	Mishra et al., 2014
SVA/cont-08	FJ232693.1	South America	2009	Liu et al., 2009
Brz_buf_9	AY895010.1	Brazil	1996	Stalder et al., 2005

† Collection date, if it is unknown or uncertain, the detection year is stated.

4.3 Capítulo 3: Serological survey for antibodies against pestiviruses in Wyoming domestic sheep

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Serological survey for antibodies against pestiviruses in Wyoming domestic sheep

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ABSTRACT

Pestiviruses including Bovine viral diarrhea virus type 1 (BVDV-1), BVDV-2 and Border disease virus (BDV) have been reported in both sheep and cattle populations, together with the HoBi-like, an emerging group of pestiviruses. Pestivirus control programs in the United States have focused on the control of BVDV-1 and 2. The incidence of pestivirus infection in sheep in the United States and the risk of transmission between cattle and sheep populations are unknown. The aim of this study was to perform serological surveillance for pestivirus exposure in sheep from an important sheep producing state in the Unites States, Wyoming. For this, sera from 500 sheep, collected across the state of Wyoming (US) in 2015–2016, were examined by comparative virus neutralization assay against four species/proposed species of pestiviruses: BVDV-1, BVDV-2, BDV and HoBi-like virus. Rates of exposure varied between geographic regions within the state. The overall pestivirus prevalence of antibodies was 5.6%. Antibodies were most frequently detected against BVDV-1 (4%), and the highest antibody titers were also against BVDV-1. Data from this study highlights understanding of the dynamics of sheep pestivirus exposure, consideration of reference strains used for VN assays, transmission patterns, and potential vaccination history should be taken into account in implementation of control measures against pestiviruses in sheep and for successful BVDV control programs in cattle.

1. Introduction

Ruminant pestivirus infections cause severe economic losses to the livestock production worldwide. The genus *Pestivirus* consists of four species: *Bovine viral diarrhea virus type 1* (BVDV-1), BVDV-2, *Border disease virus* (BDV) and *Classical swine fever virus* (CSFV). In addition, unclassified emerging pestiviruses have been identified in ruminants, such as HoBi-like viruses (Simmonds et al., 2017).

Pestiviruses are not strictly host specific and can infect even-toed ungulates such as sheep, cattle, goats, swine and wild ruminants (Schweizer, Peterhans, 2014). BVDV-1, BVDV-2, BDV and HoBi-like viruses have been identified in both sheep and cattle. Clinical manifestations are variable and include respiratory, reproductive, and enteric problems (MacLachlan and Dubovi, 2011; Shi et al., 2016; Partida et al., 2017; Silveira et al., 2017). There are a number of control programs aimed at the eradication of BVDV-1 and BVDV-2 infections in cattle (Pinior et al., 2017), but no control programs are in place for pestivirus infection in domestic sheep. In order to have successful control programs for eradication of BVDV, it is necessary to understand potential transmission patterns between different domestic livestock species. Pestivirus infections in sheep have been reported worldwide, with seroprevalence varying between 4.5 and 76.5% for various countries and regions within countries (Mishra et al., 2009, Krametter-Frotscher et al., 2007; Yelsibag and Gungor, 2009; Martin et al., 2015). However, few information is available regarding the seroprevalence of pestiviruses in domestic sheep flocks from the United States.

The aim of this study was to perform a serosurvey for pestivirus antibodies in domestic sheep from Wyoming, one of the major producing states in the US, to determine how the serological titers compare to those of cattle from the country. To this end neutralizing antibodies titers against BVDV-1, BVDV-2, BDV and HoBi-like viruses in 500 sera were determined.

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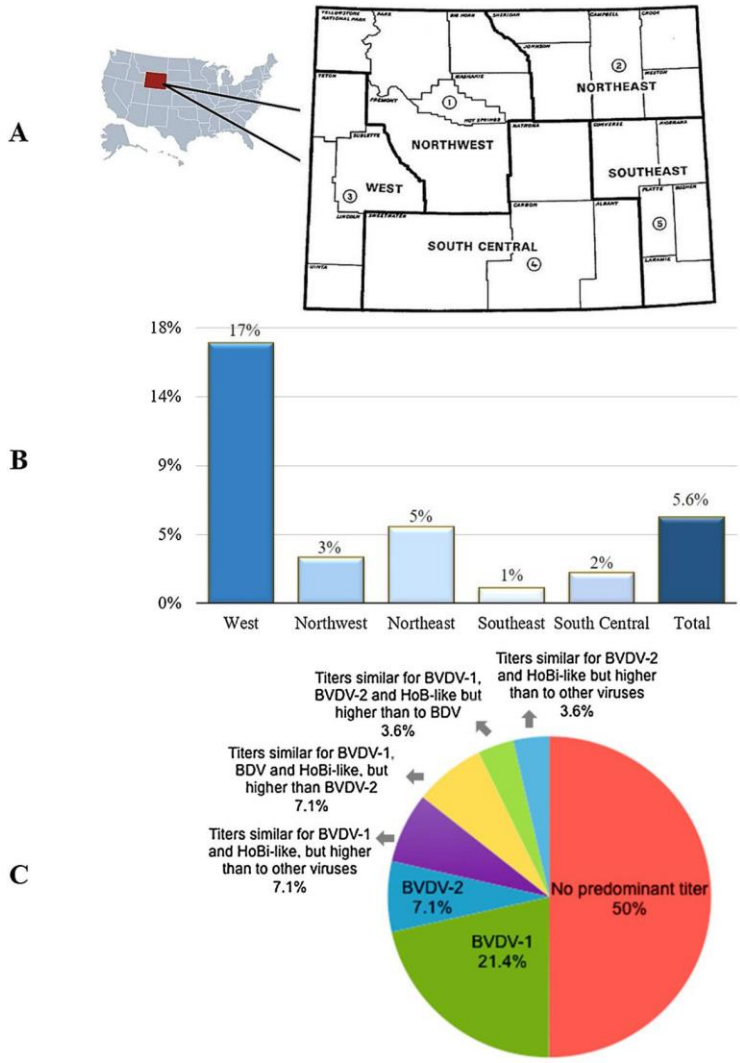

Fig. 1. Map of Wyoming State adapted from USDA (2017) showing the five geographic regions (A). Overall percentage of seropositive animals stratified by region (B). Determination of predominant titer within the positive sera (C).

2. Materials and methods

2.1. Sera collection

Sera from 500 adult domestic sheep, were collected across the state of Wyoming (US) in 2015–2016. Blood samples were collected by jugular venipuncture in 10 mL blood collection tubes (BD Vacutainer, Franklin Lakes, NJ USA). The animals were handled in accordance with the Animal Welfare Act Amendments (7 U.S. Code §2131–§2156) and all procedures were approved by the Institutional Animal Care and Use Committee of Wyoming University. Following the collection, the samples were allowed to clot, centrifuged at 2500 rpm for 10 min at room temperature, and sera were decanted and stored at −20 °C until further testing. One hundred samples from each of five distinct geographic regions in Wyoming (West, Northwest, Northeast, Southeast and Southcentral) were used in this study (Fig. 1A). Sixty-four samples came from small flocks (1–15 rams), 336 samples from medium sized flocks (16–100 rams) and 100 from large flocks (> 101 rams). Flock size

categories were based on known number of mature breeding rams in the flock, which usually represents about 5% of the total flock size (USDA, 2017). Approximately 50% of the animals were 3 years of age, and age was based on known numbers (i.e. ear tags, producer knowledge, etc.) or estimated ages (i.e. based on number and condition of teeth). Of the 500 samples, 303 were collected from females and 197 were collected from males.

2.2. Viruses and cells

Pestivirus strains used were all cytopathic and included BVDV-1a Singer, BVDV-2a 296c, BDV CB5 and HoBi-like pestivirus Italy-1/10-1. The BDV strain was propagated and titrated in ovine turbinate (OFTu) cells, while the BVDV-1, BVDV-2 and HoBi-like pestivirus strains were amplified and tittered in Madin-Darby bovine kidney (MDBK) cells. Fetal bovine serum (FBS) and cell lines were tested negative for pestivirus RNA by RT-PCR (Bauermann et al., 2013). In addition, FBS was also tested and found to be free of pestivirus antibodies by virus neutralization assay (VN) (Bauermann et al., 2014).

2.3. Virus neutralization tests

The sheep sera were tested initially in 50 pools of 10 samples each (10 pools per region). Samples from pools testing positive were then tested individually. VN was performed under the procedure described by OIE (OIE, 2016). First, sera were heat inactivated at 56 °C for 60 min. Sera was then diluted in 2-fold serial dilutions (1:8 to 1024) in media (MEM) and tested in triplicate against each of the four pestiviruses (BVDV-1, BVDV-2, BDV, HoBi-like pestivirus). Virus equivalent to a 100 tissue culture infectious dose ($TCID_{50}$) was added to each well. Plates containing virus and serum dilutions were incubated for 60 min at 37 °C in 5% CO_2. Thereafter, the cell suspension was added to the wells (10^4 cells/well). Plates were incubated for another 4 days at 37 °C in 5% CO_2. Test results were evaluated using an optical microscope. Titer was determined by the reciprocal of endpoint serum dilution that prevented viral replication as visualized by cytopathic effect.

2.4. Data analysis

The titers were converted to a $\log_2$ value according to the methods of Spearman-Karber. Titers equal or greater than 8 were considered positive (OIE, 2016). A chi-square test was used to test the significance of overall seroprevalence and regional differences, with $p < 0.05$ as the minimum level for statistical significance. To compare the predominance of titers against the different pestivirus, a formula was established for determining the comparative ratio (R) for each serum sample: $R_{pestivirusA}$ = (4 X titer against pestivirus A)/(titer against pestivirus A + titer against pestivirus B + titer against pestivirus C + titer against pestivirus D). If the ratio value for one pestivirus was > 0.231 than the value of the ratios for the other pestivirus, the sample was considered to have predominant titer for the respective virus. If the ratio value for all pestivirus was less than 0.231 between them, the sample was considered without a predominant titer.

3. Results

The virus neutralization tests were first performed with sera pools against the four pestiviruses (BVDV-1, BVDV-2, BDV, HoBi-like pestivirus). Each of the five geographic regions of Wyoming had 10 pools, consisting of 10 serum samples each pool. Thus, totaling one hundred serum samples per region and 50 pools in total. Pestivirus antibodies were detected in 14 (28%) out of 50 pools. The frequency of positive pools ranged from 10 to 70% between the regions, with the Western region the highest percentage. Segregating the positive samples by virus tested, the frequencies ranged from 14 to 22% (Table 1).

Then, samples from all positive pools were tested individually against the four pestiviruses. The overall prevalence was 5.6%, however, it varied significantly by region ($p < 0.05$). A marked regional difference was observed, with the west region containing the highest number of positive animals (17%). In contrast, other regions showed lower and similar percentage of positives (1–5%) (Fig. 1B).

Table 1
Percentage of positive pools stratified by region and virus.

Region	BVDV-1	BVDV-2	HoBi- like	BDV	Total
West	6/10 (60%)	7/10 (70%)	6/10 (60%)	6/10 (60%)	7/10 (70%)
Northwest	1/10 (10%)	1/10 (10%)	1/10 (10%)	1/10 (10%)	1/10 (10%)
Northeast	2/10 (20%)	2/10 (20%)	4/10 (40%)	0/10 (0%)	4/10 (40%)
Southeast	0/10 (0%)	1/10 (10%)	0/10 (0%)	0/10 (0%)	1/10 (10%)
South-central	1/10 (10%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	1/10 (10%)
Total	10/50 (20%)	11/50 (22%)	11/50 (22%)	7/50 (14%)	14/50 (28%)

a Positive pools were considered as pools with titer ≥ 1:8.
b 10 pools per region (100 sera).

Regardless of the region all seropositive sera showed low (≤32) or medium (64–256) neutralization titers. Titers were higher against BVDV-1 when compared to the other pestiviruses as well as the frequency of seropositive was higher for BVDV-1 (4%). On the other hand, the lowest seropositive rate (2%) and the lowest antibody titers were against BDV. The frequencies of positive animals for BVDV-2 and HoBi-like pestivirus were 3% and 3.8%, respectively.

As pestiviruses are antigenically cross reactive, a formula was devised to allow the determination of comparative strength of neutralization. Within the positive samples, 50% did not have a predominant titer against one specific pestivirus, in 24.1% BVDV-1 titer predominated and in 7.1% BVDV-2 titer predominated. Regarding the 21.5% of the remaining sera, they showed predominant titers for more than one species/proposed species of pestivirus. No sample had predominant titers for BDV and HoBi-like pestivirus (Fig. 1C).

4. Discussion

The overall pestivirus seroprevalence (5.6%) found in Wyoming domestic sheep is similar to the results from other countries (O'Neil et al., 2004; Fernández-Aguilar et al., 2016; Paniagua et al., 2016). While the seropositive rate of this study could be somewhat underestimated due to pooling of samples, it is not anticipated that pooling would change the overall conclusions of the study. The prevalence of positive samples and the antibody titers detected in sheep samples were both lower than those detected in previous surveys of cattle sera performed in the US (Houe et al., 1995; Chase et al., 2003; Bauermann et al., 2017). In addition, the predominance of antibodies against BVDV-1 and lack of predominant antibodies against BDV and HoBi-like virus found in the present study is similar to the results of a nationwide seroprevalence study conducted on bovine samples (Bauermann et al., 2017). Previous studies showed that cattle may transmit pestiviruses to sheep, mainly BVDV-1 (Mishra et al., 2009; Graham et al., 2001; Schiefer et al., 2006; Krametter-Frotscher et al., 2007). Besides, virus transmission from sheep to cattle may occur (Carlsson and Belák, 1994). Interspecies transmission between sheep and cattle is of particular importance in regions, such as the western United States, where cattle and sheep may share grazing areas (USDA, 2017). In a nationwide pestivirus serologic study performed in cattle, the Mountain West region of the United States, which includes Wyoming, showed the highest number of antibody positive animals and tended to have higher titers (Bauermann et al., 2017). Wyoming is the fourth largest sheep producing state and is the twenty-third largest cattle producer.

