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FACULDADE DE VETERINÁRIA - FAVET
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ANÁLISES DA EPIDEMIOLOGIA MOLECULAR E EVOLUÇÃO DE PESTIVÍRUS

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Porto Alegre
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Matheus Nunes Weber

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

Orientador: Dr. Cláudio Wageck Canal

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Matheus Nunes Weber

Análises da epidemiologia molecular e evolução de pestivírus

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RESUMO

O gênero *Pestivirus* da família *Flaviviridae*, compreende vírus de genoma RNA fita simples e polaridade positiva que comumente estão associados a infecções em ruminantes e suídeos. Possui quatro espécies reconhecidas: o vírus da diarreia viral bovina tipo 1 (BVDV-1), o BVDV-2, o vírus da doença da fronteira (BDV) e o vírus da peste suína clássica (CSFV). Além disso, possui possíveis espécies atípicas, sendo o vírus 'HoBi'-*like* a mais reconhecida na literatura. Com o objetivo de gerar mais informações acerca da epidemiologia molecular de pestivírus no País e dos mecanismos na evolução de vírus do gênero, o presente trabalho será apresentado sob a forma de seis artigos científicos. Os trabalhos visam a caracterização de pestivírus detectados em bovinos e javalis no Rio Grande do Sul, a identificação de cepas resultantes de provável recombinação homóloga, a análise das variantes virais intrahospedeiro (quasispécies) de animais infectados pelo vírus 'HoBi'-*like*, e a comparação da multiplicação *in vitro* de diferentes cepas de pestivírus em cultivos celulares oriundos de diferentes raças bovinas. No primeiro trabalho, amostras de soro bovino de animais do Rio Grande do Sul foram obtidas junto ao serviço veterinário oficial e submetidas a RT-PCR para detecção de pestivírus seguida de sequenciamento e análise filogenética das amostras positivas, onde 57,6% foram classificadas como BVDV-1 e 42,4% como BVDV-2, sendo constada frequência elevada de BVDV-2 quando comparada a outras regiões no mundo. No segundo trabalho, 40 amostras de javalis cativos obtidas de abatedouro foram testadas utilizando metodologia semelhante a anteriormente mencionada, e foi possível a detecção de uma amostra positiva, que foi classificada como BVDV-2, sendo a primeira evidência molecular do BVDV em javalis na literatura. No terceiro artigo científico, foi pesquisado a ocorrência de recombinação homóloga, utilizando metodologia *in silico*, em sequências de pestivírus presentes em banco de dados públicos, onde foram descritos novos possíveis eventos de recombinação entre BVDV-1a e BVDV-1b, BVDV-2a e BVDV-2b e CSFV-2.2 com outro genogrupo indefinido. No quarto e quinto trabalhos, animais persistentemente infectados com o vírus 'HoBi'-*like* foram gerados experimentalmente e, utilizando RT-PCR seguida de clonagem, sequenciamento e análises *in silico*, a composição e variações intrahospedeiro das quasispécies virais em circulação foram avaliadas, onde foi possível observar que características individuais do hospedeiro são importantes na seleção de variantes virais e que a nuvem de mutantes aumenta com o passar do tempo, não havando quasispécie dominante. Por fim, no sexto artigo científico, a multiplicação de cepas de BVDV-1a e 1b, BVDV-2a e vírus 'HoBi'-*like* foram avaliadas *in vitro* em células de cultivo primário obtidas de gado europeu, zebuíno e raça mista e comparadas utilizando modelos estatísticos, onde foi possível a observação de ausência de diferenças entre as raças ($P=0,88$), mas presença de diferença entre indivíduos da mesma raça ($P<0,05$). Os resultados aqui apresentados somam informações acerca da epidemiologia molecular, biologia e evolução dos pestivírus, sendo importantes para embasar futuras campanhas de controle e erradicação das doenças causadas por pestivírus.

Palavras-chave: pestivírus, recombinação homóloga, quasispécies, BVDV, 'HoBi'-*like*.

ABSTRACT

The genus Pestivirus of the family Flaviviridae, comprises single stranded RNA-viruses that are commonly associated with infections in ruminants and swine. It has four recognized species: bovine viral diarrhea virus 1 (BVDV-1), BVDV-2, border disease virus (BDV) and classical swine fever virus (CSFV). It also has putative atypical species where the 'HoBi'-like virus is the most highlighted in the literature. In order to generate more information about molecular epidemiology of pestiviruses and important mechanisms in the evolution of the virus genus, this work will be presented in six scientific articles form. The works aimed in the characterization of pestivirus detected in cattle and wild boar in Rio Grande do Sul state, identification of strains resulting from putative homologous recombination, analysis of intrahost viral variants (quasispecies) of animals infected with 'HoBi'-like viruses, and the comparison of in vitro growth of different pestivirus strains in cell cultures derived from different cattle breeds. In the first study, bovine serum samples of cattle from Rio Grande do Sul state were collected by the official veterinary service and submitted to RT-PCR for detection of pestivirus followed by DNA sequencing and phylogenetic analysis of the positive samples, where 57.6% were classified as BVDV-1 and 42.4% as BVDV-2, which revealed high frequency of BVDV- 2 when compared to other regions worldwide. In the second work, 40 captive wild boar lung samples obtained from slaughterhouse were tested using similar methodology described above, and one sample resulted positive and was classified as BVDV-2, which represented the first molecular evidence of BVDV in wild boars. In the third scientific article, the occurrence of homologous recombination was investigated using in silico methods applied to sequences available in public databases, which are described putative new events between BVDV-1a and BVDV-1b, BVDV-2a and 2b and CSFV-2.2 and undetermined genogroup. In the fourth and fifth studies, animals persistently infected with the 'HoBi'-like viruses were experimentally generated and using RT-PCR followed by cloning, sequencing and in silico analysis, the composition and intrahost variations of viral quasispecies in circulation were evaluated, where it was observed that individual characteristics of the host are important in the selection of viral variants and the mutant clouds increased overtime. Finally, in the sixth scientific article, the growth of BVDV-1a and 1b, and BVDV-2b, 'HoBi'-like virus strains were evaluated in vitro using primary cell cultures obtained by taurine, indicine and mixed breed cattle using statistical models, it was possible to observe the absence of differences between cattle breeds ($P=0.88$), but presence of difference between individuals within the breed ($P<0.05$). The results presented herein add information about the molecular epidemiology, biology and evolution of pestiviruses, being important to support future control and eradication programs of diseases caused by pestiviruses.

Keywords: *pestivirus, homologous recombination, quasispecies, BVDV, 'HoBi'-like.*

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

APV:	Pestivírus de antílope
APPV:	Pestivírus atípico de porcinos
BDV:	Vírus da doença da fronteira
BVDV-1:	Vírus da diarreia viral bovina tipo 1
BVDV-1a:	Vírus da diarreia viral bovina tipo 1 subtipo a
BVDV-1b	Vírus da diarreia viral bovina tipo 1 subtipo b
BVDV-2:	Vírus da diarreia viral bovina tipo 2
BVDV-2a:	Vírus da diarreia viral bovina tipo 2 subtipo a
BVDV-2b:	Vírus da diarreia viral bovina tipo 2 subtipo b
C:	Capsídeo
cp:	Citopático
CSFV:	Vírus da peste suína clássica
DM:	Doença das mucosas
ELISA:	Ensaio imunoenzimático
GPV:	Pestivírus de girafa
ICTV:	Comitée Internacional de Taxonomia Viral
MLV:	Vacina viva modificada
ncp:	Não-citopático
NGS:	Sequenciamento de alto desempenho
N^{pro}:	Autoprotease N terminal
NrPV	Pestivírus de *Rattus novergicus*
NS:	Não-estrutural
ORF:	Fase aberta de leitura
PI:	Persistentemente infectado
RaPestV:	Pestivírus de *Rhinolophus affinis*
RdRP:	RNA polimerase RNA dependente
SN:	Soroneutralização

SUMÁRIO

1 INTRODUÇÃO

O Brasil é um importante produtor mundial de alimentos e o agronegócio é um dos principais segmentos da economia nacional. Atualmente, o Brasil é o maior exportador de carne bovina e quarto de carne suína, além de ser o quarto maior produtor mundial de leite (USDA-FAS, 2014). O agronegócio desempenha um papel relevante no suprimento de alimentos e na geração de emprego e renda do Brasil. As doenças virais são preocupações constantes em qualquer programa de sanidade, devido aos grandes prejuízos econômicos que causam (HOUE, 2003) e o seu diagnóstico correto depende de técnicas laboratoriais eficientes (HOUE, 1999).

O gênero *Pestivirus*, da família *Flaviviridae*, possui quatro espécies reconhecidas pelo Comitée Internacional de Taxonomia Viral (ICTV): vírus da diarreia viral bovina tipo 1 (BVDV-1), vírus da diarreia viral bovina tipo 2 (BVDV-2), vírus da peste suína clássica (CSFV) e vírus da doença da fronteira (BDV) (SIMMONDS et al., 2011). Além das espécies estabelecidas, outros grupos de pestivírus foram identificados e são definidos como pestivírus atípicos, como o vírus 'HoBi'-*like* (SCHIRRMEIER et al., 2004), pestivírus de girafa (GPV) (AVALOS-RAMIREZ et al., 2001), pestivírus de antílope (APV) (VILCEK et al., 2005), vírus Bungowannah (KIRKLAND et al., 2007), vírus 'Aydin'-*like* em ovinos na Turquia (OGUZOGLU et al., 2009), isolados de ovinos da Tunísia (THABTI et al., 2005), pestivírus atípico de porcinos (APPV) (HAUSE et al., 2015), pestivírus de roedores *Rattus novergicus* (NrPV) e pestivírus de morcegos *Rhinolophus affinis* (RaPestV) (WU et al., 2012).

A maior importância econômica das infecções por pestivírus é a redução do desempenho reprodutivo. Além disso, elas reduzem a produtividade, diminuindo o ganho de peso e aumentando o tempo para abate e produção de leite (HOUE, 2003; MACLACHLAN; DUBOVI, 2011). Para aumentar a competitividade no mercado internacional é preciso atentar especialmente da sanidade de criações comerciais através da prevenção e controle. Contudo, estas só são possíveis quando existe uma estrutura eficiente para o diagnóstico e informações adequadas sobre a sua epidemiologia. O conhecimento das variantes virais em determinada região é importante para o estabelecimento de métodos

de diagnóstico e de controle adequados, visto que há relatos de falhas de protocolos comumente utilizados em casos de novas variantes virais (SCHIRRMEIER et al., 2004; PELETTO et al., 2012; WEBER et al., 2016b) e baixa reatividade cruzada entre espécies e subtipos virais (PIZARRO-LUCERO et al., 2006; BACHOFEN et al., 2008; NAGAI et al., 2008; RIDPATH et al., 2010; BIANCHI et al., 2011; BAUERMANN; FLORES; RIDPATH, 2012; BAUERMANN; FALKENBERG; RIDPATH, 2016). Além disso, o estudo de hospedeiros não usuais é importante para o conhecimento do papel epidemiológico dessas espécies em determinadas regiões (NETTLETON, 1990; VILCEK; NETTLETON, 2006; SEDLAK; BARTOVA; MACHOVA, 2008; SEDLAK; GIRMA; HOLEJSOVSKY, 2009; KAUTTO et al., 2012; WOLFF et al., 2016).

Assim como outros vírus de genoma RNA, os pestivírus existem como quasispécies em animais infectados (COLLINS; DESPORT; BROWNLIE, 1999; JONES; ZANDOMENI; WEBER, 2002; TÖPFER et al., 2013; DOW et al., 2015; RIDPATH et al., 2015; WEBER et al., 2016a, 2017) e estudos acerca da dinâmica da variabilidade viral intrahospedeiro podem auxiliar no conhecimento de determinantes evolucionárias na emergência de novas variantes. Além disso, a recombinação homóloga, também já relatada em vírus do gênero *Pestivirus* (JONES; WEBER, 2004; HE et al., 2007; WEBER et al., 2015), é um importante mecanismo de diversidade viral para vírus de diferentes famílias com genoma DNA e RNA (BALL, 1987; LAI, 1992; PADIDAM; SAWYER; FAUQUET, 1999; BRUYERE et al., 2000; WOROBEY, 2000; MOROZOV; PISAREVA; GROUDININ, 2000; CHARE; GOULD; HOLMES, 2003; HEATH et al., 2006; HE et al., 2009; PRAMESH; BARANWAL, 2013; BUDASZEWSKI et al., 2016).

Com base nas informações acima, e com o intuito de gerar mais informações acerca da epidemiologia molecular de pestivírus no País e de importantes mecanismos na evolução de vírus do gênero, o presente trabalho será apresentado sob a forma de seis artigos científicos. Os trabalhos visam a caracterização de pestivírus detectados em bovinos e javalis no Rio Grande do Sul, a identificação de cepas resultantes de provável recombinação homóloga, a análise das variantes virais intrahospedeiro de animais infectados pelo vírus ‘HoBi’, e a comparação do crescimento de diferentes cepas de pestivírus em cultivo celular oriundo de diferentes raças bovinas.

2 REVISÃO BIBLIOGRÁFICA

2.1 Classificação e caracterização

O gênero *Pestivirus*, pertencente à família *Flaviviridae*, compreende pequenos vírus esféricos de 40 a 60 nm, que contêm um nucleocapsídeo icosaédrico revestido externamente por um envelope derivado das membranas da célula hospedeira (FIGURA 1) (SIMMONDS et al., 2011)

O genoma dos pestivírus consiste de uma cadeia linear de RNA de cadeia positiva de 12,3 a 12,5 kb. Esta molécula de RNA apresenta duas regiões não traduzidas: uma na extremidade 5' e outra na 3'. Além disso, possui uma única fase aberta de leitura (ORF) que contém aproximadamente quatro mil códons, de onde é traduzida uma poliproteína, 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 e E2), proteína 7 (p7) e as proteínas não-estruturais (NS) NS2, NS3, NS4A, NS4B, NS5A e NS5B (FIGURA 2) (MACLACHLAN; DUBOVI, 2011; SIMMONDS et al., 2011; NEILL, 2012).

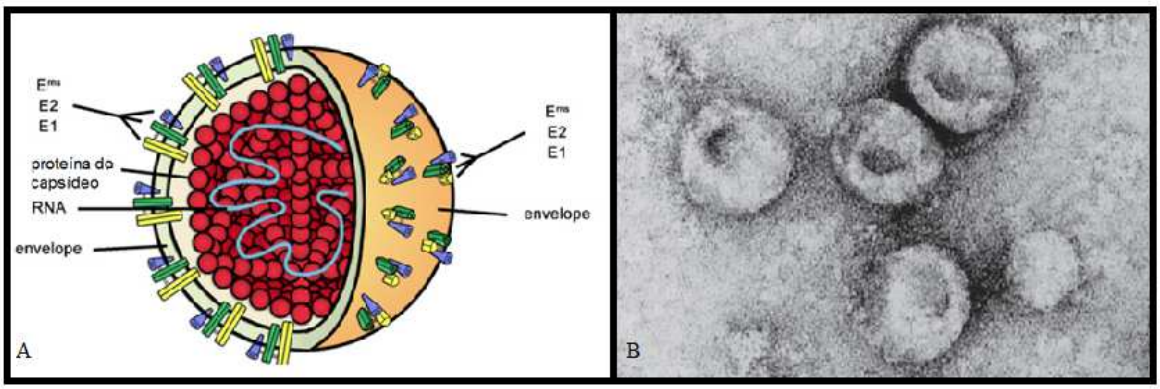

Figura 1. Morfologia e estrutura vírica dos vírus do gênero *Pestivirus*. A) Ilustração esquemática de um vírion com seus principais componentes; B) Foto de microscopia eletrônica de vírions do vírus da diarreia viral bovina. Adaptado: BEER et al., 2007; SIMMONDS et al., 2011.

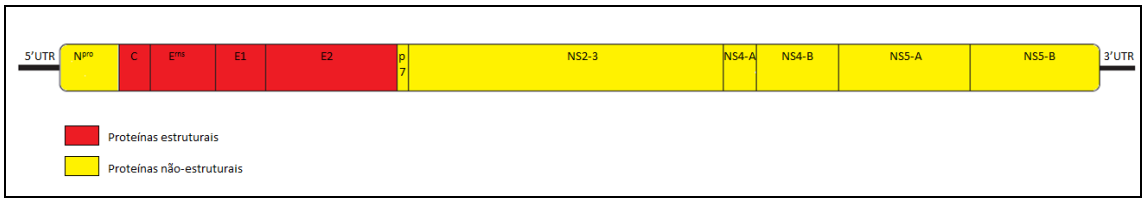

Figura 2. Estrutura e organização genômica de pestivírus. Adaptado de SIMMONDS et al., 2011.

A adsorção à célula hospedeira dos membros da família *Flaviviridae*, independentemente do gênero, parece ser mediada por ligantes presentes nas glicoproteínas do envelope. Os vírions adentram as células via endocitose mediada por receptores e replicam no citoplasma, interrompendo parcialmente as funções de transcrição e tradução da célula hospedeira (MACLACHLAN; DUBOVI, 2011). A replicação envolve a síntese de RNA complementar de sentido negativo (antigenômico), que serve como molde para a síntese do RNA de polaridade positiva que serão encapsidados como genoma da progênie viral. A morfogênese das novas partículas virais ocorre na região perinuclear do citoplasma, em associação com as membranas do complexo de Golgi e do retículo endoplasmático liso. As partículas recém-formadas aparecem em vacúolos no citoplasma e a sua liberação ocorre pela fusão dessas vesículas com a membrana plasmática. A ruptura da célula não é um pré-requisito para a liberação dos vírions, podendo ocorrer ou não dependendo do biótipo envolvido (RIDPATH; BAUERMANN; FLORES, 2012).

Os pestivírus podem apresentar dois biótipos diferentes, dependendo do seu efeito em cultivo celular: o não-citopático (ncp) e o citopático (cp). O biótipo ncp é o mais encontrado na natureza, apresenta a capacidade de replicar em células em cultura sem causar destruição celular, sendo o único biótipo encontrado em animais persistentemente infectados que ainda não apresentam doença das mucosas. Já o biotipo cp é originário de mutações, deleções e rearranjos genéticos mais frequentemente encontrados na proteína viral não estrutural NS2-3, que passa a ser expressa sob a forma de duas proteínas: NS2 e NS3 (POCOCK et al., 1987). Várias formas de geração da NS3 já foram relatadas: inserções de sequências celulares do hospedeiro (recombinação heteróloga) (QI et al., 1992; MENDEZ et al., 1998; BECHER; TAUTZ, 2011), duplicações de sequências do próprio genoma (MEYERS et al., 1992; QI et al., 1992; TAUTZ et al., 1996), deleções de parte do genoma (TAUTZ et al., 1994; KUPFERMANN et al., 1996), mutações em ponto (KUMMERER; MEYERS, 2000) ou rearranjos dentro do genoma (MEYERS et al., 1992). Essa alteração de proteína origina uma enfermidade gastrentérica invariavelmente fatal, denominada doença das mucosas (BAKER, 1995; MACLACHLAN; DUBOVI, 2011).

Cabe ressaltar que há descrição de inserções em outras regiões do genoma de pestivírus, que não a NS2-3 (MÜLLER et al., 2003; BECHER; TAUTZ, 2011).

O gênero *Pestivirus* possui quatro espécies reconhecidas pelo Comitée Internacional de Taxonimia Viral (ICTV): vírus da diarreia viral bovina tipo 1 (BVDV-1), vírus da diarreia viral bovina tipo 2 (BVDV-2), vírus da peste suína clássica (CSFV) e vírus da doença da fronteira (BDV) (SIMMONDS et al., 2011). Além das espécies estabelecidas, outros grupos de pestivírus foram identificados e são definidos como pestivírus atípicos, como o vírus 'HoBi'-*like* (SCHIRRMEIER et al., 2004), pestivírus de girafa (GPV) (AVALOS-RAMIREZ et al., 2001), pestivírus de antílope (APV) (VILCEK et al., 2005), vírus Bungowannah (KIRKLAND et al., 2007), vírus 'Aydin'-*like* em ovinos na Turquia (OGUZOGLU et al., 2009), isolados de ovinos da Tunísia (THABTI et al., 2005), pestivírus atípico de porcinos (APPV) (HAUSE et al., 2015), pestivírus de roedores *Rattus novergicus* (NrPV) e pestivírus de morcegos *Rhinolophus affinis* (RaPestV-1) (WU et al., 2012). A Figura 3 representa a relação filogenética das espécies estabelecidas e atípicas de pestivírus.

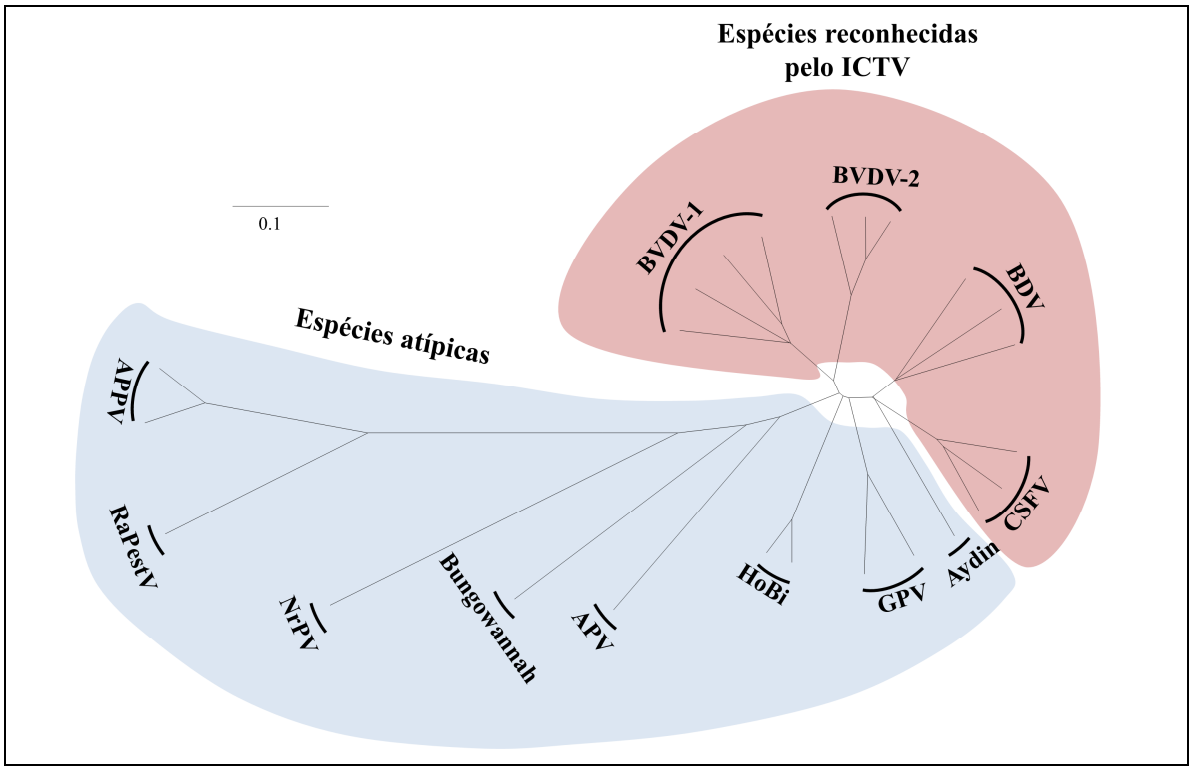

Figura 3: Agrupamento filogenético de pestivírus. A árvore filogenética foi construída com base nas sequências de nucleotídeos de sequências parciais da poliproteína compreendendo região da glicoprotepina E1 até NS4B, alinhados com o programa MAFFT, utilizando a inferência *neighboor joining*, modelo estatístico Kimura-2 e 1000 réplicas com o software MEGA6 (TAMURA et al., 2013). Inserções presentes em cepas citopáticas utilizadas na hipótese foram excluídas.

Há ainda importantes variações genéticas e antigênicas dentro das espécies de pestivírus. Com base em trabalhos prévios, o BVDV-1 pode ser dividido em pelo menos 21 subtipos (1a a 1u) (VILCEK et al., 2001; DENG et al., 2015a, 2015b), o BVDV-2 em três (2a a 2c) (FLORES et al., 2002; JENCKEL et al., 2014), o vírus 'HoBi'-*like* em quatro subtipos (3a a 3d) (GIAMMARIOLI et al., 2015), o BDV em oito genótipos (BDV-1 até BDV-8) (BECHER et al., 2003; GIAMMARIOLI et al., 2011; MARCO et al., 2011; KAWANISHI et al., 2014; PELETTO et al., 2016) e o CSFV em três genogrupos (1, 2 e 3) que ainda apresentam subgrupos (1.1, 1.2, 1.3, 1.4, 2.1a, 2.1b, 2.1c, 2.2, 2.3, 3.1, 3.2, 3.3 e 3.4) (LOWINGS et al., 1996; GREISER-WILKE et al., 1998; PATON et al., 2000; PAN et al., 2005; POSTEL et al., 2012, 2013). Aparentemente não há implicações clínicas na classificação em subtipos, genótipos ou genogrupos, contudo alguns apresentam baixa reatividade sorológica cruzada entre eles, podendo possivelmente levar a falhas vacinais (RIDPATH, 2003; PIZARRO-LUCERO et al., 2006; BACHOFEN et al., 2008; BIANCHI et al., 2011; FERNÁNDEZ-SIRERA et al., 2011).

A origem da diversidade genética de pestivírus, assim como de outros vírus, pode ser atribuída principalmente a erros da enzima RNA polimerase RNA dependente (RdRP) (DOMINGO; SHELDON; PERALES, 2012; NEILL et al., 2012). Este mecanismo leva a ocorrência de quasispécies virais, já relatadas para pestivírus (COLLINS; DESPORT; BROWNLIE, 1999; JONES; ZANDOMENI; WEBER, 2002; TÖPFER et al., 2013; DOW et al., 2015; RIDPATH et al., 2015; WEBER et al., 2016a, 2017), e seu estudo pode elucidar determinantes que agem na patogênese viral e efeitos da infecção (DOMINGO; SHELDON; PERALES, 2012). Outro mecanismo envolvido na diversidade de vírus de diversas famílias é a recombinação homóloga (BALL, 1987; LAI, 1992; PADIDAM; SAWYER; FAUQUET, 1999; BRUYERE et al., 2000; WOROBEY, 2000; MOROZOV; PISAREVA; GROUDININ, 2000; CHARE; GOULD; HOLMES, 2003; HEATH et al., 2006; HE et al., 2009; PRAMESH; BARANWAL, 2013; BUDASZEWSKI et al., 2016). Discrepâncias observadas em filogenias de pestivírus quando comparadas com diferentes regiões do genoma dos mesmos sugerem a ocorrência de recombinação homóloga entre subtipos (JONES; WEBER, 2004; NAGAI et al., 2004) e espécies (LIU et al., 2010).

2.2 Epidemiologia

Quanto ao espectro de hospedeiros das espécies reconhecidas pelo ICTV, o BVDV-1 e BVDV-2 foram primeiramente identificados em bovinos que são considerados seus hospedeiros naturais, assim como os ovinos do BDV e os suínos do CSFV (MACLACHLAN; DUBOVI, 2011). Contudo, com exceção do CSFV, que parece se restringir a suínos e javalis, o BVDV-1, BVDV-2 e BDV já foram detectados em infecções naturais em outros ruminantes domésticos e silvestres, além de suínos (ROEHE; WOODWARD; EDWARDS, 1992; CARLSSON; BELÁK, 1994; EDWARDS; ROEHE; IBATA, 1995; BECHER et al., 1999; MAKOSCHEY et al., 2002; MARCO et al., 2011; DENG et al., 2012; GAO et al., 2013; TAO et al., 2013; KAWANISHI et al., 2014; WOLFF et al., 2016).

Quanto a pestivírus de espécies ainda não estabelecidas, o GPV foi detectado apenas em uma girafa no Quênia em 1967 que apresentava sintomatologia semelhante à doença das mucosas dos bovinos (AVALOS-RAMIREZ et al., 2001) e em um cultivo de células de origem bovina na década de 90 (BECHER et al., 2003). O APV foi detectado apenas em um antílope (*Antilocapra americana*) cego nos Estados Unidos (VILCEK et al., 2005), o vírus Bungowannah em suínos com miocardite na Austrália (KIRKLAND et al., 2007), o vírus ‘Aydin’-*like* em pequenos ruminantes na Turquia (OGUZOGLU et al., 2009), os isolados da Tunísia em pequenos ruminantes na Tunísia, França e Itália (THABTI et al., 2005; MARTIN et al., 2015; CIULLI et al., 2016), o RaPestV em morcegos-ferradura-de-Java (*Rhinolophus affinis*) na China (WU et al., 2012), o NrPV em ratazanas (*Rattus novergicus*) nos Estados Unidos (FIRTH et al., 2014) e o APPV em suínos com tremores congênitos nos Estados Unidos e Alemanha (HAUSE et al., 2015; BEER et al., 2016; POSTEL et al., 2016). O vírus ‘HoBi’-*like* é o mais frequentemente relato dos pestivírus atípicos, sendo encontrado principalmente em soro fetal bovino (SCHIRRMEIER et al., 2004; STALDER et al., 2005; LIU et al., 2009; XIA et al., 2011, 2012; MAO et al., 2012; PELETTO et al., 2012; SILVEIRA et al., 2015), mas também em bovinos naturalmente infectados no Brasil, Itália, Tailândia e Bangladesh (CORTEZ et al., 2006; STÅHL et al., 2007; BIANCHI et al., 2011; DECARO et al., 2011, 2012a, 2012b; HAIDER et al., 2014; SILVEIRA et al., 2015; WEBER et al., 2016b), ovinos e caprinos na China (SHI et al., 2016) e búfalos no Brasil (STALDER et al., 2005).

A infecção por pestivírus em ruminantes apresenta distribuição mundial e muitos países relatam ou já relataram a sua presença (RIDPATH; BAUERMANN; FLORES,

2012). Além da circulação em espécies de ruminantes domésticos, a infecção também já foi descrita em diversas espécies silvestres (BECHER et al., 1999; VILCEK; NETTLETON, 2006; SEDLAK; GIRMA; HOLEJSOVSKY, 2009; VILCEK et al., 2010; FERNÁNDEZ-SIRERA et al., 2011; MARCO et al., 2011; GAO et al., 2013; GRANT et al., 2015; WOLFF et al., 2016), muitas vezes com isolados bastante distintos geneticamente aos vírus detectados em animais domésticos (BECHER et al., 1999; VILCEK et al., 2010; MARCO et al., 2011), sugerindo uma adaptação desses vírus aos hospedeiros silvestres (MARCO et al., 2011). Contudo, a relevância desses achados para epidemiologia em animais domésticos ainda permanece incerta (RIDPATH; FLORES, 2007; MARCO et al., 2011; GRANT et al., 2015; RIDPATH; NEILL, 2016).

O BDV é frequentemente relatado em ruminantes domésticos e silvestres no continente europeu (VILCEK et al., 1997, 2010; VILCEK; NETTLETON, 2006; OGUZOGLU et al., 2009; GIAMMARIOLI et al., 2011; MARCO et al., 2011; MARTIN et al., 2015), tendo também já sido reportado nos Estados Unidos (RIDPATH; BOLIN, 1997; NEILL; RIDPATH, 2014), Austrália (BECHER; ORLICH; THIEL, 1998), e em suínos no Japão (KAWANISHI et al., 2014). O BDV ainda não foi detectado no Brasil, apesar de um relato de ovino com sinais neurológicos e positivo no teste de imunohistoquímica usando o anticorpo monoclonal 15C5, amplamente utilizado na detecção de pestivírus (PESCADOR et al., 2004). Cabe ressaltar que os trabalhos com ovinos no País são escassos, havendo relato somente de pesquisa de anticorpos anti-BVDV-1 (GAETA et al., 2016), sendo este um fator que dificulta um maior conhecimento acerca da situação do BDV.

O BVDV tem sido descrito no Brasil desde o final dos anos 1960 (CORREA; NETO; BARROS, 1968). Relatos têm demonstrado a ampla distribuição da infecção nos rebanhos brasileiros, com índices de soropositividade variando entre 43 e 90% dos rebanhos (CANAL et al., 1998; POLETTO et al., 2004; THOMPSON et al., 2006; QUINCOZES et al., 2007; ALMEIDA et al., 2013). Quanto às espécies de pestivírus detectadas em ruminantes no País, o BVDV-1, BVDV-2 e vírus ‘HoBi’-*like* são frequentemente relatados (CANAL et al., 1998; FLORES et al., 2000, 2002; STALDER et al., 2005; CORTEZ et al., 2006; BIANCHI et al., 2011; WEBER et al., 2013, 2016b; OTONEL et al., 2014; SILVEIRA et al., 2015).

O CSFV já foi erradicado de países como Estados Unidos, Canadá, Austrália e Nova Zelândia. Na Europa, muitos países conseguiram a erradicação, contudo, apresentam

casos esporádicos oriundos de suídeos silvestres. A infecção pelo CSFV tem permanecido endêmica em vários países da América Central e do Sul, embora a vacinação sistemática tenha reduzido drasticamente a sua ocorrência nas últimas décadas. No Brasil, a infecção era endêmica em várias regiões até a década de 1980. Programas oficiais de controle e erradicação que envolveram o uso maciço da vacina viva modificada (cepa chinesa), obtiveram sucesso e reduziram drasticamente a ocorrência da doença. Atualmente, a infecção está em vias de erradicação, e o país pode ser dividido em uma área livre da doença (regiões Sul, Sudeste, Centro-Oeste e parte da região Nordeste) e uma região onde ainda ocorrem focos isolados da doença, porém com baixa densidade suína e sem expressão comercial e industrial (parte da região Nordeste e região Norte) (FREITAS et al., 2007; RIDPATH; BAUERMANN; FLORES, 2012).

As infecções persistentes apresentam grande importância principalmente na epidemiologia dos pestivírus em bovinos, visto que os animais persistentemente infectados (PI) se constituem nos principais reservatórios e fontes de disseminação do vírus (BAKER, 1995; HOUE, 1999; LINDBERG; ALENIUS, 1999). As outras espécies animais também são susceptíveis a infecção persistente, mas esses animais geralmente são menos viáveis e acabam vivendo menos tempo que os PIs bovinos (NETTLETON, 1990; BAKER, 1995; MACLACHLAN; DUBOVI, 2011). Os animais PIs excretam o vírus continuamente em altos títulos em secreções e em quantidades um pouco inferiores em excreções (BROCK et al., 1998; LINDBERG; ALENIUS, 1999; ARENHART et al., 2009; BAUERMANN et al., 2014). Durante a infecção aguda, os animais também excretam o vírus, porém em títulos inferiores e por menos tempo, geralmente durante 3 a 10 dias (MACLACHLAN; DUBOVI, 2011; RIDPATH; BAUERMANN; FLORES, 2012). O vírus é transmitido entre animais principalmente por contato direto e indireto (HOUE, 1999; RIDPATH; BAUERMANN; FLORES, 2012), porém também pode ser transmitido de forma iatrogênica (agulhas, material cirúrgico, luvas de palpação, tatuadores, aplicadores de brinco), por sêmen (GIVENS et al., 2003; ALMEIDA et al., 2013) e embriões contaminados (BIELANSKI et al., 2009).

2.3 Patogenia e sinais clínicos

2.3.1 Diarreia viral bovina

A apresentação clínica e a severidade da doença dependem de características importantes, como amostra viral, idade, *status* imunológico e reprodutivo do hospedeiro e infecções concomitantes com outros patógenos (RIDPATH, 2010). Apesar do termo “diarreia” compor o nome da enfermidade (diarreia viral bovina), sinais respiratórios e reprodutivos são mais comumente descritos (MOERMAN et al., 1994; BAKER, 1995; RIDPATH, 2010; MACLACHLAN; DUBOVI, 2011).

A infecção com pestivírus em bovinos pode resultar em duas categorias principais de infecção: infecção aguda de animais não-prenhes e infecção aguda de fêmeas prenhes, que pode levar a formação de animais PI (MACLACHLAN; DUBOVI, 2011; RIDPATH; BAUERMANN; FLORES, 2012).

As infecções agudas de animais não-prenhes geralmente são assintomáticas, mas podem cursar com quadros febris leves, muitas vezes imperceptíveis (BAKER, 1995; RIDPATH; BAUERMANN; FLORES, 2012). Algumas variantes virais de maior virulência podem provocar sialorréia, hiperemia, descarga nasal, tosse, diarreia, lesões ulcerativas na mucosa oral e lesões de pele (MOERMAN et al., 1994; MACLACHLAN; DUBOVI, 2011; SANTOS et al., 2011; RIDPATH; BAUERMANN; FLORES, 2012). Por serem imunodepressores, os pestivírus podem predispor os animais infectados a infecções por outros agentes patogênicos. O período de incubação varia entre 3 e 7 dias e é seguido de hipertermia transitória e leucopenia, com o vírus podendo ser detectado no sangue entre 4 e 6 dias após a infecção e podendo persistir por até 15 dias. O tecido linfóide (tonsilas, linfonodos, tecido linfóide associado a mucosas, placas de Peyer) se constitui em importante sítio de replicação viral. A enfermidade geralmente é autolimitante, cursando com morbidade alta e mortalidade muito baixa ou nula (RIDPATH; BAUERMANN; FLORES, 2012).

As infecções agudas de fêmeas prenhes podem levar a transmissão vertical do vírus, sendo os efeitos dependentes do biótipo, amostra viral e estágio da gestação (FIGURA 4). Quando abortados, os fetos geralmente estão autolizados e exibem poucas lesões características, embora alguns possam mostrar malformações características, tais como hipoplasia cerebelar, lesões cavitárias no córtex cerebral (hidrocefalia ou porencefalia), desmielinização/hipomielinogênese e defeitos esqueléticos. O estabelecimento da infecção persistente ocorre quando o feto é infectado entre os 40 e 120

dias de gestação, com o biótipo ncp. Os fetos infectados nesse período desenvolvem imunotolerância ao vírus e o seu organismo jamais consegue erradicar o vírus. Esses animais geralmente são animais refugo, com desenvolvimento retardado, e tornam-se portadores permanentes que excretam o vírus continuamente em secreções e excreções. A maioria dos bezerros PIs morre durante seu primeiro ou segundo ano de vida, em decorrência de enfermidades induzidas pela imunodepressão causada pelo vírus, ou em decorrência da DM. Os fetos que são infectados após o 125º dia de gestação são considerados imunocompetentes e podem desenvolver uma resposta imunológica que resulta na erradicação do agente (FIGURA 5) (RIDPATH; BAUERMANN; FLORES, 2012).