Furthermore, Wyoming is geographically located in the center of the predominate region for sheep production in the United States. Due to the nature of grazing systems in Wyoming, the likelihood of shared pastures between cattle and sheep is high. While sheep and cattle distribution differ across the state, the highest combined numbers of cattle and sheep are in the Southeast region (USDA, 2017). Given the higher numbers of cattle and sheep, it is surprising that this region had the lowest seroprevalence of pestivirus antibodies in sheep.

Regional variation in pestivirus antibody prevalence in sheep has been observed worldwide and also in the present study. These differences can be attributed to or associated with differences in flock size, animal density, management practices, type of production, frequency of trade, transfer of animals between herds, or even the presence of wildlife susceptible to pestivirus infection (Graham et al., 2001; Krametter-Frotscher et al., 2007). Wildlife could play an important role in pestivirus transmission especially in Wyoming, which has a large wild ruminant population. The Western region, that had greater seroprevalence, has national parks and borders with other parks.

This study suggests that the incidence of BVDV-1 and BVDV-2 exposure is lower among sheep than cattle. However, it should be noted that titers observed in cattle may also be due to widespread vaccination. To date, there are no commercial vaccines licensed for use in sheep. While the number of positive animals and titers were low, it appears that sheep flocks were exposed to pestiviruses, most likely BVDV-1 or BVDV-2. While this data cannot demonstrate if sheep can act as a reservoir of pestiviruses for cattle or that cattle are more likely to act as a reservoir for sheep, it does appear that, as a population, sheep are less likely to be exposed/infected. Similar to the previous study in cattle, there was no evidence of HoBi-like virus exposure in the sheep population tested.

Half of the positive samples did not have a predominant titer against any of the pestivirus strains used in the test. This could be the result of exposure to multiple pestiviruses or the use of strains in the VN that do not match those viruses circulating in the field. The reference strains used in this study were selected because they represented the most prevalent species and subgenotypes that circulate in cattle together with the BDV strain recommended by OIE (OIE, 2008). However, little is known about which pestiviruses are circulating in the sheep population. Data from this study highlights understanding of the dynamics of the sheep pestivirus exposure and consideration of reference strains used for VN, transmission patterns, and potential vaccination history should be taken into account when designing sheep animal programs and for successful BVDV control program in cattle.

Conflict of Interest

None declared.

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4.4 Capítulo 4: *In vitro* method to evaluate virus competition between BVDV-1 and BVDV-2 strains using the PrimeFlow RNA assay.

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In vitro method to evaluate virus competition between BVDV-1 and BVDV-2 strains using the PrimeFlow RNA assay

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ABSTRACT

Bovine viral diarrhea viruses (BVDV), segregated in BVDV-1 and BVDV-2 species, lead to substantial economic losses to the cattle industry worldwide. It has been hypothesized that there could be differences in level of replication, pathogenesis and tissue tropism between BVDV-1 and BVDV-2 strains. Thus, this study developed an *in vitro* method to evaluate virus competition between BVDV-1 and BVDV-2 strains. To this end the competitive dynamics of BVDV-1a, BVDV-1b, and BVDV-2a strains in cell cultures was evaluated by a PrimeFlow RNA assay. Similar results were observed in this study, as was observed in an earlier *in vivo* transmission study. Competitive exclusion was observed as the BVDV-2a strains dominated and excluded the BVDV-1a and BVDV-1b strains. The *in vitro* model developed can be used to identify viral variations that result in differences in frequency of subgenotypes detected in the field, vaccine failure, pathogenesis, and strain dependent variation in immune responses.

Keywords: Bovine viral diarrhea virus; Pestivirus; Viral competition; Flow cytometry

1. Introduction

Ruminant pestiviruses are globally-distributed pathogens responsible for a broad range of clinical presentations, which range from mild to severe and may affect the respiratory, digestive and/or the reproductive system (MacLachlan and Dubovi, 2011). Bovine viral diarrhea virus-1 (BVDV-1) and BVDV-2 are grouped in two different species, Pestivirus A and B, respectively, with multiple subgenotypes and belong to the genus *Pestivirus*, family *Flaviviridae*. Pestiviruses are single-stranded, positive-sense RNA viruses. The viral genome is approximately 12.3 Kb long and contains a unique open reading (ORF) frame that is flanked by 5' and 3' untranslated regions (UTRs). The ORF encodes a long polyprotein that is processed into the following polypeptides: Npro-C-Erns-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B (Smith et al., 2017).

Pestiviruses are notable for their broad host range and cell tropism and the presence of two biotypes, noncytopathic (ncp) and cytopathic (cp). In addition, clinical presentations include a wide spectrum of virulence including acute and persistent infections invariably accompanied by immunosuppressive effects. These viruses are also frequent contaminants of biological products and vaccines (Barkema et al., 2001; Fulton, 2003; Palomares et al., 2013; Schweizer and Peterhans, 2014; Kelling et al., 2002). Based on genetic variability, BVDV-1 and BVDV-2 can be further segregated into several subgenotypes, BVDV-1a-u and BVDV-2a-c (Yeşilbağ et al., 2017). Current licensed vaccines in the US only contain BVDV-1a and BVDV-2a strains (Fulton, 2015), but the most recent surveillance would suggest that BVDV-1b strains are the most prevalent in the US (Fulton et al., 2006; Workman et al., 2016).

While it is possible that the emergence and increased prevalence of BVDV antigenically and genetically different from vaccine strains is driven by vaccination

escapes, other factors such as virulence, contact between cohorts and viral fitness could also contribute to emergence and increased prevalence of a particular strain or subgroup. Previous research has reported predominance of viral strains in simultaneous experimental challenge and *in vivo* transmission studies (Brock and Chase, 2000; Frey et al., 2002; Makoschey and Janssen, 2011; Peddireddi et al., 2018; Walz et al., 2018; Zimmer et al., 2002). Most recently two BVDV exposure studies using simultaneous exposure to PI's infected with either BVDV 1a, 1b or 2a subgenotypes reported that the 2a strain was most frequently isolated from exposed animals (Peddireddi et al., 2018; Walz et al., 2018).

Viral fitness is used to describe the ability of a virus to replicate in a given environment and to be transmitted to new hosts to survive. Differences in viral fitness may impact the prevalence and emergence of different BVDV species and subgenotypes. There are a variety of methods and parameters that can be used to assess strains that have the potential for increased fitness. One method that is commonly used as a measure of viral fitness is an *in vitro* viral competition assay. This type of assay is accomplished by competition experiments, in which cells or hosts are infected with two or more viral isolates. Viral competition may be evaluated at one time point or at multiple time points post-infection (Domingo, 1997; Wargo and Kurath, 2012). Traditional methods that can be used to characterize virus competition include, DNA sequencing, PCR, and monoclonal antibodies. A drawback of these methods is that they provide a consensus of the overall population but cannot define viral dynamics at the single cell level. Therefore, an *in vitro* model was developed that allows comparison of infection at the single cell level for BVDV-1a, -1b and -2a strains. To accomplish this, BVDV-1a, -1b, and -2a strains isolated from persistently infected calves previously used in an *in vivo* transmission experiment (Walz et al., 2018), were used in a novel *in*

vitro competition assay. This assay was based on a flow cytometry technique that allows a multiplex detection of RNA at cellular level using in situ hybridization-based branched DNA amplification (Falkenberg et al., 2017; Falkenberg et al., 2019), as well as PCR and sequencing methods for viral detection for comparisons.

2. Material and methods

2.1 Viruses

Four non-cytopathic (ncp) BVDV strains, AU-PI-34 (BVDV-1a), AU-PI-285 (BVDV-1b), AU-PI-28 (BVDV-2a) and AU-PI-12 (BVDV-2a) (henceforth referred to as PI34, PI285, PI28 and PI12, respectively), were used in the study. The strains were isolated during an *in vivo* transmission study in which naïve pregnant cattle were exposed to PI calves infected with different BVDV strains (Walz et al., 2018). The BVDV-2a subgenotype predominated in live-born calves, accounting for 56% of the subgenotypes isolated. It is unknown why the most frequently isolated subgenotype was from the BVDV-2a PI's. Viral titers from serum and nasal swabs of all the PI calves used were similar. While more fetuses were positive for BVDV-1b isolates (5 fetuses) rather than BVDV 2a isolates (4 fetuses), the total number of aborted fetuses was minimal (11) (Walz et al., 2018). Thus, for the *in vitro* competition assay the representative strain of each subgenotype that resulted in the greatest number of PI animals, PI34 (BVDV-1a), PI285 (BVDV-1b), PI28 (BVDV-2a) were selected. Since the BVDV-2a strains predominated and also accounted for a significant portion of the aborted fetuses, the BVDV-2a strain (PI12) that generated the lowest number of BVDV2a PI calves were also selected to evaluate if this predominance was strain rather than subgenotype dependent. The approximately percent of the total number of PI progeny each PI contributed to is as follows; PI34 10%, PI285 14%, PI12 10% and PI28 34%.

All strains were recovered from the serum of the PI's used to expose pregnant cows. Genome sequences for isolated strains were *determined using a sequence-independent method using the MiSeq platform* to confirm their identity (Neill et al.,

2014). Then, the full length sequences were assembled and analyzed using Lasergene 12 package (DNAstar, Inc., Madison, WI) and Aligner (Codoncode, Inc., Centerville, MA, USA). The GenBank accession number of the sequences are MH23114 (PI12), MH231141 (PI28), MN188073 (PI34), MN188074 (PI285). No other viral sequences than the BVDV strains mentioned above were found.

The four ncp BVDV strains were passed four times in Madin-Darby bovine kidney (MDBK) cell line prior to use in the assay, and titrated using an immunoperoxidase staining with the monoclonal antibody N2 (Bauermann et al., 2012; Ridpath and Neill, 2000).

2.2 Cells

MDBKs were used to propagate the viral stocks used in the study, while two other cell types were used for the virus competition assay. The two other cell types used consisted of a primary bovine turbinate epithelial adherent cell culture (BTu) and a bovine lymphoma B cell suspension cell line (BL-3). These cell types were chosen to represent epithelial and lymphoid cells, which are the primary sites of replication of the BVDV in acute infection (Liebler-Tenorio et al., 2003). The BL-3 cell line (CRL-8037) received from the American Type Culture Collection (ATCC) was contaminated with BVDV. Subsequently, a BVDV free NADC-BL-3-SF cell line was derived from the contaminated line by limiting dilution (Falkenberg et al., 2017; Ridpath et al., 2006). The BTu cells were primary cells derived from bovine fetal tissues in our lab.

The NADC-BL3-SF (BL-3) cell line was maintained in RPMI-1640 medium, MDBK and BTu cells in MEM medium (Sigma Aldrich), both media supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (PAA Laboratories Inc. Ontario, Canada), L-Glutamine (Thermo Fisher Scientific, Waltham, MA) and

antibiotic-antimycotic. Cells were incubated at 37°C in a humid atmosphere with 5% CO_2. The cells and FBS were confirmed to be free of pestivirus RNA by RT-PCR (Bauermann et al., 2013). In addition, FBS was also tested and found to be free of pestivirus-antibodies by virus neutralization assay using the strains Singer (BVDV-1a), 296c (BVDV-2a), and Italy 1/10-1 (HoBi-like virus) (Vilcek et al., 1994).

2.3 Viral infection and experiment design

The competitive assay was conducted as single, dual, and triple infections in the two cell types, using the PI34 (BVDV-1a), PI285 (BVDV-1b), PI28 (BVDV-2a) and PI12 (BVDV-2a) strains, obtained and titered from passage four in MDBK as described above. The experiment was repeated three different times. The procedures used to inoculate the cells are as follows and a schematic of the experiment design can be found in Fig. 1. Approximately, 10^7 BL-3 cells (1 flask-75 cm^2) were spun down (300 × *g*, 10 min), spent (conditioned) media was harvested (for later use), and cells were inoculated with each strain (single, dual, and triple infections) then placed on a rocker at 37°C for 1 h. At the end of this time period, the cells were spun down, the supernatant discarded and a 50/50 ratio of spent/fresh media was added back to the cells (30 mL total). BTu cells (10^6) at ~70% confluence were inoculated with each strain (3 flasks-25 cm^2) and incubated at 37°C for 1 h with rocking, followed by replacement of the inoculum with fresh media. Both cell types were incubated at 37°C in a humid atmosphere with 5% CO_2 and infected at an MOI of 1.0 and 0.1 to evaluate differences that may be observed due to MOI. Absolute cell numbers for both cell types were determined by flow cytometry using counting beads (SPHERO AccuCount-Spherotech) to determine the respective MOI for each cell type and for each respective virus. In addition, both cell types were mock-infected using the respective cell media from negative control cells.

The flasks of BVDV infected cells that were not used for analysis at each respective time point (days 2, 9, and 30) were maintained as follows: the BL-3 cells (1 flask - 75cm^2) were passed 1/3 twice a week and the BTu cells were trypsinized and passed 1/4 once a week (3 flasks – 25 cm^2) (Figure 1).

On 2, 9, and 30 days post infection (dpi), cells were harvested (BL-3 – 10 mL from the one 75 cm^2 flask) or trypsinized (BTu - 2 flasks 25 cm^2) for evaluation of viral competition dynamics. At these time points, cells were used in the PrimeFlow RNA assay as well as, cells (~10^5) were collected for RNA extraction to perform RT-qPCR and RT-PCR for sequencing. After performing the PrimeFlow RNA assay, remaining cells not used for flow cytometric analysis were spun down onto glass slides for immunofluorescence (IF) microscopy.

2.4 BVDV RNA quantification by RT-qPCR and identification by DNA sequencing

The relative RNA viral load present in the cells was determined by RT-qPCR and the predominant BVDV strain present was determined by DNA sequencing.

An aliquot of 140 µL of cell culture suspension (~10^5 cells) was submitted to RNA extraction using QIAcube® (Viral RNA kit) according to the manufacture's recommendations (Qiagen, Valencia, CA). Extracted RNA was used for both RT-qPCR as well as conventional RT-PCR for DNA sequencing. For virus quantification, a commercial RT-qPCR kit was used to detect a fragment of the BVDV-1 and BVDV-2 5'UTR (BVDV VetMax Gold – Thermo Fisher Scientific). Samples were analyzed using QuantStudio™ 5 System (Applied Biosystems) with the respective software. For BVDV identification, a conventional RT-PCR that targeted the 5`UTR was performed (Ridpath et al., 1994). Amplification products were purified using a QIAquick PCR Purification kit (Qiagen), according to the manufacturer's instructions, followed by

DNA quantification using a Qubit fluorometer (Invitrogen Corporation, Carlsbad, CA). Both strands were sequenced in duplicate using a Big Dye Terminator cycle sequencing kit, on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were edited and aligned using Geneious software (Biomatters Inc, Newark, NJ). Final phylogenetic analyses were performed using Molecular Evolutionary Genetics Analysis software package 7 (MEGA7) (Tamura et al., 2011).

2.5 BVDV identification and quantification by the PrimeFlow RNA assay

The PrimeFlow RNA assay was performed to identify and quantify individual cells positive for each respective BVDV strain, using a different fluorochrome for each strain (Table 1). This technique allowed the quantification of BVDV RNA, by determination of geometric mean fluorescence intensity (gMFI). Thus, the PrimeFlow RNA assay provided the number of single, dual and triple cells positive for each strain, as well as the comparative amount of viral RNA present.

2.5.1 Cell preparation

Both cell types infected with the respective viruses, virus combinations, and mock-infected cells were harvested at 2, 9, and 30 dpi as previously described (Fig. 1). Cells harvested from each respective cell type were centrifuged (300 × *g*, 10 min), resuspended in PBS and plated at ~ 2×10^6 cells/well of a 96-well plate. Plated cell suspensions were centrifuged (300 × *g* for 2 min) at 4°C, washed with PBS again to remove any residual medium and centrifuged prior to further use in the assay. Subsequently, to identify and select live cells, plated cells were stained with fixable viability dye eFluor 450 (eBioscience, San Diego, CA) as described by the

manufacturer, which was followed by two wash steps with stain buffer per the manufacturer's recommendation (BD Biosciences, San Jose, CA).

2.5.2 The PrimeFlow RNA assay

After the live/dead staining, the 96-well plates were submitted to the PrimeFlow RNA assay, which was performed according to the manufacturer's instructions (Thermo Fischer Scientific, Carlsbad, CA). The target probes corresponding to 20 gene-specific oligonucleotide (RNA) probes, were designed based on the sequence of the genomic region coding for N^{pro}-C-E^{rns} (~1500 nt) of each strain. Probes were conjugated to a specific fluorescent dye to specifically detect the respective BVDV strain (Table 1). Probe pairs were designed by Thermo Fisher Scientific, and procedures used for the design and generation of the probes as well as the probe sequences are considered proprietary by the company (Thermo Fischer Scientific, Carlsbad, CA).

Multi-color flow cytometric analysis was performed using a BD LSRII flow cytometer (BD Biosciences). Cells were visualized in forward (FSC) and side scatter (SSC) and single cells were gated and dead cells were eliminated based on eFluor 450 dye staining. The eFluor 450 dye was excited at 405 nm laser line (violet laser) and the emission signal was detected using a 450/50 nm band-pass filter.

Single BVDV infected cells and mock-infected cells were used to set the gates and as positive and negative controls, respectively. Fluorescence-minus-one controls (lack of one target probe) and no-probe controls (lack of target probe addition), were included to set up the gates for positive cells and compensation for each fluorochrome/channel. Approximately 20,000 events per gate were acquired using LSR II equipped with violet (405 nm), blue (488 nm), and red (633 nm) lasers and data analysis was performed using FlowJo software (FlowJo LLC). In addition, probe

specificity controls were employed that consisted of adding all probes to single infected cells to verify the cross-reactivity between them, or any background. That is, all probes were included, excluding the probe to detect the single virus used to infect the cells to ensure no non-specific binding was observed with any other probes.

The gMFI, expressed on the log scale, was calculated for the RNA positive cells for each strain. The gMFI was compared within each strain, over time and between single, dual or triple infections. Comparisons could only be made for each respective fluorochrome within each strain since as the fluorescent intensity varied between each fluorochrome.

2.6 BVDV identification by immunofluorescence *(IF) microscopy*

Cells from the PrimeFlow RNA assay were subjected to the IF to verify the results observed in the PrimeFlow RNA assay, and to assess the cellular localization of each respective strain. The cell suspension (50 µL) was spun down onto glass slides using a Cytospin cytocentrifuge (Thermo Shandon Cytospin 3) at 10.16 × *g* for 3 min and coverslips were mounted using ProLong Gold antifade mount medium with DAPI (ThermoFisher, Carlsbad, CA). Fluorescent imaging was performed on a Nikon A1R+ Confocal System microscope (Nikon Instruments, Melville, NY), with three fluorescent channels (AF488, AF647, AF750) using a 40 X oil-immersion objective. NIS-Elements Advanced Research software was used for image analysis and metadata files were saved as proprietary Nikon ND files.

3. Results

The competitive assay was conducted as single, dual, and triple infections in two cell types (BL-3 and BTu). Four BVDV strains were used to infect the cells: one strain of BVDV-1a (PI34), one of BVDV-1b (PI285) and two strains of BVDV-2a (PI12 and PI28). Cells were infected using equal MOIs of each virus, harvested at three time points (2, 9, and 30 dpi) and submitted to the RT-qPCR for virus quantification, DNA sequencing, PrimeFlow RNA assay and IF. Each experiment was conducted in triplicate and analogous results were observed between each of the replicates. As there was no difference in trends or frequency of virus positive cells between the replicates, the data from one replicate is reported. Further, an MOI of 1 and 0.1 were evaluated for differences in viral dynamics due to MOI, but no difference was observed as the same trends were observed for each respective MOI. Therefore, only results using an MOI of 1 will be reported.

3.1 BVDV RNA quantification by RT-qPCR

Differences in virus quantification within the cell types, over time and between the types of infection were analyzed using an RT-qPCR assay that did not differentiate between strains. The cycle threshold (Ct) values of single, dual or triple infections in BL-3 cells at day 2 ranged from 19.9-23.1. On day 2, all types of infection containing strain PI12 (BVDV-2a) had higher Ct values (22.6-23.1) than the other types of infection (19.9-21.4; Table 2). A gradual increase in Ct values was observed on day 9 (23.1-24.6) and again on day 30 (24.6-27.7). However, at day 30, single infections had higher Ct values (26.8-27.7) than dual and triple infections (24.6-25.2; Table 2).

The Ct values observed in BTu cells at day 2 infection were very similar among the single, dual and triple infections, ranging from 15.0-16.1, but these values were lower than Ct values of BL-3 cells (19.9-23.1). Similar to BL-3 cells, a gradual

increased in Ct values was observed on day 9 (23.1-24.6) and again on day 30 (24.6-27.7). The single infections with 1a-strain and 1b-strain had the highest Ct values (23.2 and 25) among all types of infection at day 9, which decreased at day 30 (20.1-20.8; Table 3).

Collectively, these observations suggest that these BVDV strains have a higher replication rate in BTu cells when compared to BL-3 cells. In both cell types, the Ct values were lower on day 2 when compared to day 9 (Table 2, 3). From day 9 to day 30 a slight Ct increase was observed in all types of infection in BL-3 cells and in the majority of the Btu cells (Table 2, 3). All mock-infected samples harvested from the two cell types in all time points had Ct values higher than 36, which was considered negative.

3.2 BVDV identification by DNA sequencing

While RT-qPCR confirmed the presence as well as the relative amount of BVDV in each cell type at three different time points, this assay could not be used to determine the strain(s) of BVDV prevalent in dual and triple infected cells since universal forward and reverse BVDV primers were used. Therefore, DNA sequencing was performed (with amplified DNA products) to determine which BVDV strains were present in the cells at the respective time points. While all viruses could be identified for single infections at all time points, infections with multiple viruses either provided sequence that appeared to be mixed due to multiple peaks, or identified the more frequent sequence if strains were present in unequal amounts.

In the BL-3 cells, from day 2 onward, if a BVDV-2 strain was used to infect the cells, only the BVDV-2 strains were identified by sequencing (Table 2). No clear

sequence was obtained in the dual infection with 1a and 1b-strains at day 2 and 9, but the 1b-strain was identified at day 30 (Table 2).

Similar results were also observed in the BTu cells at day 30 (Table 3). However, on day 2, no unique BVDV sequence was observed in the following three infection types: (1a/1b), [2a (PI12)/2a (PI28)], [1a/1b/2a (PI28)] (Table 3). In contrast to dual and triple infections including 2a-strains in BL-3 cells, in BTu infections, the sequence of either the 1a or 1b-strain could be identified in dual and triple infections on day 2 (Table 3). By day 9, the 2a-strains were identified by sequencing in the dual and triple infected cells if a 2a-strain was used to infect BTu cells and this continued through day 30 (Table 3). The 1a-strain was identified on day 2 and day 9 in the infection with 1a/1b dual infection, but the 1b-strain was identified on day 30 (Table 3).

3.3 BVDV identification and quantification by the PrimeFlow RNA assay

In contrast to PCR amplification followed by DNA sequencing, the PrimeFlow RNA assay provided simultaneous identification of the strains and quantification of prevalence based on the percentage of infected and co-infected cells. Moreover this assay allowed comparison of BVDV RNA load based on the gMFI.

3.3.1 BL-3 cells

On day 2, the total frequency of virus positive cells in single infections ranged from 43.8-96.4% (Table 4). The total frequency of virus positive cells for dual and triple infections ranged between 39.1-94.8%, with the range of percent infected with each respective strain from 0-94.4% (Table 4). Within the dual and triple infections, the frequency of virus positive cells with two or more viruses ranged from 0.8-33.1% (Table 4).

On day 9, the total frequency of virus positive cells from single infections ranged from 12.1-31.1% (Table 4). The total frequency of virus positive cells for dual and triple infections ranged between 12.1-33%, with the range of percent infected with each respective strain from 0-26.8% (Table 4). Within the dual and triple infections, the frequency of virus positive cells with two or more viruses ranged from 0-3.6% (Table 4). The total percentage of virus positive cells decreased from day 2 to day 9, and the gMFI as a relative measure of the amount of virus in the cells also decreased substantially from day 2 to day 9 (Table 4).

On day 30, the total frequency of virus positive cells from single infections ranged from 15%-40.5% (Table 4). The total frequency of virus positive cells for dual and triple infections ranged between 15.4%-36.5%, with the range of percent virus positive cells with each respective strain from 0-34.5% (Table 4). Within the dual and triple infections, the frequency of virus positive cells with two or more viruses ranged from 0%-1% (Table 4). The total percentage of virus positive cells remained similar from day 9 to day 30, and the gMFI was also similar on day 9 to day 30 (Table 4).

The lowest frequency of positive cells following inoculation was observed with the BVDV-2a PI12-strain. However, while this strain had the lowest frequency of positive cells, the 2a-strains (PI28 and PI12) began to outcompete 1a and 1b strains early in dual and triple infections, at day 2, with the PI28 (2a)-strain being the most competitive. However, when the cells were co-infected with both two 2a-strains, the PI28-strain outcompeted and virtually eliminated the PI12-strain at day 9 (Table 4).

Since the two 2a-strains had a similar behavior in dual and triple infections with the 1a and 1b-strains, only the infections with the PI28 (2a)-strain were maintained until the end of the study.

The dual infection 1a/1b showed more dual positive cells at the three-time points, with the maximum of 33.1% at day 2 (Table 4). The PI34 (1a)-strain and PI285 (1b)-strain were those that coexisted best in dual infection. Moreover, this type of infection had one of the highest total percentage of virus positive cells at the three time points, followed by their single infections and the dual infection with the two 2a-strains PI12 and PI28. The similar frequency of 1a and 1b virus positive cells in the dual 1a/1b infection remained until day 9; however, afterwards the 1b-strain predominated at day 30.

3.3.2 BTu cells

On day 2, the total frequency of virus positive cells from single infections was 99.9-100% (Table 5). The total frequency of virus positive cells for dual and triple infections ranged between 99.4-99.9%, with the range of virus positive cells with each respective strain from 42.7-99.2% (Table 5). Within the dual and triple infections, the frequency of viral positive cells with two or three viruses ranged from 29.4-89.5% (Table 5). The frequency of BVDV positive cells in dual and triple infections at day 2 were higher in BTu cells compared to BL-3 cells.

On day 9, the total frequency of virus positive cells ranged from 41.6-60.3% (Table 5). The total frequency of virus positive cells for dual and triple infections ranged between 51.7-60.9%, with the range of percent virus positive with each respective strain from 11.9-52.5% (Table 5). Within the dual and triple infections, the frequency of virus positive cells with two or more viruses ranged from 0.7-7.8% (Table 5). As observed in the BL-3 cells, the total percentage of infected cells decreased from day 2 to day 9, and similarly the gMFI also decreased substantially from day 2 to day 9 (Table 5).