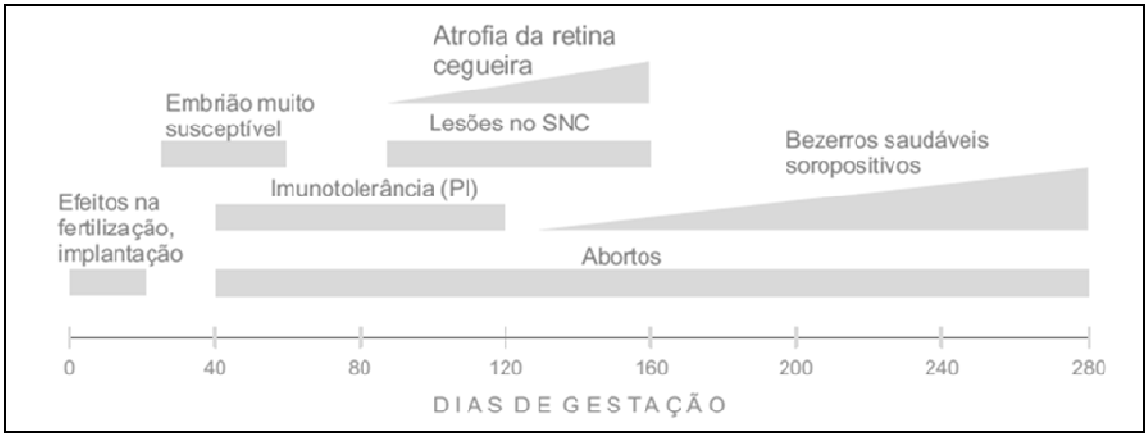

Figura 4. Consequências da infecção de fêmeas bovinas prenhes de acordo com o estágio de gestação. Fonte: RIDPATH; BAUERMANN; FLORES (2012).

A DM ocorre quando o PI portador é superinfectado com o biótipo cp do vírus, de origem exógena ou mesmo derivado do biótipo ncp do próprio animal (MEYERS et al., 1992; QI et al., 1992; TAUTZ et al., 1994; KUPFERMANN et al., 1996; MENDEZ et al., 1998; KUMMERER; MEYERS, 2000; PETERHANS et al., 2010; BECHER; TAUTZ, 2011; DARWEESH et al., 2015). A principal forma dessa variação decorre do fato da proteína NS2-3, que é expressa como um único polipeptídeo em uma cepa ncp, ser clivada em proteínas individuais nas cepas cp (POCOCK et al., 1987). Cabe ressaltar que há descrição de inserções em outras regiões do genoma de pestivírus, que não a NS2-3 (MÜLLER et al., 2003; BECHER; TAUTZ, 2011).A DM leva a letalidade próxima de 100%, ocorrendo mais frequentemente entre os seis meses a dois anos de idade. Caracteriza-se por febre, leucopenia, diarreia hemorrágica, inapetência, desidratação, lesões erosivas nas narinas e na boca e morte dentro de poucos dias. Na necropsia, erosões

e ulcerações podem ser encontradas no trato gastrintestinal, particularmente nas placas de Peyer. No esôfago, essas lesões apresentam-se no sentido longitudinal, com aspecto de “arranhão de gato”. As placas de Peyer apresentam-se edematosas, hemorrágicas e necróticas. O conteúdo intestinal é escuro e aquoso e observa-se enterite catarral ou hemorrágica (RIDPATH; BAUERMANN; FLORES, 2012).

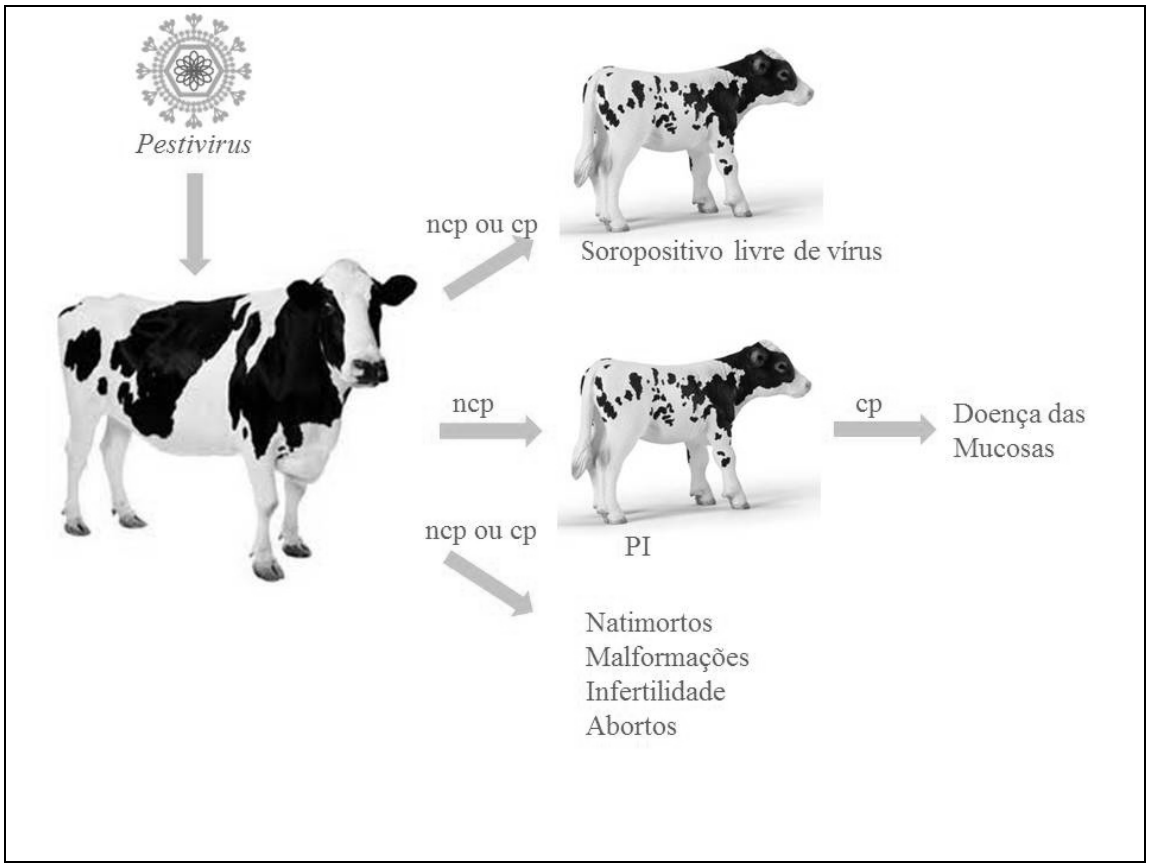

Figura 5. Consequências da infecção de fêmeas bovinas prenhes de acordo com o biótipo do vírus. Adaptado de RIDPATH; BAUERMANN; FLORES (2012).

2.3.2 Doença da fronteira

A doença da fronteira é uma doença reprodutiva de ovinos causada pelo BDV. A infecção de ovelhas não prenhes é geralmente subclínica, mas pode cursar com febre e leucopenia transitória. Em ovelhas prenhes, o vírus é capaz de atravessar a barreira transplacentária e infectar o feto, resultando em abortamentos, nascimento de cordeiros PIs, além de malformações congênitas (RIDPATH; BAUERMANN; FLORES, 2012). Quando a infecção ocorrer após os 80 dias de gestação, pode ocorrer o nascimento de cordeiros PIs, que possuem cobertura escassa e anormal de lã, geralmente pequenos, fracos e com graus variáveis de tremor, chamados de *hairy shakers*. Similarmente ao BVDV em

bovinos, os cordeiros PIs podem excretar o vírus continuamente. No entanto, sabe-se que cordeiros que nascem PI do BDV apresentam um tempo de vida menor devido a viabilidade reduzida do animal quando comparados aos bezerros PIs do BVDV (MACLACHLAN; DUBOVI, 2011; RIDPATH; BAUERMANN; FLORES, 2012). Quando um cordeiro PI for superinfectado com biótipo cp, pode desenvolver síndrome semelhante a doença das mucosas em bovinos (NEILL; RIDPATH, 2014).

2.3.3 Peste suína clássica

Casos típicos da doença são acompanhados com sintomatologia como febre alta, depressão, anorexia e conjuntivite nos animais afetados. Os sinais aparecem após um período de incubação de 2-4 dias e são seguidos por vômito, diarreia e/ou constipação, pneumonia bacteriana secundária e sinais nervosos como paresia, paralisia, letargia, tremores e convulsões. Lesões na pele, como hiperemia e púrpura no abdômen e orelhas podem ser observadas. Nos rebanhos susceptíveis, os sinais clínicos são vistos inicialmente em poucos animais, e após um tempo médio de 10 dias, até todos os animais do rebanho podem adoecer. A mortalidade no rebanho pode atingir 100% (MOERMAN et al., 1994; MACLACHLAN; DUBOVI, 2011).

Formas não tão graves, subaguda e crônica da doença geralmente possuem um período de incubação maior e um curso prolongado ou intermitente da doença com sintomas de diarreia crônica, dermatite e púrpura, infecções bacterianas secundárias e morte após algumas semanas ou meses. Estas formas da doença geralmente são associadas com cepas de virulência moderada. Suínos infectados com cepas de baixa virulência podem permanecer assintomáticos. Animais imunocompetentes que sobrevivem a infecção aguda, desenvolvem imunidade rapidamente. Contudo, animais com formas crônicas da doença eliminam o vírus via secreção continuamente ou de forma intermitente (MOERMAN et al., 1994; MACLACHLAN; DUBOVI, 2011).

A infecção em animais prenhes com cepas de baixa virulência pode levar a infecção fetal com morte embrionária, abortamento, mumificação fetal ou natimortos. Animais nascidos vivos são PIs, imunologicamente tolerantes e excretam o vírus por toda sua vida (RIDPATH; BAUERMANN; FLORES, 2012).

2.4 Diagnóstico

Como a maioria das cepas virais de pestivírus são de baixa virulência e mesmo as mais virulentas não produzem sintomatologia patognomônica, o diagnóstico clínico é bastante difícil. Deve-se suspeitar de infecção por pestivírus em ruminantes sempre que houver ocorrência de problemas reprodutivos (perdas embrionárias, abortamentos, malformações, nascimento de animais fracos ou morte perinatal). Além disso, casos de doença entérica e respiratória, com componentes hemorrágicos, erosões e ulcerações no trato digestivo também são altamente sugestivos da presença da infecção. Animais jovens fracos, com crescimento retardado e predisposição a outras enfermidades devem ser considerados potencialmente suspeitos de serem PI (BAKER, 1995; MURPHY et al., 1999; MACLACHLAN; DUBOVI, 2011).

O teste de diagnóstico padrão é o isolamento do agente em cultivos celulares seguido por identificação de antígenos virais por imunofluorescência ou imunoperoxidase, pois a grande maioria das amostras são ncp. Por ser um teste laborioso e demorado, não é indicado para análise de uma grande quantidade de amostras (SANDVIK, 2005; BRUM; WEIBLEN, 2012).

A imunohistoquímica é útil para a detecção de antígenos em tecidos (fetos abortados, placentomas, fragmentos de tecidos coletados na necropsia e biópsias de pele), especialmente quando anticorpos monoclonais adequados são utilizados. Os reagentes e procedimentos selecionados devem ter sido inteiramente validados e reações não específicas eliminadas (OIE, 2008). A presença de antígenos virais em estruturas da pele é restrita a infecções persistentes, possibilitando a diferenciação entre animais PI e dos com infecção transitória (GROOMS; KEILEN, 2002).

Os métodos de biologia molecular, principalmente a RT-PCR e RT-qPCR, podem ser adotados com fins diagnósticos, tendo vantagens de detectar pequenas quantidades de vírus e partículas virais não íntegras (BRUM; WEIBLEN, 2012). Por ser altamente conservada, protocolos baseados em amplificação de fragmentos da região 5’UTR têm sido utilizados historicamente na detecção de pestivírus (VILCEK et al., 1994; LETELLIER et al., 1999; BAXI et al., 2006; DECARO et al., 2012c), enquanto que os de amplificação do gene da proteína N^{pro} são mais adequados para estudos de filogenia (VILCEK et al., 1997, 2001).

Com o maior acesso e uso da metagenômica através do sequenciamento de alto desempenho (NGS), geralmente não utilizada como diagnóstico, mas sim pesquisa, a

etiologia de determinadas doenças e novos agentes torna-se possível (METZKER, 2010; DENG et al., 2015c; WU et al., 2015). Cabe ressaltar que seu uso possibilitou a descoberta e detecção do APPV como causador de tremores congênitos em suínos (HAUSE et al., 2015; POSTEL et al., 2016), além da descoberta do RaPestV e NrPV em quirópteros e roedores, respectivamente (WU et al., 2012; FIRTH et al., 2014).

O diagnóstico sorológico geralmente é realizado pela técnica de soroneutralização (SN) ou ensaio imunoenzimático (ELISA) (OIE, 2008). Animais infectados de forma aguda soroconvertem em 10-14 dias após a infecção natural, sendo então, a sorologia pareada uma excelente alternativa de indicação da infecção ativa. Animais PI geralmente não apresentam anticorpos no soro, já que não são capazes de responder imunologicamente ao vírus (RIDPATH; BAUERMANN; FLORES, 2012). Os testes sorológicos são excelentes para utilização em levantamentos epidemiológicos e monitoria de rebanhos (LINDBERG; ALENIUS, 1999), contudo, podem ter seu valor limitado devido ao uso de vacinas (RIDPATH; BAUERMANN; FLORES, 2012).

Os principais antígenos virais envolvidos na resposta imune são as glicoproteínas do envelope E2 e E^{rns} e a proteína não estrutural NS2-3 e, por isso, são usados em testes sorológicos. A E2 é imunoprotetora e estimula a produção de anticorpos neutralizantes como resposta a infecção viral, enquanto que a E^{rns} e a NS2-3 participam da resposta imune (CORAPI; DONIS; DUBOVI, 1990; NEILL, 2012). A NS2-3 é mais conservada antigenicamente, enquanto que a E^{rns} mostra menos diversidade antigênica que a E2 (FULTON et al., 2003; NEILL, 2012).

A SN é uma prova de referência por ser muito específica e facilmente adaptável ao tipo ou subtipo de pestivírus desejado. Apesar disso, detecta somente anticorpos neutralizantes, é demorada, laboriosa e envolve obrigatoriamente o cultivo celular e manipulação de vírus (SANDVIK, 2005; OIE, 2008; BRUM; WEIBLEN, 2012).

Os testes de ELISA para detecção de anticorpos são provas práticas, rápidas e biosseguras (BRUM; WEIBLEN, 2012). Kits comerciais de ELISA que utilizam NS2-3 e E^{rns} já demonstraram capacidade de detectar anticorpos anti-vírus ‘HoBi’-*like* em bovinos (DECARO et al., 2011; BAUERMANN; FLORES; RIDPATH, 2012; LARSKA et al., 2013; WEBER et al., 2016b), apesar de terem sido validados apenas para detecção de anticorpos anti-BVDV-1 e 2.

2.5 Controle

2.5.1 Diarreia viral bovina

O controle da infecção pode ser efetuado com ou sem o uso de vacinas, dependendo do histórico do rebanho, do risco de introdução do agente e de outros fatores epidemiológicos. O controle sem vacinação é indicado para rebanhos fechados, sem o ingresso frequente de animais e, consequentemente, de baixo risco. Esse tipo de controle é também indicado para rebanhos cujos parâmetros reprodutivos e clínicos não registrem eventos sugestivos da infecção. Para evitar a introdução da infecção, deve-se recorrer a medidas básicas de biossegurança e testar todos os animais antes de ingressarem na propriedade. Com esta medida, é possível manter rebanhos livres da infecção, pois a principal forma de introdução da infecção é por meio de animais infectados (RIDPATH; BAUERMANN; FLORES, 2012).

No início da década de 90, países escandinavos implementaram programas de erradicação do BVDV, baseados no não uso de vacinação, monitoramento sorológico e remoção de animais PI (LINDBERG; ALENIUS, 1999). Com base no sucesso inicial desses programas, outros países europeus também iniciaram programas de erradicação do BVDV, e conseguiram eliminar ou diminuir a presença do vírus nas criações (STÅHL; ALENIUS, 2012; GRAHAM et al., 2014; SCHIRRMEIER, 2014). A Suíça baseou-se no mesmo programa, contudo utilizando uma rápida identificação de animais PIs baseada na detecção de antígenos, sem o uso de testes sorológicos, além de rigoroso controle de trânsito animal (PRESI; HEIM, 2010; PRESI et al., 2011). No entanto, a erradicação do vírus pode ser bastante difícil devido às características dos locais, dos tipos de criação, das espécies de hospedeiros e das cepas envolvidas em cada região (MOENNIG; HOUE; LINDBERG, 2005; RIDPATH; BAUERMANN; FLORES, 2012).

O controle com vacinação é indicado para rebanhos com alta rotatividade de animais, rebanhos com sorologia positiva, com histórico de doença clínica ou reprodutiva e com confirmação da presença do BVDV (RIDPATH; BAUERMANN; FLORES, 2012). Sua utilização é recomendada principalmente para a imunização de fêmeas suscetíveis antes da temporada de cobertura, prevenindo a transmissão via transplacentária e consequente geração de animais PI (MOENNIG et al., 2005). A estratégia tem sido bastante utilizada nos Estados Unidos e Canadá, especialmente através do uso de vacinas vivas modificadas (MLV) (RIDPATH, 2012, 2013; GIVENS; NEWCOMER, 2015). Cabe ressaltar que neste tipo de protocolo, há a necessidade de constante atualização acerca das

variantes virais em circulação, que devem constar nas vacinas (MAHONY et al., 2005), visto às diferenças de imunidade cruzada entre diferentes espécies e subtipos de pestivírus (PIZARRO-LUCERO et al., 2006; BACHOFEN et al., 2008; NAGAI et al., 2008; RIDPATH et al., 2010; BIANCHI et al., 2011; BAUERMANN; FLORES; RIDPATH, 2012; BAUERMANN; FALKENBERG; RIDPATH, 2016). Isto pode ser exemplificado pela seleção de BVDV-1b em relação a BVDV-1a, através do uso de MLV contendo BVDV-1a no rebanho bovino americanos nos últimos 25 anos (RIDPATH et al., 2011).

No Brasil, apenas vacinas inativadas contendo BVDV-1a ou BVDV-1a e BVDV-2b são licenciadas, apesar do conhecimento de que vacinas vivas são mais eficientes. Muitas dessas vacinas são polivalentes e contém também antígenos do herpesvírus bovino tipo 1, vírus respiratório sincicial bovino e vírus parainfluenza tipo 3 bovino. Seu uso ainda é incipiente e realizado de forma desigual em diferentes regiões e sistemas de produção (FLORES et al., 2005). Além disso, trabalhos realizados com as vacinas a disposição no País têm demonstrado pouca eficiência destas frente a isolados brasileiros, além da verificação da produção de baixos títulos de anticorpos e ausência de proteção fetal (VOGEL et al., 2002; ANZILIERO et al., 2015). Com isso, as vacinas em utilização no Brasil necessitam ser revisadas, devendo conter as variantes em circulação no País, já descritas em trabalhos prévios (BIANCHI et al., 2011; WEBER et al., 2014a, 2014b; SILVEIRA et al., 2015), além da substituição das vacinas inativadas por formas mais eficientes, como as MLV. Vacinas de subunidade, expressas em diferentes sistemas, também têm sido desenvolvidas e podem ser opção (AGUIRREBURUALDE et al., 2013; PECORA et al., 2015).

2.5.2 Doença da fronteira

Apesar de os cordeiros PI serem menos viáveis que os PI bovinos, o controle também passa pela sua remoção do rebanho. Em alguns países europeus, tanto vacinas atenuadas quanto inativadas contra o BVDV-1 tem sido utilizadas, contudo, essas medidas não tem se mostrado economicamente viáveis (MACLACHLAN; DUBOVI, 2011).

2.5.3 Peste suína clássica

A enfermidade é altamente transmissível e de difícil controle em regiões de alta concentração de criações suínas e também em áreas que possuem populações de suídeos silvestres (LEIFER et al., 2010; ROSSI et al., 2015). A alimentação de suínos com restos

de alimentos permanece sendo um importante fator para a introdução da infecção em áreas livres, pois o agente pode permanecer viável por vários dias em uma variedade de subprodutos suínos (RIDPATH; BAUERMANN; FLORES, 2012).

A vacinação maciça tem sido utilizada em muitos países, reduzindo drasticamente a ocorrência e impacto econômico-sanitário. Ela ainda pode representar uma etapa de transição entre o controle com o seu uso e sem seu uso, visto que localidades que obtém a erradicação do vírus só a utilizam em casos de emergência (MOENNIG; FLOEGEL-NIESMANN; GREISER-WILKE, 2003; RIDPATH; BAUERMANN; FLORES, 2012).

3 ARTIGOS CIENTÍFICOS

Os resultados, bem como os materiais e métodos empregados para a realização dos experimentos que compõem esta tese, serão apresentados a seguir na forma de seis artigos científicos. O material suplementar correspondente a cada trabalho será apresentado na sessão “Anexos” da presente tese.

3.1 Artigo 1: High frequency of bovine viral diarrhea virus type 2 in Southern Brazil

O presente experimento já foi concluído e um artigo científico foi publicado no periódico *Virus Research*. O artigo científico será apresentado a seguir, tal qual foi publicado.

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High frequency of bovine viral diarrhea virus type 2 in Southern Brazil

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ABSTRACT

Ruminant pestiviruses can infect cattle populations worldwide and cause significant economic losses due to their impact on productivity and health. Knowledge of pestivirus diversity is important for control programs and vaccine development and for determining probable sources of infection. In this paper, we describe a search for ruminant pestiviruses with RT-PCR in sera of 9078 calves from 6 to 12 months of age. The calves were first analyzed in pools and then analyzed individually. Thirty-three RT-PCR positive animals were detected (0.36%) from 6.9% (24) of the 346 herds. The sequencing analysis of the 5′ non-coding region and N terminal autoprotease showed the presence of BVDV-1a (15 isolates), -1b (3), -1d (1) and -2b (14), with a higher frequency (42.4%) of BVDV-2 in comparison with other countries. The presence of sheep was significantly associated with BVDV infection. Our results also suggested that a BVDV control program based only on the investigation of cattle would not be successful, especially in regions with farms harboring multiple animal species. This study may also serve as a reference for future control programs in Southern Brazil because it reports the prevalence of cattle with active infections and the genetic background of the circulating strains.

1. Introduction

The genus *Pestivirus* of the family *Flaviviridae* consists of four recognized species: *Bovine viral diarrhea virus 1* (BVDV-1), BVDV-2, *Border disease virus* (BDV) and *Classical swine fever virus* (CSFV) (Simmonds et al., 2011). Moreover, an atypical group of pestiviruses, initially detected in fetal calf serum and putatively named 'HoBi'-like virus (Schirrmeier et al., 2004) has been associated with clinical disease in cattle (Decaro et al., 2011; Weber et al., 2014).

Ruminant pestiviruses (BVDV-1, BVDV-2, BDV and 'HoBi'-like viruses) can cause acute or persistent infections in cattle (MacLachlan and Dubovi, 2011; Weber et al., 2014). Acute infections are generally not apparent; when they are symptomatic, the principal clinical manifestations are mild diarrhea, fever and respiratory signs that are terminated by a vigorous immune response. Persistent infection occurs due to the ability of the virus to cross the placenta and infect non-immunocompetent fetuses, thus generating persistently infected (PI) calves that show retarded growth and excrete variable amounts of virus throughout their lives, spreading the infection in the herd. PI calves usually die during the first two years of life from mucosal disease or due to other diseases, most likely as a consequence of virus-induced immune depression (Baker, 1995; MacLachlan and Dubovi, 2011).

Pestiviruses have a single-stranded positive-sense RNA genome that contains one open reading frame, flanked by non-coding regions (NCR) at the 5′ and 3′ ends, that encodes a polyprotein that is processed into 12 polypeptides (Simmonds et al., 2011). The 5′NCR and N terminal autoprotease (N^{pro}) are widely used to characterize the genus, species and subtypes of new strains using phylogenetic approaches (Vilcek et al., 2001; Mahony et al., 2005; Pizarro-Lucero et al., 2006; Xue et al., 2010; Deng et al., 2012; Strong et al., 2013). In addition, they can be used to divide BVDV-1 into at least 17 subtypes (1a through 1q) (Vilcek et al., 2001; Stalder et al., 2005; Deng et al., 2012) and BVDV-2 into two subtypes (2a and 2b) (Flores et al., 2002). Knowledge of the circulating genetic variants in the genus *Pestivirus* has significance for establishing correct diagnostic tools and control programs because there are reports of the failure of commonly used detection techniques at the species level (Schirrmeier et al., 2004; Weber et al., 2014) and because significant antigenic changes at the species and subtype levels have been

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shown by cross-neutralization (Pizarro-Lucero et al., 2006; Ridpath et al., 2010).

A few studies have investigated BVDV risk factors (Valle et al., 1999; Presi et al., 2011; Machado et al., 2014). The principal risk factors detected to date are related to biosecurity measures (Humphry et al., 2012), reproduction management (Houe, 1999; Humphry et al., 2012), herd size (Presi et al., 2011), animal introduction (Houe, 1999; Valle et al., 1999; Presi et al., 2011), direct contact with other animals (from the same species or not) (Lindberg and Alenius, 1999; Valle et al., 1999), communal grazing (Valle et al., 1999; Presi et al., 2011), or the age of the animals (Presi et al., 2011).

The analysis of pooled sera allows the simultaneous testing of a large number of samples and has been proposed as a rapid and cost-efficient approach for the detection of pestivirus in cattle (Weinstock et al., 2001; Hanon et al., 2012). Thus, the goal of the present study was to investigate the herd prevalence of active infections by pestivirus in calves up to one year old from farms located in regions with high livestock movement in Southern Brazil, to determine the genetic diversity of the current viruses and to assess the risk factors for infection based on RT-PCR results at the herd level.

2. Materials and methods

2.1. Study area, target population and sample size

Located in Brazil's Southern region, the state of Rio Grande do Sul (RS) has an area of 268,781.896 km^2 (3.16% of the country) and is bordered by Argentina and Uruguay. It is divided into seven regions. The regions are subdivisions of Brazilian states and group together various counties by proximity and according to common agro-ecological characteristics (Fig. 1). According to official data from the State Veterinary Office (SEAPA-RS), the State has more than 13 million cattle distributed on approximately 346 thousand farms. The majority of the bovine population consists of females whose age is greater than 36 months. The predominant activity of the farms extends from breeding to fattening. Beef cattle are predominant, although these characteristics change depending on the region of the State. Statistics on the number of cattle per property show that 88% of the farms have up to 50 bovines and that approximately 1% of the properties have more than 500 animals.

The sera analyzed in this study were collected for the biannual surveillance study performed in Brazil in 2010 to demonstrate the absence of *Foot-and-mouth disease virus* (FMDV) circulation. The target population of the present study included all bovine farms that harbor FMDV-susceptible species enrolled in the national FMDV control and eradication program (http://www.agricultura.gov.br/arq_editor/file/Serological_Monitoring_efficiency_vaccination_FMDfreezone_2010_final_report_2.pdf). The units addressed by sampling were bovines from six to 12 months of age grouped on farms, which were considered the primary units for sampling purposes. The population from which the sample was randomly drawn comprised farms located in counties with high cattle movement according to State Veterinary Office data on livestock movement. To define the sampling process, 93 counties were identified. A frame list containing the farm's identification was provided and a representative sample obtained from each of the identified counties. Subsequently, 346 farms and 9078 cattle aged from 6 to 12 months were randomly sampled.

The sample size needed to detect the disease was calculated. This procedure was performed by the Ministry of Agriculture, Livestock and Food Supply-Epidemiology Division using FreeCalc version 2 (http://www.ausvet.com.au/content.php?page=software#freecalc), recommended by OIE. The methodology applied was that recommended by Cannon and Roe (Cannon and Roe, 1982) and Martin and others (Martin et al., 1992). The statistical and epidemiological parameters applied to determine the sample size were as follows: confidence level, 95%; minimal prevalence detected on affected farms, 1%; and minimal prevalence detected in affected animals on each farm, 10%.

Table 1
Nucleotide sequence of the primers used for RT-PCR.

Primer	Sequence (5′–3′)	Position
PanPesti F	GAG ATG CYA YGT GGA CGA GGG C	227–248[a]
PanPesti R	GYC TCT GCS RCA CCC TAT CAG G	345–324[a]
LV Pesti F	CTG TAC ATG GCA CAT GGA GTT G	373–394[b]
LV Pesti R	AAT CTG TTG TAT ACC CAT TT	861–842[b]

[a] Position in BVDV-1 strain NADL (GenBank accession number: NC_001461.1).
[b] Position in BVDV-2 strain 890 (GenBank accession number: U18059.1).

2.2. Primer design and validation of the RT-PCR

For primer design, 1412 pestivirus sequences of 5′-NCR in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) with sizes ranging between 118 and 424 nucleotides were selected. The sequence alignment was performed using Muscle version 3.8.31, and Genedoc software version 2.7.001 (http://www.genedoc.us) was used to identify the most conserved regions. The primers resulted in an amplification product of 118 bp (Table 1).

The selected primers were used to test representative samples of pestivirus: BVDV-1 (NADL, Singer, Oregon C24V and Osloss), BVDV-2 (Soldan and SV260) BDV (137/4 and BD Weybridge) and 'HoBi'-like viruses (LV01/12, LV02/12, LV03/12 and LV04/12). Furthermore, the bovine herpesvirus type 1 (BoHV-1) strain Los Angeles and BoHV-5 strain EVI88 were tested to assess specificity.

The detection limit was calculated in triplicate by spiking a negative serum with 10-fold dilutions of BVDV-1 strain NADL ($10^{6.8}$ $TCID_{50}$/mL) to reach a dilution of 10^{-8}.

The validation of the test (sensitivity, specificity, predictive values and accuracy) was assessed by comparing the results from the set of primers used in the present study with the results from the classical pair of primers 324 and 326 (Vilcek et al., 1994).

2.3. Sample preparation, RNA isolation and RT-PCR

The blood collected was centrifuged at 2000 × *g* for 10 min, and the sera were stored at −80 °C prior to analysis. To obtain the pools, equal volumes of the 9078 individual sera (30 μL) were mixed in 227 pools of up to 44 samples. The positive pools resulting from this stage of the analysis were further analyzed individually.

Viral RNA was isolated from 250 μL of sample using TRIzol® LS Reagent (Life Technologies, Carlsbad, CA, USA) and was suspended in 50 μL of ultrapure water according to the manufacturer's instructions. The cDNA was synthesized with SuperScript® III Reverse Transcriptase Kit (Life Technologies) using the reverse primer in a total volume of 20 μL following the manufacturer's recommendations.

The amplification of cDNA by PCR was conducted in a total volume of 25 μL containing 1× PCR buffer, 1 mM of $MgCl_2$, 0.5 mM of dNTP mix, 0.24 mM of PanPesti F and PanPesti R and 1 unit of Platinum® Taq DNA Polymerase (Life Technologies). Reactions were performed in a Veriti 60-well Thermal Cycler (Applied Biosystems, Foster City, USA) under the following conditions: 3 min at 95 °C, followed by 35 cycles of 45 s at 95 °C, 45 s at 60 °C and 45 s at 72 °C, with a final extension at 72 °C for 7 min.

2.4. Sequencing and phylogenetic analysis

The amplification of partial sequences of 5′-NCR for sequencing was performed using the primers 324 and 326 (position in BVDV-1

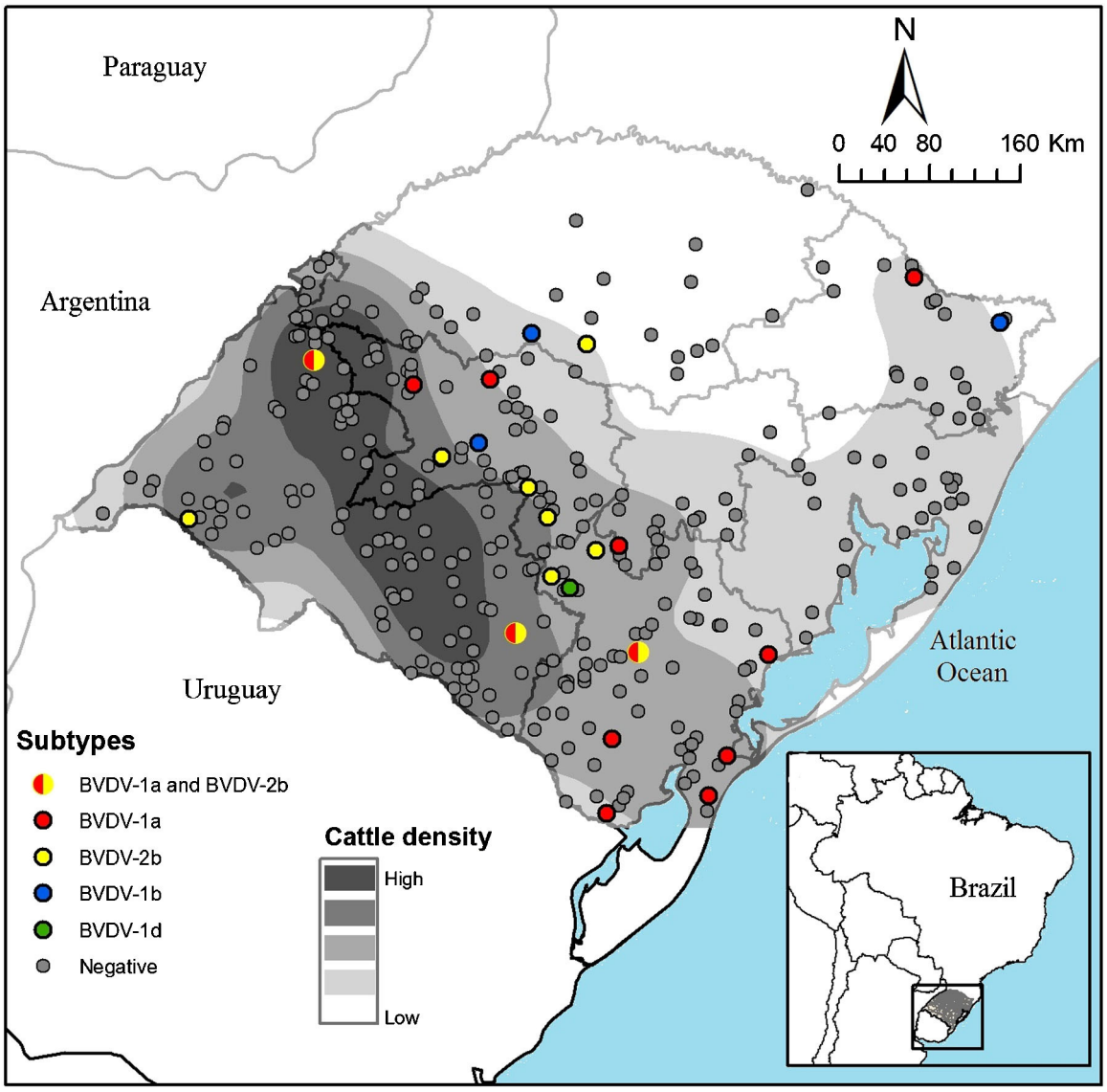

Fig. 1. Spatial distribution of the sampled farms investigated for the presence of ruminant pestiviruses in Rio Grande do Sul state, South Brazil. The cattle density and pestivirus species and subtypes are shown on the map.

strain NADL: 108–128 and 395–375) (Vilcek et al., 1994). One sample (LV20-85/13) did not amplify using this protocol, and the protocol described by Deregt et al. (2006) was utilized (position in BVDV-1 strain NADL: 107–127 and 387–368). Partial sequences of N^{pro} were amplified using primers BD1 and BD3 for BVDV-1 (position in BVDV-1 strain NADL: 367–388 and 795–771) (Vilcek et al., 2001) and LV Pesti F and LV Pesti R for BVDV-2 (Table 1). The PCR amplification products were purified using the NucleoSpin Extract II Kit (Macherey-Nagel, Düren, Germany), and both strands were sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) using a BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems).

The partial 5′-NCR and N^{pro} sequences of the present study were assembled using SeqMan (DNASTAR Lasergene® 11, Madison, USA). Sequences of 36 pestiviruses, including reference and representative strains within species and subtypes, were retrieved from GenBank® (http://www.ncbi.nlm.nih.gov/genbank/) and aligned with BioEdit version 7.1.3 software using CLUSTAL W. Molecular Evolutionary Genetics Analysis version 6 (MEGA6) (Tamura et al., 2013) was used for phylogeny inference according to the neighbor-joining criterion and the Kimura 2-parameter model. The robustness of the hypothesis was tested with 1000 non-parametric bootstrap analyses. The sequence data from the present study were deposited in GenBank® under accession numbers KM007106–KM007130 and KM217386–KM217409. The subtypes from the BVDV-1 isolates were determined according to Vilcek et al. (2001); those from the BVDV-2 isolates were determined according to Flores et al. (2002).

2.5. Questionnaire and interview

The questionnaire designed to gather information about potential risk factors associated with the occurrence of BVDV infection in the studied herds is summarized in Table 2. The structured questionnaire had ten “closed-ended” questions used to identify the risk of FMDV in the state of RS. The questionnaire was previously tested on nonparticipating farmers to identify potential sources of misinterpretation and to further refine the questions. Farm owners/managers were interviewed face to face to complete the questionnaire and to facilitate blood sampling from the herds.

Table 2
Questionnaire designed to gather information about potential risk factors associated with the occurrence of BVDV infection in the studied herds.

Subject	Factor/variables
General data on the farm	Mean age of the herd animals: based on the sampled animals. Total farm area: number of hectares of the property. Pasture area: number of hectares used for animal pasture.
Stock in the herds	Total number of animals: number of bovines and buffalo. Presence of buffalo: sharing pasture between bovines and buffalo. Presence of sheep: sharing pasture between bovines and sheep. Number of goats on the farm. Number of pigs on the farm.

2.6. Data analysis

A multivariable logistic regression was applied to identify risk factors for the presence of actively infected herds according to the RT-PCR.

All variables collected by the questionnaire were tested for sufficient variability to ensure a biologically plausible basis for the outcomes. The distribution of continuous variables was tested by calculating the histogram, mean, standard deviation and range. Frequency distributions and bar charts were used to examine categorical variables. All analyses were performed in R software v.2.15.2 (http://www.r-project.org/).

Explanatory variables were first screened based on the response rate and the frequency of the responses. Variables with large amounts of missing data (>10%) and limited variability (<20%) were not included in the analysis ($n = 10$). The remaining variables were entered individually into a univariable logistic regression model (Table 3) and selected for inclusion in the multivariable model if $P < 0.35$ (Table 4). Subsequently, all the screened variables were subjected to a correlation analysis. The criterion for non-collinear pairs of variables was a correlation <0.7. Interactions between all pairwise variables suitable for the final model were examined and, if significant ($P < 0.05$), were submitted to further analysis. Subsequently, the selected variables ($n = 5$) were included in the multivariable model (mean age of the herd animals, presence of buffalo, presence of sheep, number of goats in the herd, number of swine in the herd). Multivariable models were constructed using a manual forward method; each remaining variable was added to the best previous model, selected according to the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC). Finally, a backward elimination step was used, resulting in a final model in which only variables with $P < 0.05$ were retained.

Table 4
Multivariate analysis of risk factors for BVDV active infection by RT-PCR in cattle from Rio Grande do Sul, Brazil.

Variables	Estimate (β)	*P*-value	PR (95% CI)
Intercept	5.10	0.06	–
Confounder			
Mean age of the herd animals	−0.24	0.29	0.77 (0.52–1.15)
Risk factor			
Presence of sheep			
Yes	2.04	0.04	7.69 (1.41–41.74)
No	–	–	–

Hosmer test, $P = 0.89$.

Confounding effects were investigated by checking changes in the point estimates of the variables that were kept in the model. Changes in parameter estimates >25% were considered to indicate the presence of a confounding factor. The goodness-of-fit of the final model was evaluated with a Hosmer–Lemeshow test (Dohoo et al., 2009).