On day 30, the total frequency of virus positive cells from single infections ranged from 58.5-69.3% (Table 5). The total frequency of virus positive cells for dual and triple infections ranged between 56.2-65.7%, with the range of percent virus positive cells with each respective strain from 0-63.7% (Table 5). Within the dual and triple infections, the frequency of virus positive cells with two or more viruses ranged from 0-11.6% (Table 5). While total percentage of virus positive cells remained similar from day 9 to day 30, and the gMFI was also similar on day 9 to day 30, by day 30 2a-strains (PI12 or PI28) outcompeted the BVDV-1 isolates (Table 5). This was similar to the trend seen in dual and triple infections in BL-3 cells. Although DNA sequencing detected 2a sequence at day 9 in triple infected BTu cells [1a/1b/2a (PI12)] (Table 3), results from the PrimeFlow RNA assay revealed that the frequency of 1a strain infected cells was highest (33.2%) as compared to 1b (17.6) and 2a-PI12 (18.1%) in triple infected BTu cells (Table 5). Similar difference was also observed for 1a/1b infected BTu cells on day 30, with the 1a-strain having a greater number of virus positive cells, but the 1b strain was detected by sequencing.

While differences in viral strain replication were observed in both cell types, these differences were evident earlier in the BL-3 cells (day 9) compared to BTu cells (day 30). Specifically, the PI28 (2a)-strain excluded 1a-strain and 1b-strain in dual and triple infections. While the PI12 (2a)-strain did not eliminate other BVDV strains, at 30 days, there were a greater number of cells positive for the PI12 (2a)-strain as compared to the 1a-strain and 1b-strain, although there were a small percentage of cells positive for the 1a-strain and the 1b-strain. The PI28 (2a)-strain also outcompeted the PI12 (2a)-strain in dual infections (Table 5).

As in the BL-3 cells, the dual infection 1a/1b was responsible for the greatest percentage of dual positive cells. Whereas the 1b-strain outcompeted the 1a-strain in the

BL-3 cells, in the BTu's, a similar number of cells were virus positive for each respective strain.

3.4 Immunofluorescence (IF) microscopy

IF was used to corroborate the results observed using the PrimeFlow RNA assay. IF provided the opportunity to discern if viral strains were co-localized in cells that were positive for both or all viruses. Images obtained for IF did not suggest that BVDV strains were co-localized within the same area of the cytoplasm. The BVDV straining was dispersed throughout the cytoplasm of cell rather than concentrated in one particular area. No fluorescence signal was detected in negative (mock-infected) cells. Given that the IF images were unremarkable, data is not shown.

4. Discussion

The competitive dynamics of BVDV-1a, BVDV-1b, and BVDV-2a strains were evaluated in two cell types using multiple methods of viral detection/characterization. The same strains had been previously been used in an *in vivo* transmission study (Walz et al., 2018). Similar results were observed in both studies, regardless of the cell type or method of viral characterization used, the 2a strains outcompeted 1a and 1b-strains.. Furthermore, in *in vitro* studies, a 2a-strain(PI28) that had a high rate of transmission *in vivo,* outcompeted a 2a-strain (PI12) that had also a lower transmission rate *in vivo*.

Multiple cell lines were chosen to represent an important site for replication of the virus as well as cells that were important in establishment of initial infection in the respiratory tract (BTu) followed by systemic viral replication in lymphoid tissues (BL-3). Multiple methods of viral detection and characterization were used to represent

traditional methods, RT-qPCR for quantification and DNA sequencing for characterization of the specific viral strain, and the PrimeFlow RNA assay as a novel method that combines both the identification of the specific strain at the cellular level but also provides the ability to quantify the virus.

RT-qPCR is frequently used to compare viral loads in cultures and tissues. RT-qPCR provides a general characterization of total amount of virus but is unable to determine the number or ratio of individual cells that are virus positive. However, the Ct values observed between cell types and over time corroborated the results observed with the PrimeFlow RNA assay. RT-qPCR values were lower, indicating more virus, in the BTu cell than in BL3 cell type, which was in agreement with frequency of virus positive cells in the PrimeFlow RNA assay. Regarding the difference between the time points, samples from day 2 resulted in the lowest Ct values in both cell types and the greatest number of virus positive cells. Therefore, the general trends associated with amount of virus was similar regardless of the method used.

-In this study, DNA sequencing identified the sequence of the most abundant strain in a mixed infection. If strains were present in similar amounts, sequence results could not be used to differentiate which strains were present. In contrast, the PrimeFlow RNA assay was able to identify the frequency of cells that were positive for each respective virus at each time point whether by single, dual or triple infection at the individual cell level. Thus, the PrimeFlow was the best tool to study viral competition because incorrect assumptions could be made about the viral dynamics with regard to exclusion if DNA sequencing was the only method used to characterize the viral population. Furthermore, while both the Ct values and sequencing allowed for similar conclusions and corroborated the PrimeFlow results, neither RT-qPCR or DNA sequencing provided the opportunity to clearly define the minor virus populations.In

addition, RT-qPCR and sequencing are unable to describe the percentage of infected cells that are single, dual, or triple virus positive. Thus, results from this study would suggest that the PrimeFlow RNA assay provides a better picture of viral dynamics and is the best method currently available for evaluating infections at the cellular level following exposure with multiple pathogens.

While similar trends existed between studies in BL-3 and BTu cells, competitive exclusion was observable at earlier time points in BL-3 cells. Moreover, the BL-3 cells showed a higher variability in RNA viral load, as measured by the gMFI in the PrimeFlow RNA assay. While variation was observed in the frequency of virus positive cells for single, dual and triple infections between cell types, 2a-strains became predominant over time regardless of cell type. Whereas the 2a (PI28) strain outcompeted the 2a (PI12)-strain in BL-3 cells. In BTU cells a small percentage of strain PI12 positive cells did exist on day 30 in dual infection with 2a (PI28)- strain. It is not known if further passage and evaluation would have led to reemergence and predominance of strain PI12. This study clearly demonstrated that the 2a-strains predominated and corroborated the *in vivo* results in which the 2a-strains accounted for over 50% of the live born PI calves (Walz et al., 2018). The PrimeFlow RNA assay also allowed the observation of the dynamics between the 1a and 1b isolates in dual infections. Results from this study suggest limited competition between the 1a and 1b-strains in dual infections in BTu cells as demonstrated by the large proportion of dual positive cells and similar infection rates for each virus. In contrast, by day 30 the 1b-strain had risen to predominate in the BL-3 cells. However, there was a discrepancy between the Prime Flow assay and sequencing results in dual BVDV1 infections in BTu cells. This discrepancy could be explained by the increased number of 1b virus positive cells from day 9 to day 30. Since the gMFI cannot be compared between strains due to

differences in fluorescent intensity, the authors cannot confirm the amount of virus present in each respective cell. One possible explanation was there were more viral transcripts for the 1b virus in addition to the increase in the number of 1b virus positive cells from day 9 to day 30 leading to a higher detection of the 1b-strain by sequencing. It is unknown if continued passage of the BTu cells dual infected with the 1a and 1b-strains would have resulted in the 1b strain predominating as observed in the BL-3 cells.

Based on *in vivo* studies it appears that there is viral exclusion between BVDV strains. Only one pestivirus strain can be detected in most animals that had been inoculated with a mixture of two or three pestivirus strains (Brock and Chase, 2000; Makoschey and Janssen, 2011; Peddireddi et al., 2018; Zimmer et al., 2002). While one of those studies demonstrated simultaneous experimental infection with BVDV-1 and BVDV-2 resulted in dual persistent infections as determined by detection of both viruses, both viruses could not be isolated from the same tissue (Brock and Chase, 2000). Numerous studies have described a greater number of animals or tissues to be positive for BVDV-2 strains when used in dual exposure with BVDV-1 strains (Frey et al., 2002; Makoschey and Janssen, 2011; Peddireddi et al., 2018; Walz et al., 2018). It has been hypothesized that there could be differences in level of replication, adaptation, pathogenesis and tissue tropism between BVDV-1 and BVDV-2, during fetal infections (Bielefeldt-Ohmann et al., 2008; Brock and Chase, 2000; Makoschey and Janssen, 2011). Previous studies have described some differences between strains, including a higher degree of viremia, more pronounced lesions and more extensive distribution of viral antigen in calves inoculated with BVDV-2 when compared with calves infected with BVDV-1 (Walz et al., 2001; 2001b). Further experimentation using a greater number of strains needs to be done to determine if these differences are true of all strains within each species.

This study demonstrated that the PrimeFlow assay is a superior tool for studying coinfections. RT-PCR and DNA sequencing were unable to distinguish two or more strains in coinfections, where only the strain in a greater proportion was identified and the strain with a low viral load could not be detected. The results from the PrimeFlow RNA assay highlights the issue of the inability of traditional methods to detect mixed infections in contaminated cell cultures. Additionally, another practical application of this novel technique is the evaluation of interactions between strains that can be used in multivalent modified-live virus vaccine, but also as a model for predicting which strains could predominate in the field. The current assay may provide opportunities to evaluate potential vaccine failures or isolates that may be more prone to induce vaccine failure. Finally, these findings may help explain variations observed in the frequency of subgenotypes detected in the field.

Conflict of Interest

None declared.

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Figures

Fig. 1. Study workflow to evaluate competitive fitness between the strains of BVDV-1a, BVDV-1b, and BVDV-2a. The time points and details of virus inoculation, cell passages and techniques performed are depicted.

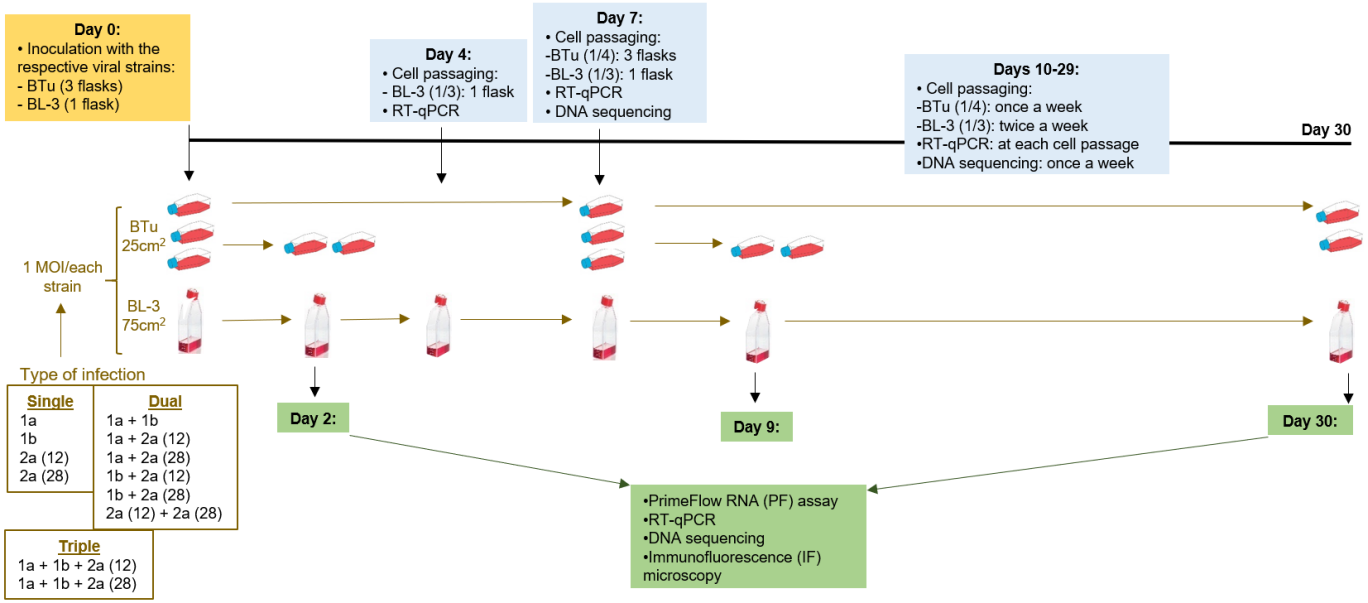

Table 1
PrimeFlow target probe types.

Strain	Subgenotype	Fluorochrome	Excitation (max)	Emission (max)
PI34	BVDV-1a	Type 1 probe (AF647)®	647 nm	668 nm
PI285	BVDV-1b	Type 4 probe (AF488)®	488 nm	519 nm
PI12	BVDV-2a	Type 6 probe (AF750)®	749 nm	775 nm
PI28	BVDV-2a	Type 6 probe (AF750),	749 nm	775 nm
		Type 1 probe (AF647)*	647 nm	668 nm

*probe used for the PI28 strain in dual infections that contained the PI12 strain, both BVDV-2a.

Table 2
RT-qPCR Ct values and DNA sequencing identification in BL-3 cells.

BL-3	Day 2		Day 9		Day 30	
*Virus used for infection	RT-qPCR Ct value	DNA sequencing	RT-qPCR Ct value	DNA sequencing	RT-qPCR Ct value	DNA sequencing
1a	20.8	1a	23.1	1a	27.4	1a
1b	19.9	1b	24.3	1b	27.7	1b
2a (12)	23.1	2a	24	2a	NT	2a
2a (28)	20.5	2a	24.4	2a	26.8	2a
1a/1b	20.3	UND	24	UND	24.6	1b
1a/2a (12)	22.6	2a	23.9	2a	NT	NT
1a/2a (28)	20.9	2a	24.6	2a	24.6	2a
1b/2a (12)	22.7	2a	23.9	2a	NT	NT
1b/2a (28)	21.1	2a	24.3	2a	24.7	2a
2a (12)/2a (28)	21.4	2a	23.9	2a	NT	NT
1a/1b/2a (12)	22.9	2a	24	2a	NT	NT
1a/1b/2a (28)	20.4	2a	24.2	2a	25.2	2a

*Bovine viral diarrhea virus strains; PI34 (1a), PI285 (1b), PI28 and PI12 (2a).
NT- Not tested.
UND – undetermined.

Table 3
RT-qPCR Ct values and DNA sequencing identification in BTu cells.