3. Results

3.1. Risk factor for BVDV based on RT-PCR results

In the univariate analysis, the number of water buffalo, sheep, goats and pigs in the farm were selected for inclusion in the multivariable model ($P < 0.35$) (Table 3). In the multivariable model, the mean age of the herd animals was found to be a confounding factor, and it was controlled during the whole regression analysis, finally the variable selected according to the criteria used for model building was the presence of sheep on the farm, which was significantly associated ($P < 0.05$) as risk factor for active BVDV infection (PR = 7.69; 95% CI: 1.41–41.74) (Table 4).

3.2. Validation of the test

The protocol with the selected primers could successfully detect all the representative strains of BVDV-1, BVDV-2, BDV and 'HoBi'-like viruses tested. The BoHV-1 strain Los Angeles and BoHV-5 strain EVI88 were not detected.

The detection limit was calculated by spiking a negative serum with 10-fold dilutions of BVDV-1 strain NADL. Positive results were obtained until a dilution of 10^{-7} (1.2 $TCID_{50}$/mL) was reached.

The test was validated by comparing the results obtained from the analysis of 227 pools of bovine sera with the RT-PCR protocol developed in the present study with the commonly used protocol described by Vilcek et al. (1994) using primers 324 and 326. The sensitivity (100%), specificity (99.5%), accuracy (99.6%), positive predictive value (96.3%) and negative predictive value (100%) of the evaluated RT-PCR methods were adequate. One

Table 3
Univariate analysis of risk factors for BVDV active infection by RT-PCR in cattle from Rio Grande do Sul, Brazil.

Variables	No. of herds	Frequency (%)/median	*P*-value	PR (95% CI)
Mean age of the herd animals	346	12	0.35	0.80 (0.54–1.18)
Total farm area	346	79.5	0.63	0.90 (0.99–1.00)
Pasture area	346	53	0.94	0.99 (0.99–1.00)
Total number of animals	346	140.5	0.68	0.99 (0.99–1.00)
Presence of buffalo	346		0.30	
Yes		90		2.89 (0.52–15.85)
No		10		–
Presence of sheep	346		0.03	
Yes		73		9.06 (1.67–48.98)
No		27		–
Number of goats in the herd	346	2	0.10	0.62 (0.39–1.01)
Number of pigs in the herd	346	2	0.26	0.96 (0.90–1.01)

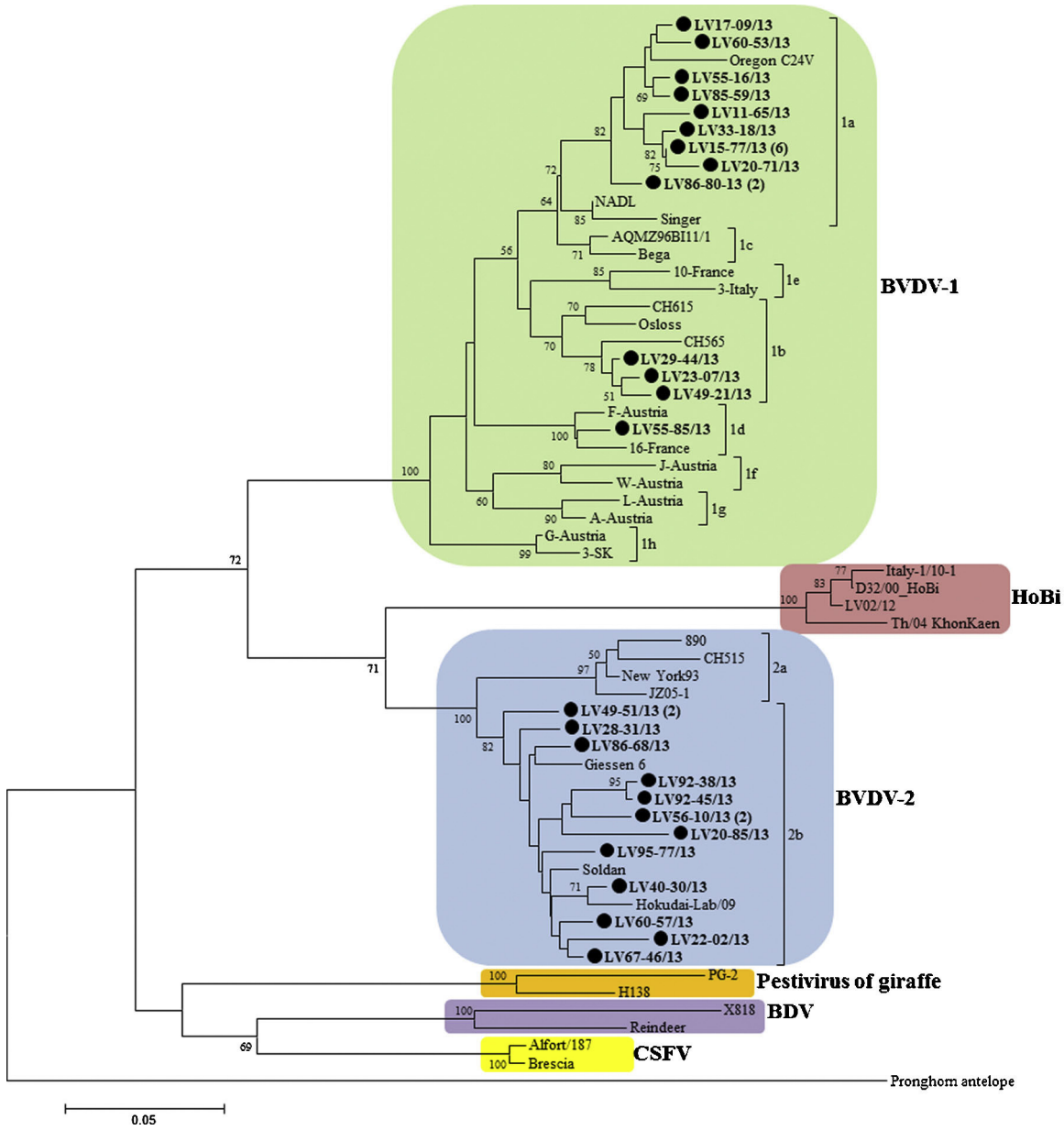

Fig. 2. Rectangular phylogenetic tree of 5′-NCR. Sequences from Brazil and representative and reference pestivirus strains were analyzed by the neighbor-joining method and the Kimura 2-parameter model. Bootstrap (1000 replicates) values >50 are indicated at the internal nodes. Brazilian isolates are highlighted in bold and with a symbol (•). The length of each pair of branches represents the distance between sequence pairs in the rectangular tree. The scale bar represents the percentage of nucleotide differences in the rectangular tree. Genbank accession numbers: BVDV-1a: NADL (AJ133738.1), Singer (L32875.1), Oregon C24V (AF091605.1); BVDV-1b: Osloss (M96687.1), CH615 (AY671982.1), CH565 (AY671981.1); BVDV-1c: Bega (AF049221.2), AQMZ96BI11/1 (AB300674.1); BVDV-1d: 16-France (AF298056.1), F-Austria (AF298065.1); BVDV-1e: 10-France (AF298054.1), 3-Italy (AF298062.1); BVDV-1f: J-Austria (AF298067.1), W-Austria (AF298073.1); BVDV-1g: A-Austria (AF298064.1), L-Austria (AF298069.1); BVDV-1h: G-Austria (AF298066.1), 3-SK (AF298068.1); BVDV-2a: 890 (U18059.1), New York93 (AF502399.1), JZ05-1 (GQ888686.2), CH515 (AY671985.1); BVDV-2b: Giessen 6 (AY379547.1), Soldan (U94914.1), Hokudai-Lab/09 (AB567658.1); HoBi: D32/00_HoBi (AB871953.1) Italy-1/10-1 (HQ231763.1), LV02/12 (KC465389.1), Th/04 KhonKaen (FJ040215.1); BDV: X818 (NC_003679.1), Reindeer (AF144618.2); CSFV: Alfort/187 (X87939.1), Brescia (M31768.1); Pestivirus of giraffe: H138 (NC_003678.1), PG-2 (KJ660072.1); Pronghorn antelope (AY781152.1).

sample was not amplified with the primers 324 and 326 (Vilcek et al., 1994) but was positive with the protocol described in the present study and was further characterized as BVDV-2 after DNA sequencing.

3.3. RT-PCR and genetic typing

The sera from 9078 cattle from six to 12 months of age were analyzed by the RT-PCR described in the present study on a pooled-sample basis using three analysis steps. This screening procedure resulted in 33 positive pestivirus samples, corresponding to a value of 0.36% of active infections in the cattle population from the farms located in the region of high livestock movement. Twenty-four out of 346 herds [6.9% (95% CI: 4.6–9.9%)] presented active infections. Seventeen of the positive herds presented only one positive, and five herds had two positive animals. Three herds contained animals with both BVDV-1 and 2. Positive herds were from 21 counties out of the 93 selected. Fig. 1 shows the location of the farms sampled in the present study as well as the location of the farms with positive animals and the species and subtype of the pestivirus detected. It was found that differences in BVDV prevalence among regions or introduction of virus in herds previously free of BVDV are often

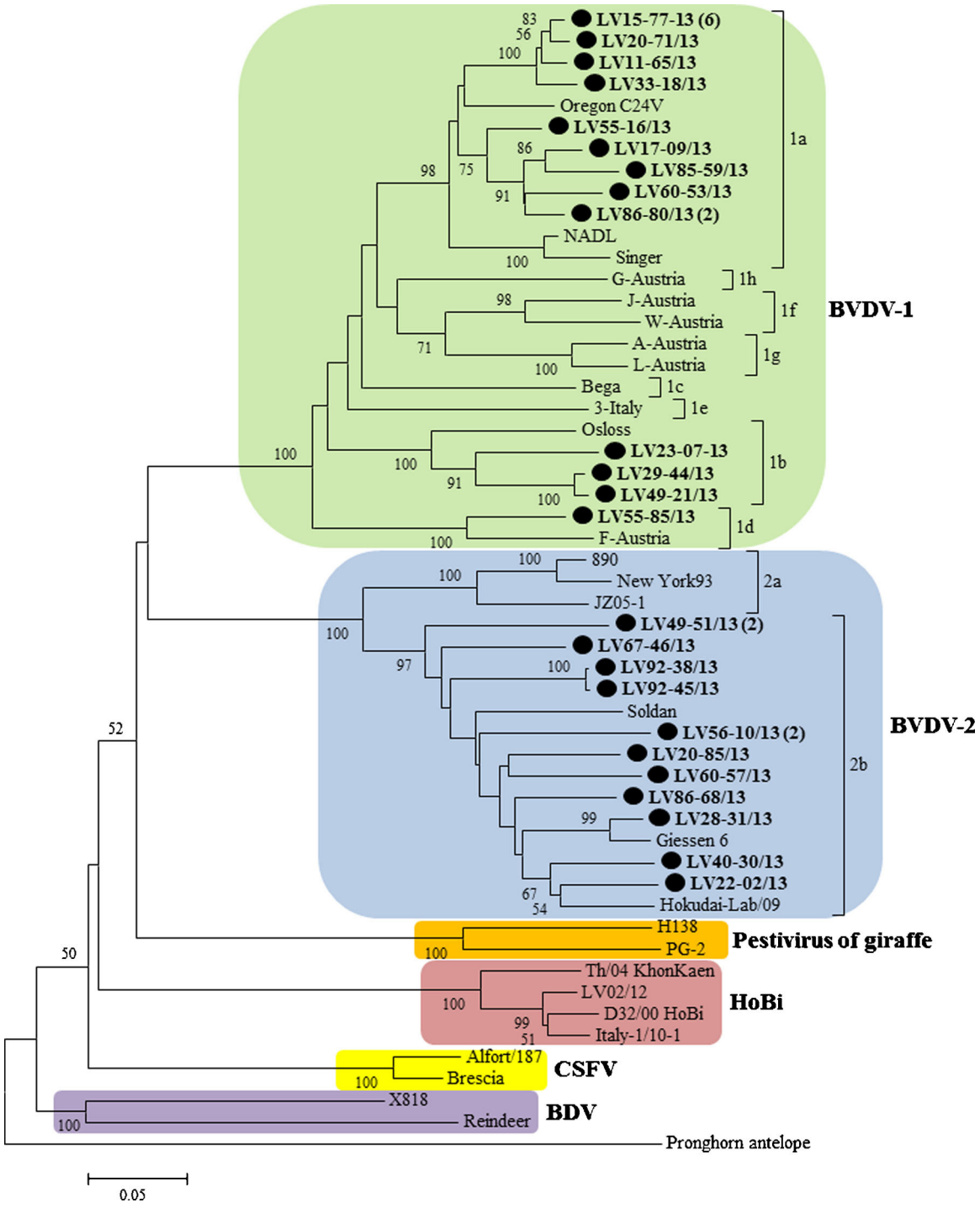

Fig. 3. Rectangular phylogenetic tree of N^{pro}. Sequences from Brazil and representative and reference pestivirus strains were analyzed by the neighbor-joining method and the Kimura 2-parameter model. Bootstrap (1000 replicates) values >50 are indicated at the internal nodes. Brazilian isolates are highlighted in bold and with a symbol (•). The length of each pair of branches represents the distance between sequence pairs in the rectangular tree. The scale bar represents the percentage of nucleotide differences in the rectangular tree. Genbank accession numbers: BVDV-1a: NADL (AJ133738.1), Singer (AF145364.1), Oregon C24V (AF091605.1); BVDV-1b: Osloss (M96687.1); BVDV-1c: Bega (AF049221.2); BVDV-1d: F-Austria (AF287284.1); BVDV-1e: 3-Italy (AF287282.1); BVDV-1f: J-Austria (AF287286.1), W-Austria (AF287290.1); BVDV-1g: A-Austria (AF287283.1), L-Austria (AF287287.1); BVDV-1h: G-Austria (AF287285.1); BVDV-2a: 890 (U18059.1), New York93 (AF502399.1), JZ05-1 (GQ888686.2); BVDV-2b: Giessen 6 (AF144470.1), Soldan (AY735495.1), Hokudai-Lab/09 (AB567658.1); HoBi: D32/00_HoBi (AB871953.1) Italy-1/10-1 (HQ231763.1), LV02/12 (KC465393.1), Th/04 KhonKaen (FJ040215.1); BDV: X818 (NC_003679.1), Reindeer (AF144618.2); CSFV: Alfort/187 (X87939.1), Brescia (M31768.1); Pestivirus of giraffe: H138 (NC_003678.1), PG-2 (KJ660072.1); Pronghorn antelope (AY781152.1).

associated with particular epidemiological determinants such as cattle population density, animal trade and pasturing practices (Houe, 1999).

The nucleotide sequence of the 5′NCR (237 bp including gaps) and N^{pro} (363 bp) was obtained for all 33 pestivirus-positive samples, with 19 (57.6%) demonstrating more similarity with BVDV-1 and 14 (42.4%) with BVDV-2. In the phylogenetic trees (Figs. 2 and 3), the viruses classified as BVDV-1 clustered as subtype 1a (15 samples), 1b (three samples) and 1d (one sample). All the BVDV-2 grouped with subtype 2b strains. The strain LV95-77/13

was not amplified in the N^{pro} fragment with the RT-PCR protocols used. Isolates with 100% identity in 5′-NCR and N^{pro} were detected in more than one animal in four situations: BVDV-1a LV15-77/13 was detected in six different calves from four different farms (two of these four herds contained two animals infected with the same strain), BVDV-1a LV86-80/13 was detected in two calves from the same farm, BVDV-2b LV49-51/13 was detected in two calves from the same farm, and BVDV-2b LV56-10/13 was also detected in two calves from the same farm. This information is shown in Figs. 2 and 3 in parentheses following the sample identification.

The nucleotide similarity of 5′-NCR among Brazilian strains ranged from 88.8 to 100% among the BVDV-1 isolates, 91.2–100% among the BVDV-2 isolates and 74.1–79.6% between BVDV-1 and BVDV-2. The sequence identity of the Brazilian isolates within the same subtypes BVDV-1a (NADL, Singer, Oregon C24V), 1b (Osloss, CH565, CH615), 1d (16-France, F-Austria) and BVDV-2b (Hokudai-Lab/09, Soldan, Giessen 6) was 93.6–100%, 93.9–98.7%, 96.3–97.1% and 91.9–100%, respectively.

The nucleotide similarity of N^{pro} among Brazilian strains ranged from 77.1 to 100% among the BVDV-1 isolates, 82.4–100% among the BVDV-2 isolates and 66.3–68.3% between BVDV-1 and BVDV-2. The sequence identity of the Brazilian isolates within the same subtypes BVDV-1a (NADL, Singer, Oregon C24V), 1b (Osloss), 1d (F-Austria) and BVDV-2b (Hokudai-Lab/09, Soldan, Giessen 6) was 85.1–100%, 86.5–98.9%, 87.8% and 82.9–100%, respectively.

The obtained phylogenetic tree for 5′-NCR (Fig. 2) showed six well-separated clusters corresponding to the known BVDV-1, BVDV-2, CSFV, BDV, 'HoBi'-like viruses and giraffe pestiviruses. Each monophyletic clade was supported by a bootstrap value of 100% at the species level and a bootstrap value ranging between 70 and 100% at the subtype level, thus confirming the robustness of the tree topology. For N^{pro} (Fig. 3), six well-separated clusters corresponding to the BVDV-1, BVDV-2, CSFV, BDV, 'HoBi'-like viruses and giraffe pestiviruses supported by a bootstrap value of 100% at the species level and a bootstrap value ranging between 97 and 100% at the subtype level also confirmed the robustness of the tree topology.

4. Discussion

Testing of pooled sera has been proposed as a rapid and cost-efficient approach for detection of pestivirus in cattle (Weinstock et al., 2001; Hanon et al., 2012). PI animals present titers between 10^2 and 10^6 $TCID_{50}$/mL in their sera (Brock et al., 1998; Arenhart et al., 2009). Accordingly, the protocol described herein had a detection limit of 1.2 $TCID_{50}$/mL and can, theoretically, be used in pools of up to 83 samples without false negative results in PI cattle. However, as viremia in acute infection is transient and can be followed by lower viral titers, it can be difficult to detect or may not be detected depending on the test used (Sandvik, 2005; Hanon et al., 2012). For these reasons, we decided to designate the calves with positive RT-PCR results as actively infected because it is not possible to define them as PI or transiently infected. It is important to reinforce that the detection limit of the RT-PCR protocol described herein was determined using BVDV-1 strain NADL and differences in the detection limit may result when different subtypes and other pestivirus species are present in the sample.

The proportion of individual active infections was 0.36%, with a herd prevalence within high cattle movement regions of 6.64% (95% CI: 4.6–9.9%) in Southern Brazil. Recently, a similar study performed in Belgium detected 2.8% (140 of 4972 animals) with active infections (Hanon et al., 2012). The differences between the two studies may be explained by differences in the prevalence of active infections between the two regions, by the non-random sample collection methodology used in the study in Belgium, population sampled, and by differences in the sensitivity and specificity of the tests used because the results from Belgium were obtained with real-time RT-PCR.

The presence of sheep was significantly associated with active BVDV infection. Such other ruminants are susceptible to BVDV infection and can infect or be infected by cattle, maintaining the infection in the herd because ruminant pestiviruses are not necessarily host specific (MacLachlan and Dubovi, 2011). Contact between susceptible species during communal grazing has been previously associated with BVDV infection (Valle et al., 1999).

The genetic diversity of ruminant pestivirus species and subtypes was analyzed. At the species level, the frequency of BVDV-1 (57.6%) and 2 (42.4%) was similar to that described by Pizarro-Lucero and others (Pizarro-Lucero et al., 2006) from Chile. Apparently, the prevalence of BVDV-2 in South America is higher than that reported from North America (Kim et al., 2009; Ridpath et al., 2010), Europe (Vilcek et al., 2001; Stalder et al., 2005; Strong et al., 2013), Asia (Xue et al., 2010; Deng et al., 2012) and Australia (Mahony et al., 2005; Ridpath et al., 2010). 'HoBi'-like viruses were not detected in the present work, although the protocol described in this paper was able to detect them. Previous reports identified this putative species in cattle (Cortez et al., 2006; Weber et al., 2014) and water buffalo in Brazil (Stalder et al., 2005). The negative results for these viruses in the current study can be explained by a possible low prevalence of this atypical species in Southern Brazil. BDV was also not detected by the present study and has never been reported from South America.

Previous studies have been performed in Brazil to classify pestivirus isolates from bovines into species using phylogenetic analysis (Canal et al., 1998; Cortez et al., 2006), but a segregation into subtypes was not performed. The present study verified the predominance of BVDV-1a (45.4%) and BVDV-2b (42.4%) in addition to BVDV-1b (9.1%) and 1d (3%). One BVDV-1a isolate was detected in four different herds. The reason for this finding could be that the population from which the sample was drawn represents areas with a high level of livestock movement. Such movement can facilitate BVDV transmission between farms. Apparently, the genetic diversity of ruminant pestivirus in Southern Brazil is similar to that reported in individual European countries but is lower in comparison with all countries in Europe (Vilcek et al., 2001; Stalder et al., 2005; Strong et al., 2013) and China (Xue et al., 2010; Deng et al., 2012). Knowledge about circulating pestivirus is significant for establishing correct diagnostic tools and control programs because there are reports of the failure of commonly used molecular detection techniques (Schirrmeier et al., 2004; Weber et al., 2014) and because significant antigenic changes at the species and subtype levels have been shown by cross-neutralization (Pizarro-Lucero et al., 2006; Ridpath et al., 2010).

The present study proposed a rapid and cost-efficient tool for the detection of ruminant pestiviruses in cattle and determined the prevalence of actively infected animals aged from six months to one year in Southern Brazil. The study found that BVDV-1a and BVDV-2b were predominant. Furthermore, a high frequency of BVDV-2 in comparison to other countries was observed. Our results also suggested that a BVDV control program based only on investigations of bovines will not be successful in regions where the farms harbor multiple animal species. This research may also serve as a reference for future control programs because it mapped the presence of active infections and the genetic variability of the strains that circulate in Southern Brazil.

Conflict of interest

None declared.

Acknowledgments

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3.2 Artigo 2: Primeira evidência da infecção pelo vírus da diarreia viral bovina em javalis

O presente experimento já foi concluído e um artigo científico foi publicado no periódico *Acta Scientiae Veterinariae*. O artigo científico será apresentado a seguir, tal qual foi publicado.

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RESEARCH ARTICLE
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Primeira evidência da infecção pelo vírus da diarreia viral bovina em javalis

First Evidence of Bovine Viral Diarrhea Virus Infection in Wild Boars

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ABSTRACT

***Background*:** The farming of wild boars has growing due to the interest of the human consumption of this exotic meat. Such a development may pose an increased risk of disease transmission between boars and domestic animals. The wild boar population has increased in South America in the last years due the absence of predator causing economic losses due to direct damage to crops and risk of disease transmission. The genus *Pestivirus* within the family *Flaviviridae* are composed by four recognized species by the International Committee on the Taxonomy of Viruses (ICTV): classical swine fever virus (CSFV), border disease virus (BDV), bovine viral diarrhea virus type 1 (BVDV-1) and 2 (BVDV-2). Other putative species denoted as atypical pesitiviruses have been reported as 'HoBi'-like virus, giraffe pestivirus, Bungowannah pestivirus, Pronghorn antelope virus, atypical porcine pestivirus (APPV), Norwegian rat pestivirus (NrPV) and *Rhinolophus affinis* bat pestivirus (RaPestV-1). CSFV is commonly detected in wild boars, but despite positive serology, bovine viral diarrhea virus (BVDV) was never detected in this animal species. Thereby, the present communication describes the first detection of BVDV in the lungs of captive boars using RT-PCR and DNA sequencing.

***Materials, Methods & Results*:** Forty lung samples from farmed wild boars were collected after slaughter in a commercial abattoir. The organs were crushed separately, centrifuged, and the supernatant was stored for further analysis. The total RNA was isolated using a phenol-based protocol and RT-PCR protocol that amplified 118 bp of 5' untranslated region (5'UTR) was carried out. One out 40 samples resulted positive. The positive sample had partial fragments of 5'UTR and N terminal autoprotease (N^{pro}) sequenced and analyzed. The strain LV Java/2012 presented 99% of identity in 5'UTR and 98% in N^{pro} region with a BVDV-2 previously reported in bovines in Southern Brazil. In both 5'UTR and N^{pro} phylogenetic analysis, the strain LV Java/2015 clustered with BVDV-2 strains and was most closely related to subtype 2b identified in bovines in Southern Brazil grouping in the same terminal node.

***Discussion*:** Wild boars are commonly associated to pathogen transmission to domestic animals. This animal species is considered a reservoir of the pestivirus CSFV and important keys in CSFV control and eradication programs in Europe. Despite indirect presence of BVDV was reported in wild boars by serology tests, the direct detection of the viral agent was never reported. The present study showed the presence of BVDV-2 genomic segments obtained by RT-PCR followed by DNA sequencing in captive wild boars. The reported data suggests a possible importance of this animal species in the epidemiology of ruminant pestiviruses which could interfere in control and eradication programs of these important pathogens for cattle worldwide. The strain LV Java/2012 was closely related to BVDV-2b and presented highest identity with a strain detected in cattle from Southern Brazil. This data suggests that wild boars and bovines could be sharing this pathogen due the similarity of the strains and that both were reported in the same region. It can lead to need of inclusion of wild swines in BVDV control programs since boars can circulate between different regions and carry this pathogen to different cattle herds. The present study reported the first molecular evidence of BVDV in wild boars in the literature. The data generated herein suggests a possible importance of boars in the epidemiology of ruminant pestiviruses.

Keywords: BVDV, wild boar, pestivirus, RT-PCR.

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INTRODUÇÃO

A criação de javalis (*Sus scrofa scrofa*) em cativeiro tem crescido devido ao interesse humano pelo consumo de carnes exóticas. Além disso, animais de vida livre têm se multiplicado em descontrole no País, devido a fatores como ausência de predador natural, causando danos a lavouras e risco de transmissão de doenças a animais domésticos [3,7,9,12,19].

O gênero *Pestivirus* da família *Flaviviridae* consiste de quatro espécies reconhecidas pelo Comitê Internacional de Taxonomia Viral (ICTV): o vírus da peste suína clássica (CSFV), o vírus da diarreia viral bovina tipo 1 (BVDV-1) e 2 (BVDV-2) e o vírus da doença da fronteira (BDV) [14]. Além das espécies estabelecidas, outros grupos de pestivírus foram identificados e são definidos como pestivírus atípicos, como o vírus 'HoBi' [11], pestivírus de girafa [1], vírus Pronghorn [20], vírus Bungowannah [6], pestivírus atípico de porcinos (APPV) [4], pestivírus de roedores *Rattus novergicus* (NrPV) e pestivírus de morcegos *Rhinolophus affinis* (RaPestV-1) [22].

Suínos (*Sus scrofa domesticus*) são os hospedeiros primários do CSFV, porém também podem ser infectados por outros pestivírus, como o BVDV-1 e 2 [2,8]. A presença desses vírus em criações de suínos gera sintomatologia semelhante à causada pelo CSFV [8,16], tornando necessária a sua diferenciação. Javalis frequentemente estão relacionados à disseminação de CSFV na Europa [7,10,19]. Apesar da demonstração sorológica de BVDV em javalis já ter sido relatada [12], jamais o vírus foi detectado e caracterizado. Com isso, o objetivo do presente estudo é testar amostras de pulmões de javalis cativos obtidas em abatedouro para a presença de pestivírus e caracterizar geneticamente as positivas.

MATERIAIS E MÉTODOS

Amostras

Quarenta amostras de pulmões de javalis foram coletadas após o abate em um abatedouro comercial (Glorinha, RS, Brasil) entre os meses de agosto e outubro de 2012. Os órgãos foram macerados individualmente, suspensos em volume final de 5 mL com PBS pH 7,2, centrifugados a 5.000 *g* durante 15 min e o sobrenadante coletado e estocado a -80ºC.

Extração de RNA e RT-PCR

O RNA total foi extraído de 250 µL de amostra utilizando kit comercial (TRIzol® LS)[1] e eluído em 50 µL de água ultrapura, seguindo recomendações do fabricante. A RT-PCR foi realizada utilizando kit comercial Superscript®One Step RT-PCR with Platinum®Taq[1] e os iniciadores PanPesti F e PanPesti R que amplificam um fragmento esperado de 118 pares de base (pb) da região 5' não-traduzida (5'UTR) [21]. Para tanto, as reações foram preparadas em um volume total de 25 µL contendo 2 µL de RNA total da amostra, 20 pmol de cada iniciador e demais soluções do kit, conforme recomendações do fabricante. A síntese de cDNA foi realizada a 55°C por 30 min. As condições da PCR foram uma desnaturação inicial de 94°C por 3 min seguida de 35 ciclos de desnaturação a 95°C por 45 s, anelamento a 60°C por 45 s e extensão a 72°C por 45 s, e extensão final a a 72°C por 7 min, realizadas em um termociclador automático modelo Veriti®96-well[2]. Na etapa seguinte, os produtos de amplificação foram submetidos à eletroforese em gel de agarose 2,0% com 0,1 µg/mL de Blue Green Loading Dye I[3] e visualização dos produtos de amplificação sob lâmpada ultravioleta.

Sequenciamento e análise filogenética

As amostras positivas foram submetidas a novas reações de RT-PCR para amplificação de fragmento de 288 pb da 5'UTR com os iniciadores 324 e 326 [18] e de 488 pb com os iniciadores LV Pesti F e LV Pesti R [21] nas condições descritas na sessão anterior. Os produtos de amplificação foram purificados utilizando o kit comercial NucleoSpin Extract II Kit[4]. Ambas as fitas foram sequenciadas utilizando-se dos iniciadores diretos e reversos em sequenciador automático (ABI-PRISM 3100 Genetic Analyzer)[2] munido com capilares de 50 cm e polímero POP6[2]. Os DNAs-molde (30 a 60 ng) foram marcados utilizando-se 3,2 pmol do iniciador 5'-NNNNNNNNNNNNN-3' e 2 L do reagente BigDye Terminator v3.1 Cycle Sequencing RR-1002 em um volume final de 10 L. As reações de marcação foram realizadas em termociclador GeneAmp PCR System 97002 com uma etapa de desnaturação inicial a 96ºC por 3 min seguida de 25 ciclos de 96ºC por 10 s, 55ºC por 5 s e 60ºC por 4 min. Após marcadas, as amostras foram purificadas pela precipitação com isopropanol e lavagem com etanol 70%. Os produtos precipitados serão diluídos em 10 µL de formamida, desnaturados a 95ºC por 5 min, resfriados em gelo por 5 min e eletroinjetados no sequenciador automático. Os dados de sequenciamento foram coletados utilizando-se o programa Data Collection v1.0.1[2] com os parâmetros Dye Set

"Z"; Mobility File "DT3100POP6{BDv3}v1.mob"; BioLIMS Project "3100_Project1"; Run Module 1 "StdSeq50_POP6_50cm_cfv_100"; e Analysis Module 1 "BC-3100SR_Seq_FASTA.saz".

As sequências parciais da 5'UTR e N^{pro} foram montadas utilizando o programa SeqMan[5], as sequências dos iniciadores retiradas e a sequências resultantes submetidas à análise pelo *nucleotide* BLAST do GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) para confirmação da identidade. Para análise filogenética, 18 sequências de cepas representativas de pestivírus foram coletadas do GenBank (http://www.ncbi.nlm.nih.gov/genbank/) e alinhadas com CLUSTAL W [17]. As árvores filogenéticas foram construídas utilizando o modelo de inferência *Neighbor-joining* (NJ) com o programa MEGA6 [15], utilizando o modelo estatístico Kimura-2. A robustez da hipótese foi testada em 1000 réplicas.

RESULTADOS

Umas das 40 (2,5%) amostras de pulmão de javalis foram positivas na RT-PCR para detecção de fragmento da 5'UTR [21] utilizada para triagem inicial. A amostra LV Java/2012 teve porções da 5'UTR [18] e N^{pro} [21] amplificadas por RT-PCR e submetidas a sequenciamento de DNA. Na análise por *nucleotide* BLAST, apresentou 99% de identidade na região 5'UTR e 98% na N^{pro} com a cepa de BVDV-2b LV/Patol02/09 (Números de acesso do GenBank: KP715137.1 e KP743045.1, respectivamente), detectada em bovinos no Rio Grande do Sul, Brasil.

A árvore filogenética para 5'UTR (Figura 1A) mostrou seis agrupamentos distintos correspondentes à BVDV-1, BVDV-2, BDV, CSFV, vírus HoBi e pestivírus de girafa. Cada clado monofilético foi suportado por valores de réplicas de 99 e 100%, confirmando a robustez da hipótese. A cepa LV Java/2012 agrupou-se no braço correspondente ao BVDV-2, mostrando maior relação próxima com cepas do subtipo 2b e no mesmo nodo terminal que a cepa LV/Patol02/09 (Número de acesso do GenBank: KP715137.1), suportado por valor de réplica de 99%. Já na árvore para N^{pro} (Figura 1B), ocorreram seis agrupamentos semelhantes aos correspondentes às espécies de pestivírus observados na análise para 5'UTR, suportados por valores de réplicas de 100%. A cepa LV Java/2012 também agrupou-se no braço correspondente ao BVDV-2, mostrando maior relação próxima com cepas do subtipo 2b e no mesmo nodo terminal que a cepa LV/Patol02/09 (Número de acesso do GenBank: KP743045.1).

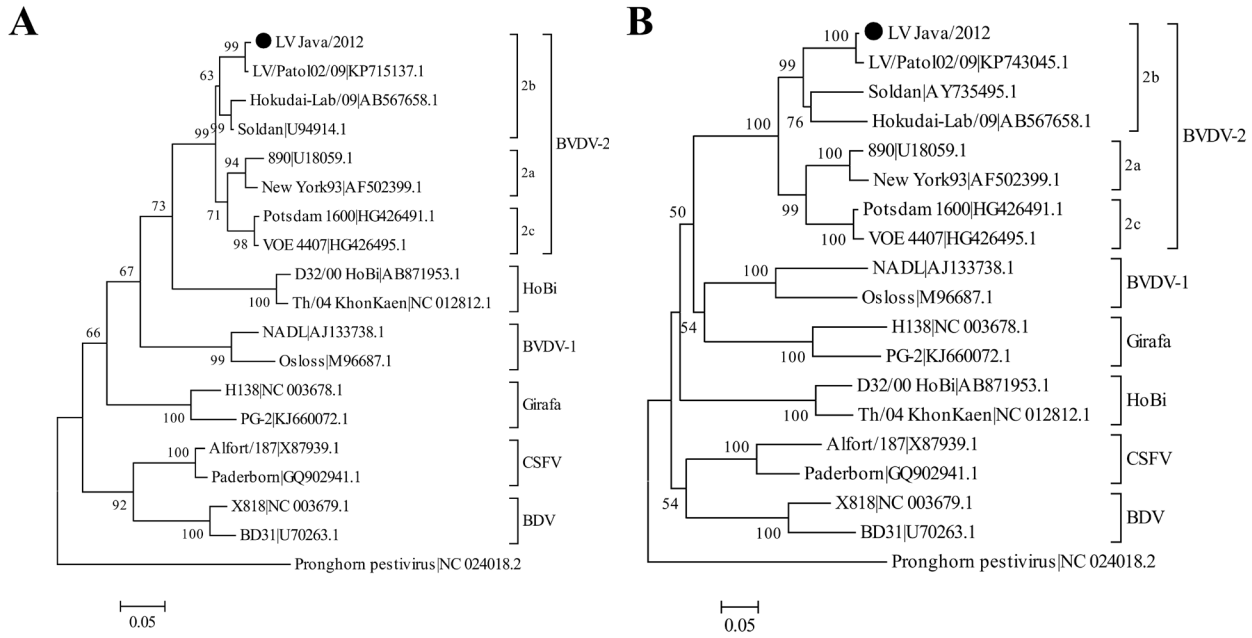

Figura 1. Árvores filogenéticas de pestivírus construídas utilizando o programa MEGA6 representando (A) análise da de porção parcial da 5'UTR e (B) da região N^{pro}. Foram utilizados o modelo de inferência *neighbor-joining* e modelo estatístico Kimura-2 com 1000 réplicas, onde somente os valores de réplicas >50% são representados. A cepa detectada no presente estudo (LV Java/2012) está destacada com o símbolo ●.

DISCUSSÃO

Javalis são comumente associados a transmissão de patógenos a espécies de animais domésticos [3,7,9,12,19], sendo comumente associados como reservatórios do CSFV na Europa [7,19], dificultando programas de controle e erradicação na região [10,19]. Apesar de já relata a sorologia positiva para BVDV em javalis [12], o agente viral jamais foi relatado na espécie na literatura. O presente estudo pesquisou a presença de pestivírus em amostras de javalis cativos obtidos em abatedouro e encontrou a presença de segmentos de genomas BVDV-2. Esses dados podem sugerir uma possível importância da espécie na epidemiologia de pestivírus de ruminantes, podendo também interferir dificultando programas de controle e erradicação desse agente viral que causa grandes prejuízos na bovinocultura ao redor do mundo [5].

O BVDV-1 e BVDV-2 são endêmicos no Brasil, sendo BVDV-1a e BVDV-2b os mais prevalentes em bovinos no Sul do Brasil [13,21]. No presente estudo, foi encontrada uma cepa de pestivírus em javali que demonstrou maior identidade e relação (Figura 1) com cepas de BVDV-2b, especialmente com a cepa LV/Patol 02/09, recentemente relatada no Rio Grande do Sul, Brasil [13]. Estes dados sugerem que possa estar ocorrendo uma possível transmissão de BVDV entre javalis e bovinos, visto a semelhança das cepas e ao fato de ambas terem sido encontradas na mesma região. Este fato pode levar a necessidade de inclusão do controle deste agente viral em suínos selvagens, que circulam livremente e podem levar o BVDV a diferentes regiões e rebanhos, atuando como mais um dispersor do vírus.

O BVDV causa sintomatologia semelhante a cepas de baixa virulência do CSFV em suínos [8,16]. Mais estudos quanto à presença de BVDV e suas consequências em javalis são necessários para averiguar possível patogenicidade do agente viral na espécie.

CONCLUSÃO

O presente estudo relatou a primeira evidência molecular da presença de BVDV em javalis na literatura. Os dados aqui relatados sugerem uma possível importância de suínos selvagens na epidemiologia de pestivírus de ruminantes, devido ao livre trânsito desses animais, que tem tido sua população crescendo na América do Sul nos últimos anos, principalmente devido à ausência de predador dessa espécie exótica na região.

MANUFACTURERS

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3.3 Artigo 3: Homologous recombination in pestiviruses: Identification of three putative novel events between different subtypes/genogroups

O presente experimento já foi concluído e um artigo científico foi publicado no periódico *Infection, Genetics and Evolution*. O artigo científico será apresentado a seguir, tal qual foi publicado.

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Homologous recombination in pestiviruses: Identification of three putative novel events between different subtypes/genogroups

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ABSTRACT

Viruses from the genus *Pestivirus* of the family *Flaviviridae* have a non-segmented, single-stranded RNA genome and can cause diseases in animals from the order Artiodactyla. Homologous recombination is rarely reported in this virus family. To detect possible recombination events, all complete pestivirus genomes that are available in GenBank were screened using distinct algorithms to detect genetic conversions and incongruent phylogenies. Three putative recombinant viruses derived from recombination from different pestivirus subtypes/genogroups were detected: *Bovine viral diarrhea virus 1* (BVDV-1) strain 3156, BVDV-2 strain JZ05-1 and *Classical swine fever virus* (CSFV) strain IND/UK/LAL-290. The present study demonstrated that the pestivirus classification cannot be based only on the analysis of one fragment of the genome because genetic conversions can lead to errors. The designation of the recombinant forms (RF) provides a more informative structure for the nomenclature of the genetic variant. The present work reinforces that homologous recombination occurs in pestivirus populations under natural replication and describes the first evidence of recombination in BVDV-2.

1. Introduction

The viruses of the genus *Pestivirus* belong to the family *Flaviviridae*, together with the genus *Hepacivirus* (*Hepatitis C virus*, HCV) and *Flavivirus* (*Yellow fever virus*, YFV; *West Nile virus*, WNV; *Dengue virus*, DENV; and Japanese encephalitis virus, JEV). Pestiviruses have a single-stranded, positive-sense RNA genome of approximately 12.3 kilobases (kb) that contains only one open reading frame (ORF) that is flanked by non-coding regions (NCRs) at its 5′ and 3′ ends. The ORF encodes a polyprotein that is processed into twelve polypeptides: N terminal protein (N^{pro}), capsid protein (C), envelope glycoprotein (E^{rns}, E1 and E2), protein 7 (p7) and the non-structural proteins (NS) NS2, NS3, NS4A, NS4B, NS5A and NS5B (Simmonds et al., 2011).

Similar to many RNA viruses, pestiviruses exhibit high genetic heterogeneity and can be divided in four species that are recognized by the International Committee on the Taxonomy of Viruses (ICTV): *Bovine viral diarrhea virus 1* (BVDV-1), BVDV-2, *Classical swine fever virus* (CSFV) and *Border disease virus* (BDV) (Simmonds et al., 2011). These viruses also present species variants, with BVDV-1 presenting at least 17 subtypes (1a–q) (Deng et al., 2012; Vilcek et al., 2001), BVDV-2 presenting three subtypes (2a–c) (Flores et al., 2002; Jenckel et al., 2014), BDV presenting seven genotypes (Becher et al., 2003; Giammarioli et al., 2011), and CSFV presenting three genogroups (1, 2 and 3) that can each be divided into three or four subgenogroups (Greiser-Wilke et al., 1998; Lowings et al., 1996; Pan et al., 2005). In addition to the established species, new putative viruses with significant genetic and antigenic differences were already detected, i.e., the pestivirus of giraffe (Avalos-Ramirez et al., 2001), 'HoBi'-like viruses (Schirrmeier et al., 2004), Pronghorn virus (Vilcek et al., 2005), Bungowannah virus (Kirkland et al., 2007) and two other viruses that were detected in small ruminants from Tunisia (Thabti et al., 2005) and Turkey (Oguzoglu et al., 2009).

Heterologous recombination (or non-homologous recombination) in pestiviruses was already reported to generate the cytopathic (cp) biotype that can evolve from non-cytopathic (ncp) viruses and cellular sequences (Becher and Tautz, 2011; Hughes, 2004).

Instead, homologous recombination is an important evolutionary process for many viruses (Simon-Loriere and Holmes, 2011) and was previously identified in *Flaviviridae* members such as DENV (Chen et al., 2008; Tolou et al., 2001; Villabona-Arenas et al., 2013) and HCV (Kalinina et al., 2002; Shi et al., 2012), as well as BVDV-1 (Jones and Weber, 2004) and CSFV (He et al., 2007) from

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the pestivirus genus. Previous studies demonstrated that virus classification cannot be based only on the analysis of one fragment of the genome since genetic conversions can lead to errors and proposed the designation of the recombinant forms (Kalinina et al., 2002; Jones and Weber, 2004). Thus, the goal of the present study was to search, identify and determine the distribution of putative recombination events using several algorithms to detect genetic conversions in all complete pestivirus genomes that are available in GenBank.

2. Materials and methods

The available whole-genome sequences of 125 pestiviruses were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) on July 13th, 2014 (Table A.1). The genome sequences were from strains of recognized species and their subdivisions as well as atypical species. The dataset was aligned using ClustalW and the BioEdit version 7.2.5 software (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

Putative recombination events were verified using the Recombination Detection Program version 4 (RDP4) software (http://web.cbio.uct.ac.za/~darren/rdp.html) with the default settings. The software used several algorithms, including RDP (Martin and Rybicki, 2000), GENECONV (Padidam et al., 1999), BootScan (Martin et al., 2005), MaxChi (Smith, 1992), Chimaera (Posada and Crandall, 2001), SiScan (Gibbs et al., 2000) and 3Seq (Boni et al., 2007). The beginning and end breakpoints of the potential recombinant sequences were also defined by the RDP4 software. Putative recombinant events were considered significant when $P \leqslant 0.01$ was observed for the same event using four or more algorithms. The already-described, recombinant pestivirus BVDV-1 strain ILLNC (GenBank accession number U86600.1) (Jones and Weber, 2004), CSFV strain 39 (GenBank accession number AF407339.1) (He et al., 2007) and CSFV strains ALD (GenBank accession number D49532.1) and SWH (GenBank accession number DQ127910.1) (Ji et al., 2014) were used to analyze the performance of the applied methodology.

Phylogenetic analysis was performed to visualize possible relationships between the putative recombinant strains and other pestiviruses. The beginning and end breakpoints of the potential recombinant strains were used to define the cutoff and to segregate the genomes into three or four segments to perform independent analyses. Molecular Evolutionary Genetics Analysis version 6 (MEGA6) (Tamura et al., 2013) was used for phylogeny inference according to the maximum likelihood algorithm. The nucleotide substitution model was defined by the tool "find best DNA/Protein model (ML)" of MEGA6. The robustness of the hypothesis was tested with 1000 non-parametric bootstrap analyses.

All the sequence alignments used to perform all the analysis for RDP4 and to construct the phylogenetic trees are available in Figshare (http://figshare.com/) with the DOI number http://dx.doi.org/10.6084/m9.figshare.1272825.

3. Results

Three novel putative events were found with *P* values lower than 0.01 (Table 1). The results showed that three pestivirus strains (3156/BVDV-1, JZ05-1/BVDV-2 and IND/UK/LAL-290/CSFV) are potential recombinant viruses derived from parental viruses with different subtypes/genogroups. The possible major and minor parents of the putative recombinants as well as the beginning and end breakpoints were also defined by RDP4 and are shown in Table 1 and Fig. 1. The BVDV-1 strain ILLNC and the CSFV strains 39, ALD and SWH which were analyzed as controls of known recombination events, confirmed the previous findings (He et al., 2007; Ji et al., 2014; Jones and Weber, 2004).

The putative BVDV-1 recombinant strain 3156 (GenBank accession number JN704144.1) had the SD1 (BVDV-1a) strain (GenBank accession number M96751.1) as its major parent and GX4 (BVDV-1b) as its minor parent (GenBank accession number KJ689448.1). In the putative recombination event, the SD1 sequence had replacements of two homologous regions derived from GX4: the first one ending in the glycoprotein E2 gene and the second had its beginning and end breakpoints between the NS2 and NS5A genes (Fig. 1A).

The putative recombinant strain JZ05-1 (GenBank accession number GQ888686.2) had the 11F011 (BVDV-2a) strain (GenBank accession number KC963968.1) as its major parent. However, the RDP4 software could not find the minor parent in the sequences available in Genbank and defined it as unknown. The putative region of recombination between 11F011 and the unknown parent strain was located between the p7 and NS4B genes (Fig. 1B). The fragment of the genome of JZ05-1 that was obtained from the unknown origin (located between the breakpoints) was also submitted to a MegaBlast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) to find a possible minor parent, and the result showed only 89.5% identity with BVDV-2b strain SD1301 (GenBank accession number KJ000672.1).

The putative recombinant strain IND/UK/LAL-290 (GenBank accession number KC851953.1) had the Bergen strain CSFV-2.2 (GenBank accession number KJ619377.1) as its major parent. The RDP4 software could also not define the minor parent in the sequences available in GenBank. The putative recombination region between Bergen and this unknown parent strain was located between the NS3 and the NS5A genes (Fig. 1C). The

Table 1
Putative recombination events in pestiviruses detected using the RDP4 software.

Recombinant		3156 (JN704144.1)	3156 (JN704144.1)	JZ05-1 (GQ888686.2)	IND/UK/LAL-290 (KC851953.1)
Major parent		SD1 (M96751.1)	SD1 (M96751.1)	11F011 (KC963968.1)	Bergen (KJ619377.1)
Minor parent		GX4 (KJ689448.1)	GX4 (KJ689448.1)	Unknown strain	Unknown strain
P-values determined by seven different programs	RDP	5.085×10^{-201}	9.299×10^{-154}	7.195×10^{-17}	7.902×10^{-7}
	GENECONV	4.770×10^{-192}	1.924×10^{-135}	ND	7.093×10^{-3}
	BootScan	1.330×10^{-199}	8.486×10^{-152}	6.248×10^{-16}	1.896×10^{-4}
	MaxChi	7.433×10^{-54}	5.133×10^{-57}	4.824×10^{-16}	1.355×10^{-7}
	Chimaera	8.489×10^{-58}	2.318×10^{-57}	5.358×10^{-15}	1.223×10^{-6}
	Siscan	2.985×10^{-55}	3.079×10^{-78}	1.353×10^{-23}	5.966×10^{-6}
	3Seq	2.684×10^{-303}	6.752×10^{-170}	7.320×10^{-10}	5.445×10^{-4}
Beginning breakpoint (position in alignment)		–	4469 (99% CI: 4436–4490)	3637 (99% CI: 3622–3694)	5995 (99% CI: 5880–6145)
End breakpoint (position in alignment)		2871 (99% CI: 2852–2874)	8715 (99% CI: 8698–8718)	7360 (99% CI: 7342–7372)	7296 (99% CI: 7224–7363)

ND: Recombination not detected with this algorithm.

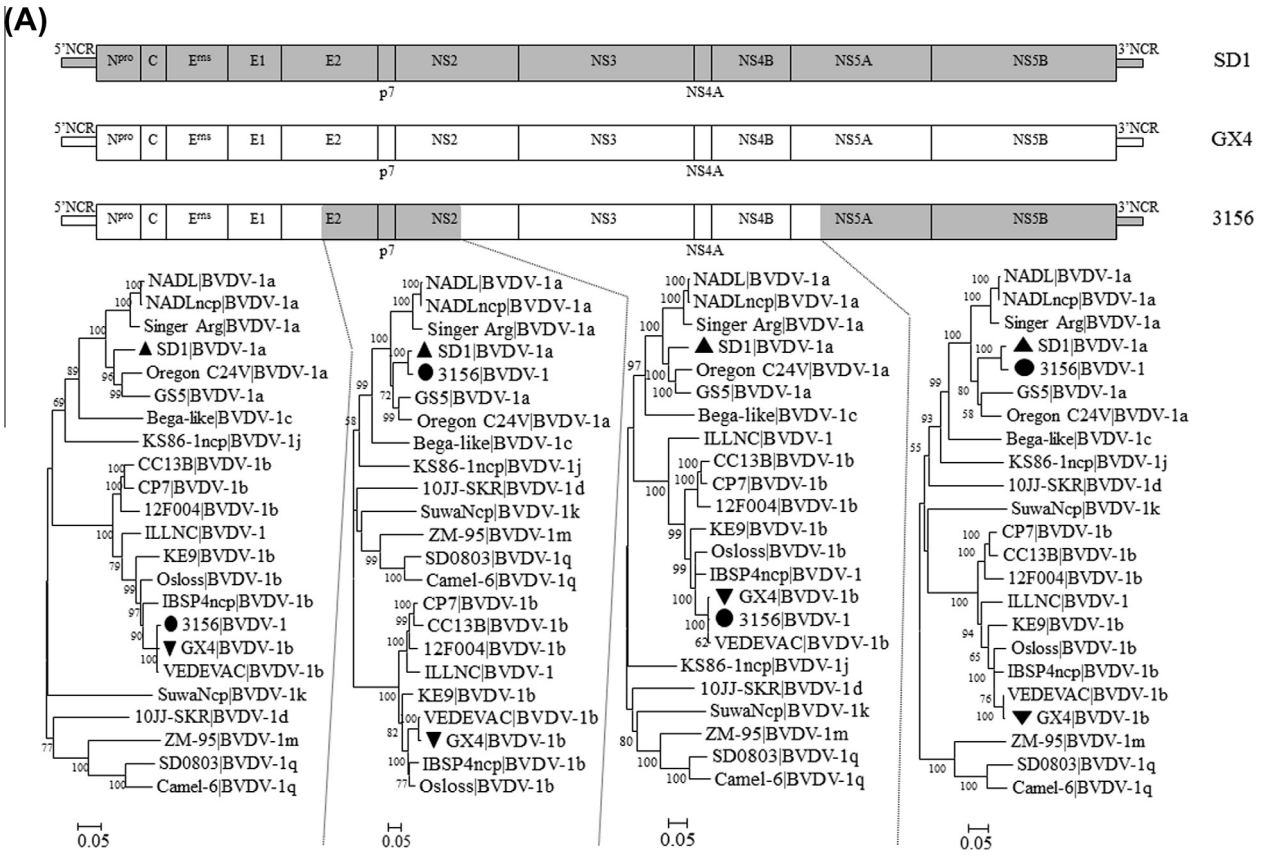

Fig. 1. Schematic representation of the potential recombination events in pestiviruses. The genome organization of the potential recombinant viruses, with the breakpoints and segments of the genome derived from the major and minor parents, are schematically represented. The relationship between the putative recombinant (●) and major (▲) and minor (▼) parents in the different segments of the genome is shown in the maximum likelihood rectangular trees, where bootstrap values ⩾50% are represented for strains 3156 (A), JZ05-1 (B) and IND/UK/LAL-290 (C).

fragment of the genome of IND/UK/LAL-290 with unknown origin (located between the breakpoints) was also submitted to a Mega-Blast search to find a possible minor parent, and this analysis resulted in only 84% identity with the recombinant CSFV strain 39 (GenBank accession number AF407339.1).

To visualize the incongruences in the phylogenetic analysis, ten different estimations from different genomic regions between potential recombinant strains and their putative major and minor parents were constructed. The nucleotide substitution model selected by MEGA6 was General Time Reversible with gamma distributed with invariant sites (GTR + G + I) for all the analysis with exception of the third segment of the BVDV-2 analysis where GTR + G was selected.

The analysis of strain 3156 (BVDV-1) showed that this strain clustered with BVDV-1a strains in two trees (Fig. 1A). In the other two trees, the putative recombinant clustered with BVDV-1b strains. All of these nodes were supported by 100% bootstrap values.

The analysis of strain JZ05-1 (BVDV-2) showed that, in trees constructed using the downstream region of the beginning breakpoint and upstream region of the end breakpoint, this strain clustered in a branch formed by its major parent (11F011) and other BVDV-2a strains (890, C413, p11Q and New York93) (Fig. 1B). These branches were also supported by 100% bootstrap values. However, in the tree constructed with the segment of the genome between the beginning and the end breakpoints, JZ05-1 clustered in the same terminal node as BVDV-2b strains Hokudai-Lab/09 (GenBank accession number: AB567658.1) and SD1301 (GenBank accession number: KJ000672.1), and this result was supported by a 90% bootstrap value. This information led to the conclusion that the other non-recombinant parent is the ancestor of the clade formed by Hokudai-Lab/09 and SD1301.

For CSFV, strain IND/UK/LAL-290 clustered in the branch of CSFV-2.2 (90% bootstrap value) and in the same terminal node as CSFV strain 39, and this result was supported by a bootstrap value of 100%. In the analysis of the region between the breakpoints, strain IND/UK/LAL-290 clustered in a unique branch with strain 39, and this result was supported by a 100% bootstrap value. In the tree constructed with the segment upstream the end breakpoint, IND/UK/LAL-290 clustered in the same branch of the Bergen strain, and this result was supported by a 100% bootstrap value (Fig. 1C). The location of strain 39, however, changed.

4. Discussion

Natural recombination has been described in members of *Flaviviridae*, i.e., *Flavivirus* and *Hepacivirus* (Chen et al., 2008; Kalinina et al., 2002; Shi et al., 2012; Tolou et al., 2001; Villabona-Arenas et al., 2013); however, there are only four reports of natural recombination for the genus *Pestivirus*, i.e., one in BVDV-1 and three for CSFV (He et al., 2007; Ji et al., 2014; Jones and Weber, 2004). In the present report, strong evidence for the occurrence of homologous recombination in BVDV-1 and BVDV-2 from different subtype strains and of CSFV from the same or different genogroups was supported by at least six of the seven algorithms that were used to detect genetic conversion (Table 1) and by the phylogenetic analysis. The data reveals that strain 3156, a BVDV-1 detected in cattle from China in 2011, is a putative recombinant between BVDV-1a and BVDV-1b (Fig. 1). A recombination between BVDV-1a and 1b

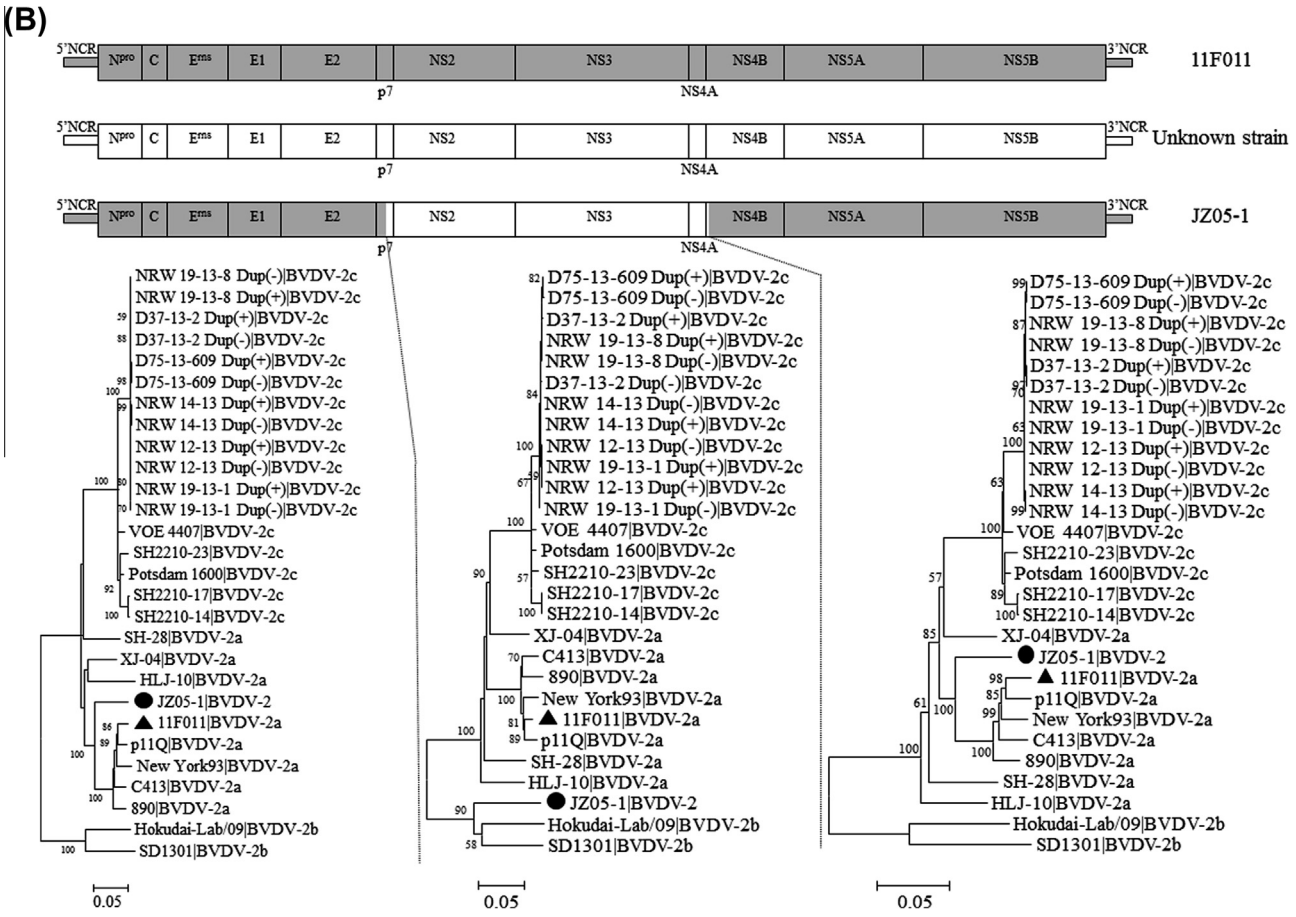

Fig. 1 (*continued*)

was previously reported (Jones and Weber, 2004), and the data reported in the present study reinforces that this phenomena can take place in natural viral populations as an important evolutionary event. Furthermore, it is important to highlight that strain 3156 is deposited in GenBank as a BVDV-1b strain, although this virus can be classified as BVDV-1a or 1b according to the genomic region that is analyzed.

The present data also reveals that strain JZ05-1, a cp virus detected in cattle from China in 2005, is a possible recombinant between the BVDV-2a and BVDV-2b strains (Fig. 1B). However, it can be observed in the maximum likelihood trees that an ancient recombination (strain JZ05-1) also occurred where the exact parental strains cannot be pinpointed, and also a case where it cannot be certain about which are the parental and which are the recombinants. This hypothesis can be better visualized in the phylogenetic trees since JZ05-1 is not located in the same terminal node as the sequences classified as major and minor parents, but apparently emerged in independent terminal nodes in all the trees. This is the first evidence of recombination for BVDV-2.

For CSFV, homologous recombination from different genogroups has been reported (He et al., 2007). The present findings point that strain IND/UK/LAL-290, a CSFV detected in a backyard pig from India, generated by a homologous recombination between a CSFV-2.2 and a CSFV other than from genogroup 2 (Fig. 1C). In two of the maximum likelihood trees it can be observed that IND/UK/LAL-290 is located in the CSFV-2 branch closely related to strain Bergen and in the third one in a branch that has the same ancestor of the CSFV-2 genogroup. Moreover, it evolved independently creating a branch where IND/UK/LAL-290 is closely related to strain 39, another putative recombinant strain (He et al., 2007). It can be deduced that an ancestor of strain 39 participated in the generation of strain IND/UK/LAL-290. Furthermore, this virus was classified as CSFV-2.2 (Kumar et al., 2014), despite the incongruences that could be observed in its phylogeny.

It is important to reinforce that RDP4 define the parental sequences based on pairwise comparisons between the query (putative recombinant) and reference sequences used in the analysis. This means that the putative recombinant and the possible parental sequences are both descendants of an acestor that was possibly better represented by the common node at the phylogeny.

Potential recombination events between different pestivirus species were not found. Interspecies recombination in pestiviruses can be an extremely rare process because gene transfer between different genomes require physical cohabitation of both genomes in the same cell (Simon-Loriere and Holmes, 2011) and viremia lasts at least 10 days and results in a sterile immunity (MacLachlan and Dubovi, 2011). Furthermore, to be able to spread further, the recombinant virus must not only be viable but also have to compete with both parental strains (Simon-Loriere and Holmes, 2011). It is important to reinforce that the homologous recombinations observed in the present work may have emerged artificially, intentionally or not since the study is based on data from a public database and all these putative recombinants are single rather than a group.

The BVDV-1, BVDV-2, CSFV and BDV are classified into variants within their respective species (Becher et al., 2003; Flores et al., 2002; Lowings et al., 1996; Vilcek et al., 2001), but recombinant strains carry incongruences in these classifications because of their evolutionary origin. The present study demonstrated that the pestivirus classification cannot be based only on the analysis of one fragment of the genome because genetic conversions can lead to errors. For example, the designation of the recombinant forms

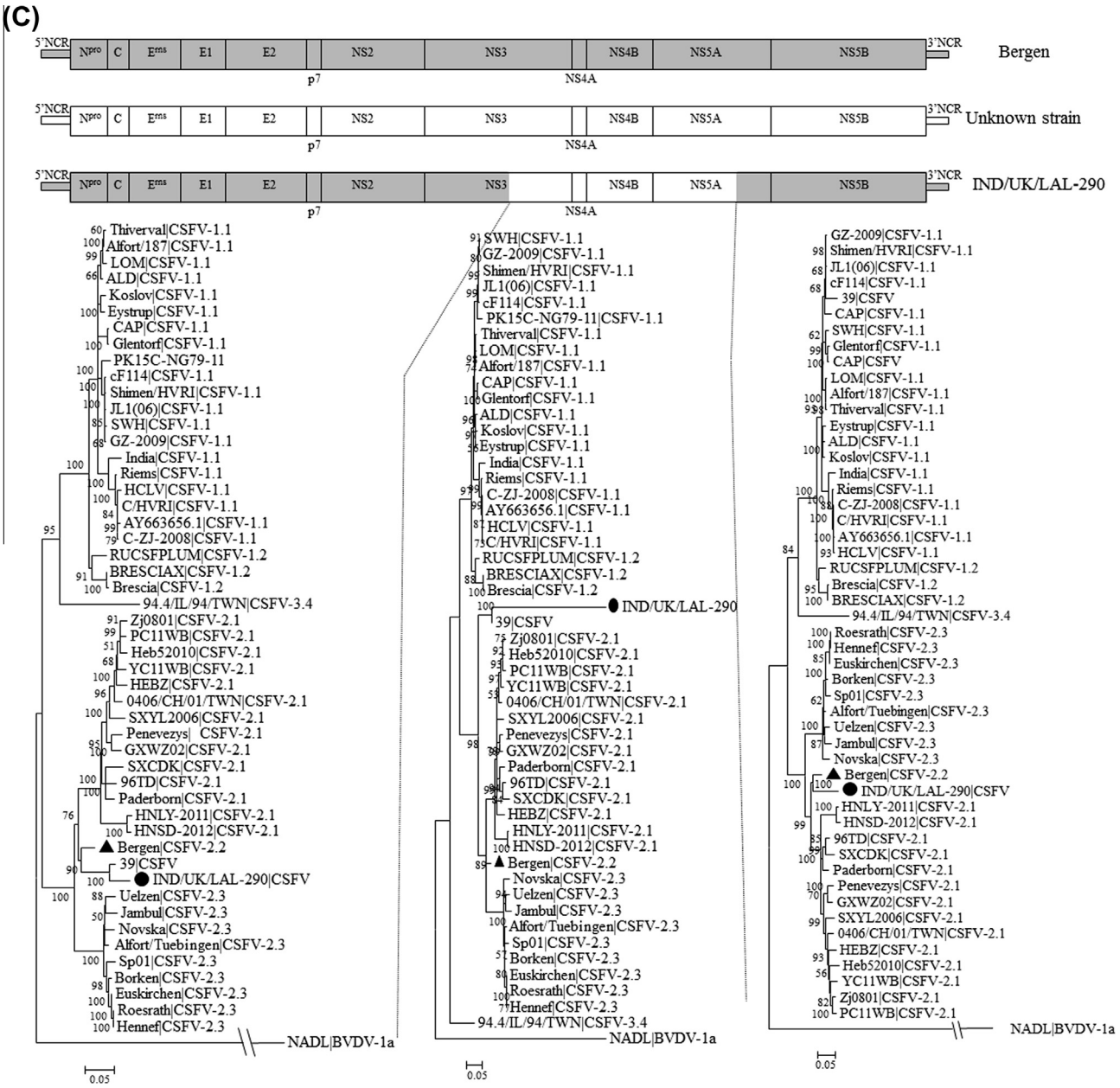

Fig. 1 (*continued*)

(RF), following the proposal of Jones and Weber (2004) for BVDV-1 and of Kalinina et al. (2002) for HCV, provides a more informative structure for the nomenclature of the genetic variant. According to this nomenclature, we propose the designation: RF1a1b and RF2a2b for viruses exhibiting the inter-subtype recombination in BVDV-1 and BVDV-2, respectively. The nomenclature for the CSFV from different genogroups reported herein is difficult to establish because the genogroup of the minor parent was not identified, and therefore, it was not possible to define it in the phylogeny.

Because the present study found that 5.6% (7 of 125) of the full-length analyzed genomes are potential recombinant viruses, homologous recombination in pestiviruses is more frequent than what has been reported for the other *Flaviviridae* members (Shi et al., 2012). Furthermore, as could be observed for HCV and DENV (Chen et al., 2008; Kalinina et al., 2002; Shi et al., 2012; Tolou et al., 2001), the location of the homologous recombination in the genome of the potential recombinant pestiviruses was random.

In the present study, three putative novel recombination events in pestiviruses were detected, showing the first evidence of recombination in BVDV-2 and reinforcing the role of these horizontal gene transfer events in the evolution of BVDV-1 and CSFV. For pestiviruses, these events are apparently even more frequent than in other *Flaviviridae* members. Moreover, the existence of recombinant strains can represent a challenge for phylogenetic and taxonomic studies. The evolutionary consequences of viral homologous recombination must be further understood to determine the extent to which recombination plays a role in pestivirus evolution and to establish adequate theoretical frames for the study of viral phylogenies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2014.12.032.

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3.4 Artigo 4: Comparison of ‘HoBi’-like viral populations among persistent infected calves generated under experimental conditions and to inoculum virus

O presente experimento já foi concluído e um artigo científico foi publicado no periódico *Virology*. O artigo científico será apresentado a seguir, tal qual foi publicado.

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Comparison of 'HoBi'-like viral populations among persistent infected calves generated under experimental conditions and to inoculum virus

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ABSTRACT

Like other members from the *Pestivirus* genus, 'HoBi'-like pestiviruses cause economic losses for cattle producers due to both acute and persistent infections. The present study analyzed for the first time PI animals derived from a controlled infection with two different 'HoBi'-like strains where the animals were maintained under conditions where superinfection by other pestiviruses could be excluded. The sequence of the region coding for viral glycoproteins E1/E2 of variants within the swarms of viruses present in the PI calves and two viral inoculums used to generate them were compared. Differences in genetic composition of the viral swarms were observed suggesting that host factors can play a role in genetic variations among PIs. Moreover, PIs generated with the same inoculum showed amino acid substitutions in similar sites of the polyprotein, even in serum from PIs with different quasispecies composition, reinforcing that some specific sites in E2 are important for host adaptation.

1. Introduction

Pestivirus infections of cattle can cause significant economic losses due to their impact on productivity and health (Houe, 2003). A putative new species named 'HoBi'-like virus has been proposed in the genus *Pestivirus* within the family *Flaviviridae* (Schirrmeier et al., 2004). 'HoBi'-like virus infections have been reported in cattle from Brazil (Bianchi et al., 2011; Cortez et al., 2006; Silveira et al., 2015; Weber et al., 2016), Thailand (Ståhl et al., 2007), Italy (Decaro et al., 2011), Bangladesh (Haider et al., 2014) and India (Mishra et al., 2014). Recent reports suggest that 'HoBi'-like virus share many characteristics with bovine viral diarrhea virus (BVDV-1 and BVDV-2) such the clinical signs (Decaro et al., 2011; Ridpath et al., 2013; Weber et al., 2016) and generation of persistent infected calves (PI) (Bauermann et al., 2014; Decaro et al., 2013; Weber et al., 2016).

Persistent infection occurs as a consequence of *in utero* exposure during the first trimester of gestation generating PI animals. Persistent infections are maintained because the fetal immune system develops a tolerance for viral proteins that lasts for the life of the animal. These animals shows different clinical presentation from apparently normal to a wide range of clinical signs including congenital defects such as reduced growth rates and abnormalities of hair, skin, skeletal and nervous system (Baker, 1995; MacLachlan and Dubovi, 2011). The reason for this variation is unknown, but it is hypothesized that strain and fetal developmental stage at the time of infection may play a role. Generation and maintenance of PI calves under experimental conditions is both time consuming and expensive, thus the genetic constitution of virus populations in PIs is largely unstudied. Studies of the mechanisms involved in establishing and maintaining persistent infection has the potential to significantly increase the understanding of the biology of PI generation.

Pestiviruses have a single-stranded, positive-sense RNA genome that contains one open reading frame (ORF) that is flanked by non-coding regions (NCRs) at the 5′ and 3′ ends, which encodes a polyprotein that is processed into 12 polypeptides (Simmonds et al., 2011). The 5′NCR and N-terminal autoprotease (Npro) are the genomic regions most frequently used to characterize the genus, species and subtypes of new strains using phylogenetic methods (Giammarioli et al., 2015; Vilcek et al., 2001). However, the envelope glycoprotein 1 (E1) and 2 (E2) have higher levels of diversity and are required for cell entry and membrane fusion of pestiviruses (Simmonds et al., 2011). E2 determines cellular tropism, binds the cell-surface receptor, and contains the major neutralizing antibody epitopes, presenting a unique three-domain architecture (Li et al., 2011, 2013; Liang et al., 2003).

Like to other viruses with RNA genome, pestivirus isolates exist as quasispecies (swarms of individual viruses) in infected animals

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(Collins et al., 1999; Dow et al., 2015; Jones et al., 2002; Ridpath et al., 2015; Töpfer et al., 2013). There is limited information on the mechanisms that select for different virus genetic populations and it is not known whether polymorphisms are due to differences in viral strains, host factors or a combination of both. It has been shown the BVDV persistent infections vary in outcome and that different PI animals can carry viral populations which differ widely in size and diversity (Dow et al., 2015; Ridpath et al., 2015). In the present study size and genetic constitution of viral populations circulating in PI calves generated under experimental conditions using strains D32/00_HoBi (Schirrmeier et al., 2004) and Italy-1/2011-1 (Decaro et al., 2011) were compared. In addition comparison was made to the viruses used as inoculum. Analyses were conducted to examine changes between the inoculum and circulating persistent virus in different calves.

2. Materials and methods

2.1. Viruses, cells and 'HoBi'-like PI animals generation

Primary bovine turbinate (BTu) cells with ≥12 passages were used to propagate and titrate the two 'HoBi'-like virus strains used in this study: D32/00_HoBi, detected in Germany in a fetal bovine serum (FBS) lot from South America origin (Schirrmeier et al., 2004), and Italy-1/10-1, identified in an outbreak of respiratory disease in an Italian herd (Decaro et al., 2011). The cells used for virus amplification were grown in minimal essential medium (MEM), supplemented with L-glutamine (1.4 mM), gentamicin (50 mg/L), and 10% FBS. Cells and FBS were verified free of pestivirus antigen and antibodies by RT-PCR and a virus neutralization test (VNT), respectively (Bauermann et al., 2012). For virus propagation, 25 cm^2 flasks containing 70% confluent BTu cell monolayers were inoculated with one of the two 'HoBi'-like virus strains and incubated at 37 °C for 72–96 h. Following one freeze-thaw cycle, the suspension was centrifuged for 10 min at 1000 × *g*. Supernatants were collected, aliquoted, and stored at −70 °C until use. The virus stocks were titrated in 96-well microtiter plates by endpoint dilution using immunoperoxidase staining with the anti-E2 monoclonal antibody N2 for endpoint detection of viral antigens, as previously described (Bauermann et al., 2012). Titers were calculated and expressed as median tissue culture infective doses (TCID) (Reed and Muench, 1938).

'HoBi'-like PIs used in the present study were generated in a previous study (Bauermann et al., 2014). Briefly, twelve crossbreed heifers that were negative for 'HoBi'-like and BVDV by virus isolation, RT-PCR (Bauermann et al., 2014), and negative for antibodies (Bauermann et al., 2013) were selected. Estrus synchronization and artificial insemination were performed. Eight pregnant heifers were selected and moved into biosecurity level 3 (BSL3) containment at around 55 days of gestation (two heifers housed per room). The animals were infected at around day 70 of gestation by the instillation of 2.5 mL of infected cell culture supernatant (10^5 $TCID_{50}$/mL) into each nostril. Four heifers (two rooms) were infected with the 'HoBi'-like virus strain Italy-1/10-1, and four heifers were infected with the strain D32/00_HoBi. Two apparently healthy PI animals were generated from the heifers infected with D32/00_HoBi (PI #102 and PI #103), and two from the ones infected with Italy-1/10-1 (PI #101 and PI #104).

2.2. RNA isolation

Total RNA was prepared using 140 μL of sample from the inoculums used to infected the heifers (D32/00_HoBi and Italy-1/10-1) and serum samples from the four PI calves 30 days after birth (Bauermann et al., 2014). A robotic workstation (Qiacube, Qiagen, Hilden, Germany) was used for automated RNA purification by a spin-column system (QIAamp Viral RNA Mini Kit, Qiagen) according to the manufacture's recommendations. The extracted RNA was stored at −70 °C.

2.3. RT-PCR, Cloning and generation of viral sequences

PCR amplifications were conducted using primers 2020f 5′-GGCAGGCACATGAGGAAGTAGAC-3′ (position in 'HoBi'-like virus strain D32/00_HoBi GenBank accession number AB871953.1: 2109-2131) and 3099r 5′-GGTCCATTATTGCCACTCCATCTC-3′ (position in strain D32/00_HoBi: 3189-3168) that amplified a portion of glycoprotein E1 and E2. Briefly, the reaction mix (100 μL total) included 10 μL of total RNA and was prepared using a commercial kit (SuperScript III one-step RT-PCR system with Platinum *Taq* high fidelity; Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Cycle conditions were as previous published (Bauermann et al., 2014). PCR amplicons were detected by electrophoresis in a 1% stained (GelRed; Biotium, Hayward, CA, USA) agarose gel with visualization under UV light, excised and purified using QIAquick Gel Extraction kit (QIAgen).

Cloning was performed using a commercial kit (StrataClone Blunt PCR Cloning Kit, Invitrogen, Waltham, MA, USA). At least 100 colonies for each PCR product were grown and the DNA extracted using a commercial kit (Qigen QIAprep 96 Turbo Mini Prep, Qiagen).

Double stranded DNA were purified and concentrated using a Gene clean Spin Kit according the manufacturer's instructions (MP Biomedicals, Solon, OH, USA) followed by quantification using the Qubit® 2.0 Fluorometer for dsDNA (Invitrogen). The appropriate quantity of dsDNA was labeled in both directions using Big Dye terminator chemistries (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's instructions. The labeled products were sequenced using an ABI 3100 genetic analyzer (Applied Biosystems Inc.). All sequences were confirmed by sequencing both strands in duplicates. Sequences were submitted as batch files to GenBank. The trace identifier (TI) numbers assigned by NCBI are 2343260674 through 2343263545.

2.4. Analysis of viral sequences

Sequences were edited and aligned using Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI, USA). Amino acid phylogenetic trees were constructed using MEGA version 6.0 (Tamura et al., 2013) using the minimum-evolution test according to the Poison statistical model with 1000 replicates. D32/00_HoBi or Italian 'HoBi'-like viruses were used as outgroup.

Amino acid entropy landscapes were calculated with BioEdit version 7.1.3 software and the graphs were constructed with

Table 1
Summary of the data obtained from the sequencing of clones from the inoculums and persistently infected (PI) animals.

Sample	Number of clones	Range of variation of identity	
		Nucleotide (%)	Amino acid (%)
Inoculum D32/00_HoBi	108	99.6–100	98.8–100
Inoculum Italy-1/2011-1	125	99.6–100	99.1–100
PI 101[a]	113	99.4–100	98.5–100
PI 102[b]	122	99.1–100	97.9–100
PI 103[b]	104	98.9–100	97.1–100
PI 104[a]	143	98.8–100	97.1–100

[a] 'HoBi'-like PI animal generated from a heifer infected with Italy-1/10-1.
[b] 'HoBi'-like PI animal generated from a heifer infected with D32/00_HoBi.