*BTu	Day 2		Day 9		Day 30	
Virus used of infection	RT-qPCR Ct value	DNA sequencing	RT-qPCR Ct value	DNA sequencing	RT-qPCR Ct value	DNA sequencing
1a	15	1a	23.2	1a	20.1	1a
1b	15.3	1b	25	1b	20.8	1b
2a (12)	15.6	2a	19.9	2a	21.5	2a
2a (28)	15.5	2a	23.2	2a	22.5	2a
1a/1b	15.2	1a	20.6	1a	20.9	1b
1a/2a (12)	15.4	1a	20	UND	21	2a
1a/2a (28)	15	1a	23.6	2a	22.7	2a
1b/2a (12)	15.7	1b	20.6	2a	21.6	2a
1b/2a (28)	15.1	UND	19.7	2a	22.5	2a
2a (12)/2a (28)	15.2	UND	20.3	2a	22.8	2a
1a/1b/2a (12)	16.1	1a	19.6	2a	21.3	2a
1a/1b/2a (28)	15.4	UND	20.2	2a	21.5	2a

*Bovine viral diarrhea virus strains; PI34 (1a), PI285 (1b), PI28 and PI12 (2a).
UND – undetermined.

Table 4
Percentage of single, dual, and triple positive cells and gMFI of positive BL-3 cells.

BL-3	Day 2			Day 9			Day 30		
*Virus used for infection	gMFI per strain	% positive cells (total and per strain**)	% co-positive cells (total and per combination**)	gMFI per strain	% positive cells (total and per strain**)	% co-positive cells (total and per combination**)	gMFI per strain	% positive cells (total and per strain*)	% co-positive cells (total and per combination**)
1a	24991	95	0	14882	29.8	0	8394	40.5	0
1b	14221	96.4	0	8991	31.1	0	9186	30.5	0
2a (12)	766	43.8	0	743	15.1	0	NT	NT	NT
2a (28)	1236	85.2	0	729	12.1	0	735	15	0
1a/1b	17372/9923	93.6 = 65.8/60.9	33.1	15121/8633	33 = 20.7/15.9	3.6	9400/9387	36.5 = 3.0/34.5	1
1a/2a (12)	5490/694	48.8 = 24.5/32.5	8.2	na/701	0/13.6	0	NT	NT	NT
1a/2a (28)	8583/1175	83.5 = 1.2/83.2	0.9	na/759	0/12.1	0	na/753	0/15.8	0
1b/2a (12)	9689/716	39.1 = 1.7/38.2	0.8	na/710	0/13.9	0	NT	NT	NT
1b/2a (28)	6205/1173	83.5 = 5.4/82.7	4.6	7302/788	14.6 = 1.2/13.6	0.2	na/738	0/15.4	0
2a (12)/2a (28)	597/8287	94.8 = 4.5/94.4	4.1	701/4662	27.4 = 0.7/26.8	0.1	NT	NT	NT
1a/1b/2a (12)	5479/na/727	52.2 = 27.2/0/35	10 (1a2a)	na/na/791	0/0/17.3	0	NT	NT	NT
1a/1b/2a (28)	na/6125/1155	79.9 = 0/4.5/78.8	3.7 (1b2a)	na/6874/816	17 = 0/2.4/15.2	0.6 (1b2a)	na/na/753	0/0/16.6	0

*Bovine viral diarrhea virus strains; PI34 (1a), PI285 (1b), PI28 and PI12 (2a).
NT- Not tested.
na – not applicable, when the % of positive cells is 0.
** It refers to the total % of positive cells by a particular strain or combination of strains. However, it does not mean that % of cells are exclusively positive for the particular strains.

Table 5
Percentage of single, dual, and triple positive cells and gMFI of positive BTu cells.

BTu	Day 2			Day 9			Day 30		
*Virus used for infection	gMFI per strain	% positive cells (total and per strain**)	% co-positive cells (total and per combination**)	gMFI per strain	% positive cells (total and per strain**)	% co-positive cells (total and per combination**)	gMFI per strain	% positive cells (total and per strain**)	% co-positive cells (total and per combination**)
1a	63489	100	0	13523	60.3	0	16289	69.3	0
1b	111907	99.9	0	20889	41.6	0	25817	56.5	0
2a (12)	6450	100	0	1053	59.1	0	1188	64.8	0
2a (28)	6661	99.9	0	1177	57	0	1287	58.5	0
1a/1b	48550/ 36268	99.7 = 99.2/90	89.5	12928/ 21100	55.2 = 41.3/21.7	7.8	14531/ 23165	62.3 = 41.1/32.8	11.6
1a/2a (12)	55481/ 3520	99.9 = 94.9/53.9	48.9	12744/ 1082	59.5 = 40.9/25.5	6.9	13401/ 1191	62.3 = 11/54.3	3
1a/2a (28)	48419/ 3586	99.9 = 95.4/47.6	43.1	12336/ 1162	55.8 = 34/28.6	6.8	na/1293	0/61.1	0
1b/2a (12)	57491/ 1798	99.7 = 95.7/63.3	59.3	20859/ 1035	52.1 = 31/26.8	5.7	21718/ 1096	56.2 = 7.3/50.9	2
1b/2a (28)	49551/ 1453	99.4 = 87.2/65.4	53.2	19348/ 1131	51.7 = 15.4/41	4.7	na/1263	0/58.9	0
2a (12)/2a (28)	2413/ 56699	99.9 = 75.5/89.5	65.1	922/14516	60.9 = 16.2/52.5	7.8	724/16247	65.7 = 5.8/63.7	3.8
1a/1b/2a (12)	43260/ 30818/ 2956	99.9 = 95.9/76.3/42.7	85.6 = 74.3 (1a1b), 39.7 (1a2a), 30.4 (1b2a), 29.4 (1a1b2a)	12331/ 20695/ 1057	59.8 = 33.2/17.6/18.1	10.4 = 4.2 (1a2a), 0.7 (1a1b2a), 5.4 (1a1b), 2.2 (1b2a)	13789/ 22857/ 1216	62 = 8.3/8.4/50.4	5.1 = 2.3 (1a2a), 2.3 (1b2a), 0.9 (1a1b), 0.2 (1a1b2a)
1a/1b/2a (28)	32167/ 32805/ 3418	99.9 = 90.4/73.1/53.2	84.4 = 45.5 (1a2a), 32.4 (1a1b2a), 68.5 (1a1b), 35.2 (1b2a)	11340/ 19886/ 1164	52.8 = 17.9/11.9/34.3	8.2 = 4.5 (1a2a), 0.7 (1a1b2a), 2.3 (1a1b), 2.8 (1b2a)	na/na/ 1294	0/0/58.9	0

*Bovine viral diarrhea virus strains; PI34 (1a), PI285 (1b), PI28 and PI12 (2a).
na – not applicable, when the % of infected cells is 0.
** Refers to the total % of infected cells by a particular strain or combination of strains. However, it does not mean that % of cells are exclusively infected by the particular strains.

4.5 Capítulo 5: Cross-neutralizing antibodies induced by BVDV-1a, 1b and 2a replicon particle vaccines to a broad diverse panel of BVDV strains

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Cross-neutralizing antibodies induced by BVDV-1a, 1b and 2a replicon particle vaccines to a broad diverse panel of BVDV strains

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ABSTRACT

BVDV viruses are globally-distributed pathogens of cattle that lead to substantial economic losses and are responsible for a broad range of clinical presentations. They belong to the bovine viral diarrhea virus type 1 (BVDV-1) and BVDV-2 species and can be further segregated in several subgenotypes. This great genetic and antigenic diversity is one of the main causes of vaccine failure. BVDV-1a, 1b and BVDV-2a are the predominant subgenotypes in many countries and their protein E2 is the major target of neutralizing antibodies. Venezuelan equine encephalitis virus replicon (Vrep) can be used as a delivery vector of heterologous protein and are a promising control strategy to overcome the shortcomings of the traditional vaccines. In this study we assessed the titers of cross-neutralizing antibodies induced by Vrep vaccines expressing E2 of BVDV-1a, 1b and BVDV-2a strains against 26 BVDV strains, belonging to seven subgenotypes. These results were also compared to those elicited by an MLV (BVDV-1a, 2a). Although the three Vrep vaccines induced neutralizing antibodies against virtually all strains, the BVDV-1b-Vrep and MLV induced the highest titers and neutralized a broader range of antigenic diverse BVDV strains.

Keywords:

Bovine viral diarrhea virus

Pestivirus

Vaccine

Virus replicon particle (VRP)

INTRODUCTION

BVDV infections of cattle can result in significant economic losses worldwide and lead to manifestations ranging from mild clinical signs to serious outcomes, which may manifest in the immune, respiratory, gastrointestinal and or reproductive system (Schweizer and Peterhans, 2014). The causative agent is either the Bovine Viral Diarrhea Virus type 1 (BVDV-1) or the BVDV-2, recently renamed as *Pestivirus A* and *Pestivirus B* species, belonging to the family *Flaviviridae,* genus *Pestivirus* (Smith et al., 2017). Pestiviruses are single-stranded, positive-sense RNA viruses with a genome size of 12,3 nt (Smith et al., 2017). BVDV strains shows a considerable genetic diversity and the two species can be further segregated into several subgenotypes, BVDV-1a-u (Vilček et al., 2001; Deng et al., 2015) and BVDV-2a-c (Tajima et al., 2001; Jenckel et al., 2014).

This pronounced genetic diversity an antigenic variability among pestiviruses is a well-known phenomenon that may affect the success of BVDV vaccinations programs. Thereby, BVDV vaccines must provide protection against the broad diversity of strains that are either currently in that country or might be introduced (Fulton, 2015). Different BVDV subgenotypes predominate in different countries. In general, BVDV-1b and BVDV-1a are the most predominant subgenotypes worldwide, followed by BVDV-2a in the USA (Yeşilbağ et al., 2017). As such, most vaccines contain only BVDV-1a and BVDV-2a strains and are killed virus (KV) or modified-live virus (MLV) (Fulton, 2015).

MLV provides a faster, stronger, broader and longer-term immune response than the KV (Newcomer et al., 2017). However, both types of vaccines have significant shortcomings as both are susceptible to neutralizing and complement-mediated destruction triggered by passively acquired antibodies (Griebel, 2015). While the low imunogenicity of KV often leads to immune failure, the major concerns related to the MLV refer to the intrinsic risk of virulence reversions and possibility of immunodeppression, transmission from vaccinates to susceptible contacts and contribution to the mucosal disease in persistently infected (PI) cattle (Fulton, 2015; Newcomer et al., 2017).

Thus, there is a need for developing more efficient and safer vaccines. Replicon (RP) vectors, as the Venezuelan equine encephalitis virus replicon (Vrep), have induced both innate, humoral and cell mediated responses to the heterologous proteins expressed. Therefore, Vrep vaccines represents an elegant strategy to overcome the problems

encountered by traditional vaccines. This type of vaccine is safe in terms of horizontal and vertical transmission and allows the differentiation of infected from vaccinated animals (DIVA), a long-desired need for BVDV control programs (Raue et al., 2011; Ljungberg and Liljeström, 2014). In addition, the same Vrep vector can be used for multiple vaccinations within the same animal with no decrease in vaccine efficacy (Vander Veen et al., 2012a; Ljungberg and Liljeström, 2014). As the E2 is the major BVDV protective protein, it constitutes an excellent candidate for the development of a subunit vaccine (Loy et al., 2013; Hossain and Rowland, 2018).

A Vrep vaccine based on BVDV-1b-E2 have been previously engineered (Loy et al., 2013) and in the present study, the same methodology was applied to design two vaccines based on BVDV-1a and BVDV-2a strains. Therefore, this study aimed to assess the cross-neutralizing antibodies induced by these three Vrep vaccines against representative BVDV strains, belonging to several subgenotypes; and to compare the humoral immune response elicited by these Vrep vaccines with those developed in cattle immunized with a MLV BVDV vaccine.

MATERIALS AND METHODS

1- Viruses and virus propagation

Wild-type strains used for hyperimmunization or strains used as vaccines homologous ones in virus neutralization assay (VN) included NADL cp (BVDV-1a), NY-1 (BVDV-1b) and 296c, 53637, 125c (BVDV-2a). These strains plus a panel of 26 BVDV field strains, belonging to eight subgenotypes isolated from Italy (1e, 1f, 1g, 1k), Switzerland (1e, 1h, 1k) and The United Kingdom (UK) (1a, 1d, 1i) were used for cross neutralization assays. These strains were selected as they belonged to the predominant subgenotypes detected in the countries mentioned above (**Table 1**).

All strains were amplified and tittered in Madin-Darby bovine kidney (MDBK) cells, which were grown in MEM medium (Sigma Aldrich), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (PAA Laboratories Inc. Ontario, Canada), L-Glutamine (ThermoFisher Scientific, Waltham, MA), antibiotic-antimycotic and incubated at 37°C in a humid atmosphere with 5% CO_2. FBS and cell lines were tested free for pestivirus RNA by RT-PCR (Vilcek et al., 1994). In addition, FBS was also tested

and found to be free of pestivirus antibodies by virus neutralization assay (VN) (Bauermann et al., 2014).

To define the titer of noncytopatogenic strains an imunoperoxidase using monoclonal antibody (mAb) N2 was performed as previously described (Ridpath et al., 2010). The mAb BZ32 was used for imunoperoxidase for two strains (58-1, 58-2) (Ridpath and Neill, 2000). Endpoint titers were calculated using the Spearman-Karber method of endpoint determination.

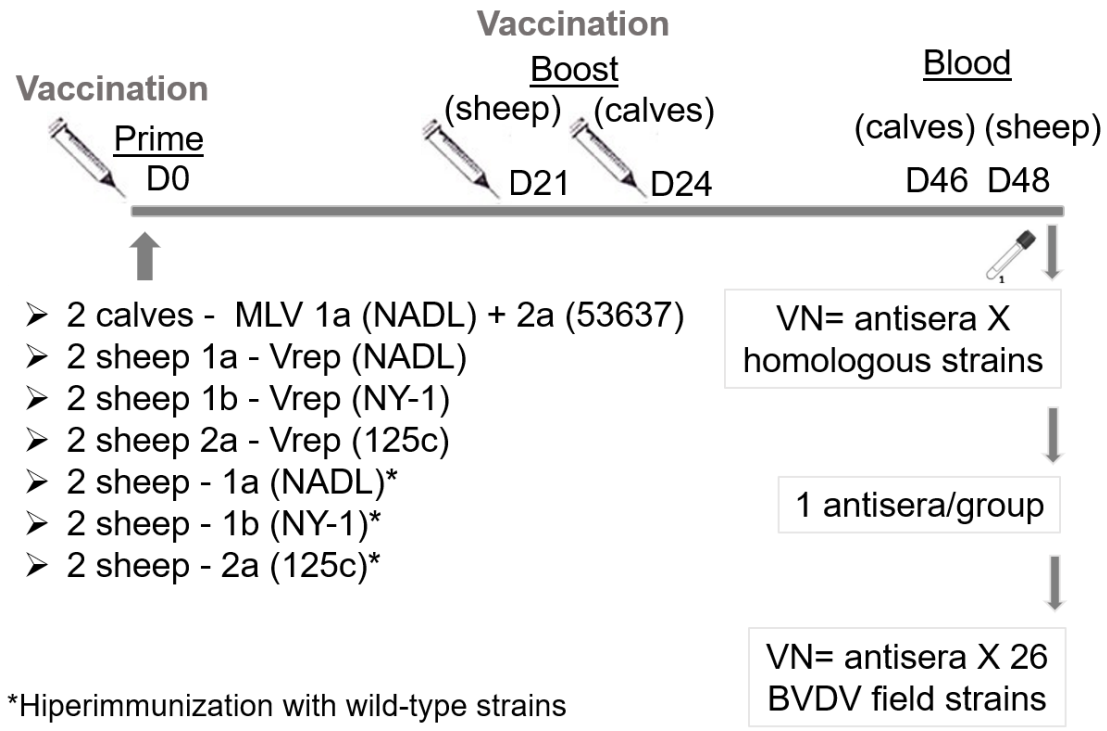
2- BVDV E2 alphavirus-derived replicon vaccine construction

The E2 glycoprotein gene was de novo synthesized (DNA2.0) based on the sequence from BVDV-1a strain NADL (Genbank: 7960755), NY1-BVDV-1b (Genbank: AY027671) (Loy et a., 2013) and 125c-BVDV-2a (Genbank: U25053). A Venezuelan equine encephalitis virus derived virus replicon particle (Vreps) vaccine was engineered to express E2 from the mentioned three strains using previously described methods (Loy et a., 2013) and they were here named: 1a-Vrep, 1b-Vrep and 2a-Vrep.

3- Immunization

Animals were either vaccinated with a commercial MLV (calves) or with the Vrep vaccines (sheep). In addition, sheep were hyperimmunized with the wild-types strains used to develop the Vreps, to compare the antibody titers with those induced by the vaccines. The experimental design is shown in Fig 1. Animals were sourced from BVD free herds. Each animal was tested and found to be seronegative to BVDV-1a (NADL), 1b (NY-1) and 2a (296c) by VN; and BVDV free by RT-PCR, as described below and previously (Vilcek et al., 1994). The use of the animal experimental protocol was reviewed and approved by the Institutional Animal Care and Use Procedure (ARS-2016-500) of the National Animal Disease Center.

Fig 1 - Experimental design to assess cross-reactive neutralizing antibodies induced by Vreps, MLV vaccines and the 26 BVDV strains from Italy, Switzerland, UK.



3.1 MLV

Two colostrum-deprived (CD) calves were vaccinated with a commercially available MLV, the One Shot® BVD vaccine (Zoetis) (BVDV-1a cp NADL, BVDV-2a cp 53637). The MLV was administered according to the manufacturer's instructions twice with an interval of 24 days. Blood samples were taken at days 0, 17, 31, 38 and 46 post-vaccination.

3.2 Vrep vaccines and infection with strains

Twelve sheep were divided in six groups, each group composed by 2 sheep that were inoculated with one of the Vrep vaccines or with the strains; NADL (BVDV-1a), NY-1 (BVDV-1b), 125c (BVDV-2a). Two consecutive (21 days apart) IM inoculation were performed with 2 mL (1 mL neck/shoulder and 1ml buttock) of the inoculum: $3.2X10^8$ infectious units (IU)/mL (Vrep 1a), $3.8X10^8$ IU/mL (Vrep 1b), $9.3X10^8$ IU /mL (Vrep 2a), or 10^6/ml of each strain. Serum was taken from animals on days 0, 21 and 48 post-vaccination.

4. Antisera

Prior to determine the cross-neutralizing titers, each antisera obtained by MLV, Vreps or wild-type strains hyperimmunization was tested against its homologous wild-type BVDV strain through the VN protocol described below. The serum from the animal with the highest antibody titer in its own group was chosen to be used in the further cross-neutralization tests. Since the 2a strain (125c) was not available for the VN, the strain 296c was chosen since it was the closest related representative 2a strain available. Since there is no multivalent replicon vaccine, a mixture 50% of 1a-Vrep and 50% of 2a-Vrep antisera was tested against the BVDV strains.

5. VNs and analysis of cross neutralizing antibody responses

The VN procedure applied to both antisera tested against its homologous BVDV strain and the panel of field strains, was performed as described below (OIE, 2016). Firstly, the sera were heat inactivated at 56ºC for 60 min. Sera were diluted in 2-fold serial dilutions (1:8 to 1024) and tested in quintuplicate. A total of 100 $TCID_{50}$/well of each virus was added and the plates containing virus and serum dilutions were incubated for 60 min at 37ºC in 5% CO2. Thereafter, the cell suspension (10^5cells/mL) was added and the plates were incubated 4 days at 37ºC in 5% CO2. Test results were evaluated using an optical microscope for cytopathic isolates. Titer was determined by the reciprocal of endpoint serum dilution that prevented viral replication. The imunoperoxidase using mAb N2 was used to determine endpoint for the majority of the ncp isolates with the exception of two strains (58-1, 58-2) that were tested using mAb BZ32, as previously described (Ridpath and Neill., 2000; Ridpath et al., 2010;). The reciprocal of the serum dilutions were log2 transformed according to the methods of Spearman-Karber, then they were back-transformed as geometric means (GMT). Sera which had a GMT ≥8 was considered as positive.

The serological relatedness was expressed as the percentage (P) of the heterologous GMT as compared with the homologous GMT using the formula P= 100 X (BA/AA); where BA is the GMT against strain B (heterologous) using antisera A; and AA is the GMT against strain A (homologous) using antisera A (Ridpath et al., 2010). The P value (≤25) represents four-fold differences in the GMT between homologous and heterologous antisera and this indicates significant antigenic differences.

6. Statistical Analysis

Statistical analysis was conducted using GraphPad Prism7 software. The Wilcoxon and Mann-Whitney test were used to analyze the differences in the GMTs between animals vaccinated with MLV and with Vreps vaccines. The significance of the variability among the subgenotypes within the immunization groups was determined using Anova 2-way and Multiple T-tests. $P< 0.05$ was considered significant.

RESULTS

1. Comparison between Vrep vaccines and MLV

Nearly all vaccines antiserum was able to neutralize (GMT≥8) all the virus strains tested (Table 1).

Exception occurred when 1a-Vrep antisera was tested against the strain R5013/96 (1k). The 1b-Vrep induced the higher GMT values, which ranged from 23-2896 against the heterologous strains, with an average of 545 (SEM=126). In 57.7% of field strains the GMT induced by the 1b-Vrep was 256 or higher, while the MLV elicited a GMT 256 or higher against 69.2% strains. The MLV raised an average GMT of 455 (SEM=76) against the heterologous strains, with titers ranging from 91-1783. These GMT averages induced by 1b-Vrep and MLV does not show significant differences between them. However, they are significantly higher ($p<0.05$) than those induced by 1a and 2a-Vrep, which elicited a GMT average of 74 (SEM=16) and 119 (SEM=31), respectively. Although the GMT ranged from 7-362 (1a-Vrep) and 14-724 (2a-Vrep), only in two (1a-Vrep) and three (2a-Vrep) strains, these Vreps induced a GMT ≥256.

As expected, the highest antibody titers were yielded by the homologous (vaccines) strains in almost all cases, with values ≥891 that increased overtime (data not shown). Notwithstanding, MLV induced higher antibody titer against 1f strains than to 1a and 2a vaccines strains. All strains showed significant antigenic differences when tested against 1a-Vrep, because this vaccine induced a GMT 4-fold lower against these strains than to the homologous vaccine strain. Similarly, 96% and 84% of the strains had significant antigenic differences against 2a-Vrep and 1b-Vrep strain, respectively. However, MLV vaccine reached the lowest antigenic differences, only against 30% of the BVDV field strains.

Table 1 - Neutralizing antibodies (GMT)[a] observed following vaccination with MLV and Vrep vaccines or hyperimmunization with wild-type strains against BVDV-1 strains, segregating by country

Neutralizing titer to		Vaccine	Vrep				infection strains		
Country/Sample ID	Sub-genotype	One shot	1a	1b	2a	1a/2a[b]	1a	1b	2a
Field strains									
Switzerland									
CH-Maria[d]	1e	181	11	181	45	23	57	91	≤8
S03-1175[d]	1e	362	45	362	14	111	29	91	≤8
Carlito[d]	1e	362	11	23	56	45	14	91	≤8
R2000-95[d]	1e	181	91	256	91	181	14	≤8	23
CH-04-01b[e]	1h	724	181	223	91	169	114	256	≤8
R3572/90[f]	1h	181	45	181	23	45	11	45	≤8
SM09-20[d]	1h	91	45	223	23	91	11	91	≤8
CH-Suwa[g]	1k	724	45	1448	724	223	57	228	≤8
208 11 016 (R3230/95)[f]	1k	362	23	181	128	446	23	57	≤8
R5013/96[g]	1k	181	≤8	91	23	16	11	9	≤8
UK[h]									
62-2	1a	362	14	91	23	23	23	45	≤8
63-1	1a	362	45	45	23	91	57	45	≤8
67-1	1d	362	91	724	91	181	57	181	14
67-2	1d	362	45	362	45	91	114	114	≤8
68-1	1e	724	56	114	45	64	14	91	≤8
58-1	1i	362	45	873	23	45	≤8	14	≤8
58-2	1i	362	91	512	34	45	≤8	91	≤8
69-1	1i	362	181	362	114	181	228	362	11
Italy[f]									
D7219	1e	181	45	630	181	91	10	228	≤8
D14688	1f	1783	362	1448	56	455	304	910	10
D18648	1f	1448	256	1448	362	724	79	1024	14
D7182	1f	362	45	362	228	724	114	181	9
D23284	1g	181	11	45	23	23	11	45	≤8
D58731	1k	362	23	724	91	114	≤8	57	≤8
D18892	1k	724	91	2896	362	724	362	362	11
D59460	1k	181	11	362	181	14	≤8	91	≤8
Vaccines strains									
NADL	1a	891	1448	-	-	724	111	-	-
NY-1	1b	-	-	3641	-	-	-	111	-
296c[c]	2a	-	-	-	1448	676	-	-	362
53637	2a	1663	-	-	-	-	-	-	-

a- Reciprocal of the highest dilution of serum capable of completely neutralizing 100-300 TCID-50 of the respective virus strain

b- mixture of 50% of 1a antiserum + 50% 2a antiserum

c - Since the 2a replicon vaccine strain that was not available for the neutralization tests, we used the strain 296c as a closely related representative 2a strain.

d - Stalder et al., 2005

e- Bachofen et al., 2008
f- not published?
g- Schweizer et al., 2001
h - Booth, 2013
The absence of VN is expressed as <8.
GMT lower (<4-fold) than the homologous are underlined.

Although there were variations in GMT between strains, even within the same subgenotype, there is no significant difference between the subgenotypes. Nonetheless, the highest cross neutralizing titer achieved by the MLV and 1a-Vrep antiserum was against two strains of 1f subgenotype, whereas the highest cross neutralizing titer achieved by 1b-Vrep and 2a-Vrep antiserum was against two different strains of 1k subgenotype. The lowest titer produced by the MLV, 1a-Vrep, 1b-Vrep, 2a-Vrep antisera was against strains of the following subgenotypes: 1h, 1k, 1a and 1g; 1e, respectively.

2. Comparison between Vrep vaccines and the infection with the strains

To compare the cross-neutralizing antibodies against different BVDV strains induced by Vreps vaccines to those induced by the infection with the strains, animals were infected with the same 1a, 1b and 2a strains used for the construction of Vreps. An agreement between the strain and their Vrep counterpart in relation to the ranking of which strains induced the highest titers was found in 26.9% of the BVDV field strains tested. Besides, 1b vaccine antisera raised the highest titer against 82.1% of the strains, when compared to 1a and 2a vaccine strain antisera, which induced the highest titer only against 14% and 3% of the strains, respectively.

Differently of the GMT raised by Vreps antisera, the highest cross neutralizing titer achieved by 1a, 1b and 2a wild-type strains was against strains of the subgenotypes 1k, 1f and 1e, respectively. The lowest titers induced by 1a and 1b wild-type strains was against strains of 1i and 1k; and 1e, sequentially. BVDV- 2a wild-type antisera was negative for 69.2% of the strains tested.