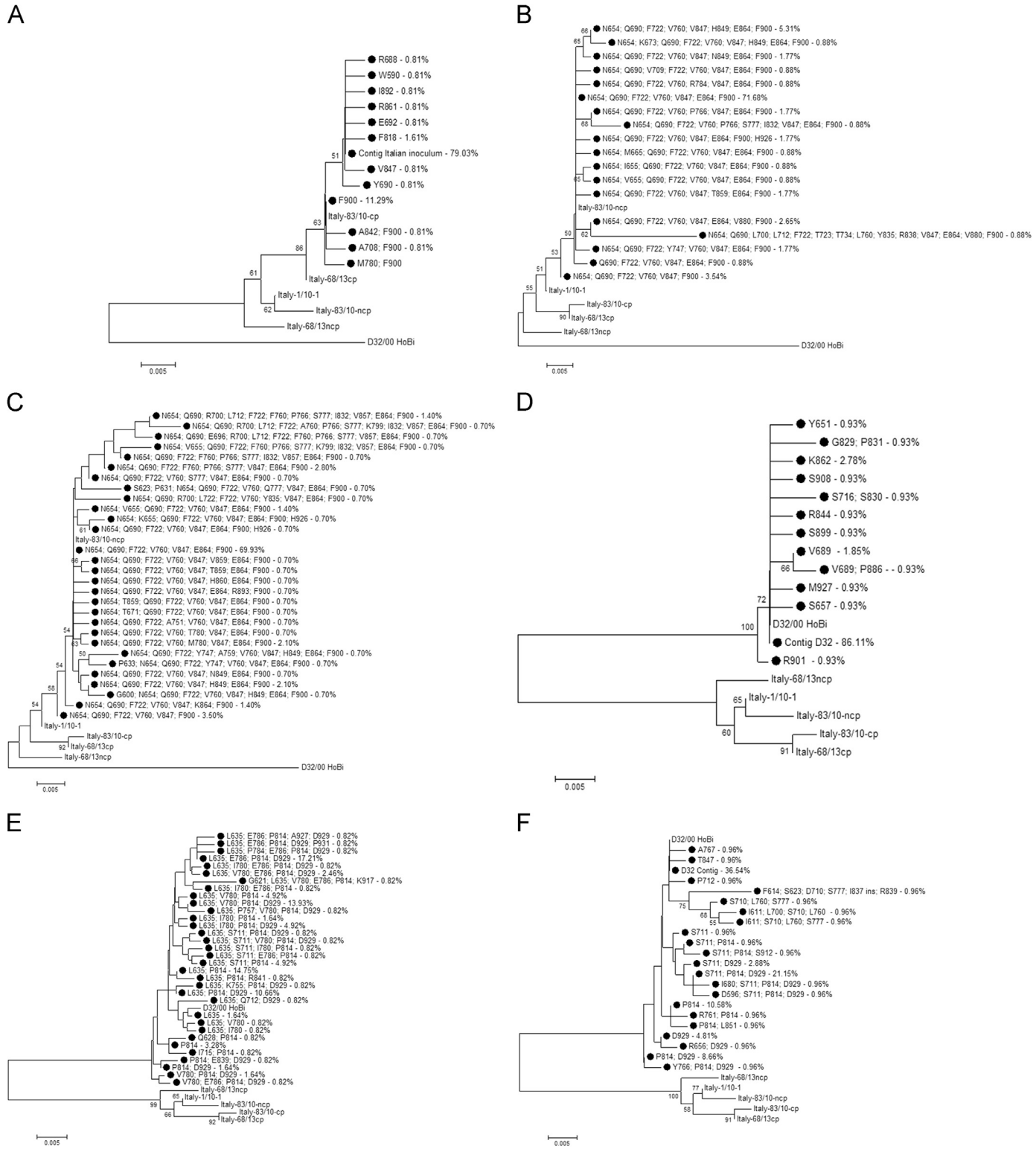

Fig. 1. Dendograms of the viral population present in the inoculum Italy-1/10-1 (A) used to generate the calves persistently infected (PI) #101 (B) and PI #104 (C), and inoculum D32/00_HoBi (D) used to generate PI #102 (E) and PI #103 (F). The amino acid phylogenetic trees using portion of the glycoprotein E1 and E2 were constructed using minimum-evolution inference poison parameter model. Bootstrap (1000 replicates) values >50 are indicated at the internal nodes. The most frequent viral variant in inoculum D32/00_HoBi and Italy-1/2011-1 were named as "Contig D32" and "Contig ITA" respectively. The other viral sequences were named according the amino acid substitution and position in the polyprotein compared with the dominant quasispecie in the respective inoculum. The percentage of each viral sequence presence is represented with the sequence name. GenBank accession numbers: D32/00_HoBi (AB871953.1), Italy-1/10-1 (HQ231763.1), Italy-83/10-ncp (JQ612704.1), Italy-83/10-cp (JQ612705.1), Italy-63/13ncp (KJ627179.1) and Italy-63/13cp (KJ627180.1).

GraphPad Prism 5 Software (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

Amino acid phylogenetic trees were constructed from at least 100 clones (Table 1) generated from both 'HoBi'-like virus strains inoculum (D32/00_HoBi and Italy-1/10-1) and serum from the PIs calves 30 days after birth (PIs #101 and #104: generated with Italy-1/10-1 inoculum; PIs #102 and #103: generated with D32/00_HoBi inoculum) (Fig. 1). Nucleotide and amino acid identity within clones of each sample are described in Table 1.

The most frequent viral swarm variant present in inoculum Italy-1/10-1 was named as "Contig ITA" and had 99% sequence identity at both the nucleotide and predicted amino acid level with

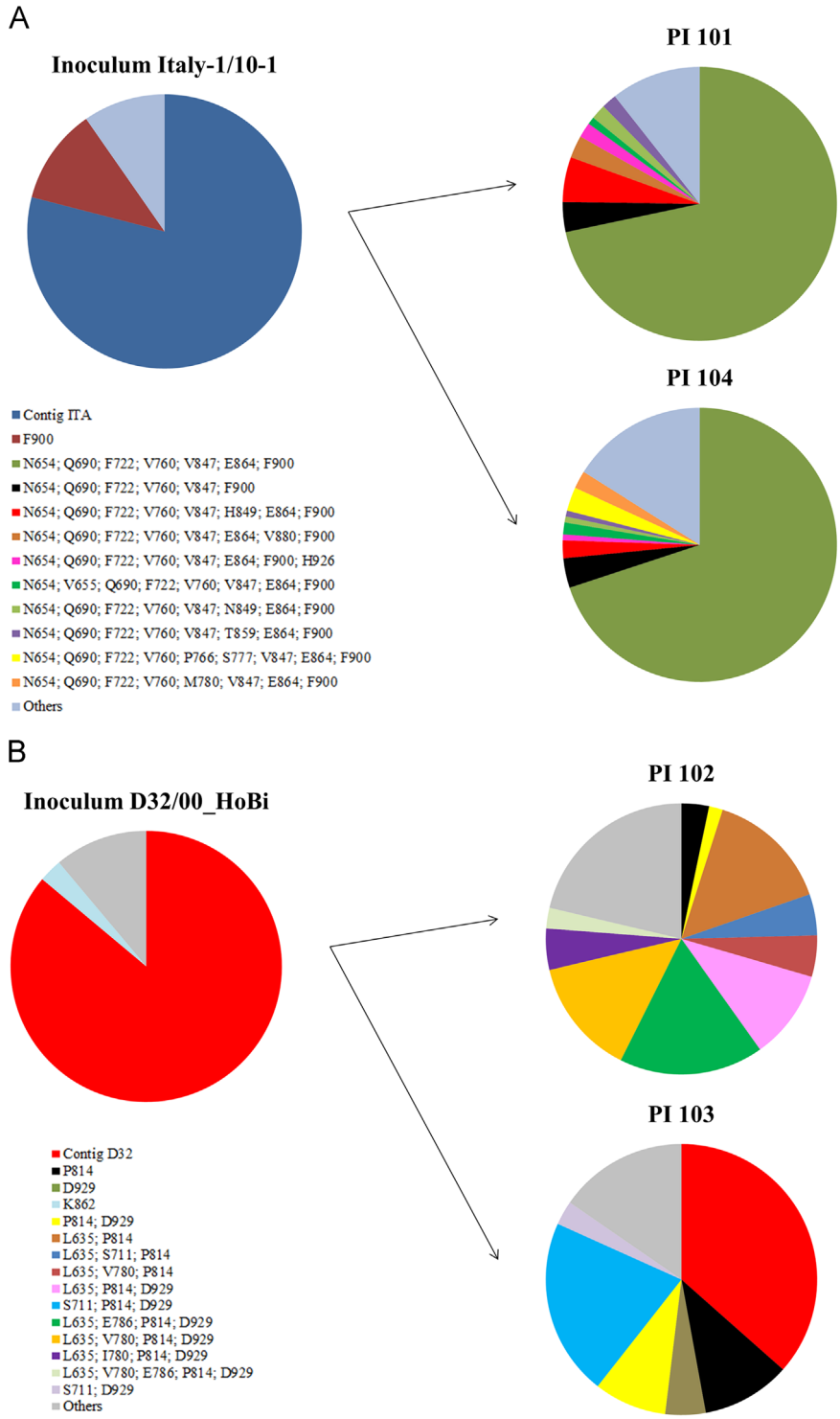

Fig. 2. Viral populations present in the inoculum Italy-1/10-1 and persistently infected (PI) calves #101 and #104 (A), and inoculum D32/00_HoBi and PIs #102 and #103 (B). The most frequent viral variant in inoculum D32/00_HoBi and Italy-1/10-1 were named as "Contig D32" and "Contig ITA" respectively, and the other samples named according the amino acid substitution and position in the polyprotein compared with the dominant quasispecie in the respective inoculum. Viral populations composing less than 2% and that are not present as viral population in any other swarm are represented in the category named as "Others".

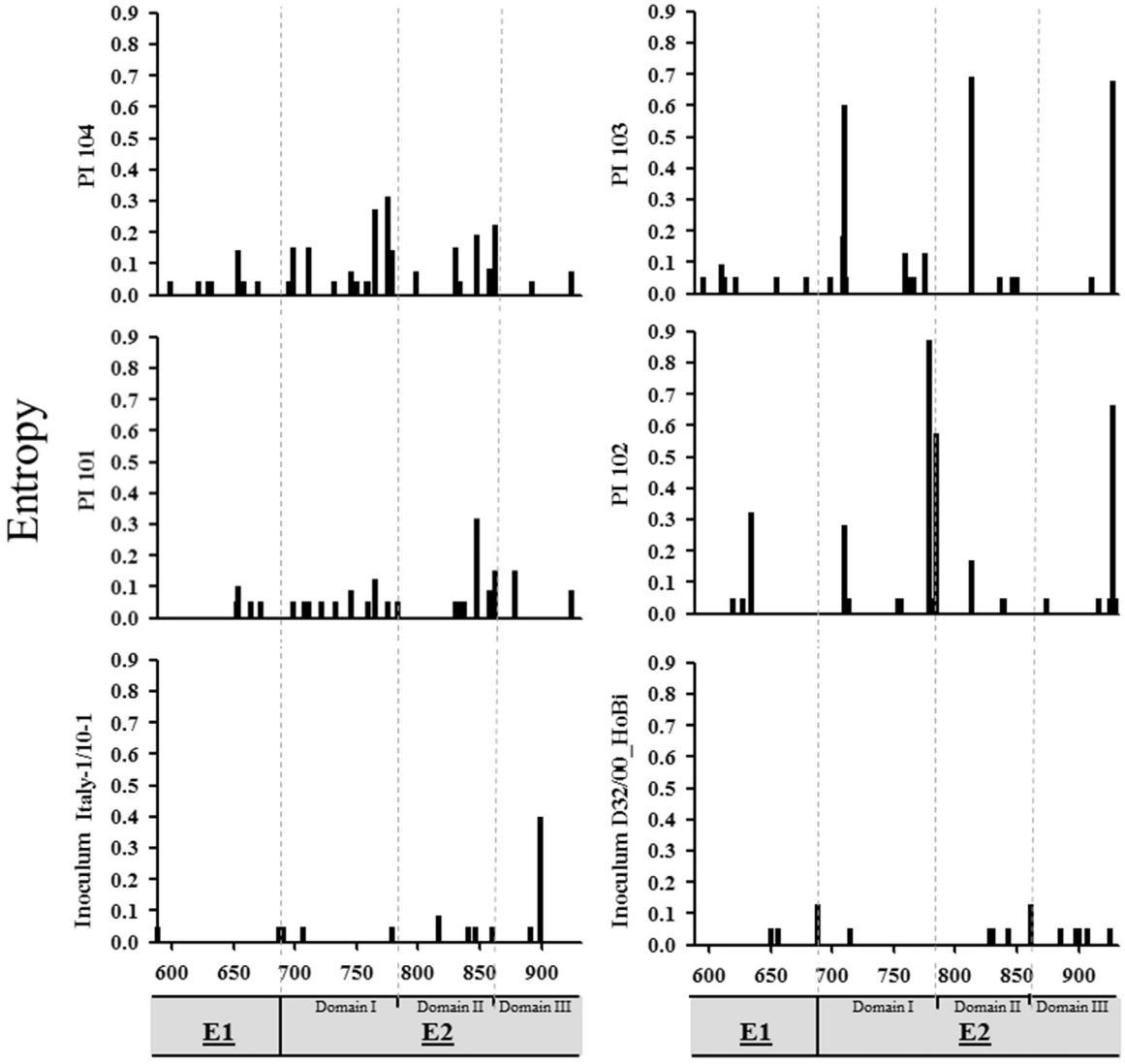

Fig. 3. Position-wise entropy of amino acid distribution in the glycoprotein E1 and E2 encoding regions. For each sample (inoculums Italy-1/10-1 and D32/00_HoBi, and PIs #101, #102, #103 and #104), the entropy landscape (vertical axis) computed from error-corrected reads is shown at each position in polyprotein (horizontal axis) represented as single genomic site for glycoprotein E1 and E2 (domains I, II and III) encoding sequences. Inoculum Italy-1/10-1 used to infect the heifers that generated persistently infected (PI) calves #101 and #104, and inoculum D32/00_HoBi used to infect the ones that generated PIs #103 and #104.

the Italian strains Italy-1/2011-1, Italy-83/10-cp, Italy-83/10-ncp, Italy-68/13cp and Italy-68/13ncp (GenBank accession numbers HQ231763.1, JQ12705.1, JQ612704.1, KJ627180.1 and KJ627179.1, respectively). The most frequent viral swarm variant present in inoculum D32/00_HoBi was named as "Contig D32" and had 100% of sequence identity with strain D32/00_HoBi (GenBank accession number AB871953.1). The viral swarm present in PI animals #101 and #104 included a dominant variant that had 100% sequence identity with strain Italy-83/10-ncp. The circulating swarm within PI animal #102 did not contain one dominant variant but at least five different ones that while differing from each other had 99% sequence identity with strain D32/00_HoBi. In PI #103 the same variant detected as the dominant variant in inoculum D32/00_HoBi (Contig D32) was also dominant (36.54%), but made up a lower percentage of the viral swarm.

The viral quasispecies composing the inoculum Italy-1/10-1 and the PIs #101 and #104 is shown in Fig. 2A while the inoculum D32/00_HoBi and PIs #102 and #104 viral composition is represented in Fig. 2B. As the consensus sequences in the inoculums were named as "Contig ITA" and "Contig D32", the individual variant sequences were named according the amino acid substitution and position in the polyprotein compared with the dominant variant in the respective inoculum. The viral variant "Contig ITA" was observed as 79.03% of the viral population present in inoculum Italy-1/10-1 but was not observed in PIs #101 and #104. The "Contig D32"was observed as 86.11% and 36.54% of the viral population in inoculum D32/00_HoBi and PI #103, respectively, but was not detected in PI #102. Table A. 1 summarizes the viral populations detected in the inoculum Italy-1/10-1 and the PIs generated from it. Table A. 2 shows the population for the inoculum D32/00_HoBi and PIs generated from it.

Entropy landscapes along the glycoproteins E1 and E2 (including positions in the domains I, II and II) (Fig. 3) were generated. Higher variability, as demonstrated by more clear-cut peaks within E1 and E2 was observed in the PI animals' graphs when compared to the inoculums. Moreover, the graphs from PIs generated with the same inoculum appear more similar other than to graphs from the PIs generated with the other inoculum.

4. Discussion

PI animals represent an unique opportunity to study the biology of pestiviruses because they can harbor viral populations that

differ widely in size and diversity (Dow et al., 2015; Ridpath et al., 2015). In the present study, the composition of viral populations from the viral inoculums and experimentally generated PIs were compared. The inoculums and PI animals generated with the strain Italy-1/10-1 carried viral swarms with one dominant variant representing about 70% or more of the viral population (Fig. 1). In contrast the PIs generated with strain D32/00_HoBi, presented with higher diversity with none of variants composing more than 50% of the sequences. The same pattern of quasispecies presentation without any dominant variant was previously reported for hepatitis C virus (Navas et al., 1998), another member for *Flaviviridae* family (Simmonds et al., 2011), and reinforces the need for further efforts to understanding the evolution mechanisms of these viruses.

Efficient adaptation to the host was observed with BVDV-1 strains, where viral populations are genetically distinct between different body compartments due to local selection pressures (Dow et al., 2015). This explanation can be applied to the results obtained in the present work where the dominant variant observed in the PIs generated with the strain Italy-1/2011-1 changed when compared with its inoculum (Fig. 2A). Moreover, the genetic composition of the viral swarms circulating within the PIs generated with strain D32/00_HoBi differed from the inoculum that generated them and from each other (Fig. 2B). Difference observed between the swarms circulating within PI animals generated using the same inoculum suggests that host factors can play a role in the selection of genetic variants within PIs swarms.

The strain Italy-1/10-1 (Decaro et al., 2011) was obtained from the laboratory that isolated it and used in previously published studies (Bauermann et al., 2014). It was observed in both the previously published studies and this study that the consensus sequences of the inoculum (Fig. 1A) and the viral swarms within PIs generated with it (PIs #101 and #104) (Fig. 1B and C) were more close-related with other Italian'HoBi'-like viruses (Decaro et al., 2014, 2012) than to the strain Italy-1/10-1. These findings suggest that strains reported in Italy (Decaro et al., 2014, 2012, 2011) probably originated from the same strain and illustrate the same genetic adaptation to each host seen in the present study.

Determinants on glycoprotein E2 function in cell tropism and entry (Li et al., 2011; Liang et al., 2003; Simmonds et al., 2011) and probably play a role in virus-host adaptation. In the present work, the single-site entropy landscapes showed that the polyproteins from the inoculum along E1/E2 are more uniform when compared with the ones from PI animals. Moreover, the graphs for PIs generated with the same inoculum were more close-related to each other than to the ones generated with the other inoculum (Fig. 3). This may reflect the genetic population from PIs generated with the same strain evolving in similar sites in order to obtain the genetic variant with better fitness. On the other hand, inoculums may show lower amino acid variability in the entropy graphs because they were propagated in cell culture and thus were not exposed to the selective pressure imposed by to the host immune system and other intrinsic host factors acting upon viral swarms replicating in the infected animal (Baker, 1995; MacLachlan and Dubovi, 2011).

This is the first study that looked at the nature of the swarms of within animals persistently infected with'HoBi'-like viruses. Previous studies reported the analysis of BVDV (Dow et al., 2015; Ridpath et al., 2015) and presented similar observation when compared with the present work, particularly in reference to the impact of host factors on viral swarm diversity and breath. These findings suggested the presence of broad similarities within the biology of bovine pestiviruses. It is important to reinforce that this work reveals differences in viral swarms but did not reveal the reason/factors behind them. Further study is needed because elucidating the interaction between the selecting factors imposed by the host and the evolutionary tendencies of the virus in maintenance of persistent pestivirus infections has the potential to uncover truths about both the bovine immune system and viral evolution.

Conflict of interest

None declared.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2016.03.001.

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3.5 Artigo 5: Temporal dynamics of ‘HoBi’-like pestivirus quasispecies in persistently infected calves generated under experimental conditions

O presente experimento já foi concluído e um artigo científico foi publicado no periódico *Virus Research*. O artigo científico será apresentado a seguir, tal qual foi publicado.

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Temporal dynamics of 'HoBi'-like pestivirus quasispecies in persistently infected calves generated under experimental conditions

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ABSTRACT

'HoBi'-like virus is an atypical group within the *Pestivirus* genus that is implicated in economic losses for cattle producers due to both acute and persistent infections. Pestivirus strains exist as quasispecies (swarms of individual viruses) in infected animals and the viral populations making up the quasispecies differ widely in size and diversity in each animal. In the present study the viral quasispecies circulating in persistently infected (PI) calves, generated and maintained under experimental conditions using two different 'HoBi'-like strains, was observed over time. An increase in genetic variability and the development of certain mutations was observed over time. Mutations observed included the loss of a putative N-linked glycosylation site in the E2 region and the change of specific residues in E1/E2. It is hypothesized that these changes may be the results on continued adaption of the pestivirus to individual hosts. This is the first study characterizing variation in the viral swarms of animals persistently infected with HoBi-like viruses over time. Studies of the shifts in PI viral swarms will contribute to our understanding of the host and viral mechanisms that function in the maintenance of pestivirus persistent infections.

1. Introduction

Viruses of the genus *Pestivirus* of the family *Flaviviridae* have a single-stranded positive-sense RNA genome and consists of four recognized species: *Bovine viral diarrhea virus 1* (BVDV-1), BVDV-2, *Border disease virus* (BDV) and *Classical swine fever virus* (CSFV) (Simmonds et al., 2011). Bovine viral diarrhea (BVD) includes an array of reproduce, enteric and respiratory clinical presentations that is associated with infection with BVDV-1 and 2 (MacLachlan and Dubovi, 2011). BVD causes significant economic loss to dairy and beef producers worldwide. An atypical group of pestiviruses isolated from cattle and water buffalo, putatively named 'HoBi'-like virus (Schirrmeier et al., 2004; Stalder et al., 2005), is associated with clinical presentations undistinguishable from those caused by BVDV-1 and 2 (Bauermann et al., 2015; Ridpath et al., 2013; Weber et al., 2016b). While genetically and antigenically distinct, 'HoBi'-like viruses share many characteristics with BVDV1 and BVDV2 including the ability to establish persistent lifelong infections in cattle (Bauermann et al., 2014; Decaro et al., 2013; Weber et al., 2016b).

Persistent infection of the fetus is a consequence of *in utero* exposure approximately between days 30 and 120 of gestation. Persistent infections are maintained because the fetal immune system develops tolerance for viral proteins. This immune tolerance lasts for the life of the animal. Within the persistently infected (PI) host, the causative pestivirus replicates in the absence of selective pressure from adaptive immune system. However, as the PI animal can respond to and eliminate pestiviruses immunologically different from the virus it carries, the persistent virus must maintain an immunologic profile that does not break immunotolerance. Therefore the PI animal represents an unique evolutionary model. PI animals have different clinical presentations, from apparently normal to a wide range of lesions, including congenital defects such as reduced growth rates and abnormalities of hair, skin, skeletal and nervous system (Baker, 1995; MacLachlan and Dubovi, 2011). The reason for this variation is unknown, but it is hypothesized that strain and fetal developmental stage at the time of infection may play a role. Generation and maintenance of PI calves under experimental conditions is both time consuming and expensive, thus the

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variability of the genetic constitution of virus populations within PIs is largely unstudied. Studies of the mechanisms involved in establishing and maintaining persistent infection have the potential to significantly increase the understanding of the biology of immune tolerance.

Like other viruses with RNA genomes, pestivirus strains exist as quasispecies (swarms of many individual viruses) in infected animals (Collins et al., 1999; Dow et al., 2015; Jones et al., 2002; Ridpath et al., 2015; Töpfer et al., 2013; Weber et al., 2016a). The analysis of this intra-host genome variability can provide insights into evolutionary drivers and determinants of viral pathogenesis that contribute to the outcome of infection. Virus population diversity is integral to RNA virus evolution and survival and is attributed to error prone genome replication by the RNA-dependent RNA polymerase (RdRP) (Domingo et al., 2012; Neill, 2012).

The envelope glycoprotein 1 (E1) and 2 (E2) are required for cell entry and membrane fusion of pestiviruses (Liang et al., 2003; Simmonds et al., 2011). E2 presents a unique three-domain architecture (domains I, II and III) (Li et al., 2011, 2013; Liang et al., 2003) that determines cellular tropism, binding to the cell-surface receptor, and contains the major neutralizing antibody epitopes. When the consensus sequences of different BVDV strains and species are compared, the E1 and E2 coding regions show higher variability that the rest of the genome. It is postulated that variability in these genomes is due to selective pressure due to the host immune response. However, a higher rate of variation is observed in this region even in the presence of the immunotolerance the exits in PI cattle (Ridpath et al., 2015). The analysis of variation in E1/E2 coding regions over time in PI cattle could potentially provide information regarding the pestiviral quasispecies evolution dynamics (Ridpath et al., 2015; Weber et al., 2016a).

The present study is a continuation of a previously published study (Weber et al., 2016a), in which differences in genetic composition of the viral swarms between animals persistently infected with 'HoBi'-like virus were observed. Variations in the diversity of 'HoBi'-like virus swarms in PI cattle, generated using the same inoculum, observed in that study suggested that host factors play a role in genetic variations among PIs. In this work, the quasispecies circulating in cattle persistently infected with 'HoBi'-like viruses were evaluated over time to determine if the quasispecies shifted with time and if so, to characterize the genetic changes associated with these shifts.

2. Materials and methods

2.1. Generation, housing and sampling of 'HoBi'-like PI calves

'HoBi'-like PIs used in the present study were described previously (Bauermann et al., 2014). PI calves #101 and #103 were individually housed and not allowed contact with other animals. PI #102 and #104 were co-housed as part of another study (Bauermann et al., 2015) where they were exposed, by commingling, to five calves, four sheep, four goat and five pigs housed by species for 30 min, twice a day in order to study transmission. The period of that study comprised 30 days (days ~40–70 of life of each PI calf).

Serum samples were collected from the four PI calves at day of birth (day 0) and days 30, 60 and 90 days of life.

2.2. RNA isolation, RT-PCR, cloning and generation of viral sequences

Total RNA was prepared using 140 μL of sample from the inoculums used to infect the heifers (D32/00_HoBi and Italy-1/10-1) and from serum samples from the four PI calves collected at day of birth (day 0) and days 30, 60 and 90 days of life. A robotic workstation (Qiacube, Qiagen, Hilden, Germany) was used for automated RNA purification by a spin-column system (QIAamp Viral RNA Mini Kit, Qiagen) according to the manufacturer recommendations and the extracted RNA stored at −70 °C.

The PCR amplification followed by cloning and DNA sequencing method was chosen over the next generation sequencing (NGS) in order to analyze small variations in E1–E2 gene. NGS generates short sequences that must be assembled to yield a consensus sequence. This limits the usefulness of NGS in determining proportions of single nucleotide polymorphisms (SNPs) (He et al., 2013; Nielsen et al., 2011; Nowrousian, 2010) or in detecting multiple variations distantly spaced that occur in tandem within a gene. PCR amplifications were conducted using primers 2020f 5′-GGCAGGCACATGAGGAAGTAGAC-3′ (position 2109–2131 at 'HoBi'-like virus strain D32/00_HoBi, GenBank accession number AB871953.1) and 3099r 5′-GGTCCATTATTGCCACTCCATCTC-3′ (position 3189-3168 at strain D32/00_HoBi) that amplified a portion of glycoprotein E1 and E2 according Weber et al. (2016a).

Cloning was performed using a commercial kit (StrataClone Blunt PCR Cloning Kit, Invitrogen, Waltham, MA, USA). At least 100 colonies for each PCR product were grown and the DNA extracted using a commercial kit (Qigen QIAprep 96 Turbo Mini Prep, Qiagen). Double stranded DNA was purified and concentrated using a Gene Clean Spin Kit according the manufacturer's instructions (MP Biomedicals, Solon, OH, USA) followed by quantification using the Qubit® 2.0 Fluorometer for dsDNA (Invitrogen). The appropriate amount of dsDNA was labeled in both directions using Big Dye terminator chemistries (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's instructions. The labeled products were sequenced using an ABI 3100 genetic analyzer (Applied Biosystems Inc.). All sequences were confirmed sequencing both strands in duplicate. Sequences were submitted as batch files to GenBank. The trace identifier (TI) numbers assigned by NCBI are 2343260674 through 2343263545 and 2343530384 through 2343537603.

2.3. Analysis of viral sequences

Sequences were edited and aligned using Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI, USA). Amino acid sequence alignments were performed using BioEdit version 7.1.3. N-linked glycosylation patterns were predicted using the software NetNGlyc 1.0 Server (www.cbs.dtu.dk/services/NetNGlyc). Amino acid phylogenetic trees were constructed using MEGA 6 (Tamura et al., 2013) using the minimum-evolution test according to the Poison statistical model with 1000 replicates. Nucleotide sequences were used to calculate the average mean diversity using the Tajima-Nei model with 500 replicates. The non-synonymous (d_{NS}) and synonymous (d_S) coefficients were calculated using the Nei-Gojobori (Jukes-Cantor) model with 500 replicates. Amino acid entropy landscapes were determined with BioEdit version 7.1.3 software and the graphs were constructed with GraphPad Prism 5 Software (GraphPad Software Inc., La Jolla, CA, USA). All the sequence alignments are available in Figshare (http://figshare.com/) with DOI number https://dx.doi.org/10.6084/m9.figshare.3410095 .v1.

2.4. Real-time RT-PCR (RT-qPCR)

Virotype BVDV test kit (Qiagen) was used to assay 'HoBi'-like virus load in serum samples from the four PIs at day of birth, 30, 60 and 90 of life. The 25 μL reaction mixture contained 19.75 μL of RT-qPCR mix, 0.25 μL of enzyme mix, and 5 μL of extracted RNA. Neither the oligonucleotide sequences nor the PCR target gene were disclosed by the company. The thermal protocol was as follows: reverse transcription at 50 °C for 20 min and RT inactivation/initial

denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 45 s, and extension at 68 °C for 45 s.

2.5. Statistical analysis

Correlation between virus load obtained by RT-qPCR and average mean diversity values for the four PI calves in the four points of serum collection was assayed using the Spearman rank coefficient ($p < 0.05$) using GraphPad Prism 5 Software (GraphPad Software Inc., La Jolla, CA, USA).

2.6. Virus neutralization tests (VNT)

Sera from the 'HoBi'-like PI calfs in day of birth and days 30, 60 and 90 of life were tested for antibodies against 'HoBi'-like virus strains D32/00_HoBi and Italy-1/10-1 by virus neutralization test (VNT). Briefly, serum samples were filtered through 22 μm membranes, incubated at 56 °C for 90 min, and serial dilutions from 1:2 to 1:256 were prepared. Dilutions were then incubated individually with 100 $TCID_{50}$ of each virus for 90 min at 37 °C and 5% CO_2. Suspensions of bovine turbinate (BT) cells were added, and after four days of incubation monolayers were fixed and subjected to immunoperoxidase monolayer assay using monoclonal antibody N2 anti-E2 as previously described (Bauermann et al., 2012).

3. Results

The inoculums and 'HoBi'-like PI serum samples could be amplified with primers 2020f and 3099r with exception of sera from animal #101 at day of birth (day 0) and 90 days of age. Real-time PCR testing of these two samples demonstrated low levels of virus in these serum samples (Table 1).

VNTs of serum from PIs #101, #102, #103 and #104 at days 0, 30, 60 and 90 did not detect antibodies against either HoBi-like virus strain.

Amino acid phylogenetic trees were constructed from at least 100 clones (Table 1) generated from the two inoculums of 'HoBi'-like strains (D32/00_HoBi and Italy-1/10-1) used to generate the PI calves and serum from the PIs calves at days 0, 30, 60 and 90 for PIs #102, #103 and #104 and days 30 and 60 for PI #101. PIs #101 and #104 were generated with Italy-1/10-1 inoculum and PIs #102 and #103 were generated with D32/00_HoBi inoculum (Fig. 1). The quasispecies of the two PI animals that were housed individually was comprised of viruses with sequences similar to those seen in the inoculum that was used to generate them. The two PI's that were co-housed (PI #102 and #104) had quasispecies on day 60 that included viruses that were similar to the D32/00_HoBi inoculum and viruses that were similar to the Italy-1/10-1 inoculum. The quasispecies of PI #104 presented 21.1% (30 out of 142 clones) of the viral variants grouped in the D32/00_HoBi-like cluster and 78.9% (112 clones out of 142) viral variants group in the Italy-1/10-1 (the inoculum virus used to generate it) while PI#102 had 88.7% (126 out of 142 clones) that clustered with the D32/00_HoBi-like strain (the inoculum virus used to generate it and 11.3% (16 out of 142 clones)) in the Italy-1/10-1 cluster. Average mean diversity, nucleotide, and amino acid identity within clones of each sample are described in Table 1. Average mean diversity values and the virus load obtained by RT-qPCR did not correlate using Spearman rank coefficient ($r = 0.09021$; $p = 0.7591$; 95% CI: −0.4762–0.6038).

The viral quasispecies composing the inoculum Italy-1/10-1 and the PIs #101 and #104 are shown in Fig. 2A while viral composition for the inoculum D32/00_HoBi and PIs #102 and #104 are represented in Fig. 2B. The consensus sequences in the inoculums were named as "Contig ITA" and "Contig D32", and the individual variant sequences were named according to the amino acid substitution and position in the polyprotein compared with the dominant variant in the respective inoculum. Table A.1 summarizes the viral populations detected in the inoculum Italy-1/10-1 and the PIs generated from it. Table A.2 shows the viral populations for the inoculum D32/00_HoBi and PIs generated from it.

Within the quasispecies of calves PI generated with the Italy-1/10-1 inoculum there were a group of viruses that had 100% identity to the sequence Italy-83/10-ncp strain (GenBank accession number JQ612704.1) (Fig. 1A). The proportion of this group of viruses varied by calf and by sample date (Fig. 2A). The inoculum Italy-1/10-1 contained a dominant quasispecies named as "Contig ITA" that had 99% sequence identity at both the nucleotide and predicted amino acid levels with the sequences of Italian strains Italy-1/10-1, Italy-83/10-cp, Italy-83/10-ncp, Italy-68/13cp and Italy-68/13ncp entered in GenBank (GenBank accession numbers HQ231763.1, JQ12705.1, JQ612704.1, KJ627180.1 and KJ627179.1, respectively). Contig ITA could not be observed in the viral swarms in PIs #101 and #104 and was substituted by a group of viruses 100% identical to the sequence Italy-83/10-ncp strain. These variants were dominant at day 30 for PI #101 (71.7%) and at day of birth (59%) and days 30 (69.9%) and 60 (44.6%), presenting a decrease in the total proportion of viral swarm composition at day 60 (4.7%) for PI #101 and day 90 for PI #104 (22.7%).

The inoculum D32/00_HoBi contained a dominant quasispecies named as "Contig D32" that had 100% of sequence identity with the sequence of strain D32/00_HoBi entered in GenBank (GenBank accession number AB871953.1) (Fig. 1B). The circulating swarm within PI animal #102 at day of birth and 30, 60 and 90 of age did not contain one dominant variant but at least five different clusters that while differing from each other had 99% sequence identity with the published sequence of strain D32/00_HoBi. In PI #103 the same variant detected as the dominant variant in inoculum D32/00_HoBi (Contig D32) was also dominant in the day of birth (43.4%) and day 30 (36.54%) (Fig. 2B). This variant decreased in frequency at days 60 (11.1%) and 90 of life (14.8%). In general, PIs #102 and #103 did not presented a dominant variant with frequency higher than 50% at any point analyzed, but a mixture of variants with similar levels of frequency in the viral population composition.

Entropy landscapes along the glycoproteins E1 and E2 (including positions in the domains I, II and II) (Fig. 3) were generated. Higher variability, as demonstrated by more clear-cut peaks within E1 and E2 was observed in the PI animals when compared to the inoculums. Moreover, the graphs within each PI appear similar in all days and presented an increased variability along the analyzed days for both PIs generated with both strain Italy-1/10-1 (Fig. 3A) and D32/00_HoBi (Fig. 3B).

Values of nucleotide diversity were estimated independently for each region of E1/E2 glycoprotein (Table 2). The inoculums had d_{NS}/d_S ratio ≤1 or borderline in all analyzed regions. In general, the d_{NS}/d_S ratio was ≤1 or borderline for the E1 region from all PIs in all points analyzed. In E2 domain I, the higher ratios could be observed in all PIs with exception of PI #103 where the values were >1 but borderline when the standard errors are analyzed. Analysis of domain II of E2 region showed d_{NS}/d_S ratio >1 in day of birth in all calves with decrease of this rate over time. In E2 domain III, the values were variable but the ratios >1 in PIs #101 and 103 at 30 and 60 days of life and PI # 104 in the day of birth and 30 days of life.

Amino acid alignment from the E1/E2 region of the most prevalent viral population present in inoculums D32/00_HoBi and Italy-1/10-1, and in 'HoBi'-like PIs at day 90 were compared with other 'HoBi'-like pestivirus genomes available in GenBank (Fig. 4). Amino acid substitutions were more frequent in E2 domain I (11 out of 27) in comparison with four in E1 and six for both domains II and III of the glycoprotein E2. In general, the amino acid substitutions resulted in conversion to amino acid residues that could be observed 'HoBi'-like genomes available in GenBank. Table A.3

Table 1
Summary of the data obtained from the real-time PCR (RT-qPCR) and the sequencing of clones from the inoculums and persistently infected (PI) animals.

Sample	Day of life	RT-qPCR (Ct)	Mean diversity ($\times 10^{-4}$)*	Number of clones	Range of variation of identity	
					Nucleotide	Amino acid
Inoculum Italy-1/2011-1		NP	6.53	125	99.6–100%	99.1–100%
PI #101[d]	Birth	36.8	ND	ND	ND	ND
	30	31.3	9.96	113	99.4–100%	98.5–100%
	60	28.8	26.76	106	98.9–100%	97.7–100%
	90	34.9	ND	ND	ND	ND
PI #104[a]	Birth	20.6	13.86	100	98.9–100%	97.4–100%
	30	21.2	13.07	143	98.8–100%	97.1–100%
	60[c]	22.7	19.26	112	99.2–100%	98.0–100%
	90	25.2	27.91	119	98.6–100%	96.8–100%
Inoculum D32/00_HoBi		NP	5.88	108	99.6–100%	98.8–100%
PI #102[b]	Birth	22.5	24.96	114	99.2–100%	98.3–100%
	30	24.6	27.95	122	99.1–100%	97.9–100%
	60[d]	25.8	21.61	126	99.3–100%	98.3–100%
	90	26.2	27.58	116	99.1–100%	98.0–100%
PI #103[b]	Birth	22.3	23.06	106	98.9–100%	97.7–100%
	30	25.4	21.75	104	98.9–100%	97.1–100%
	60	25.8	18.90	126	99.3–100%	98.6–100%
	90	25.1	30.25	101	98.9–100%	97.4–100%

NP: not performed. ND: not determined.

* Standard error of the means are reported in Table A.5.

[a] 'HoBi'-like PI animal generated from a heifer infected with Italy-1/10-1.

[b] 'HoBi'-like PI animal generated from a heifer infected with D32/00_HoBi.

[c] Animal #104 exposed to PI #102 and presented a D32/00_HoBi-like swarm (30/142 clones) in day 60 of life where the average mean diversity was 21.4×10^4, and nucleotide and amino acid identity ranged between 99.4–100% and 98.8–100%, respectively.

[d] Animal #102 was exposed to PI #104 and presented a Italy-1/2011-1-like swarm (16/142 clones) in day 60 of life where the average mean diversity was 18.7×10^4, and the nucleotide and amino acid identity ranged between 99.3–100% and 98.3–100%, respectively.

describes the percentage of viral variants that contain amino acid substitutions for the inoculums and 'HoBi'-like PIs.

Putative N-linked glycosylation patterns in E1/E2 regions were predicted using the software NetNGlyc 1.0 Server and compared with representative 'HoBi'-like and other pestivirus genomes (Table A.4). Putative *N*-glycosylation sites present in amino acid positions 596 (located in E1 region), 807 (E2 domain II), 876 and 920 (both in E2 domain III) were present in all sequences. BVDV-1 strain NADL (GenBank accession number CAB91846.1) and BDV strain X818 (GenBank accession number NP_620062.1) presented the same putative four sites. An extra putative site was observed at position 812 (E2 domain II) of Contig D32 and D32/00_HoBi (GenBank accession number: BAO04453.1). The same five putative sites found in Contig D32 and D32/00_HoBi can be seen in CSFV

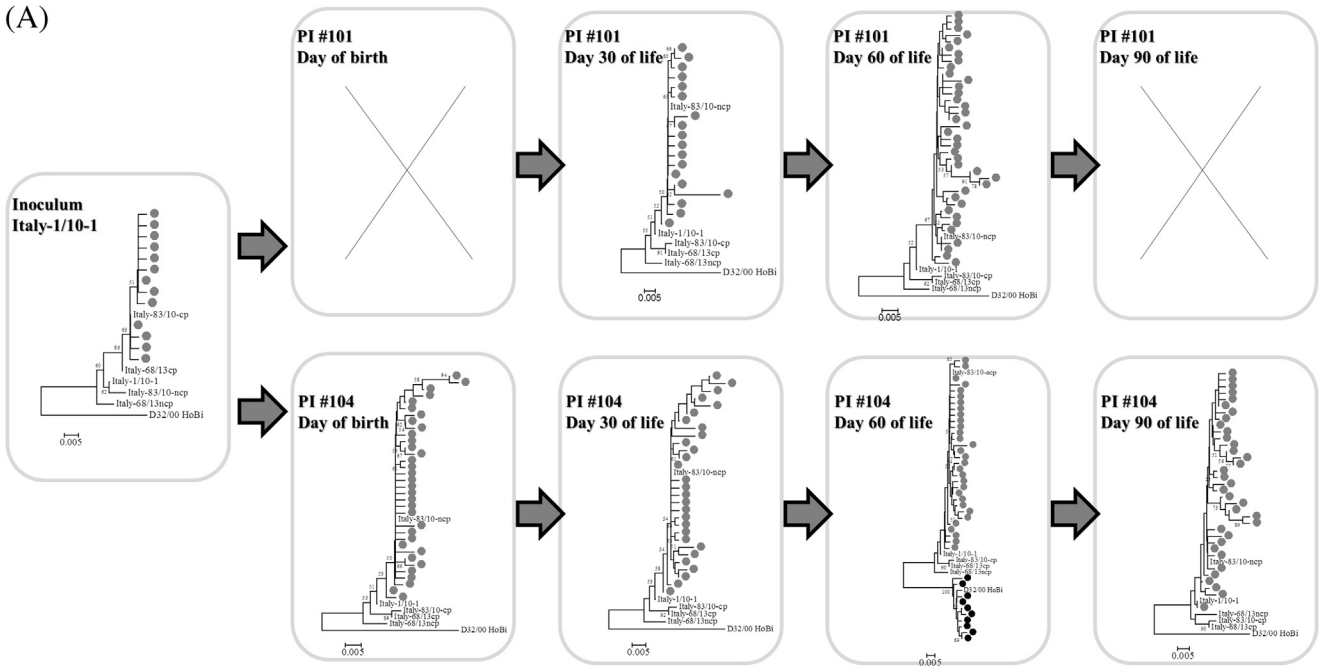

Fig. 1. Dendograms of the viral population present in the inoculum Italy-1/10-1 and PIs #101 and #104 in day of birth and days 30, 60 and 90 of life (A), and inoculum D32/00_HoBi and PIs #102 and #103 in day of birth and days 30, 60 and 90 of life (B). The amino acid phylogenetic trees using portion of the glycoprotein E1 and E2 (residues 589–933) were constructed using minimum-evolution inference poison parameter model. Bootstrap (1000 replicates) values >50 are indicated at the internal nodes. Viral swarms detected that grouped in Italy cluster are represented by ●, and the ones that grouped in D32/00_HoBi cluster by ●.

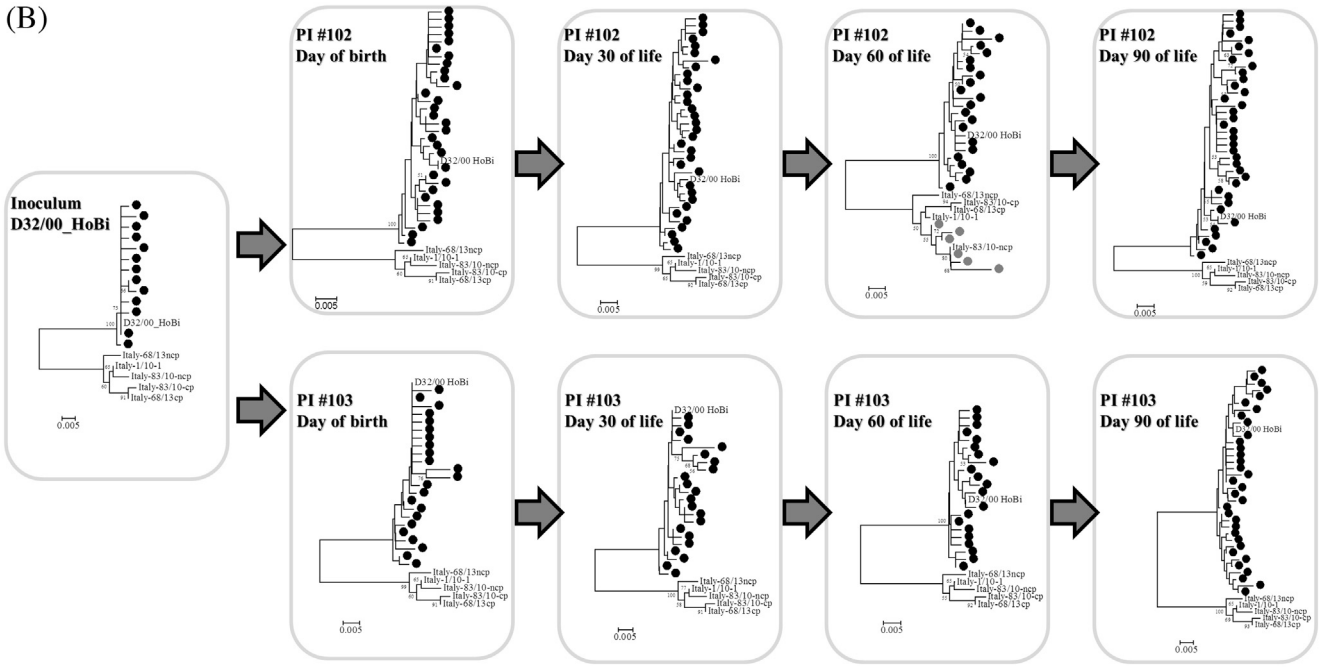

Fig. 1. (*Continued*)

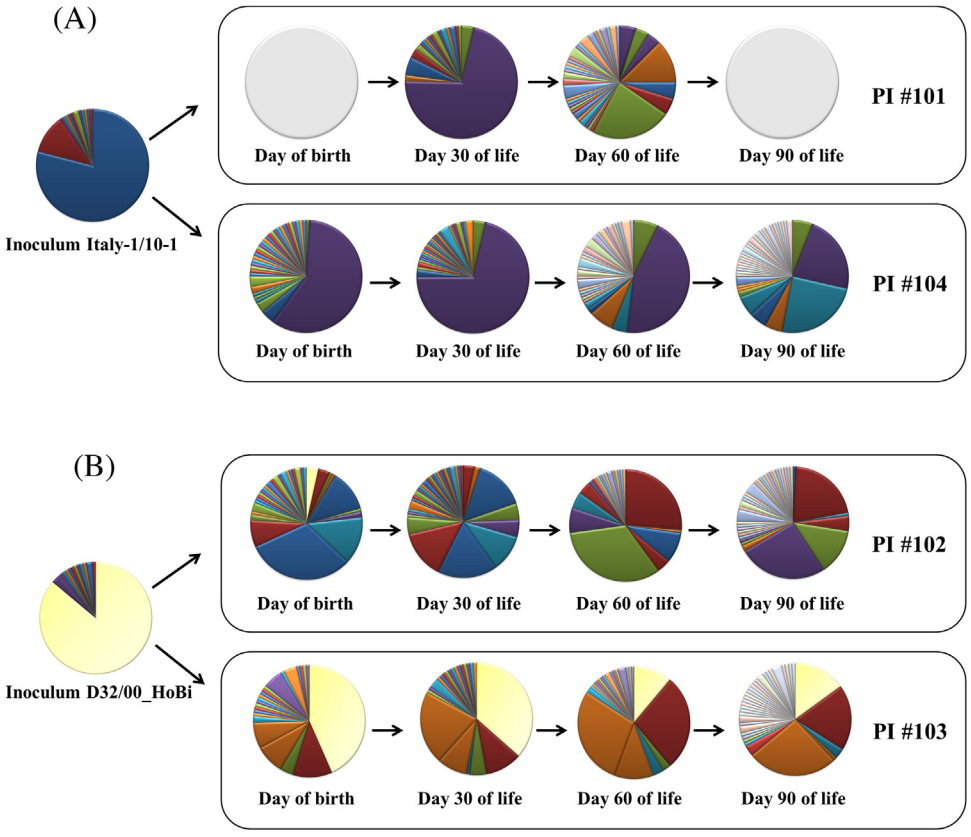

Fig. 2. Proportion and composition of viral populations present in the inoculum Italy-1/10-1 and persistently infected (PI) calves #101 and #104 (A), and inoculum D32/00_HoBi and PIs #102 and #103 (B). PI# 101 was negative in the RT-PCR performed in day of birth and day 90 of age and could not be analyzed. Table A.1 denotes the exact percentage of viral variants that comprise inoculum Italy-1/10-1 and PIs #101 and #104, and Table A.2 for and inoculum D32/00_HoBi and PIs #102 and #103.

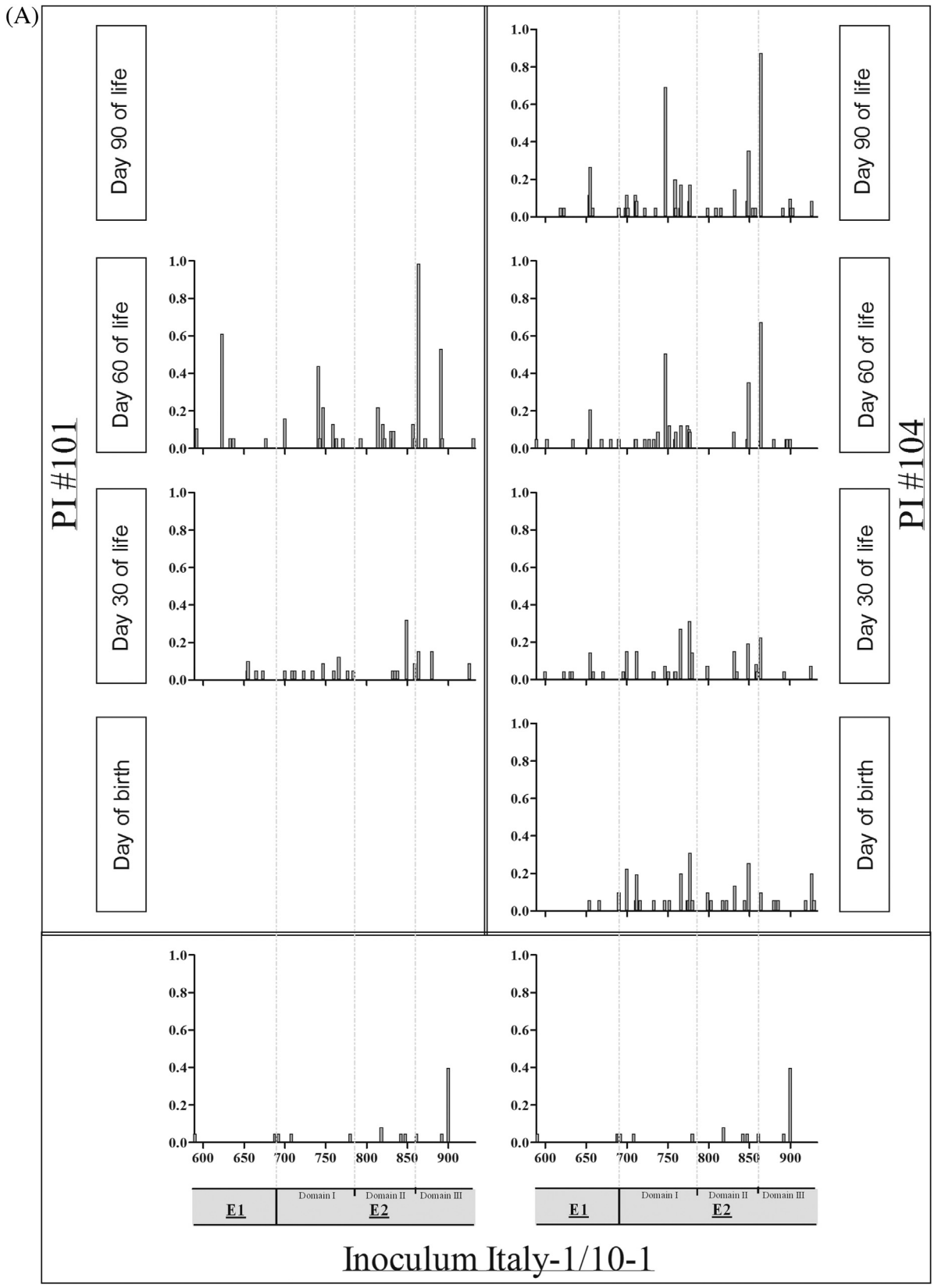

Fig. 3. Position-wise entropy of amino acid distribution in the glycoprotein E1 and E2 encoding regions (residues 589–933) for inoculum Italy-1/10-1 and persistently infected (PI) calves #101 and #104 (A), and inoculum D32/00_HoBi and PIs #102 and #103 (B) in day of birth and days 30, 60 and 90. For each sample, the entropy landscape (vertical axis) computed from error-corrected reads is shown at each position in polyprotein (horizontal axis) represented as single genomic site for glycoprotein E1 and E2 (domains I, II and III) encoding sequences. Inoculum Italy-1/10-1 used to infect the heifers that generated persistently infected (PI) calves #101 and #104, and inoculum D32/00_HoBi used to infect the ones that generated PIs #103 and #104.

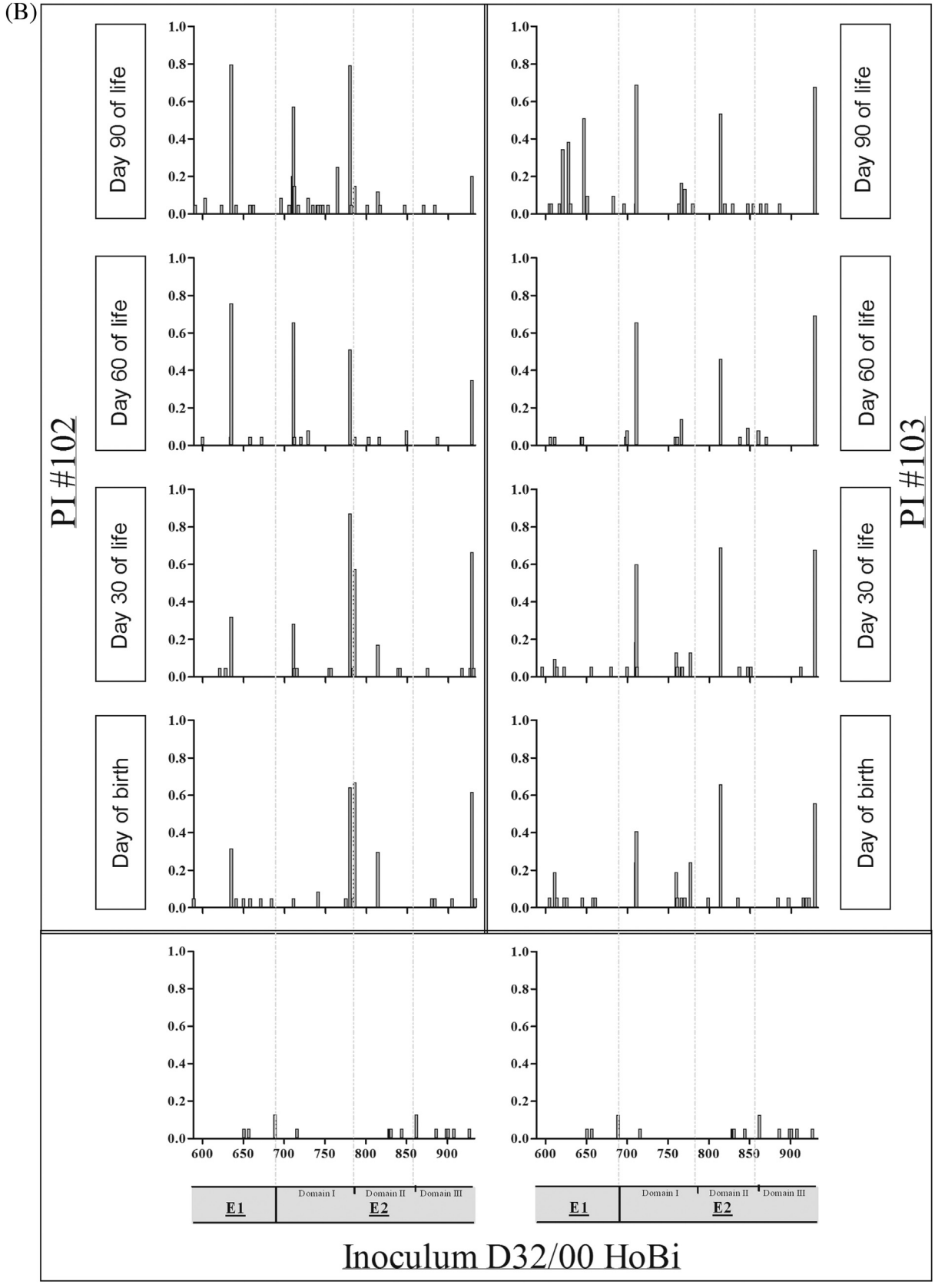

Fig. 3. (*Continued*)

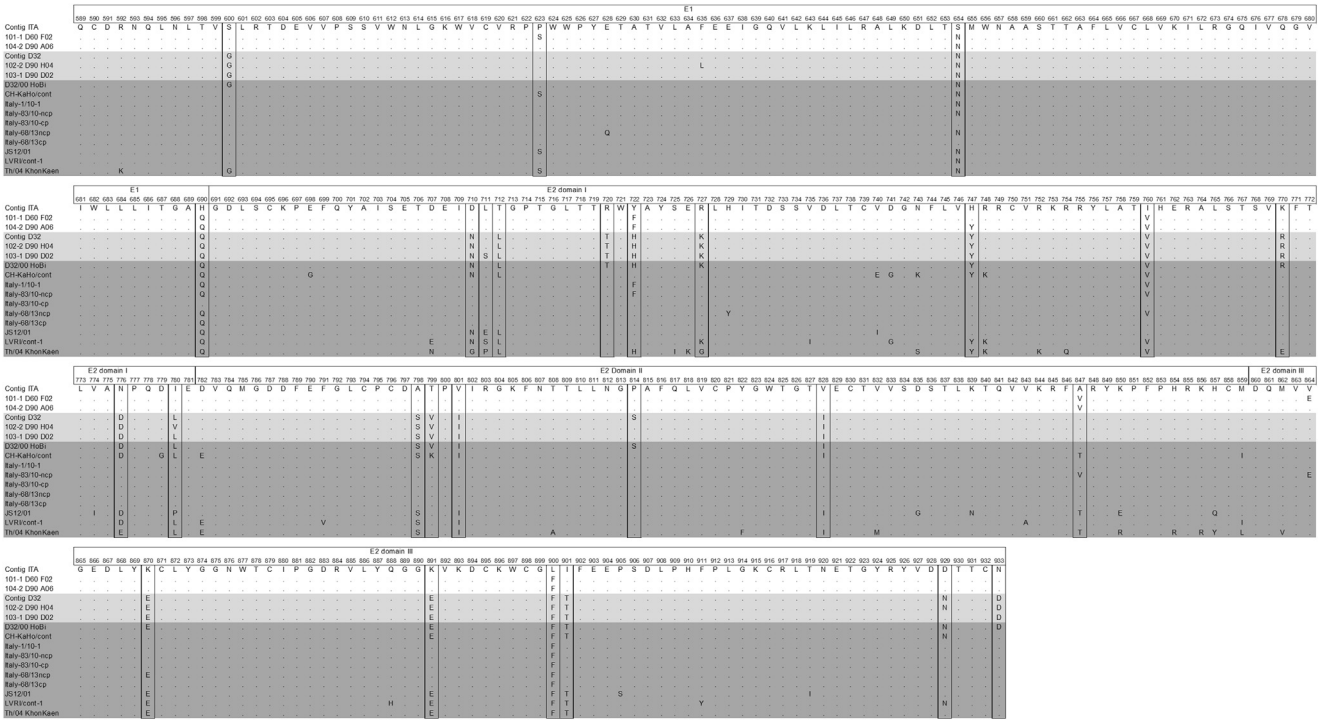

Fig. 4. Amino acid alignment from the E1/E2 region (amino acid residues 589–933) of the most prevalent viral population present in inoculums D32/00_HoBi and Italy-1/10-1 and in 'HoBi'-like PIs in the last day of serum collection compared with ten 'HoBi'-like strains. The 27 amino acid residue positions that varied between the six most prevalent viral populations generated in the present study and the representative 'HoBi-like sequences are boxed. GenBank accession numbers: D32/00_HoBi (AB871953.1), CH-KaHo/cont (JX985409.1), Italy-1/10-1 (HQ231763.1), Italy-83/10-ncp (JQ612704.1), Italy-83/10-cp (JQ612705.1), Italy-63/13ncp (KJ627179.1), Italy-63/13cp (KJ627180.1), JS12/01 (JX469119.1), LVRI/cont-1 (KC297709.1) and Th/04 KhonKaen (NC_012812.1).

strain Alfort/187 (GenBank accession number CAA61161.1). In contrast, BVDV-2 strain 890 (GenBank accession number AAA82981.1) and giraffe pestivirus strain H138 (GenBank accession number NP_620053.1) had three putative sites corresponding to positions 596, 807 and 876 of the 'HoBi'-like sequences.

4. Discussion

Major events in the biology of RNA viruses, such as their capacity to change cell tropism/host range or avoid elimination by the host immune response have their origin in quasispecies dynamics (Domingo et al., 2012). In the present study, the composition of viral quasispecies present in inoculums and in the serum samples collected at different time points from experimentally generated 'HoBi'-like PI calves were compared. One of the PIs (PI #101) presented a low viremia in two sampling points (day of birth and day 90) (Table 1) impeding further viral analyzes but demonstrated that viral load as well as quasispecies characteristics may vary over time. While PI animals never completely clear themselves of virus (MacLachlan and Dubovi, 2011), fluctuation in viremia levels can be observed (Arenhart et al., 2009; Bauermann et al., 2014; Brock et al., 1998) which can result in negative results in diagnostic assays (Bauermann et al., 2014; Fulton et al., 2009). The reason for this fluctuation is unknown but is probably dues to an interaction of virus and host factors involved in the maintenance of immunotolerance.

Two of the 'HoBi'-like PIs (#102 and #104), that were persistently infected with different 'HoBi'-like strains, had contact during a concomitant study (Bauermann et al., 2015). Superinfection of these animals, with the strain carried by the other contact PI calf, could be demonstrated in samples collected at 60 days. However, at 90 days, the quasispecies present no longer included sequences from the superinfecting virus. These calves did not develop neutralizing antibody titers detectable by the VNT applied, thus the process for clearance of the superinfecting virus probably was not based on a humoral response. It is important to highlight that innate immune response was not evaluated in the present study. One possible explanation could be the elimination of the superinfecting virus due to virus competition and exclusion (Soller and Epstein, 1965) where the virus that was the source of the fetal infection and was present during embryogenesis was better fit to the immunotolerance host than the superinfecting virus. Similar results have been observed in studies of animals persistently infected with CSFV (Muñoz-González et al., 2016). In those studies, CSFV animals were protected from infection with a secondary CSFV strain. More studies are required in order to elucidate these differences.

The direction and degree of the selective constraints operating in a coding region can be estimated by the ratio between nucleotide diversity values in non-synonymous and synonymous positions (d_{NS}/d_S ratio) (Nei and Gojobori, 1986; Nelson and Hughes, 2015; Yang and Bielanski, 2000). For the 'HoBi'-like viral populations analyzed in the present study, the analysis suggested consistent positive selection could be observed in domain I of glycoprotein E2 in all PI calves, with exception of PI #103 (Table 2). These findings were supported by the higher number of amino acid variability pressure in domain I observed in Fig. 3 and amino acid substitutions in Fig. 4 and in contrast with results obtained in a similar work performed with BVDV-2 where the more variable region in glycoprotein E2 was the domain II (Ridpath et al., 2015). E2 domains I and II are distant from the viral membrane anchor and based on putative folding are likely to be exposed on the virus surface (Li et al., 2013; Omari et al., 2013). Further, sequencing of epitope mapping suggests that these domains contain sequences coding for neutralizing epitopes (Deregt et al., 1998). Thus variability in these regions could contribute to antigenic diversity and function in driving pestivirus evolution.

Table 2
Non-synonymous (d_{NS}) and synonymous (d_S) rates per site of amino acid of E1/E2 (nucleotide residues 2132–3167) region among inoculums and 'HoBi'-like persistently infected cattle.

Sample	Day of life	E1			E2 Domain I			E2 Domain II			E2 Domain III		
		$d_{NS} \pm SEM$ ($\times 10^{-4}$)	$d_S \pm SEM$ ($\times 10^{-4}$)	d_{NS}/d_S	$d_{NS} \pm SEM$ ($\times 10^{-4}$)	$d_S \pm SEM$ ($\times 10^{-4}$)	d_{NS}/d_S	$d_{NS} \pm SEM$ ($\times 10^{-4}$)	$d_S \pm SEM$ ($\times 10^{-4}$)	d_{NS}/d_S	$d_{NS} \pm SEM$ ($\times 10^{-4}$)	$d_S \pm SEM$ ($\times 10^{-4}$)	d_{NS}/d_S
Inoculum Italy-1/2011-1		2.1 ± 1.2	13.1 ± 6.2	0.16	2.3 ± 1.4	12.2 ± 5.3	0.19	3.5 ± 2.2	3 ± 2.1	1.15	16.3 ± 14.9	9.6 ± 5.6	1.69
PI #101[a]	Birth	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	30	3.8 ± 2.1	19.3 ± 6.7	0.19	11.2 ± 3.9	10.8 ± 5.3	1.01	14.2 ± 8.5	10.2 ± 5.9	1.39	10.1 ± 5.9	3.6 ± 2.6	2.75
	60	18.2 ± 13.9	25.6 ± 9.4	0.71	25.3 ± 12.5	11.6 ± 5.6	2.19	20.6 ± 7.8	21.9 ± 10.6	0.94	55.3 ± 40.2	26.1 ± 23.2	2.12
	90	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PI #104[a]	Birth	3.4 ± 2.1	8.2 ± 4.4	0.42	30.4 ± 11.3	9.1 ± 5.4	3.32	17.4 ± 7.9	7.7 ± 5.1	2.27	13.7 ± 6.3	4.1 ± 2.3	3.32
	30	6.1 ± 2.6	9.5 ± 4.2	0.63	32.2 ± 13.3	6.4 ± 3.7	5.00	12.1 ± 6.4	10.7 ± 5.3	1.13	8.8 ± 5.8	2.8 ± 1.6	3.05
	60[c]	8.3 ± 4.5	16.9 ± 7.7	0.49	34.1 ± 16.4	10.8 ± 6.3	3.15	10.4 ± 8.8	23.9 ± 10.3	0.43	26.9 ± 21.4	25.9 ± 10.1	1.03
	90	9.7 ± 6.2	36.4 ± 10.8	0.26	51.8 ± 26.1	12.7 ± 5.6	4.06	19.4 ± 10.8	19.3 ± 7.9	1.01	39.2 ± 19.2	24.1 ± 10.7	1.62
Inoculum D32/00_HoBi		2.1 ± 1.2	12.9 ± 6.2	0.16	2.3 ± 1.4	12.2 ± 5.3	0.19	3.5 ± 2.2	3.1 ± 2.6	1.15	16.3 ± 14.9	9.6 ± 5.5	1.69
PI #102[b]	Birth	20.7 ± 15.9	32.9 ± 26.1	0.63	20.3 ± 16.1	16.1 ± 5.7	1.54	36.6 ± 29.1	13.4 ± 8.3	2.72	29.2 ± 25.4	24.8 ± 9.7	1.18
	30	17.2 ± 15.5	44.1 ± 25.6	0.39	36.2 ± 26.7	14.7 ± 6.1	2.46	28.7 ± 21.9	31.2 ± 15.9	0.92	31.7 ± 27.9	19.8 ± 8.1	1.59
	60[d]	25.6 ± 22.6	13.2 ± 8.3	1.94	40.6 ± 26.1	18.8 ± 8.5	2.15	4.4 ± 2.2	32.7 ± 18.4	0.13	12.6 ± 11.2	25.5 ± 12.1	0.49
	90	26.9 ± 21.8	24.4 ± 10.7	1.10	68.7 ± 32.8	12.9 ± 5.6	5.32	10.4 ± 4.8	32.9 ± 14.8	0.32	7.8 ± 5.8	13.9 ± 6.7	0.55
PI #103[b]	Birth	9.7 ± 4.2	27.4 ± 14.1	0.35	30.7 ± 14.8	24.3 ± 19.2	1.26	29.2 ± 26.2	7.2 ± 5.1	4.02	28.5 ± 20.5	22.9 ± 9.1	1.23
	30	5.8 ± 2.4	15.4 ± 6.9	0.38	34.3 ± 19.8	31 ± 14.7	1.10	31.4 ± 27.6	7.2 ± 4.3	4.32	29.8 ± 17.1	3.9 ± 2.8	7.65
	60	2.8 ± 1.2	14.6 ± 7.6	0.19	29.7 ± 22.4	25.6 ± 15.2	1.16	18.7 ± 15.5	12.1 ± 7.5	1.54	32.2 ± 28.9	9.6 ± 5.2	3.35
	90	39.9 ± 19.5	26.5 ± 9.9	1.51	35 ± 25.4	17.2 ± 14.9	2.03	24.1 ± 19.8	18.9 ± 9.4	1.27	32.1 ± 30.9	2 ± 10.6	1.60

ND: Not determined.
[a] 'HoBi'-like PI animal generated from a heifer infected with Italy-1/10-1.
[b] 'HoBi'-like PI animal generated from a heifer infected with D32/00_HoBi.
[c] This animal was exposed to PI #104 and presented a D32/00_HoBi-like swarm that was excluded from the present analysis.
[d] This animal was exposed to PI #104 and presented a Italy-1/10-1-like swarm that was excluded from the present analysis.

The existence of positive selection driving gene diversity is suggested by changes in the E2 domain II observed when comparing day of birth quasispecies to the quasispecies in later samples (Table 2). Virus-host adaptation and differences determined by intra-host characteristics in viral variants selection have been suggested by previous studies of BVDV-1 (Dow et al., 2015), BVDV-2 (Ridpath et al., 2015) and 'HoBi'-like virus (Weber et al., 2016a) infections. However, this is the first report of temporal analysis of viral swarm composition for pestiviruses. A shift in diversity in viral variants was observed over time in PIs generated with inoculum Italy-1/10-1 (Fig. 2A) and D32/00_HoBi (Fig. 2B).

The dominant quasispecies and the predominance of quasispecies changed in each PI. Added to this, the increase in the number of virus variants in PIs along time was observed (Fig. 3) suggesting a putative increase in mutation pressure. The same change in temporal pattern was previously reported for other RNA viruses as HIV and HCV (Canobio et al., 2004; Farci et al., 2006; Mccutchan et al., 2005) that also cause persistent and chronic infections (Carter and Saunders, 2007; MacLachlan and Dubovi, 2011). It is important to highlight that variation in viral swarms occurs over time and is dependent on host factors. While variations seen in viral swarms may represent adaptation from cell growth to growth in *in vivo*, the adaptation and variation over time was different in each animal.

One of the most common forms of protein modification is N-linked glycosylation, in which a high mannose core is attached to the amide nitrogen of asparagine in the context of the conserved motif Asn-X-Ser/Thr (Vigerust and Shepherd, 2007). Change in N-linked glycosylation sites in E1/E2 regions of CSFV were associated to virulence attenuation (Fernandez-Sainz et al., 2009; Risatti et al., 2007) and immune system evasion (Gavrilov et al., 2011). Moreover, change in *N*-glycosylation patterns are associated with entry in host cell for influenza virus (Londrigan et al., 2011). Comparison of predicted *N*-glycosylation sites (Table A.4) revealed that the majority of the 'HoBi'-like sequences had putative *N*-glycosylation sites while the strain D32/00_HoBi (GenBank accession number BAO04453.1) and the dominant quasispecies present in inoculum D32/00_HoBi had five (position 814 in 'HoBi'-like polyprotein). This fifth site was also observed in CSFV strain Alfort/187 (GenBank accession number CAA61161.1) but not in sequences from other reference pestiviruses. Viral variants containing this extra site decreased in number over time in the PIs generated with inoculum D32/00_HoBi. More studies are required to understand if the change of *N*-glycosylation sites can function in 'HoBi'-like virus virulence and evasion of immune system.

Virus population diversity is considered important in RNA virus evolution and survival and is attributed to random error in prone genome replication by the RdRP (Domingo et al., 2012; Neill, 2012). In the present study, a tendency for viral quasispecies to shift to variants containing the same amino acid residue at certain positions was noted (Fig. 4). This may be related to virus-host adaptation as glycoprotein E2 is known to determine virus tropism for pestiviruses (Liang et al., 2003). More studies are required to elucidate if intrinsic host factors and/or random error by RdRP are responsible for the differences over time.

5. Conclusion

This is the first study that looked at a temporal nature of the viral swarms within pestivirus PI animals. It was observed that genetic variability in viral mutant clouds increased along time. The lack of a putative N-linked glycosylation site in the E2 region and the change of specific residues in E1/E2 suggest an enhancement in the pestivirus adaptation to the host. Further studies are required to explain how these changes implies in pestivirus biology, virulence and immune evasion since maintenance of persistent pestivirus

infections has the potential to uncover truths about both the bovine immune system and viral evolution.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2016.09.018.

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3.6 Artigo 6: Variation in pestivirus growth in testicle primary cell culture is more dependent on the individual cell donor than cattle breed

O presente experimento já foi concluído e um artigo científico foi submetido e aceito para publicação no periódico *Veterinary Research Communications* em 08 de novembro de 2016. O artigo científico será apresentado a seguir, tal qual foi aceito para publicação.

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ORIGINAL ARTICLE

Variation in pestivirus growth in testicle primary cell culture is more dependent on the individual cell donor than cattle breed

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Abstract The causes of bovine respiratory disease complex (BRDC) are multifactorial and include infection with both viral and bacterial pathogens. Host factors are also involved as different breeds of cattle appear to have different susceptibilities to BRDC. Infection with bovine pestiviruses, including bovine viral diarrhea virus 1 (BVDV1), BVDV2 and 'HoBi'-like viruses, is linked to the development of BRDC. The aim of the present study was to compare the growth of different bovine pestiviruses in primary testicle cell cultures obtained from taurine, indicine and mixed taurine and indicine cattle breeds. Primary cells strains, derived from testicular tissue, were generated from three animals from each breed. Bovine pestivirus strains used were from BVDV-1a, BVDV-1b, BVDV-2a and 'HoBi'-like virus. Growth was compared by determining virus titers after one passage in primary cells. All tests were run in triplicate. Virus titers were determined by endpoint dilution and RT-qPCR. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by the Tukey's Multiple Comparison Test (P 0.05). Significant differences in virus growth did not correlate with cattle breed. However, significant differences were observed between cells derived from different individuals regardless of breed. Variation in the replication of virus in primary cell strains may reflect a genetic predisposition that favors virus replication.

Keywords Pestivirus · Bovine · Taurine · Indicine · Cell culture

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Introduction

Bovine viral diarrhea viruses (BVDV) are segregated into two species, BVDV-1 and BVDV-2, within the genus *Pestivirus* of the family *Flaviviridae* (Simmonds et al. 2011). Infection with an emerging species within the pestivirus genus, 'HoBi'-like viruses (Schirrmeier et al. 2004), results in clinical symptoms in cattle that are indistinguishable from those caused by BVDV-1 and BVDV-2 infections including reproductive, respiratory and digestive disorders (Decaro et al. 2011; Weber et al. 2016b). Pestiviruses can cross species barriers and infect a variety different hosts within the order Artiodactyla (Liess and Moennig 1990; Nettleton 1990; Becher et al. 1997; Krametter-Froetscher et al. 2010). However, not all pestivirus strains grow to the same efficiency in cells derived from different species (Roehe and Edwards 1994; Liang et al. 2003). This suggests that there are selective host factors involved in the control of viral growth.

Cattle belong to the *Bos taurus* species (Order Artiodactyla, Family Bovidae, Subfamily Bovinae). There are three subspecies within this species: *Bos taurus primigenius* (the extinct aurochs), *Bos taurus indicus* (indicine cattle) and *Bos taurus taurus* (taurine cattle) (Grubb 2005). While taurine cattle have greater growth rates in the absence of stressful situations, indicine exhibit

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higher heat tolerance and resistance to some internal and external parasites (Frisch and Vercoe 1984; Léger et al. 2013) and pathogens such as bovine tuberculosis (Ameni et al. 2007).

Bovine respiratory disease complex (BRDC) causes significant economic losses to beef and dairy producers worldwide. The development of BRDC is the result of the interaction of multiple factors including infection with pathogens and physiological or physical stressors. While the heritability of BRDC is low, breed differences have been observed (Snowder et al. 2005; Taylor et al. 2010). A study comparing the incidence of BRDC in several breeds of *Bos taurus* to the incidence in two different breeds of *Bos indicus* found the taurine cattle to be at a greater risk for BRDC than indicine cattle (Cusack et al. 2007). As stated above, infection with viral and/or bacterial pathogens has been associated with the development of BRDC. In particular, infection with BVDV is often suspected as an initiating event for BRDC (Fulton et al. 2000). Consequently variation in BVDV replication could translate into variation in susceptibility to BRDC.