3. Comparison between 1a/2a Vrep vaccine and MLV

To compare MLV to Vrep vaccines, since there is no multivalent replicon vaccine, an equal mixture of 1a replicon antisera with 2a replicon antisera (1a/2a-Vrep) was prepared and tested against the BVDV field strains. Comparing the average of all GMT, the MLV (mean= 455) antiserum raised a higher titer than 1a/2a-Vrep (mean= 190,

SEM=44) ($p<0.01$). However, the 1a/2a-Vrep replicon antisera induced a higher GMT than that elicited by 1a-Vrep (74) and 2a-Vrep (119) ($p<0.05$) individually. Only in 19.2% of the strains the 1a/2a-Vrep reached a GMT ≥256.

Additionally, both MLV and 1a/2a-Vrep raised the same GMT in only 11.5% of the BVDV field strains tested. The 1a/2a-Vrep induced a higher GMT than MLV against 7.7% of the strains, but MLV raised the highest GMT against the other 80.7% of the strains. The comparison of the response against 1a and 2a-Vrep individual or combined (1a/2a), found that the last was able to reach a greater GMT in 69.2% of BVDV field strains. Interestingly, the highest and the lowest cross neutralizing titer achieved by 1a/2a-Vrep antisera was against strains of 1k. In general, there is no significant difference among the subgenotypes. About half of the BVDV field strains showed significant antigenic differences when tested against the 1a/2a replicon antisera.

DISCUSSION

BVDV remains a worldwide animal health concern and vaccination is one important component of prevention. Although many vaccines (KV and MLV) are commercially available, there are many drawbacks associated with those, as mentioned previously (Newcomer, 2017). To overcome these disadvantages, Vrep vaccines showed to be an elegant alternative (Loy, 2013; Hossain, 2018). Thus, this study aimed to assess the humoral immune response induced by 1a, 1b or 2a Vrep vaccines against several BVDV subgenotypes; and compare to those elicited by a MLV.

Both three Vreps were designed based on the E2, the main target of neutralizing antibodies and the main protective mediator for BVDV disease (Donis et al., 1988; Paton et al., 1992). Since the E2 is highly-variable (Chernick et al., 2018), there is a great antigenic diversity among the BVDV species, subgenotypes and even between strains of the same subgenotype (Nagai et al., 2001; Becher et al., 2003; Bachofen et al., 2008). These differences of cross-neutralizing antibodies against different strains, even belonging to the same subgenotype, were also observed in the present study. The practical significance of these antigenic differences is undetermined and there is no strict correlation between diversity and protection. However, it is well known that antigenic differences may lead to vaccine failure or a partial protection (Fulton, 2015; Newcomer et al., 2015). Therefore, vaccines would be improved by taking into consideration the

BVDV subgenotypes/strains prevalent in a particular region or country. Besides, that vaccines strains must provide a broad cross-protection against the BVDV diversity (Kelling, 2004; Griebel, 2015). This characteristic was reached by 1b-Vrep, since this vaccine elicited higher titers of neutralizing antibodies against the wide spectrum of antigenic diverse strains tested, similar to the MLV. Thus, BVDV strains and subgenotypes tested in this study probably display a greater antigenic similarity to the 1b vaccine strain than to 1a and 2a vaccine strains, which was also confirmed by the immunization of sheep with the wild-type BVDV strains used to construct the Vreps.

Humoral antibodies are widely used to measure the efficiency of vaccines as they are correlated to protection in BVDV. Despite the antibody titer threshold for protection being uncertain, a titer at least 256 showed to be sufficient to prevent clinical signs, viremia and virus shedding (Howard et al., 1989; Bolin and Ridpath, 1995; Fulton and Burge, 2000; Wang et al., 2014; Falkenberg et al., 2015; Downey-Slinker et al., 2016). The 1b-Vrep was the Vrep vaccine that elicited the greatest number of antibody titers higher than 256. Although, the 1a and 2a Vrep raised in general lower neutralizing titers against all heterologous strains, they elicited high antibody titers against the homologous strains. Thereby, the 1a and 2a Vrep are suitable for further testing to address the homologous protection. On the other hand, a multivalent Vrep, constituted by 1a and 2a strains, could be an alternative to improve the robustness of the humoral response and to provide a broader antigenic response.

Although the BVDV-1b is the predominant subgenotype in many countries as USA and Europe, only few commercial vaccines contain it in its composition (Yesilbag, 2017; Newcomer, 2017). Thus, the efficacy of BVDV-1a and BVDV-2a vaccines against BVDV-1b and the needed to include this subgenotype in the current BVDV vaccines have been discussed (Fulton, 2015). The 1b-Vrep previously developed and evaluated in this study, had already induced cross-neutralizing titers against BVDV-1a and BVDV-2a strains. In addition, it mitigated clinical disease when calves were challenged with the homologous strain (Loy, 2013). Another study also demonstrated that the 1b-Vrep, also based in E2, could be a future strategy, since it elicited BVDV-E2 specific IgA, IgM, IgG in pigs (Hossain, 2018).

Although, the current study does not address whether heterologous neutralization titers indicate efficacy against heterologous challenge, BVDV Vrep is a promising control strategy and can overcame the shortcoming of traditional vaccines. Although the three Vrep induced antibody titers against virtually all strains, the 1b Vrep vaccine was the one

that induced the highest antibody titers against a broad range of heterologous strains. Thus, it is a candidate vaccine suitable for further testing to address the heterologous protection, the extent and duration of immunity. Whereas, the 1a and 2a RP vaccine raised lower titers of neutralizing antibodies, they may be optimized to provide a strong and broad immune response as well as the 1a and 2a Vrep multivalent could be an alternative. Thereby, improving the vaccine efficacy against BVDV makes the vaccination an even more important control method for BVDV in cattle population.

Conflict of Interest

None declared.

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5. CONCLUSÕES

Os cinco trabalhos que compõem a presente tese de doutorado levaram às seguintes conclusões descritas abaixo:

1) O HoBi-like pestivírus foi a espécie viral mais prevalente detectada em amostras de soro de bovinos dos estados do Rio Grande do Norte e Maranhão, Brasil. Nenhum pestivírus foi detectado em amostras de pequenos ruminantes.
2) Análise filogenéticas, genéticas e evolutivas dos HoBi-like pestivírus revelaram que o gene da glicoproteína E2 é o que mais varia e o que mais sofre seleção positiva, o que acontece principalmente no domínio associado à imunogenicidade. Essas análises demonstraram que as cepas brasileiras são mais semelhantes geneticamente às europeias e às detectadas em soro fetal bovino. Ademais, esses estudos sugerem que o HoBi-like pestivírus pode ter se originado na Ásia, mais especificamente na Índia, de onde foi introduzido no Brasil e deste, posteriormente, foi introduzido na Europa.
3) A soroprevalência de anticorpos anti-pestivírus em ovinos foi de 5,6%, contudo, variou entre as regiões do estado do Wyoming, Estados Unidos. Esse inquérito também indicou que a maioria dos animais soropositivos possuem anticorpos devido a infecções por BVDV-1.
4) A exclusão competitiva foi observada entre cepas de BVDV em um estudo *in vitro* que foi realizado em dois tipos celulares bovinos e avaliado pela técnica de PrimeFlow RNA Assay. As cepas de BVDV-2a excluíram as cepas de BVDV-1a e BVDV-1b em coinfecções. Esse mesmo resultado de predominância de BVDV-2a foi observado em um estudo *in vivo* realizado com as mesmas cepas.
5) As três vacinas de replicon (Vreps), baseadas na E2 de cepas de BVDV-1a, BVDV-1b, BVDV-2a, induziram anticorpos neutralizantes contra 26 cepas de BVDV pertencentes a sete subgenótipos. Contudo, os animais vacinados com a BVDV-1b Vrep geraram títulos maiores do que aqueles vacinados com BVDV-1a e BVDV-2a Vrep e similares aqueles induzidos por uma vacina com vírus vivo modificado, contendo uma cepa de BVDV-1a e uma de BVDV-2a.

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7. ANEXO

Autoria de artigo científico não relacionado ao tema da tese

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RESEARCH

Serosurvey for Influenza D Virus Exposure in Cattle, United States, 2014–2015

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Influenza D virus has been detected predominantly in cattle from several countries. In the United States, regional and state seropositive rates for influenza D have previously been reported, but little information exists to evaluate national seroprevalence. We performed a serosurveillance study with 1,992 bovine serum samples collected across the country in 2014 and 2015. We found a high overall seropositive rate of 77.5% nationally; regional rates varied from 47.7%–84.6%. Samples from the Upper Midwest and Mountain West regions showed the highest seropositive rates. In addition, seropositive samples were found in 41 of the 42 states from which cattle originated, demonstrating that influenza D virus circulated widely in cattle during this period. The distribution of influenza D virus in cattle from the United States highlights the need for greater understanding about pathogenesis, epidemiology, and the implications for animal health.

Influenza D virus (IDV; genus Deltainfluenzavirus, family Orthomyxoviridae) is an enveloped, single-stranded, negative sense RNA virus with 7 genome segments and 1 surface glycoprotein, the hemagglutinin-esterase fusion (HEF) protein (*1*,*2*). The first detection of IDV dates back to Oklahoma, USA, in 2011 from pigs exhibiting influenza-like disease (*3*), although retrospective seroprevelance data suggests the presence of IDV in goats in the United States before 2002 (*4*). Subsequently, IDV has been identified in low frequency in pigs in Italy (*5*,*6*) and Luxembourg (*7*). In addition, evidence suggests IDV circulates in other hosts such as small ruminants, camels, and buffalo in Togo, Kenya, and China (*8*,*9*); and small ruminants, feral swine, and equids in the United States (*4*,*10*,*11*).

Although IDV has been detected in other species, cattle appear to be the main reservoir (*1*,*12*). A variety of sample types and methods of detection have been used to determine the prevalence of IDV in different regions, in various ages, breeds, and numbers of cattle evaluated. The lack of consistency between the methods and cattle evaluated may be a contributing factor to variability in prevalence of IDV in different regions. Seroprevelance data have been reported in cattle from Luxembourg (*7*), Japan (*13*,*14*), the United States (*1*,*15*,*16*), Togo, Benin, and Morocco (*9*); the highest reported seropositive rate (80.2%) was in the United States (*16*) and Luxembourg (*7*) and the lowest (1.9%) in Benin (*9*). Serologic testing provides an indication of IDV exposure but is not a measure of active infections. IDV RNA from respiratory samples of cattle has been detected in several countries; the United States (*1*,*15*,*17*,*18*), Italy (*5*), France (*19*), Ireland (*20*), China (*8*,*21*), Japan (*22*), and Mexico (*18*). Studies from Mexico (*18*) reported the highest frequency of positive samples (29.7%) and China the lowest (0.7%) (*21*).

In both experimental and field infections with IDV, mild to moderate respiratory disease have been reported (*23*,*24*). In addition, IDV-positive samples are reported not only from cattle manifesting clinical signs associated with bovine respiratory disease but also from cattle that are asymptomatic and appear to be healthy (*20*–*22*). Experimental infection of calves demonstrated that IDV caused mild to moderate respiratory disease and that peak viral shedding occurred at 4–6 days postinfection; seroconversion was detected as early as day 6 postinfection (*12*,*23*,*24*). Whereas IDV infection by itself has been associated mainly with mild respiratory illness, IDV has also been implicated as a contributor to bovine respiratory disease complex (BRDC), which is the most costly disease affecting the US cattle industry (*17*,*18*,*23*,*25*).

Because there are no commercially available vaccines against IDV, positive serologic assays reflect natural

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exposure. Given the potential of IDV to contribute to BRDC, inclusion of IDV in vaccination programs has been debated. The frequency of IDV RNA–positive samples from US cattle is 4.8%–18% (*1*,*15*,*17*,*18*), and positive samples have been reported in the US cattle population since 2003 (*16*). The seropositive rate has been reported at 13.5%–80.2% (*15*,*16*); the upper Midwest region has the highest seroprevalence. The wide variation of seroprevalence could be caused by differences in the age of the cattle evaluated or by differences across regions because of limited sample size and the focus on the midwestern and south-central regions of the country. We conducted a national serosurvey of cattle of a similar age to fully evaluate the potential role of IDV in BRDC infections and the effect of IDV on animal health and productivity.

Materials and Methods

Samples

We assessed 1,992 banked bovine serum samples for IDV-specific antibodies. The samples, collected between August 2014 and December 2015 as part of the US brucellosis surveillance program, were previously used to screen for ruminant pestivirus and bovine leukemia virus (BLV) exposure (*26*,*27*). We aimed to determine the seropositivity rate for IDV and retrospectively compare that rate with seropositivity rates for ruminant pestivirus and BLV from the same samples to identify regional patterns or differences in the US cattle population.

The serum samples came from both male and female cattle ≥2 years of age, raised in 42 states, and were randomly collected from 5 slaughter plants. The states were categorized into 6 regions as previously defined (*26*): Pacific West (PW), Mountain West (MW), Upper Midwest (UMW), South Central (SC), Northeast (NE), and Southeast (SE) (Figure 1). The number of samples taken in each slaughter plant, listed by state (California, Florida, Nebraska, Pennsylvania, Minnesota), was proportional to the total annual number of cattle ≥2 years of age that had been processed in that plant. All samples were previously reported as negative for brucellosis.

Virus Selection and Propagation

To select the IDV strain used for the hemagglutination inhibition (HI) assay, we performed phylogenetic analysis on HEF genes with IDV strains that circulated in the United States during the same period in which the samples used for this study were collected (Figure 2). We downloaded

Figure 1. Number of samples collected from each state in study of influenza D virus in cattle, United States, 2014–2015. Asterisks (*) indicate states with 1 slaughter plant that contributed samples.