Castration is a common practice in beef production. Primary cell strains are easily generated from the testicles that are removed during castration. Replication of virus in testicular tissue occurs during natural infections (Givens et al. 2009) and commonly used protocols for BVDV viral amplification use testicle cell lines (OIE 2008). The aim of the present study is to compare growth of different bovine pestivirus species and subtypes in testicle cell cultures obtained from different individuals of taurine, indicine and mixed taurine and indicine breeds.

Materials and methods

Virus propagation and titration

Madin-Darby bovine kidney (MDBK) cells (CCL-22; ATCC) were used to propagate and titrate three cytopathic bovine pestivirus strains: BVDV-1a strain NADL (GenBank accession number AJ133738.1), BVDV-1b strain TGAC (Z54175.1) and BVDV-2a strain 296c (AF268172.1). All three strains were isolated from US cattle suffering from mucosal disease. The cytopathic 'HoBi'-like virus strain used was a clone of Italy-1/10–1 (Decaro et al. 2011) kindly provided by Dr. Nicola Decaro (Faculty of Veterinary Medicine of Bari, Bari, Italy). This strain was cloned by limiting dilution and a clone, selected, based on faster and clearer expression of cytopathic effect, was used for the studies detailed here. The MDBK cells were grown in minimal essential medium (MEM), supplemented with L-glutamine (1.4 mM), gentamicin (50 mg/L), and 10 % fetal bovine serum (FBS). Cells were confirmed free of pestivirus based on RT-PCR, and FBS was confirmed free of pestivirus and antibodies against pestiviruses by RT-PCR and virus neutralization test (VNT), respectively (Bauermann et al. 2014b). For virus propagation, 75-cm^2 flasks containing 70 % confluent MDBK cell monolayers were inoculated with one of the four pestivirus strains and incubated at 37 °C for 72 to 96 h. Following one freeze-thaw cycle, the suspension was centrifuged for 10 min at 1000 x g. Supernatants were collected, aliquoted, and stored at −80 °C until use. The virus stocks were titrated in 96-well microtiter plates by endpoint dilution. Titers were calculated and expressed as median tissue culture infective doses ($TCID_{50}$) (Reed and Muench 1938).

Bovine testicle (BTe) cells preparation, virus inoculation and titration

Testicle were collected, following castration, from three animals each from the following breed: Angus (taurine cattle) (#B.TAURUS 314c, #B.TAURUS 352c and B.TAURUS 392c), Brahman (indicine cattle) (#B.INDICUS 524c, #B.INDICUS 528c and #B.INDICUS 578c) and mixed Nelore and Angus (mixed taurine/indicine cattle) (#MIXED 299c, #MIXED 442c and #MIXED 461). Aseptically, the epididymis and serosa were removed, and the primary BTe cell cultures were prepared (Burleson et al. 1992). Cells were verified to be free of pestivirus by RT-PCR (Bauermann et al. 2014b).

The resulting nine cell strains were passed once every four days. No differences were observed among the nine strains in the replication rate or cell morphology. At passage seven, cells were plated into a 12-well plate and, when approximately 70 % confluent, three wells were each infected with one of the four bovine pestivirus strains, at a multiplicity of infection (MOI) of 0.1 and incubated for seven days. At harvest cultures (including cell monolayer and culture fluid of 2.0 mL/well) were frozen at -80 °C for 24 h. The resulting lysates were then thawed, transferred to a centrifuge tube and centrifuged for 10 min at 1000×g. Supernatants were collected, aliquoted, and stored at −80 °C until testing. Virus titrations were performed in MDBK by endpoint dilution as described above.

RNA isolation and quantitative RT-PCR (RT-qPCR)

Total RNA was prepared using 140 μL of sample from the supernatants generated as described above. A robotic workstation (Qiacube, Qiagen, Hilden, Germany) was used for automated RNA purification by a spin-column system (QIAamp Viral RNA Mini Kit, Qiagen) according to the manufacturer recommendation. The extracted RNA was stored at −80 °C.

VetMax-Gold-bovine virus diarrhea RNA test kit (Life Technologies, Austin, TX, USA) was employed for RNA quantification. Samples were run in triplicate and each time samples were run NADL, TGAC, 296c or Italy-cp positive samples were run as controls. The 25-μL reaction mixture used for the test consisted of 12.5 μL of 2X RT-PCR buffer, 1 μL of 25X BVDV primer-probe mix, 1 μL of 25X RT-PCR enzyme mix, and 8 μL of extracted RNA. Quantification of virus RNA was

performed using a standard curve based on ten-fold serial dilutions (10^{-1} to 10^{-4}) of a positive control of known titer.

Sequencing

In order to rule out cross-contamination between infected BTe cultures, the sequence of passed virus was compared to the sequence of inoculation virus as follows. Total RNA was isolated from cell lysates, and RT-PCR was performed followed by DNA sequencing of the amplicon. Briefly, RNA isolation was performed as described above, and RT-PCR used the primers 324 and 326 (Vilcek et al. 1994). Amplification product was purified and concentrated using QIAquick® PCR Purification Kit (Qiagen) according the manufacturer's instructions followed by quantification using the Qubit® 2.0 Fluorometer for dsDNA (Life Technologies). The appropriate amount of dsDNA was labeled in both directions using Big Dye terminator chemistries (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's instructions. The labeled products were sequenced using an ABI 3100 genetic analyzer (Applied Biosystems Inc.). The sequences of all samples were confirmed by sequencing both strands in duplicate.

Statistical analysis

Correlation of virus load obtained using titration by endpoint dilution and RT-qPCR was evaluated using the Pearson's correlation coefficient. For statistical comparison, one way analysis of variance (ANOVA) followed by the Tukey's Multiple Comparison Test (MCT) were used. Growth of each viral strain in BTe cells originating from different bovine breeds, growth of different viral strains in BTe cell originating from the same breed and growth of different viral strains in the different animals from the same breed were compared using the results obtained in virus titration by endpoint dilution. *P*-values less than 0.05 were considered significant. Graphs were drawn using the GraphPad Prism 5 Software (GraphPad Software Inc., San Diego, USA).

Results

Bovine testicle cells from different individuals of taurine, indicine and mixed taurine and indicine breeds were prepared and inoculated with different bovine pestivirus species and subtypes. Based on time to reach confluency, no difference in cell growth rate was observed. Viral titration by endpoint dilution and RT-qPCR for the different animals within the taurine, indicine and mixed cattle breed BTe cells are shown in Fig. 1a and b, respectively. The results obtained by virus titration using both methods in each animal showed negative correlation using the Pearson's correlation coefficient ($r = -0.5186$; $P = 0.0012$; 95 % CI: -0.7239 to −0.2290).

ANOVA was used to evaluate differences in of virus growth between different bovine breeds, between animals within each breed group and between the different virus strains in cells from same donor. Titration by endpoint dilution showed no significant difference ($P = 0.8889$) in growth of each virus based on breed (Fig. 2a). However, there were significant differences in growth rate among animals within each breed group (Fig. 2a). Significant differences were also observed in growth rate of NADL and TGAC in cells derived from different animals within breed groups (Fig. 1a). The differences between the triplicates in each animal expressed as the standard error of the means (SEM) were generally low, and null in some cases (Fig. 1). While variation in viral growth was observed between cells derived from individual animals, there was no one individual who cells grew all viruses tested to universally higher or lower levels (Fig. 1a).

The same analyses performed using the RT-qPCR yielded similar results. No significant difference was observed when the growth of each virus in BTe cells originated from the different bovine breeds when measured by RT-qPCR ($P = 0.2618$) (Fig. 2b). As observed in the statistical analysis with the endpoint dilution, there were significant differences for the viral growth rate between animals within the breed groups (Fig. 2b) and between strains NADL and TGAC in different individuals within same bovine breeds (Fig. 1b).

DNA sequencing confirmed that the virus tittered in BTe cell cultures matched the inoculation virus confirming absence of cross contamination.

Discussion

In the present study, cattle ($n = 3$) of taurine, indicine or taurine/indicine cross breed, of similar age and from the same location were castrated and their testicles used to prepare primary cell cultures. These primary cell cultures were then inoculated with the different pestiviral strains and viral replication compared. The virus titers revealed differences in viral replication between individuals, but that these differences are not associated with breed. Previously differences in the viral load have also been reported in persistently infected (PI) cattle generated using the same pestivirus strain (Bauermann et al. 2014a) and that viral titer can vary over time (Arenhart et al. 2009). Variation has also been observed in the width and breath of the viral swarms circulating in persistently infected cattle (Ridpath et al. 2015; Weber et al. 2016a; Weber et al. 2017). Cattle infected in the same outbreak can have greatly different outcomes (Carman et al. 1998). It has been proposed

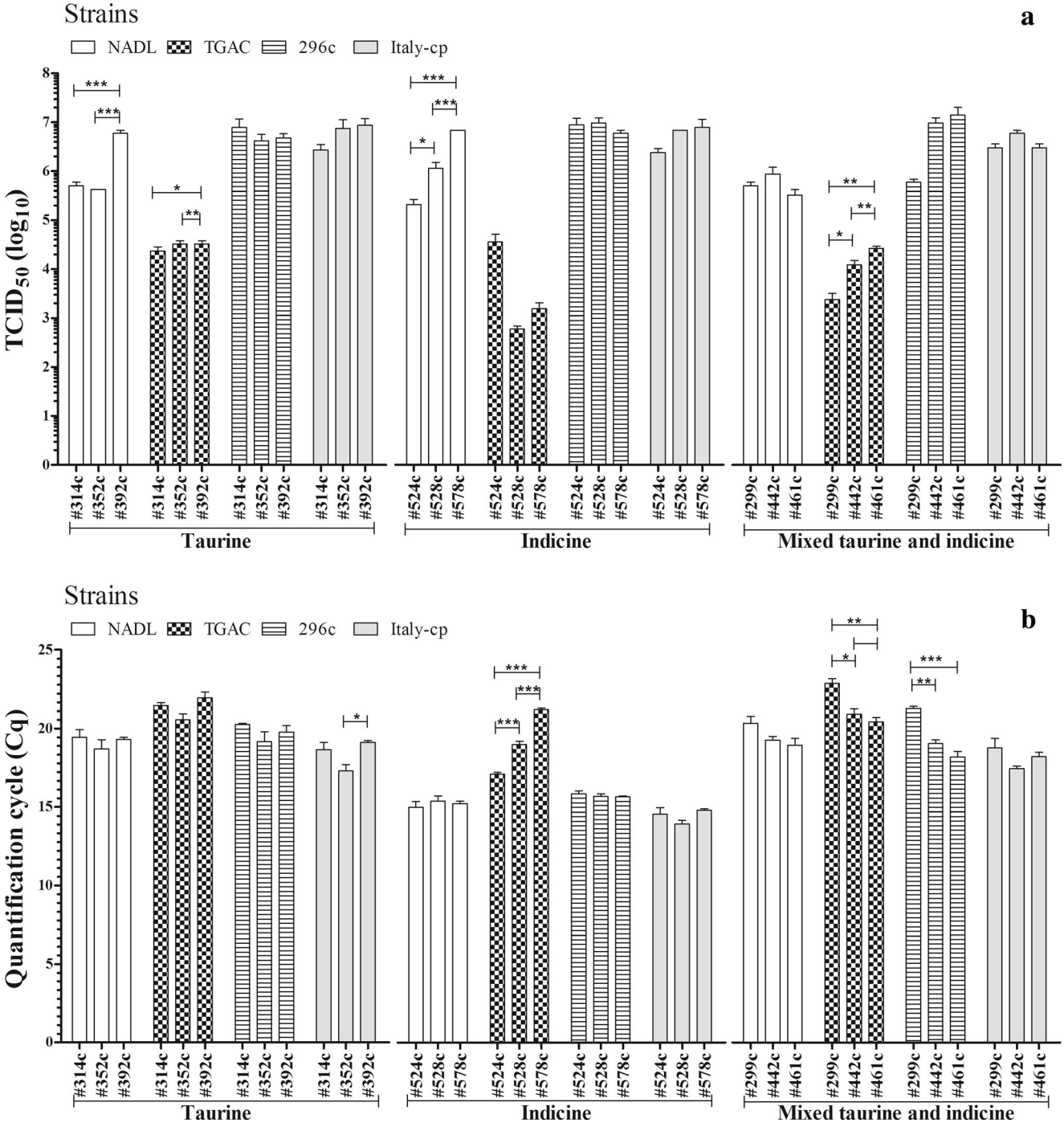

Fig. 1 Virus load (*vertical axis*) obtained by endpoint dilution (A) and RT-qPCR (B) of bovine testicle (BTe) cells obtained from different taurine, indicine and mixed breed cattle infected with different bovine pestivirus strains (*horizontal axis*). All assays were performed in triplicate. Data are presented as means with standard errors indicated that resulted for the triplicate combination of each individual cell donor. Comparisons of the growth of different viruses in the different animals from same breed were made by ANOVA, followed by Tukey's MCT. **P* 0.05, ***P* 0.01, ****P* 0.001

that these differences are associated with host factors, particularly host factors that control immune response (Peterhans and Schweizer 2013; Lussi and Schweizer 2016). Immune response was not a factor in the in vitro system used in the present study, thus variation observed in viral replication are due to host factors that directly impact viral uptake, replication, assembly or release.

Previously published research has indicated that pestiviruses replicate at different rates in host cell from different species (Liess and Moennig 1990; Roehe and Edwards 1994; Liang

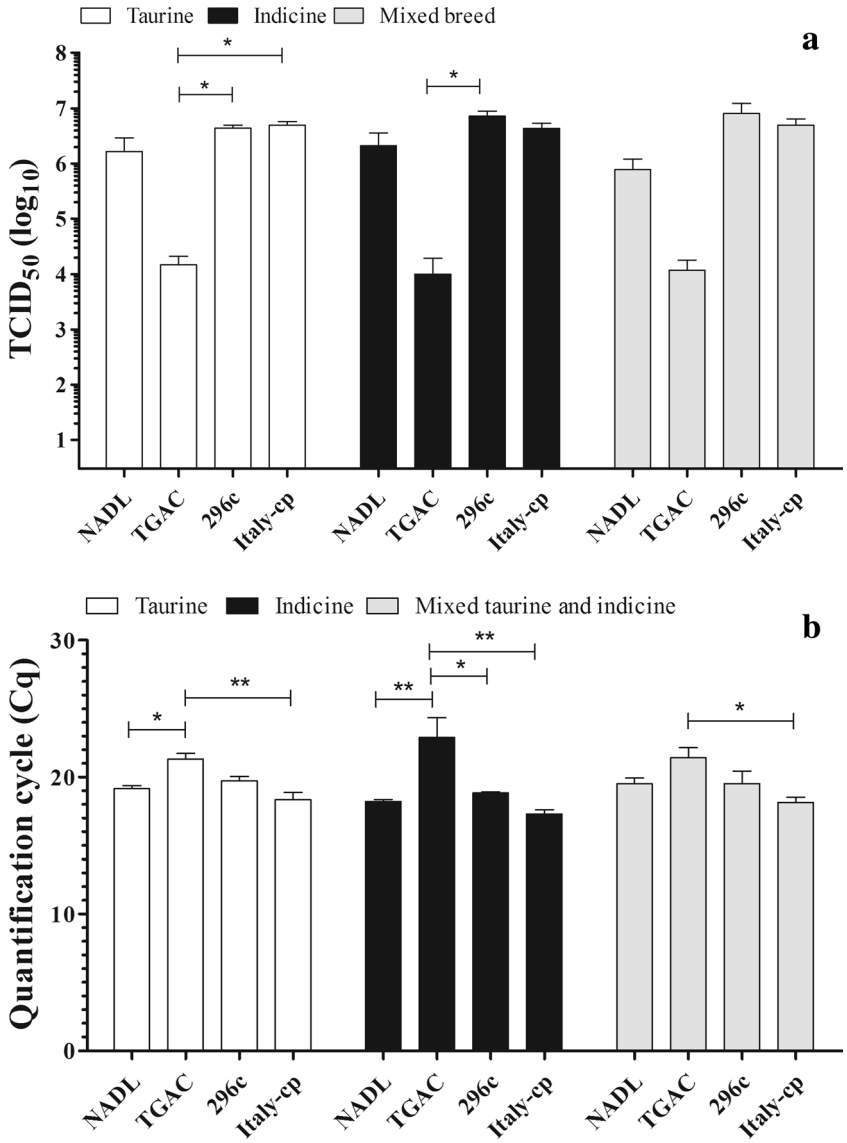

Fig. 2 Bovine pestiviruses titer in bovine testicle (BTe) cells obtained from taurine, indicine and mixed cattle breeds obtained by endpoint dilution (A) and RT-qPCR (B). Vertical axis represents the mean of the virus titers obtained by the triplicate of the three different animals that compose each breed group. Horizontal axis represents the pestivirus strain used to infect each BTe cell group. Data represents means ± standard errors that resulted for the combination of all the triplicates of individuals within each cattle breed. Comparisons of the growth of different viruses in the different animals from same breed were made by ANOVA, followed by Tukey's MCT. **P* 0.05, ***P* 0.01, ****P* 0.001

et al. 2003). In this study, differences in replication were not associated with differences in host subspecies (taurine and indicine cattle). However, differences in viral replication were observed in cells derived from different animals belonging to the same breed, implying that individual animal traits may impact on viral replication. These findings are supported by the low SEM observed in the triplicates in two different tests from each individual within same cattle breed. The virus strains used in this study did not universally grow to higher titers in any one of the cell strains derived from the individual animals. Instead, individual viral strains grew to different titers in cells derived from different animals. Thus, not only are host factors involved but there is an interplay between viral and host factors. Clinical presentation observed in both acute and persistent BVDV infections are highly variable. The results of the current study suggest that viral strain and host factors may both play a part in viral replication and could lead to differences in clinical presentation.

Bovine pestiviruses are able to infected and induce disease in swine and ruminant species other than cattle (Nettleton 1990; Decaro et al. 2012; Tao et al. 2013; Bauermann et al. 2015; Wolff et al. 2016). The present study lacks the evaluation of the viral growth on testicular cells of other animal species that can allow comparison of in vitro observations with in vivo health outcomes. This evaluation can be the topic of future studies.

It is important to note that while the present work reveals differences between individuals in viral growth it does not reveal the reasons/factors behind them. Further study is needed in order to elucidate the mechanisms of adaptation of pestiviruses in different animals to the better understand the host and viral mechanisms involved. However, the model developed in this study, which employs cell strains generated

from the testicular tissue of castrated cattle, would allow comparison of in vitro observations with in vivo health outcomes during beef production.

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Compliance with ethical standards

Conflict of interest The authors whose names are listed below certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria, educational grants, participation in speakers' bureaus, membership employment, consultancies, stock ownership, or other equity interest, and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript. We have no conflict of interest.

Statement on the welfare of animals All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed consent Informed consent was obtained from all individual participants included in the study.

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4 CONCLUSÕES

Resumidamente, os seis trabalhos que compõem a presente tese de doutorado levaram às seguintes conclusões descritas abaixo:

A) O BVDV-1a e o BVDV-2b foram os pestivírus mais frequentes em bovinos no Rio Grande do Sul utilizando amostragem coletada pelo serviço veterinário oficial no ano de 2010;

B) O Rio Grande do Sul apresenta alta frequência de BVDV-2 quando comparado a outras regiões no mundo;

C) Foi relatada a primeira evidencia molecular da ocorrência de infecção pelo BVDV em javalis reportadas na literatura, indicando uma possível importância desta espécie animal na epidemiologia de pestivírus de ruminantes;

D) Três prováveis novos eventos de recombinação homóloga foram reladas em vírus do gênero *Pestivirus*, mostrando a primeira evidência de ocorrência desse evento em BVDV-2;

E) A análise de variantes virais intrahospedeiro (quasispécies) foi realizada pela primeira vez em vírus ‘HoBi’-*like*, onde foram observados que fatores individuais do hospedeiro são importantes na emergência e complexidade de variantes virais;

F) Quando as variantes virais em animais PI foram analisadas temporalmente, foi possível observar que a nuvem de mutantes aumento com o passar do tempo, além de que a perda de um sítio de glicosilação N-ligada pode significar a adaptação do vírus ao hospedeiro *in vivo*;

G) A multiplicação de cepas de BVDV-1a e 1b, BVDV-2a e vírus ‘HoBi’-*like* foram avaliadas *in vitro* em células de cultivo primário obtidas de gado europeu, zebuíno e raça mista e comparadas utilizando modelos estatísticos, onde foi possível a observação de ausência de diferenças de crescimento viral entre as raças, mas presença de diferença entre indivíduos da mesma raça.

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ANEXOS

Anexo A – Material suplementar referente ao Artigo 3

Table A.1

Complete genome sequences of pestiviruses available in GenBank on 13th June 2014 and analyzed in the present study.

Species/Subgroup	Strain	GenBank accession number	Year of isolation	Country / Region
BVDV-1	NADLncp	NC_001461	1963	USA
BVDV-1	NADLncp	AJ133739.1	1963	USA
BVDV-1	Oregon C24V	AF091605.1	1960	USA
BVDV-1	Singer_Arg	DQ088995.2	2006	Argentina
BVDV-1	SD1	M96751.1	1992	USA
BVDV-1	VEDEVAC	AJ585412.1	ND	Hungary
BVDV-1	KE9	EF101530.1	2007	Germany
BVDV-1	IBSP4ncp	KJ620017.1	ND	Brazil
BVDV-1	GX4	KJ689448.1	2012	China
BVDV-1	CP7	U63479.1	1985	USA
BVDV-1	12F004	KC963967.1	2012	South Korea
BVDV-1	3156	JN704144.1	ND	China
BVDV-1	GS5	KJ541471.1	2013	China
BVDV-1	CC13B	KF772785.1	2013	China
BVDV-1	Osloss	M96687.1	1967	Germany
BVDV-1	Bega-like	KF896608.1	1989	Australia
BVDV-1	10JJ-SKR	KC757383.1	2010	South Korea
BVDV-1	KS86-1ncp	AB078950.1	1986	Japan
BVDV-1	Suwancp	KC853440.1	1993	Switzerland
BVDV-1	ZM-95	AF526381.3	1995	China
BVDV-1	SD0803	JN400273.1	2008	China
BVDV-1	Camel-6	KC695810.1	2010	China
BVDV-1	ILLNC	U86600.1	1991	USA
BVDV-2	890	U18059.1	1990	USA
BVDV-2	New York'93	AF502399.1	1993	USA
BVDV-2	XJ-04	FJ527854.1	2004	China
BVDV-2	HLJ-10	JF714967.1	2011	China
BVDV-2	SH-28	HQ258810.1	2009	China
BVDV-2	P11Q	AY149215.1	ND	Canada
BVDV-2	11F011	KC963968.1	2011	South Korea
BVDV-2	C413	NC_002032.1	1997	USA
BVDV-2	Hokudai Lab/09	AB567658.1	2009	Japan

BVDV-2	SD1301	KJ000672.1	2012	China
BVDV-2	JZ05-1	GQ888686.2	2005	China
BVDV-2	VOE 4407	HG426495.1	2007	Germany
BVDV-2	SH2210-23	HG426494.1	2010	Germany
BVDV-2	SH2210-17	HG426493.1	2010	Germany
BVDV-2	SH2210-14	HG426492.1	2010	Germany
BVDV-2	Potsdam 1600	HG426491.1	2000	Germany
BVDV-2	NRW_19-13-8_Dup(-)	HG426489.1	2013	Germany
BVDV-2	NRW_19-13-8_Dup(+)	HG426490.1	2013	Germany
BVDV-2	NRW_19-13-1_Dup(+)	HG426488.1	2013	Germany
BVDV-2	NRW_19-13-1_Dup(-)	HG426487.1	2013	Germany
BVDV-2	NRW_14-13_Dup(+)	HG426486.1	2013	Germany
BVDV-2	NRW_14-13_Dup(-)	HG426485.1	2013	Germany
BVDV-2	NRW_12-13_Dup(+)	HG426484.1	2013	Germany
BVDV-2	NRW_12-13_Dup(-)	HG426483.1	2013	Germany
BVDV-2	D75-13-609_Dup(+)	HG426482.1	2013	Germany
BVDV-2	D75-13-609_Dup(-)	HG426481.1	2013	Germany
BVDV-2	D37-13-2_Dup(+)	HG426480.1	2013	Germany
BVDV-2	D37-13-2_Dup(-)	HG426479.1	2013	Germany
BDV	X818	NC_003679.1	1987	Australia
BDV	BD31	U70263.1	1978	USA
BDV	Coos Bay 5 c	KJ463423.1	1980	USA
BDV	Coos Bay 5 nc	KJ463422.1	1980	USA
BDV	JSLS12-01	KC963426.1	2012	China
BDV	Reindeer	AF144618.2	1996	Germany
BDV	Gifhorn	KF925348.1	1999	Germany
BDV	Chamois-1	GU270877.1	2001	Andorra
BDV	Aveyron	KF918753.1	1984	France
CSFV	Alfort/187	X87939.1	1987	France
CSFV	Riems	AY259122.1	ND	Vaccine
CSFV	ALD	D49532.1	ND	Japan
CSFV	Eystrup	AF326963.1	1964	Germany
CSFV	HCLV	AF091507.1	1999	China
CSFV	Koslov	HM237795.1	ND	Czech Republic
CSFV	Thiverval	EU490425.1	ND	France
CSFV	Brescia	M31768.1	ND	ND
CSFV	Paderborn 09	GQ902941.1	2009	Denmark
CSFV	96TD	AY554397.1	1996	Taiwan
CSFV	PC11WB	KC149991.1	2011	South Korea
CSFV	YC11WB	KC149990.1	2011	South Korea
CSFV	HNLY-2011	JX262391.1	2011	China
CSFV	HNSD-2012	JX218094.1	2012	China
CSFV	Bergen	KJ619377.1	1977	Netherlands
CSFV	Roesrath	GU233734.1	2009	Germany
CSFV	Borken	GU233731.1	2006	Germany

CSFV	94.4/IL/94/TWN	AY646427	1994	Taiwan
CSFV	39	AF407339.1	2001	China
CSFV	Glentorf	U45478.1	ND	ND
CSFV	CAP	X96550.1	ND	ND
CSFV	cF114	AF333000.1	ND	ND
CSFV	JL1(06)	EU497410.1	2006	China
CSFV	India	EU857642.1	ND	India
CSFV	Sp01	FJ265020.1	2001	Spain
CSFV	LOM	EU789580.1	1980	Japan
CSFV	Zj0801	FJ529205.1	2008	China
CSFV	SXYL2006	GQ122383.1	2006	China
CSFV	SXCDK	GQ923951.1	2009	China
CSFV	HEBZ	GU592790.1	2009	China
CSFV	Euskirchen	GU233732.1	2005	Germany
CSFV	Hennef	GU233733.1	2009	Germany
CSFV	Uelzen	GU324242.1	2004	Germany
CSFV	C-ZJ-2008	HM175885.1	2008	China
CSFV	GZ-2009	HQ380231.1	2009	China
CSFV	Novska	HQ148061.1	2002	Croatia
CSFV	Jambul	HQ148062.1	2007	Bulgaria
CSFV	Penevezys	HQ148063.1	2009	Lithuania
CSFV	GXWZ02	AY367767.1	ND	China
CSFV	Heb52010	JQ268754.1	2010	China
CSFV	0406/CH/01/TWN	AY568569.1	2001	Taiwan
CSFV	AY663656	AY663656.1	ND	China
CSFV	BRESCIAX	AY578687.1	2001	USA
CSFV	RUCSFPLUM	AY578688.1	2001	USA
CSFV	PK15C-NG79-11	KC503764.1	2011	India
CSFV	C/HVRI	AY805221.1	ND	China
CSFV	Shimen/HVRI	AY775178.2	ND	China
CSFV	IND/UK/LAL-290	KC851953.1	2012	India
CSFV	Alfort/Tuebingen	J04358.2	ND	Germany
CSFV	SWH	DQ127910.1	ND	China
Giraffe pestivirus	H120	NC_003678.1	1967	Kenya
Giraffe pestivirus	PG-2	KJ660072.1	1995	Africa
‘HoBi'-like	DS32/00_'HoBi'	AB871953.1	2004	Brazil
‘HoBi'-like	CH-KaHo/cont	JX985409.1	2000	South America
‘HoBi'-like	JS12/01	JX469119.1	2012	China
‘HoBi'-like	LVRI/cont-1	KC297709.1	2012	South America
‘HoBi'-like	Italy-1/10-1	HQ231763.1	2010	Italy
‘HoBi'-like	Italy-129/07	KC788748.1	2007	Italy
‘HoBi'-like	Italy-68/13cp	KJ627180.1	2013	Italy
‘HoBi'-like	Italy-68/13ncp	KJ627179.1	2013	Italy
‘HoBi'-like	Italy-83/10-cp	JQ612705.1	2010	Italy
‘HoBi'-like	Italy-83/10-ncp	JQ612704.1	2010	Italy

'HoBi'-like	Th/04_khonKaen	NC_012812.1	2004	Thailand
Turkey isolates	Aydin/04-TR	NC_018713.1	2004	Turkey
Pronghorn virus	Antilope	AY781152.3	2000	USA
Bungowannah virus	Bungowannah	EF100713.2	2003	Australia

ND: Not determined.

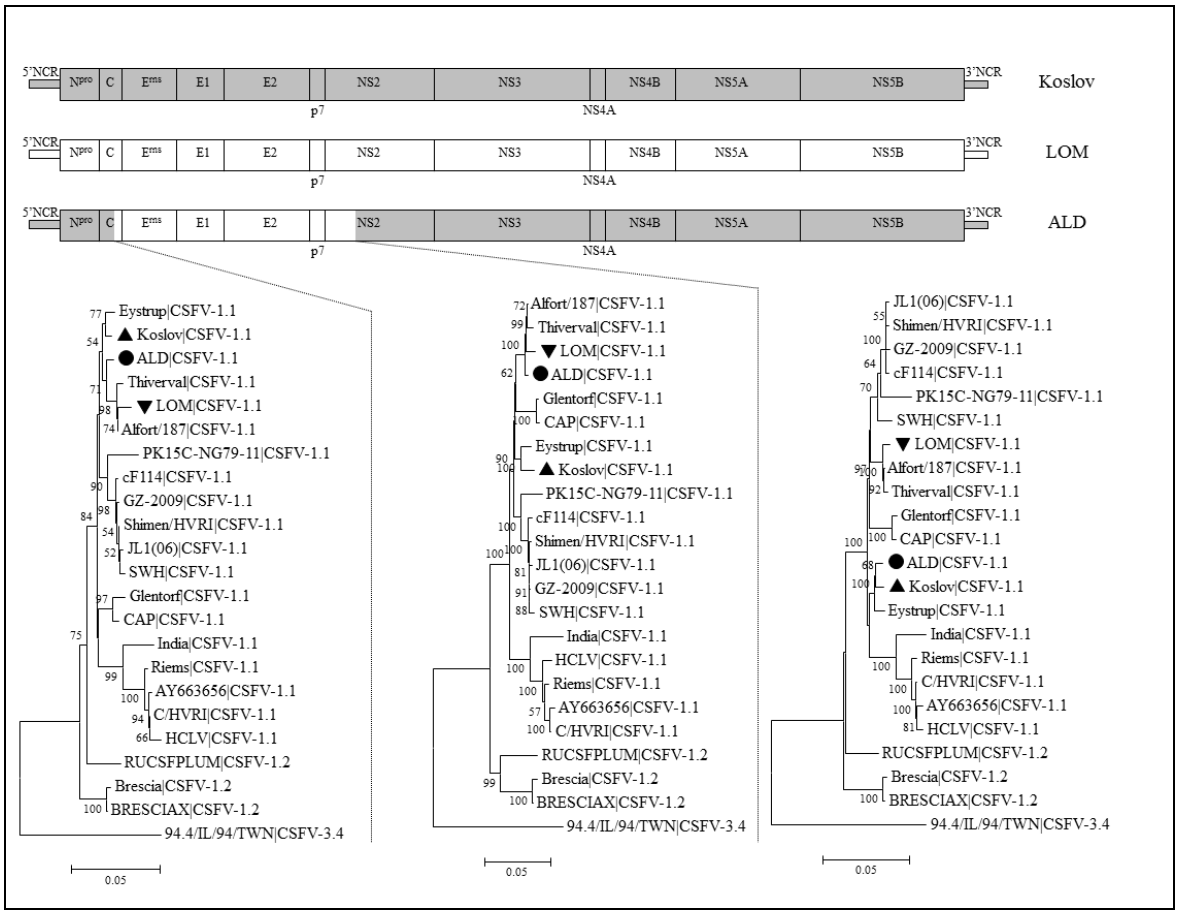

Figure A.1

Schematic representation of the potential recombination event in CSFV strain ALD. The genome organization of the potential recombinant viruses, with the breakpoints and segments of the genome derived from the major and minor parents, are schematically represented. The relationship between the putative recombinant (●) and major (▲) and minor (▼) parents in the different segments of the genome is shown in the neighbor-joining, rectangular trees, where bootstrap values ≥50% are represented.

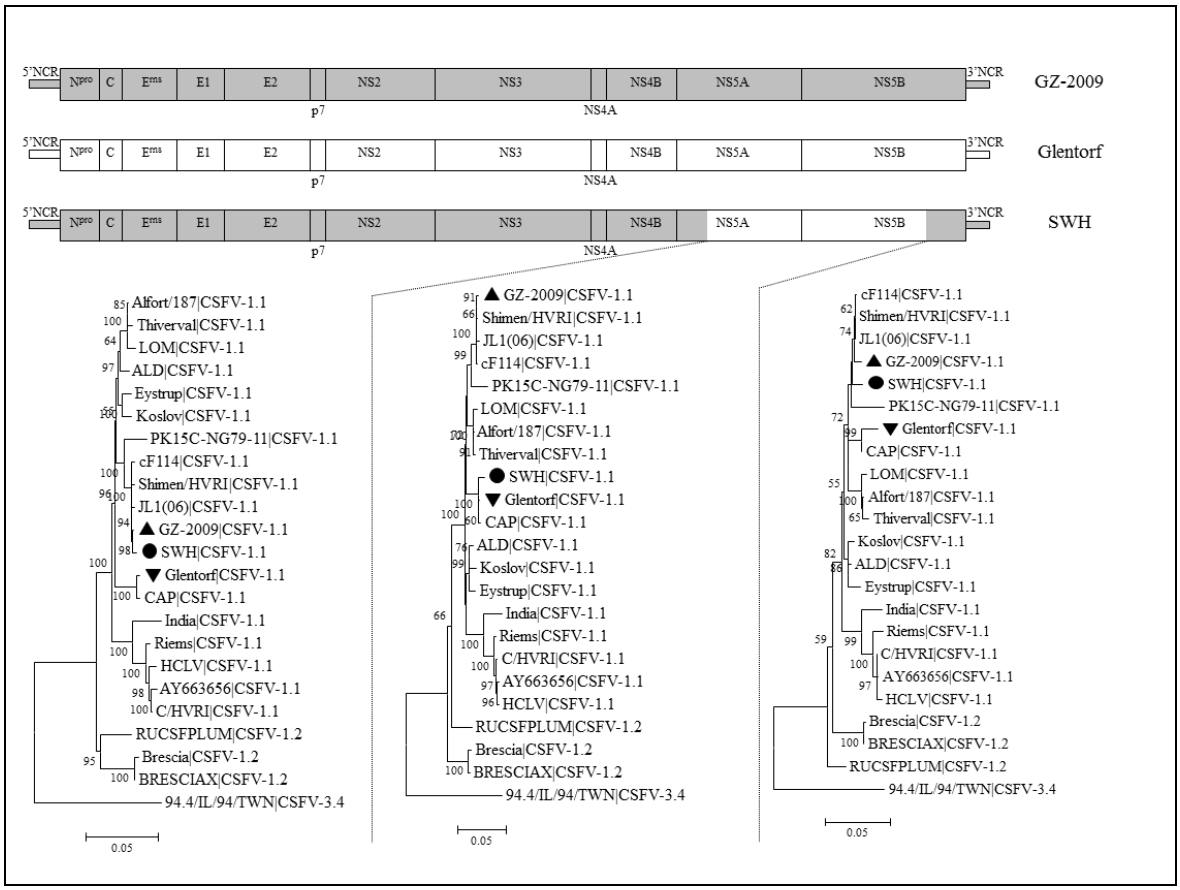

Figure A.2

Schematic representation of the potential recombination event in CSFV strain SWH. The genome organization of the potential recombinant viruses, with the breakpoints and the segments of the genome derived from the major and minor parents, are schematically represented. The relationship between the putative recombinant (●) and the major (▲) and minor (▼) parents in the different segments of the genome is shown in the neighbor-joining, rectangular trees, where bootstrap values ≥50% are represented.

Anexo B – Material suplementar referente ao Artigo 4

Table A.1

Percentage of viral populations detected in strain Italy-1/2011-1 and PIs 101 and 104 that were generated for heifers infected with this inoculum. The dominant quasispecies in inoculum Italy-1/2011-1 was named as “Contig ITA” respectively, and the other samples named according the amino acid substitution and position in the polyprotein compared with the dominant quasispecie in the respective inoculum.