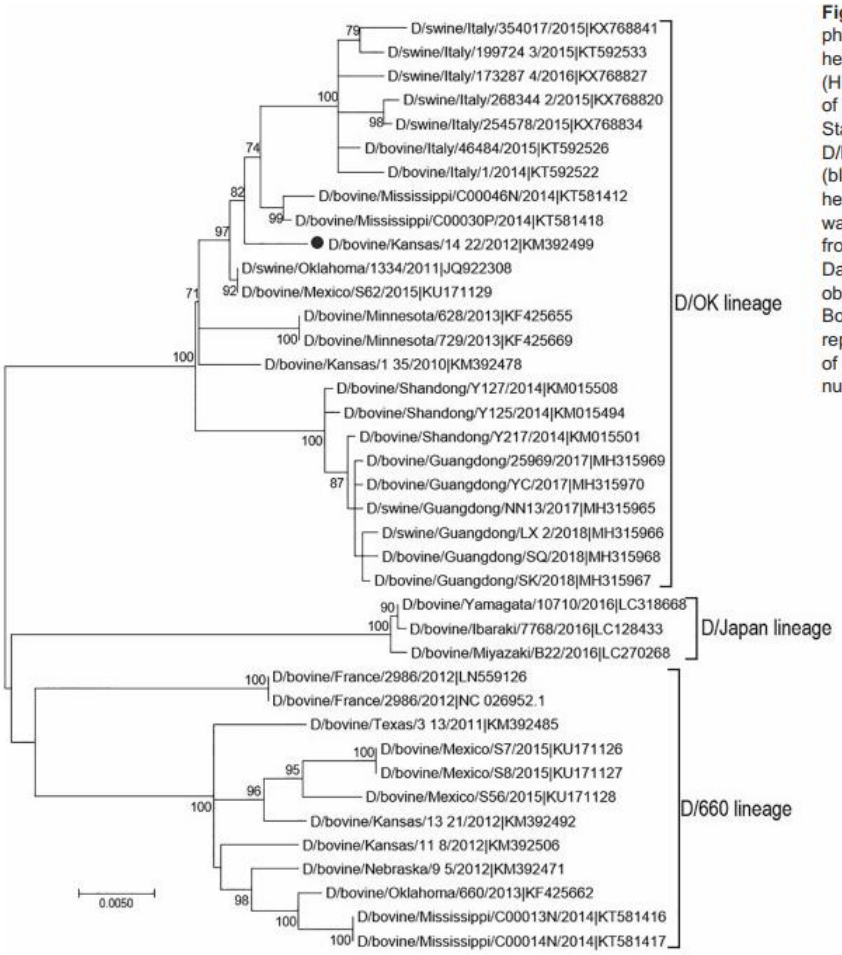

Figure 2. Maximum-likelihood phylogeny of the influenza D virus hemagglutinin-esterase fusion (HEF) gene constructed for study of influenza D virus in cattle, United States. Representative US strain D/bovine/Kansas/14-22/2012 (black dot), used as antigen in hemagglutination inhibition analysis, was aligned with reference strains from the Influenza Research Database (http://www.fludb.org) obtained on September 28, 2018. Bootstrap values >70% (1,000 replicates) are shown to the right of the nodes. Scale bar represents nucleotide substitutions per site.

full-length HEF gene segment sequences (n = 39) from the Influenza Research Database (http://www.fludb.org) on September 28, 2018. We aligned full-length segments using the MAFFT plugin for Geneious version 9.1.4 (Biomatters Ltd., http://www.geneious.com) with subsequent manual correction. We constructed a maximum-likelihood tree inferred in IQ-tree (http://www.iqtree.org) using a general time-reversible model of nucleotide substitution combined with a gamma-distributed rate variation with statistical support generated through ultrafast bootstrap analysis (*28*,*29*). We chose a representative US strain, D/bovine/Kansas/14-22/12, showing a high amino acid similarity (96%–99.2%) with US strains detected during 2014–2015, and a high hemagglutination (HA) titer.

We maintained swine testicle cells (ATCC CRL-1746) used for propagation of IDV in MEM medium (Sigma Aldrich, https://www.sigmaaldrich.com), supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (PAA Laboratories, Inc., https://www.fishersci.com) and L-glutamine (ThermoFisher Scientific, https://www.thermofisher.com) antibiotic-antimycotic solution incubated at 37°C in a humid atmosphere of 5% CO_2. We propagated the D/bovine/Kansas/14-22/12 strain, diluted 1:1,000 in swine testicle cells cultured in serum-free medium in the presence of TPCK-trypsin (0.1 µg/mL) and 5% bovine serum albumin, and incubated at 37°C for up to 4 days.

Serology

We performed the HI assay for detection of D/bovine/Kansas/14-22/12–specific antibodies in accordance with the specifications in the World Health Organization manual on animal influenza A virus diagnosis and surveillance (*30*). We treated 1:3 serum samples with receptor-destroying enzyme (Denka Seiken UK, http://www.denka-seiken.jp) at 37°C for 18 hours, heat inactivated it at 56°C for 1 h, and diluted it 1:10 with phosphate-buffered saline. We

conducted the assay in duplicate, at room temperature and in V-bottom 96 well plates, starting at 1:10 and doing 2-fold serial dilutions to reach a 1:1,280 dilution. We added the serially diluted samples to the virus (8 hemagglutination units/50 μL) for 1 h, then incubated with 0.5% turkey red blood cells for 30 min. The endpoint titer was the reciprocal of the highest dilution of serum that demonstrated partial to full inhibition of hemagglutination. We determined that a serum with an HI titer ≥40 was seropositive according to previous IDV serosurveillance studies (*4*,*15*). We used a negative control (PBS), as well as a positive control consisting of a rabbit polyclonal antiserum generated against D/swine/OK/1334/2011, in the HI assay (*1*). To exclude the possible presence of nonspecific antibodies, we also performed HI with serum samples from 10 colostrum-deprived calves; all showed titers of 0, which confirmed negativity.

Statistical Analysis

We used GraphPad Prism 7 software (GraphPad Software, LLC, https://www.graphpad.com) to statistically compare seropositive rates of IDV infection by χ^2 test and geometric mean titers (GMT) by the Kruskal-Wallis and Mann-Whitney test. We considered $p<0.05$ significant.

Results

Of the 1,992 cattle serum samples tested by HI assay for detection of IDV specific antibodies, 1,545 (77.5%) samples were positive; the overall GMT of positive samples was 230 (titers ranged from 40 to 1,280). We identified positive serum in samples from 41 of the 42 states tested (Table). The seropositivity rate was 25%–93.8% among the states and average GMT was 80–460. However, sample size was small in some of the states with low positivity, low titer, or both, which might have caused bias in the regional distribution.

We categorized the results by geographic region to compare the differences of seropositive rate and GMT. The seropositive rate range was 47.7%–84.6% ($p<0.05$) and GMT 110–260 ($p<0.05$) among the regions (Table). Mountain West region had the highest seropositive rate (84.6%) and GMT (260); Northeast region had the lowest seropositive rate (47.7%) and GMT (110).

Discussion

Although IDV was described in pigs earlier than in cattle in the United States, subsequent reports of retrospective samples suggested that cattle are the natural reservoir (*1*,*12*). Because seroprevalence surveillance in US cattle had been conducted only at state or regional levels, we undertook a nationwide serologic survey to detect IDV antibodies in cattle. Our results clearly demonstrate that IDV circulated with high frequency in cattle in all regions of the USA during 2014–2015.

We observed regional variation in seropositive rate and GMT, although all regions had relatively high frequency. Overall, the Upper Midwest and Mountain West

Table. Serosurveillance results for influenza D virus in cattle, by region and state, United States, 2014–2015*

Region and state	No. samples	Seropositive rate, %†	GMT (range)‡
Mountain West			
Idaho	187	87.2	230 (40–1,280)
Montana	86	84.9	270 (40–1,280)
Colorado	78	88.5	330 (40–1,280)
Utah	29	79.3	240 (80–1,280)
Wyoming	24	79.2	460 (80–1,280)
Arizona	21	57.1	140 (40–1,280)
New Mexico	16	93.8	210 (40–1,280)
Nevada	8	75.0	250 (80–1,280)
Total	449	84.6	260 (40–1,280)
Upper Midwest			
Nebraska	125	91.2	260 (40–1,280)
Iowa	101	92.1	270 (40–1,280)
Kansas	98	86.7	300 (40–1,280)
Missouri	94	86.2	220 (40–1,280)
South Dakota	87	90.8	300 (40–1,280)
Minnesota	83	89.2	280 (40–1,280)
Wisconsin	79	84.8	250 (40–1,280)
Ohio	47	48.9	130 (40–640)
North Dakota	41	56.1	400 (40–1,280)
Indiana	27	37.0	120 (40–640)
Michigan	19	63.2	190 (40–1,280)
Illinois	6	83.3	160 (80–320)
Total	807	84.0	260 (40–1,280)
South Central			
Oklahoma	102	79.4	230 (40–1,280)
Texas	48	75.0	170 (40–1,280)
Total	150	78.0	210 (40–1,280)
Pacific West			
California	166	77.7	190 (40–1,280)
Oregon	42	76.2	300 (40–1,280)
Washington	40	72.5	230 (40–1,280)
Total	248	76.7	210 (40–1,280)
Southeast			
Arkansas	24	83.3	180 (40–640)
Virginia	23	43.5	130 (40–640)
Kentucky	22	68.2	310 (80–1,280)
Florida	21	57.1	170 (40–1,280)
Alabama	19	68.4	140 (40–1,280)
Tennessee	10	50.0	240 (80–640)
West Virginia	9	33.3	200 (80–640)
Louisiana	7	42.9	160 (80–320)
Mississippi	4	25.0	80 (80–80)
Georgia	4	75.0	160 (80–320)
Total	143	59.5	180 (40–1,280)
Northeast			
Pennsylvania	61	50.8	120 (40–1,280)
New York	61	45.9	110 (40–1,280)
Vermont	47	51.1	110 (40–640)
Connecticut	11	0	0
Maryland	7	71.4	110 (40–1,280)
Massachusetts	5	60.0	80 (80–80)
New Hampshire	3	66.7	80 (80–80)
Total	195	47.7	110 (40–1,280)

*GMT, geometric mean titer.
†Seropositive rate was calculated using those samples with hemagglutination inhibition titer ≥40.
‡GMT was calculated using those samples with HI titer ≥40. Lowest and highest titers were measured from those samples with HIT titer ≥40.

regions showed the highest seropositive rates and the highest antibody titers, and also encompassed the states with the highest GMT. A similar result was obtained in a pestivirus serologic study performed with the same serum samples; here too, the Mountain West region showed the highest number of antibody-positive animals and higher titers (*20*). Although it is not possible to establish the cause, both pestivirus and IDV serology follow a similar trend. Potential causes include herd size, which can exceed 1,000 animals in these areas, and the potential for livestock and wildlife species to commingle and facilitate virus transmission. Evidence indicates IDV can infect nonbovine hosts, such as sheep, goats, pigs, and equids, in the United States (*4*,*10*,*31*). However, the full range of susceptible hosts for IDV is unknown, and interspecies transmission has not been demonstrated among the known hosts.

Seroprevalence of IDV in small ruminants was reported in samples collected from the Mountain West and Upper Midwest regions, whereas samples from other regions were negative (*4*). Moreover, in the Upper Midwest region, a high percentage of small ruminants with high titers was described, and the farms where they were located were in close proximity to cattle farms (*4*). This issue needs to be explored further to understand the importance of IDV as a threat for animal health and whether this is an underlying factor for the increased seroprevalence of viral pathogens in regions that have greater potential for interspecies transmission.

In general, we observed lower titers and a lower percentage of positive animals in the Northeast and Southeast regions. These results are similar to those reported from the pestivirus serosurvey that also found these 2 regions to have the lowest titers and lowest number of cattle seropositive for BVDV (*20*). On the other hand, in the BLV serosurvey, the Northeast had the highest seropositive rate for BLV, and the Mountain West, the lowest seropositive rate (*27*). Although seroprevalence differences existed between BLV and the other viruses evaluated (pestivirus and IDV), these differences could be caused by limited number of samples collected in these regions, differences in the epidemiology of these viruses, or differences in herd management practices across the regions. Previous data of IDV exposure in cattle of different ages in Mississippi (Southeast region) reported a high seroprevalence in cattle >1 year of age (*15*). Discrepancies between the current study and the previous reports could be explained by the number of samples evaluated in each of these studies; only 4 samples originated from Mississippi in our study, whereas ≥500 cattle were sampled in a previous study (*15*). Although our study encompassed the entire United States, the limited number of samples from several states, and subsequently the regions they represent, may have caused underestimation or overestimation of the seropositive rate of IDV. Despite the limitations of our study, data indicate that IDV is widespread at rates similar to the regional or state data previously reported (*15*,*16*).

Our findings, combined with those from previous serosurveillance studies (*15*,*16*), confirm a high nationwide seroprevalence of IDV in US cattle populations. Because of the potential association of IDV with BRDC (*17*,*18*,*23*,*31*) and the dearth of vaccines to prevent IDV infection (*12*,*32*), concerns have been raised regarding the negative effect of IDV on animal health. A possible explanation for the high seropositive rate is that IDV is common in the respiratory tract of cattle; times of stress, immune attack, or environmental changes that affect the respiratory tract can increase viral shedding but might not cause disease. Unpublished diagnostic data from our laboratory show that IDV is detected more frequently in samples that are also positive for other respiratory pathogens than in those positive for IDV alone. This finding indicates that IDV can either predispose the respiratory tract or act as an opportunistic pathogen in concert with other pathogens to cause BRD. Further research, including co-infection studies, is needed to elucidate the full range of susceptible hosts and the dynamics of interspecies transmission to understand the contribution of IDV to BRDC. In summary, our serosurveillance study of bovine serum samples from 2014–2015 showed a high seropositivity rate for IDV in the United States; 41 of the 42 states from which cattle originated had seropositive animals. No IDV vaccine exists. IDV infection has also been implicated in BRDC, the most costly disease affecting the US cattle industry. Therefore, our findings may indicate an ongoing risk to animal health.

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