Sequence	Inoculum	101	104
Contig ITA	79,03%	0,00%	0,00%
F900	11,29%	0,00%	0,00%
N654; Q690; F722; V760; V847; F900	0,00%	3,54%	3,50%
N654; Q690; F722; V760; V847; E864; F900	0,00%	71,68%	69,93%
N654; Q690; F722; Y747; V760; V847; F900	0,00%	0,00%	0,00%
N654; Q690; F722; Y747; V760; V847; E864; F900	0,00%	1,77%	0,00%
N654; Q690; F722; V760; V847; H849; E864; F900	0,00%	5,31%	2,10%
N654; Q690; F722; V760; V847; E864; V880; F900	0,00%	2,65%	0,00%
N654; Q690; L700; F722; V760; V847; K864; F900	0,00%	0,00%	0,00%
A623; N654; Q690; F722; V760; V847; K864; F900	0,00%	0,00%	0,00%
N654; V655; Q690; F722; Y747; V760; V847; E864; F900	0,00%	0,00%	0,00%
S623; N654; Q690; F722; V760; V847; K864; F900	0,00%	0,00%	0,00%
S623; N654; Q690; F722; V760; V847; K864; T891; F900	0,00%	0,00%	0,00%
S623; N654; Q690; F722; N741; V760; V847; K864; F900	0,00%	0,00%	0,00%
S623; N654; Q690; F722; V760; V847; E864; F900	0,00%	0,00%	0,00%
I892	0,81%	0,00%	0,00%
R688	0,81%	0,00%	0,00%
W590	0,81%	0,00%	0,00%
R861	0,81%	0,00%	0,00%
E692	0,81%	0,00%	0,00%
F818	1,61%	0,00%	0,00%
V847	0,81%	0,00%	0,00%
Y690	0,81%	0,00%	0,00%
A842; F900	0,81%	0,00%	0,00%
A708; F900	0,81%	0,00%	0,00%
M780; F900	0,81%	0,00%	0,00%
N654; Q690; F722; V760; V847; E864; F900; H926	0,00%	1,77%	0,70%
N654; Q690; F722; V760; R784; V847; E864; F900	0,00%	0,88%	0,00%
N654; M665; Q690; F722; V760; V847; E864; F900	0,00%	0,88%	0,00%
N654; Q690; V709; F722; V760; V847; E864; F900	0,00%	0,88%	0,00%
N654; I655; Q690; F722; V760; V847; E864; F900	0,00%	0,88%	0,00%
N654; V655; Q690; F722; V760; V847; E864; F900	0,00%	0,88%	1,40%
N654; Q690; F722; V760; V847; N849; E864; F900	0,00%	1,77%	0,70%
N654; K673; Q690; F722; V760; V847; H849; E864; F900	0,00%	0,88%	0,00%

N654; Q690; F722; V760; P766; V847; E864; F900	0,00%	1,77%	0,00%
N654; Q690; F722; V760; P766; S777; I832; V847; E864; F900	0,00%	0,88%	0,00%
N654; Q690; F722; V760; V847; T859; E864; F900	0,00%	1,77%	0,70%
N654; Q690; L700; L712; F722; T723; T734; L760; Y835; R838; V847; E864; V880; F900	0,00%	0,88%	0,00%
Q690; F722; V760; V847; E864; F900	0,00%	0,88%	0,00%
N654; T659; Q690; F722; V760; V847; E864; F900	0,00%	0,00%	0,70%
N654; T671; Q690; F722; V760; V847; E864; F900	0,00%	0,00%	0,70%
N654; Q690; F722; A751; V760; V847; E864; F900	0,00%	0,00%	0,70%
N654; Q690; F722; V760; V847; E864; R893; F900	0,00%	0,00%	0,70%
N654; Q690; F722; V760; V847; H860; E864; F900	0,00%	0,00%	0,70%
N654; Q690; F722; Y747; A759; V760; V847; H849; E864; F900	0,00%	0,00%	0,70%
P633; N654; Q690; F722; Y747; V760; V847; E864; F900	0,00%	0,00%	0,70%
G600; N654; Q690; F722; V760; V847; H849; E864; F900	0,00%	0,00%	0,70%
N654; Q690; F722; V760; V847; V859; E864; F900	0,00%	0,00%	0,70%
N654; K655; Q690; F722; V760; V847; E864; F900; H926	0,00%	0,00%	0,70%
N654; Q690; R700; L712; F722; V760; Y835; V847; E864; F900	0,00%	0,00%	0,70%
N654; Q690; F722; V760; S777; V847; E864; F900	0,00%	0,00%	0,70%
S623; P631; N654; Q690; F722; V760; Q777; V847; E864; F900	0,00%	0,00%	0,70%
N654; Q690; F722; V760; P766; S777; V847; E864; F900	0,00%	0,00%	2,80%
N654; V655; Q690; F722; F760; P766; S777; K799; I832; V857; E864; F900	0,00%	0,00%	0,70%
N654; Q690; F722; F760; P766; S777; I832; V857; E864; P900	0,00%	0,00%	0,70%
N654; Q690; E696; R700; L712; F722; F760; P766; S777; V857; E864; F900	0,00%	0,00%	0,70%
N654; Q690; R700; L712; F722; F760; P765; S777; I832; V857; E864; F900	0,00%	0,00%	1,40%
N654; Q690; R700; L712; F722; A760; P766; S777; K799; I832; V857; E864; F900	0,00%	0,00%	0,70%
N654; Q690; F722; V760; T780; V847; E864; F900	0,00%	0,00%	0,70%
N654; Q690; F722; V760; M780; V847; E864; F900	0,00%	0,00%	2,10%

Table A.2

Percentage of viral populations detected in strain D32/00_HoBi and PIs 102 and 103 that were generated for heifers infected with this inoculum. The dominant quasispecies in inoculum D32/00_HoBi was named as “Contig D32” respectively, and the other samples named according the amino acid substitution and position in the polyprotein compared with the dominant quasispecie in the respective inoculum.

Sequence	**Inoculum**	**102**	**103**
Contig D32	86,11%	0,00%	36,54%
P814	0,00%	3,28%	10,58%
D929	0,00%	0,00%	4,81%
K862	2,78%	0,00%	0,00%
S711; P814	0,00%	0,00%	0,96%
P814; D929	0,00%	1,64%	8,66%
L635; P814	0,00%	14,75%	0,00%
V635; P814	0,00%	0,00%	0,00%
L635; S711; P814	0,00%	4,92%	0,00%
L635; V780; P814	0,00%	4,92%	0,00%
L635; P814; D929	0,00%	10,66%	0,00%
S711; P814; D929	0,00%	0,00%	21,15%
L635; E786; P814; D929	0,00%	17,21%	0,00%
L635; V780; P814; D929	0,00%	13,93%	0,00%
L635; I780; P814; D929	0,00%	4,92%	0,00%
G829; P831	0,93%	0,00%	0,00%
M927	0,93%	0,00%	0,00%
S908	0,93%	0,00%	0,00%
R901	0,93%	0,00%	0,00%
Y651	0,93%	0,00%	0,00%
R844	0,93%	0,00%	0,00%
S716; S830	0,93%	0,00%	0,00%
S899	0,93%	0,00%	0,00%
S657	0,93%	0,00%	0,00%
V959	1,85%	0,00%	0,00%
V689; P887	0,93%	0,00%	0,00%
L635; E786; P814; A927; D929	0,00%	0,82%	0,00%
L635; E786; P814; D927; P931	0,00%	0,82%	0,00%
L635; P784; E786; P814; D929	0,00%	0,82%	0,00%
L635; V780; E786; P814; D929	0,00%	2,46%	0,00%
L635; I780; E786; P814; D930	0,00%	0,82%	0,00%
L635; K755; P814; D929	0,00%	0,82%	0,00%
L635	0,00%	1,64%	0,00%
L635; V780	0,00%	0,82%	0,00%
L635; I780	0,00%	0,82%	0,00%
L635; Q712; D929	0,00%	0,82%	0,00%

L635; I780; P814	0,00%	1,64%	0,00%
G621; L635; V780; E786; P814; K917	0,00%	0,82%	0,00%
L635; I780; E786; P814	0,00%	0,82%	0,00%
L635; S711; I780; P814	0,00%	0,82%	0,00%
L635; S711; E780; P815	0,00%	0,82%	0,00%
L635; P814; R841	0,00%	0,82%	0,00%
L635; S711; P814; D929	0,00%	0,82%	0,00%
L635; S711; V780; P814; D929	0,00%	0,82%	0,00%
L635; P757; V780; P814; D929	0,00%	0,82%	0,00%
V780; E786; P814; D929	0,00%	0,82%	0,00%
V780; P814; D930	0,00%	1,64%	0,00%
I715; P814	0,00%	0,82%	0,00%
Q628; P814	0,00%	0,82%	0,00%
P814; E839, D929	0,00%	0,82%	0,00%
I680; S711; P814; D929	0,00%	0,00%	0,96%
D596; S711; P814; D929	0,00%	0,00%	0,96%
S711; D929	0,00%	0,00%	2,88%
S711; P814; S912	0,00%	0,00%	0,96%
Y766; P814; D929	0,00%	0,00%	0,96%
R656; D929	0,00%	0,00%	0,96%
P814; L851	0,00%	0,00%	0,96%
R656; D929	0,00%	0,00%	0,96%
S711	0,00%	0,00%	0,96%
P712	0,00%	0,00%	0,96%
A767	0,00%	0,00%	0,96%
T847	0,00%	0,00%	0,96%
F614; S623; D710;S777; I837; Ins R839	0,00%	0,00%	0,96%
S710; L760; S777	0,00%	0,00%	0,96%
I611; L700; S710; L760	0,00%	0,00%	0,96%
I611; S710; L760; S777	0,00%	0,00%	0,96%

Anexo C – Material suplementar referente ao Artigo 5

Table A.1

Percentage of viral populations detected in inoculum Italy-1/10-1 and PIs #101 and #104 in day of birth and days 30, 60 and 90 of life that were generated for heifers infected with this inoculum. The dominant quasispecies in inoculum Italy-1/10-1 was named as "Contig ITA" respectively, and the other samples named according the amino acid substitution and position in the polyprotein compared with the dominant quasispecie in the respective inoculum.

Sequence	**Inoculum**	PI #101				PI #104			
		Day 0	**Day 30**	**Day 60**	**Day 90**	**Day 0**	**Day 30**	**Day 60**	**Day 90**
Contig ITA	79,03%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
F900	11,29%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
N654; Q690; F722; V760; V847; F900	0,00%	ND	3,54%	0,00%	ND	1,00%	3,50%	7,14%	5,88%
N654; Q690; F722; V760; V847; E864; F900	0,00%	ND	71,68%	4,72%	ND	59,00%	69,93%	44,64%	22,69%
N654; Q690; F722; Y747; V760; V847; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	4,46%	24,37%
N654; Q690; F722; Y747; V760; V847; E864; F900	0,00%	ND	1,77%	0,00%	ND	0,00%	0,00%	7,14%	5,04%
N654; Q690; F722; V760; V847; H849; E864; F900	0,00%	ND	5,31%	0,00%	ND	4%	2,10%	1,79%	5,04%
N654; Q690; F722; V760; V847; E864; V880; F900	0,00%	ND	2,65%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
N654; Q690; L700; F722; V760; V847; K864; F900	0,00%	ND	0,00%	3,77%	ND	0,00%	0,00%	0,00%	0,00%
A623; N654; Q690; F722; V760; V847; K864; F900	0,00%	ND	0,00%	3,77%	ND	0,00%	0,00%	0,00%	0,00%
N654; V655; Q690; F722; Y747; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	5,88%
S623; N654; Q690; F722; V760; V847; K864; F900	0,00%	ND	0,00%	12,26%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; V760; V847; K864; T891; F900	0,00%	ND	0,00%	4,72%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; N741; V760; V847; K864; F900	0,00%	ND	0,00%	4,72%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; V760; V847; E864; F900	0,00%	ND	0,00%	22,64%	ND	0,00%	0,00%	0,00%	0,00%
I892	0,81%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
R688	0,81%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
W590	0,81%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
R861	0,81%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,00%

E692	0,81%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
F818	1,61%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
V847	0,81%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
Y690	0,81%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
A842; F900	0,81%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
A708; F900	0,81%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
M780; F900	0,81%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
N654; Q690; F722; V760; V847; E864; F900; H926	0,00%	ND	1,77%	0,00%	ND	3,00%	0,70%	0,00%	1,68%
N654; Q690; F722; V760; R784; V847; E864; F900	0,00%	ND	0,88%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
N654; M665; Q690; F722; V760; V847; E864; F900	0,00%	ND	0,88%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; V709; F722; V760; V847; E864; F900	0,00%	ND	0,88%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
N654; I655; Q690; F722; V760; V847; E864; F900	0,00%	ND	0,88%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
N654; V655; Q690; F722; V760; V847; E864; F900	0,00%	ND	0,88%	0,00%	ND	0,00%	1,40%	0,00%	0,00%
N654; Q690; F722; V760; V847; N849; E864; F900	0,00%	ND	1,77%	0,00%	ND	1,00%	0,70%	0,00%	0,00%
N654; K673; Q690; F722; V760; V847; H849; E864; F900	0,00%	ND	0,88%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
N654; Q690; F722; V760; P766; V847; E864; F900	0,00%	ND	1,77%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; F722; V760; P766; S777; I832; V847; E864; F900	0,00%	ND	0,88%	0,00%	ND	2,00%	0,00%	0,00%	0,00%
N654; Q690; F722; V760; V847; T859; E864; F900	0,00%	ND	1,77%	0,00%	ND	0,00%	0,70%	0,00%	0,00%
N654; Q690; L700; L712; F722; T723; T734; L760; Y835; R838; V847; E864; V880; F900	0,00%	ND	0,88%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
Q690; F722; V760; V847; E864; F900	0,00%	ND	0,88%	0,00%	ND	0,00%	0,00%	0,00%	0,00%
N654; T659; Q690; F722; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,70%	0,00%	0,00%
N654; T671; Q690; F722; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,70%	0,00%	0,00%
N654; Q690; F722; A751; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,70%	0,00%	0,00%
N654; Q690; F722; V760; V847; E864; R893; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,70%	0,00%	0,00%
N654; Q690; F722; V760; V847; H860; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,70%	0,00%	0,00%
N654; Q690; F722; Y747; A759; V760; V847; H849; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,70%	0,00%	0,00%
P633; N654; Q690; F722; Y747; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,70%	0,00%	0,00%
G600; N654; Q690; F722; V760; V847; H849; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,70%	0,00%	0,00%

N654; Q690; F722; V760; V847; V859; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,70%	0,00%	0,00%
N654; K655; Q690; F722; V760; V847; E864; F900; H926	0,00%	ND	0,00%	0,00%	ND	0,00%	0,70%	0,00%	0,00%
N654; Q690; R700; L712; F722; V760; Y835; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,70%	0,00%	0,00%
N654; Q690; F722; V760; S777; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	3,00%	0,70%	0,00%	0,00%
S623; P631; N654; Q690; F722; V760; Q777; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,70%	0,00%	0,00%
N654; Q690; F722; V760; P766; S777; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	2,80%	1,79%	0,00%
N654; V655; Q690; F722; F760; P766; S777; K799; I832; V857; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,70%	0,00%	0,00%
N654; Q690; F722; F760; P766; S777; I832; V857; E864; P900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,70%	0,00%	0,00%
N654; Q690; E696; R700; L712; F722; F760; P766; S777; V857; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,70%	0,00%	0,00%
N654; Q690; R700; L712; F722; F760; P765; S777; I832; V857; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	1,40%	0,00%	0,00%
N654; Q690; R700; L712; F722; A760; P766; S777; K799; I832; V857; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,70%	0,00%	0,00%
N654; Q690; F722; V760; T780; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,70%	0,00%	0,00%
N654; Q690; F722; V760; M780; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	2,10%	0,00%	0,00%
N654; Q690; F722; V760; V847; E864; A885; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; S716; F722; V760; V847; E864; V880; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; F722; V760; R817; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; F722; V760; V847; E864; G883; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; F722; V760; T775; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; F722; V760; V802; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; F722; V760; K803; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; A712; F722; A746; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; A712; F722; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; R700; F722; A733; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; F722; A733; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; E700; F722; A733; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; R690; F722; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; S711; F722; V760; A774; V847; E864; F900; H926	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; F722; V760; Q777; V847; E864; F900; H926	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%

N654; R690; F722; V760; P766; S777; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; R700; L712; F722; V760; P766; S777; K799; I832; V847; 849; E864; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; R700; L712; F722; V760; P766; S777; K799; I832; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,84%
N654; Q690; F722; S752; V760; N844; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; F722; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; F722; V760; V847; E864; F900; N929	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; F722; V760; V847; E864; F900; I919	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; F722; V760; V780; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
N654; Q690; F722; V760; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,84%
N654; Q690; F722; V760; F822; V847; F900	0,00%	ND	0,00%	0,00%	ND	1,00%	0,00%	0,00%	0,00%
S623; G637; N654; Q690; F722; V760; V847; K864; F900	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; V760; A832; V847; K864; F900	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; V760; V847; K864; E891; F900	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; V760; V847; K864; I891; F900	0,00%	ND	0,00%	1,89%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; V760; S771; H822; V847; F900	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; V760; V847; F900	0,00%	ND	0,00%	1,89%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; V760; V847; M872; F900	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; V760; V847; T859; F900	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; G741; V760; V847; F900	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
S623; F633; N654; Q690; F722; Y747; V760; V847; F900	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
A623; N654; Q690; F722; Y747; V847; K864; F900	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; Y747; V760; V847; K864; F900	0,00%	ND	0,00%	3,77%	ND	0,00%	0,00%	0,00%	2,52%
S623; N654; Q690; F722; V760; V847; Q864; F900	0,00%	ND	0,00%	1,89%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; N741; V760; V847; E864; F900	0,00%	ND	0,00%	1,89%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; N741; V760; R820; V847; Y857; K864; F900	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; N741; V760; R820; S830; V847; Y857; K864; F900; P931	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; N741; V760; R820; S830; V847; Y857; K864; F900	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; V760; S814; V847; F901	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%

K592; S623; N654; Q690; F722; V760; S814; V847; E864; F900	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; V760; S814; V847; E864; F900	0,00%	ND	0,00%	2,83%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; S743; V760; S814; V847; E864; F900	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
G592; S623; N654; Q690; F722; V760; V847; E864; F900	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; V760; V847; E864; E891; F900	0,00%	ND	0,00%	2,83%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; V760; I833; V847; E864; N891; F900	0,00%	ND	0,00%	1,89%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; V760; I793; V847; E864; F900	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; V760; K763; V847; E864; N893; F900	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
S623; N654; Q690; F722; S759; V760; V847; E864; F900	0,00%	ND	0,00%	1,89%	ND	0,00%	0,00%	0,00%	0,00%
A623; N654; Q690; F722; V760; V847; E864; F900	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
A623; N654; Q690; F722; G741; V760; V847; E864; F900	0,00%	ND	0,00%	1,89%	ND	0,00%	0,00%	0,00%	0,00%
N654; I677; Q690; F722; V760; V847; E864; F900	0,00%	ND	0,00%	0,94%	ND	0,00%	0,00%	0,00%	0,00%
V634; N654; Q690; F722; V760; V847; E864;F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,89%	0,00%
N654; Q690; F722; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,89%	0,00%
M602; N654; Q690; F722; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,89%	0,00%
N654; I669; Q690; F722; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,89%	0,00%
N654; Q690; F722; A733; V760; P766; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,89%	0,00%
N654; A680; Q690; F722; V760; V847; E864; V880; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	2,68%	0,00%
N654; Q690; F722; V760; V847; E864; R896; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,89%	0,00%
N654; Q690; F722; A738; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	1,79%	0,00%
N654; Q690; F722; K727; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,89%	0,00%
N654; Q690; F722; V760; V847; N849; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	2,68%	0,00%
N654; Q690; F722; V760; V847; D849; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,89%	0,00%
N654; Q690; N710; F722; Y747; A759; V760; V847; H849; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,89%	0,00%
N654; Q690; F722; V760; N831; V847; H849; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	1,79%	0,00%
N654; V655; F722; Y747; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	2,68%	0,00%
N654; V655; F722; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	1,79%	0,00%
N654; V655; F722; V760; V847; E864; Y895; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,89%	0,00%

N654; Q690; F722; Y747; V760; I774; V847; K864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	1,79%	0,00%
N654; Q690; F722; V760; I774; S776; V847; K864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,89%	0,00%
N654; Q690; F722; Y747; V760; V847; K864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	1,79%	0,00%
N654; Q690; S711; F722; Y747; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,89%	0,00%
N654; Q690; F722; Y747; V760; V847; I863; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,89%	0,00%
R589; N654; Q690; F722; V760; V847; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,89%	0,00%
N654; Q690; F722; G752; V760; V847; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	2,68%	0,00%
N654; Q690; F722; V760; D776; V847; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,89%	0,00%
N654; T658; Q690; F722; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
N654; Q690; F722; V760; V847; R854; E864; E891; F900; T901	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
N654; Q690; F722; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
N654; Q690; R700; F722; V760; D776; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
N654; Q690; F722; Y747; V760; D776; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
N654; Q690; F722; Y747; V760; P766; S777; A809; I832; V847; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
N654; Q690; F722; V760; P766; S777; V848	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
N654; Q690; F722; V760; P766; S777; I832; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
N654; Q690; R700; L712; F722; V760; P766; S777; I832; V847; E864; Y900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
N654; V655; Q690; F722; V760; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	1,68%
N654; Q690; F722; Y747; V760; V847; N849; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
N654; Q690; F722; Y747; A759; V760; V847; Y857; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
N654; Q690; F722; Y747; A759; V760; V847; H849; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	1,68%
L623; N654; Q690; N710; F722; Y747; A759; V760; V847; H849; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
N654; Q690; N710; F722; Y747; A759; V760; V847; H849; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	1,68%
N654; Q690; F722; Y747; V760; H765; V847; E864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
R619; N654; Q690; F722; Y747; V760; V847; Q864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
N654; Q690; S711; F722; Y747; V760; V847; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	1,68%
N654; Q690; G698; F722; Y747; V760; V847; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
N654; Q690; H701; F722; Y747; V760; V847; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%

N654; Q690; F722; I735; Y747; V760; V847; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
N654; Q690; F722; Y747; V760; V847; F900; G903	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
Q690; F722; V760; V847; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
Q690; F722; Y747; V760; P815; V847; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	0,84%
N654; Q690; F722; V760; V847; Q864; F900	0,00%	ND	0,00%	0,00%	ND	0,00%	0,00%	0,00%	1,68%

ND: Not determined.

Table A.2

Percentage of viral populations detected in inoculum D32/00_HoBi and PIs #102 and #103 in day of birth and days 30, 60 and 90 of life that were generated for heifers infected with this inoculum. The dominant quasispecies in inoculum Italy-1/10-1 was named as “Contig ITA” respectively, and the other samples named according the amino acid substitution and position in the polyprotein compared with the dominant quasispecie in the respective inoculum.

Sequence	Inoculum	PI #102				PI #103			
		Day 0	Day 30	Day 60	Day 90	Day 0	Day 30	Day 60	Day 90
Contig D32	86,11%	3,51%	0,00%	0,00%	0,86%	43,40%	36,54%	11,11%	14,85%
P814	0,00%	3,51%	3,28%	26,19%	20,69%	11,32%	10,58%	27,78%	18,82%
D929	0,00%	0,88%	0,00%	0,00%	0,00%	3,77%	4,81%	2,38%	0,00%
K864	2,78%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
S711; P814	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,96%	3,17%	2,97%
P814; D929	0,00%	0,88%	1,64%	0,79%	0,00%	8,49%	8,66%	11,11%	0,99%
L635; P814	0,00%	12,28%	14,75%	8,73%	0,00%	0,00%	0,00%	0,00%	0,00%
V635; P814	0,00%	0,00%	0,00%	3,97%	4,31%	0,00%	0,00%	0,00%	0,00%
L635; S711; P814	0,00%	0,88%	4,92%	32,54%	12,93%	0,00%	0,00%	0,00%	0,00%
L635; V780; P814	0,00%	1,75%	4,92%	7,14%	25,00%	0,00%	0,00%	0,00%	0,00%
L635; P814; D929	0,00%	14,04%	10,66%	4,76%	0,00%	0,00%	0,00%	0,00%	0,00%
S711; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	7,55%	21,15%	28,57%	25,74%
L635; E786; P814; D929	0,00%	31,73%	17,21%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; V780; P814; D929	0,00%	7,89%	13,93%	3,97%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; I780; P814; D929	0,00%	1,75%	4,92%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
G831; P833	0,93%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
M929	0,93%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
S910	0,93%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
R903	0,93%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
Y653	0,93%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%

R846	0,93%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
S718; S832	0,93%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
S901	0,93%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
S659	0,93%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
V961	1,85%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
V691; P888	0,93%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; E786; P814; A927; D929	0,00%	0,00%	0,82%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; E786; P814; D927; P931	0,00%	0,00%	0,82%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; P784; E786; P814; D929	0,00%	0,00%	0,82%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; V780; E786; P814; D929	0,00%	0,88%	2,46%	0,00%	1,72%	0,00%	0,00%	0,00%	0,00%
L635; I780; E786; P814; D930	0,00%	0,00%	0,82%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; K755; P814; D929	0,00%	0,00%	0,82%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635	0,00%	2,63%	1,64%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
L635; V780	0,00%	0,00%	0,82%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; I780	0,00%	0,00%	0,82%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; Q712; D929	0,00%	0,00%	0,82%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; I780; P814	0,00%	0,00%	1,64%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
G621; L635; V780; E786; P814; K917	0,00%	0,00%	0,82%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; I780; E786; P814	0,00%	0,00%	0,82%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; S711; I780; P814	0,00%	0,00%	0,82%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
L635; S711; E780; P815	0,00%	0,00%	0,82%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; P814; R841	0,00%	0,00%	0,82%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; S711; P814; D929	0,00%	0,00%	0,82%	0,79%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; S711; V780; P814; D929	0,00%	0,00%	0,82%	0,79%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; P757; V780; P814; D929	0,00%	0,00%	0,82%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
V780; E786; P814; D929	0,00%	0,00%	0,82%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
V780; P814; D930	0,00%	0,88%	1,64%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
I715; P814	0,00%	0,00%	0,82%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%

Q628; P814	0,00%	0,00%	0,82%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
P814; E839, D929	0,00%	0,00%	0,82%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
I680; S711; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,96%	0,00%	0,00%
D596; S711; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,96%	0,00%	0,00%
S711; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	2,88%	0,00%	0,00%
S711; P814; S912	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,96%	0,00%	0,00%
Y766; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,96%	0,00%	0,00%
R656; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,96%	0,00%	0,00%
P814; L851	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,96%	0,00%	0,00%
R656; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,96%	0,00%	0,00%
S711	0,00%	0,00%	0,00%	0,00%	0,00%	1,89%	0,96%	1,59%	0,00%
P712	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,96%	0,00%	0,00%
A767	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,96%	0,00%	0,00%
T847	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,96%	0,00%	0,00%
F614; S623; D710;S777; I837; Ins R839	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,96%	0,00%	0,00%
S710; L760; S777	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,96%	0,00%	0,00%
I611; L700; S710; L760	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,96%	0,00%	0,00%
I611; S710; L760; S777	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,96%	0,00%	0,00%
L635; E786; P814; K884; D929	0,00%	0,88%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; S684; E786; P814; D929	0,00%	0,88%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; E786; P814; V880; D929	0,00%	0,88%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
R589; E786; P814; D929	0,00%	0,88%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; I783; E786; P814; D929	0,00%	0,88%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; E786; P814; D929; N933	0,00%	0,88%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; E786; P814	0,00%	1,75%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; S780; E786; P814; D929	0,00%	0,88%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; E741; V780; E786; P814; D929	0,00%	1,75%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; V658; P814; D929	0,00%	0,88%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%

L635; D929	0,00%	1,75%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; D775; V780; P814; D929	0,00%	0,88%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; E780; P814; A905; D929	0,00%	0,88%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; R650; I780; P814	0,00%	0,88%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; I780; P814	0,00%	1,75%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; M671; P814	0,00%	0,88%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; A641; P814	0,00%	0,88%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
T658	0,00%	0,00%	0,00%	0,00%	0,00%	0,94%	0,00%	0,00%	0,00%
M661	0,00%	0,00%	0,00%	0,00%	0,00%	0,94%	0,00%	0,00%	0,00%
P766	0,00%	0,00%	0,00%	0,00%	0,00%	0,94%	0,00%	0,00%	2,97%
E915	0,00%	0,00%	0,00%	0,00%	0,00%	0,94%	0,00%	0,00%	0,00%
M884	0,00%	0,00%	0,00%	0,00%	0,00%	0,94%	0,00%	0,00%	0,00%
R761	0,00%	0,00%	0,00%	0,00%	0,00%	0,94%	0,00%	0,00%	0,00%
L897; S922	0,00%	0,00%	0,00%	0,00%	0,00%	0,94%	0,00%	0,00%	0,00%
L626; K835	0,00%	0,00%	0,00%	0,00%	0,00%	0,94%	0,00%	0,00%	0,00%
A799	0,00%	0,00%	0,00%	0,00%	0,00%	0,94%	0,00%	0,00%	0,00%
F614; S623; D710; S777	0,00%	0,00%	0,00%	0,00%	0,00%	0,94%	0,00%	0,00%	0,00%
I611; F710; L760; S777	0,00%	0,00%	0,00%	0,00%	0,00%	5,72%	0,00%	0,00%	0,00%
P814; I919	0,00%	0,00%	0,00%	0,00%	0,00%	0,94%	0,00%	0,00%	0,00%
S711; P814	0,00%	0,00%	0,00%	0,00%	0,00%	3,77%	0,00%	0,00%	0,00%
S777; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,94%	0,00%	0,00%	0,00%
M645; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,94%	0,00%	0,00%	0,00%
K605; P814; F918; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,94%	0,00%	0,00%	0,00%
M770; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,94%	0,00%	0,00%	0,00%
S711; P814; K870; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,79%	0,99%
G698; S711; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,79%	0,00%
P814; E860	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,79%	0,00%
I611; S711; P814; E860	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,79%	0,00%

P814; T847	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,79%	0,99%
R761; P814	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,79%	0,00%
A606; P814	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,79%	0,00%
K700; P814	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	1,59%	0,00%
I759; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,79%	0,00%
P814; P838; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,79%	0,00%
S644; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,79%	0,00%
P766; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	2,38%	0,00%
S711; P766	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,79%	0,00%
V847	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,79%	0,00%
T645	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,79%	0,00%
P814; F887	0,00%	0,00%	0,00%	0,79%	0,00%	0,00%	0,00%	0,00%	0,00%
P814; Y816	0,00%	0,00%	0,00%	0,79%	0,00%	0,00%	0,00%	0,00%	0,00%
M712; P814	0,00%	0,00%	0,00%	0,79%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; T658; P814	0,00%	0,00%	0,00%	0,79%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; E786; K803; P814	0,00%	0,00%	0,00%	0,79%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; S711; P814; C851	0,00%	0,00%	0,00%	1,59%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; I780; P814	0,00%	0,00%	0,00%	0,79%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; A780; P814	0,00%	0,00%	0,00%	0,79%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; S711; Y729; V780; P814	0,00%	0,00%	0,00%	0,79%	0,00%	0,00%	0,00%	0,00%	0,00%
L635; M720; V780; P814	0,00%	0,00%	0,00%	0,79%	0,00%	0,00%	0,00%	0,00%	0,00%
S600; V634; L635; P672; V780; P814	0,00%	0,00%	0,00%	0,79%	0,00%	0,00%	0,00%	0,00%	0,00%
Y729; P814	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
V801; P814	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
V658; P814	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
W884	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
V635; I740; P814	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
V635; P814; R817	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%

L635; S711; M712; P814	0,00%	0,00%	0,00%	0,00%	3,45%	0,00%	0,00%	0,00%	0,00%
L635; R696; S711; E782; P814	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
A603; L635; S711; P814	0,00%	0,00%	0,00%	0,00%	1,72%	0,00%	0,00%	0,00%	0,00%
L635; S711; R753; P814	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
L635; S711; P814; V847	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
L635; D710; F765; V780; P814; K870	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
L635; D710; F765; V780; P814	0,00%	0,00%	0,00%	0,00%	3,43%	0,00%	0,00%	0,00%	0,00%
L635; D710; S711; F765; P814	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
L635; S711; F765; P814	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
L635; N706; S711; F765; P814	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
L635; C747; E786; P814; D929	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
L635; E786; P814; D929	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
L635; I735; P814; D929	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
L635; N741; P814; D929	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
S623; V780; P814	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
L635; M712; V780; P814	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
L635; R696; V780; P814	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
L635; S744; V780; P814	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
L635; M717; V780; P814	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
L635; A662; V780; P814	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
L635; A641; V780; P814	0,00%	0,00%	0,00%	0,00%	0,86%	0,00%	0,00%	0,00%	0,00%
D710	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
I647; V683; N696; P814	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
I647; P814	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	1,98%
I647	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
T621; D628; I647	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	1,98%
T641; D628; I647; P766	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
T621; D628; I647; P814	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%

T641; D628; I647; Y651; P814	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
R617; T621; D628; I647; P814	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
D628; P814	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
G763; P814	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
P814; I863	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
S641; P814	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
P814; R854; M886	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
S711; P814; A819	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
S711; K770; P814	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
T621; D628; I847; Y651; S711; P814; D829; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0.99%
T621; D628; I647; S711; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	1,98%
T621; D628; S711; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
D628; I647; V683; S711; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
D628; I647; S711; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
I647; S711; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	2,97%
K605; I647; S711; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
S711; K770; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
I647; S711; K770; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%
S711; S780; P814; D929	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%	0,99%

Table A.3

Amino acid residues in E1/E2 region that differ within the inoculums and 'HoBi'-like PI calves viral swarms. The percentage of viral variants that contain the amino acid substituted (left) and the substitute (right) are represented in each row.

	P623S	**S654N**	**H690Q**	**Y722F**	**H747Y**	**I760V**	**A847V**	**L900F**	**F635L**	**L711S**	**S814P**	**N929D**
Inoculum Italy-1/10-1	100/0	100/0	100/0	100/0	100/0	100/0	99.2/0.8	86.3/13.7				
PI #101 - Day of birth	ND	ND	ND	ND	ND	ND	ND	ND				
PI #101 - Day 30 of life	100/0	0.9/99.1	0/100	0/100	98.2/1.8	0/99.1	0/100	0/100				
PI #101 - Day 60 of life	11.3/81.1	0/100	0/100	0/100	94.3/5.7	0/100	0/100	0/100				
PI #101 - Day 90 of life	ND	ND	ND	ND	ND	ND	ND	ND				
PI #104 - Day of birth	100/0	1/99	0/98	0/100	100/0	0/100	0/100	0/100				
PI #104 - Day 30 of life	99.3/0.7	0/100	0/100	0/100	98.6/0	0/99.3	0/100	0/100				
PI #104 - Day 60 of life	100/0	0/100	0/100	0/78.9	62.7/37.3	0.7/99.3	21.1/78.9	0/100				
PI #104 - Day 90 of life	99.2/0	1.7/98.3	0/100	0/100	47.1/52.9	0/100	0.8/99.2	0/99.2				
Inoculum D32/00									100/0	100/0	100/0	100/0
PI #102 - Day of birth									9.6/90.4	99.1/0.9	8.8/91.2	30.7/69.3
PI #102 - Day 30 of life									9.8/90.2	91.8/8.2	4.1/95.9	38.5/61.5
PI #102 - Day 60 of life									37.3/59.2	67.6/32.4	0/100	78.9/21.1
PI #102 - Day 90 of life									27.6/66.4	74.1/25.9	2.6/97.4	94.8/5.2
PI #103 - Day of birth									100/0	85.8/14.2	63.2/36.8	75.5/24.5
PI #103 - Day 30 of life									100/0	71.2/28.8	52.9/47.1	58.7/41.3
PI #103 - Day 60 of life									100/0	63.5/36.5	17.5/82.5	51.6/48.4
PI #103 - Day 90 of life									100/0	54.5/45.5	22.8/77.2	58.4/41.6

ND: Not determined.

Table A.4

Putative N-glycolisation patterns in E1/E2 region of HoBi-like viruses (residues 589–933) obtained for viral variants obtained in the present study and representative 'HoBi'-like strains predicted using the software NetNGlyc 1.0 Server. Positions in polyprotein from 'HoBi'-like sequences were inferred using D32/00_HoBi (GenBank accession number: BAO04453.1).

Sequence name	Position	Sequon	Potencial	Jury agreement	N-Glyc result
Contig ITA	596	NLT	0.7409	9/9	++
	807	NTT	0.5801	7/9	+
	876	NWT	0.5614	6/9	+
	920	NET	0.5416	6/9	+
101-1 D60 F02	596	NLT	0.7404	9/9	++
	807	NTT	0.5801	7/9	+
	876	NWT	0.5617	6/9	+
	920	NET	0.5417	6/9	+
104-2 D90 A06	596	NLT	0.7410	9/9	++
	807	NTT	0.5801	7/9	+
	876	NWT	0.5618	6/9	+
	920	NET	0.5417	6/9	+
Contig D32	596	NLT	0.6937	9/9	++
	807	NTT	0.5550	6/9	+
	812	NGS	0.6714	9/9	++
	876	NWT	0.5567	6/9	+
	920	NET	0.5539	7/9	+
102-2 D90 H04	596	NLT	0.6938	9/9	++
	807	NTT	0.5581	6/9	+
	876	NWT	0.5567	6/9	+
	920	NTT	0.5539	7/9	+
103-1 D90 D02	596	NLT	0.6938	9/9	++
	807	NTT	0.5581	6/9	+
	876	NWT	0.5568	6/9	+
	920	NET	0.5418	6/9	+
D32/00_HoBi	596	NLT	0.6937	9/9	++
	807	NTT	0.5550	6/9	+
	812	NGS	0.6714	9/9	++
	876	NWT	0.5567	6/9	+
	920	NET	0.5539	7/9	+
Italy-1/10-1	596	NLT	0.7410	9/9	++
	807	NTT	0.5801	7/9	+
	876	NWT	0.5617	6/9	+
	920	NET	0.5418	6/9	+
Italy-83/10-ncp	596	NLT	0.7410	9/9	++
	807	NTT	0.5801	7/9	+
	876	NWT	0.5618	6/9	+

	920	NET	0.5417	6/9	+
Italy-83/10-cp	596	NLT	0.5417	9/9	++
	807	NTT	0.5616	7/9	+
	876	NWT	0.5801	6/9	+
	920	NET	0.7409	6/9	+
Italy-68/13ncp	596	NLT	0.7410	9/9	++
	807	NTT	0.5801	7/9	+
	876	NWT	0.5568	6/9	+
	920	NET	0.5418	6/9	+
Italy-68/13cp	596	NLT	0.7410	9/9	++
	807	NTT	0.5801	7/9	+
	876	NWT	0.5618	6/9	+
	920	NET	0.5416	6/9	+
CH-KaHo_cont	596	NLT	0.7407	9/9	++
	807	NTT	0.5672	7/9	+
	876	NWT	0.6673	9/9	++
	920	NET	0.5540	7/9	+
JS12/01	596	NLT	0.7408	9/9	++
	807	NTT	0.5719	7/9	+
	876	NWT	0.5568	6/9	+
	920	NET	0.5047	3/9	+
LVRI-cont-1	596	NLT	0.7409	9/9	++
	807	NTT	0.5719	7/9	+
	876	NWT	0.5568	6/9	+
	920	NET	0.5461	7/9	+
Th/04_KhonKaen	596	NLT	0.6989	9/9	++
	807	NTT	0.5663	7/9	+
	876	NWT	0.5569	6/9	+
	920	NET	0.5419	6/9	+
BVDV1 NADL	597	NLT	0.7648	9/9	+++
	809	NTT	0.6163	8/9	+
	878	NWT	0.5494	5/9	+
	922	NET	0.6152	8/9	+
BVDV2 890	597	NLT	0.7271	9/9	++
	807	NAS	0.5154	6/9	+
	876	NWT	0.5872	7/9	+
BDV X818	595	NLT	0.7629	9/9	+++
	806	NTT	0.6637	8/9	+
	811	NGS	0.6774	9/9	++
	875	NWT	0.5646	6/9	+
	919	NET	0.5449	5/9	+
CSFV Alfort/187	594	NLT	0.7778	9/9	+++
	805	NTT	0.6586	8/9	+
	810	NGS	0.6757	9/9	++
	874	NWT	0.5833	7/9	+
	918	NET	0.5872	6/9	+

Giraffe H138	593	NLT	0.7017	9/9	++
	805	NTT	0.6984	9/9	++
	874	NWT	0.6458	9/9	++

Table A.5

Standard error of the mean obtained from the average mean diversity calculated using Tajima-Nei model with 500 replicates.

Sample	**Day of life**	**Standard error of the mean ($x10^{-4}$)**
Inoculum Italy-1/10-1		2.47
PI #101	Birth	ND
	30	2.13
	60	6.47
	90	ND
PI #104	Birth	2.72
	30	2.74
	60	5.01
	90	7.66
Inoculum D32/00_HoBi		2.07
PI #102	Birth	7.81
	30	9.01
	60	7.71
	90	8.17
PI #103	Birth	6.72
	30	8.01
	60	9.67
	90	8.66

ND: Not determined.