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SELEÇÃO DE ALVOS GENÔMICOS PARA A FILOGENIA DE VÍRUS DA DIARREIA VIRAL BOVINA (BVDV) E IDENTIFICAÇÃO DE NOVO SUBTIPO DE BVDV-2

Santa Maria, RS
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Tese apresentada ao Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Sanidade e Reprodução Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutor em Ciência Animal.**

Orientador: Prof. Dr. Eduardo Furtado Flores

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Aprovada em 3 de março de 2023

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Santa Maria, RS
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RESUMO

SELEÇÃO DE ALVOS GENÔMICOS PARA A FILOGENIA DE VÍRUS DA DIARREIA VIRAL BOVINA (BVDV) E IDENTIFICAÇÃO DE NOVO SUBTIPO DE BVDV-2

AUTOR: Pablo Sebastian Britto de Oliveira
ORIENTADOR: Eduardo Furtado Flores

Os vírus da diarreia viral bovina 1 (*Bovine viral diarrhea virus*, BVDV-1) e BVDV-2, classificados no gênero *Pestivirus*, família *Flaviviridae*, são importantes patógenos de distribuição mundial. Os pestivírus são vírus envelopados e contêm uma molécula de RNA de sentido positivo com aproximadamente 2.3 quilobases (kb) como genoma. Até o presente, foram descritos 21 subtipos de BVDV-1 (a-u) e 4 de BVDV-2 (a-d). Apesar dessa variabilidade genética, isolados e cepas de BVDV têm sido frequentemente subtipados pela análise filogenética da sequência parcial da região não-traduzida 5' (5'UTR) e/ou de regiões codificantes, como de N^{pro} e E2. Essas análises, no entanto, podem gerar resultados equivocados ou de baixo suporte estatístico, dificultando o conhecimento sobre a real diversidade e circulação dos subtipos de BVDV. A melhor alternativa para contornar esses obstáculos seria a subtipagem dos vírus a partir da análise do genoma completo. Essa estratégia, no entanto, apresenta custo alto, além de ser inviável para uso em larga escala. Assim, o primeiro estudo investigou os alvos genômicos mais adequados para a subtipagem de BVDV-1 e BVDV-2, comparando-se a subtipagem baseada em análise de genes/regiões individuais com aquela baseada na análise do genoma completo (GC). O estudo foi realizado com 91 (BVDV-1) e 85 (BVDV-2) GC disponíveis no banco de dados GenBank. Os vírus foram subtipados analisando-se seu GC, bem como as regiões codificantes dos genes individuais e as regiões não-traduzidas (3' e 5' UTRs completas e 5' UTRs parciais). A distância geodésica entre a árvore gerada pela análise do GC (referência) e aquelas geradas pelas análises das regiões genômicas/UTRs também foi calculada. Em geral, as análises baseadas em 3'UTR e 5'UTR apresentaram as subtipagens menos confiáveis, comparando-se com análise de GC. Para o BVDV-1, a filogenia baseada em C, E^{rns}, E1, E2, p7, NS2, NS3, NS4B, NS5A e NS5B foi equivalente à do GC. Em relação à região codificante de BVDV-2, houve pelo menos uma não conformidade em comparação com a análise do GC, em todos os alvos analisados. Após calcular a distância geodésica entre as árvores de GC e da região codificante/UTRs, observou-se que a NS4B (para BVDV-1) e NS5A (BVDV-2) apresentaram topologia e extensão de haste mais próximos às árvores geradas pela análise de GC. Além desses resultados, também observou-se que 14 sequências de BVDV-2 não puderam ser classificadas como subtipos a, b ou c. Então, realizou-se um segundo estudo mais detalhado para verificar se essas sequências poderiam representar um subtipo distinto de BVDV-2. Inicialmente, realizou-se uma "prova de equivalência" entre as análises filogenéticas baseadas em 85 genomas completos/quase completos (GCQCs) e suas respectivas fases abertas de leitura (*open reading frames*, ORFs), o que comprovou que as ORFs podem ser utilizadas de forma confiável para a filogenia de BVDV-2. Isso possibilitou aumentar o conjunto de dados para 139 sequências. Dentre essas, sete sequências, que não puderam ser classificadas como BVDV-2 a-d, formaram um agrupamento distinto em todas as árvores filogenéticas analisadas. Esse agrupamento foi então sugerido como um novo subtipo, denominado BVDV-2e. As sequências de BVDV-2e também apresentaram 44 alterações de aminoácidos em comparação com o BVDV-2a-c. Em conclusão, sugere-se que as regiões de NS4B e NS5A podem ser utilizadas como alvos genômicos para a subtipagem de BVDV-1 e BVDV-2, respectivamente. Além disso, identificou-se um grupo de BVDV-2 que pode constituir um

novo subtipo (BVDV-2e), e que poderia ser investigado em futuros estudos epidemiológicos e filogenéticos de BVDV.

Palavras-chave: NS4B. NS5A. BVDV-2e. Filogenia.

ABSTRACT

SELECTION OF GENE TARGETS FOR PHYLOGENY OF BOVINE VIRAL DIARRHEA VIRUS (BVDV) AND IDENTIFICATION OF A NOVEL BVDV-2 SUBTYPE

AUTHOR: Pablo Sebastian Britto de Oliveira
ADVISOR: Eduardo Furtado Flores

Bovine viral diarrhea virus 1 (BVDV-1) and BVDV-2, classified within the genus *Pestivirus*, family *Flaviviridae*, are important pathogens of cattle worldwide. Pestiviruses are enveloped viruses and contain a positive-sense, single-stranded RNA molecule of approximately 12.3 kilobases as the genome. Up to date, 21 subtypes of BVDV-1 (a-u) and 4 of BVDV-2 (a-d) have been described. Although this genetic variability, BVDV isolates/strains have been frequently subtyped by phylogenetic analysis of the partial sequence of the 5' untranslated region (5'UTR) and/or coding regions such as N^{pro} and E2. These analyses, however, can generate erroneous results, or with low statistical support, hindering the knowledge about the real diversity and circulation of BVDV subtypes. The best alternative to circumvent this obstacle would be to subtype the viruses by analyzing the complete genome (CG), a costly and unfeasible strategy for large-scale use. Thus, the first study investigated the most suitable genomic targets for subtyping of BVDV-1 and BVDV-2, comparing subtyping based on individual gene/region analysis and that based on whole genome (GC) analysis. The study was performed with 91 (BVDV-1) and 85 (BVDV-2) GC available in the GenBank database. The viruses were subtyped by analyzing their GC as well as the coding regions of the individual genes and the untranslated regions (complete 3' and 5'UTRs and partial 5'UTRs). Also, the geodesic distance between the tree generated by GC (reference) analysis and those generated by genomic region/UTR analyses were calculated. In general, analyses based on 3'UTR and 5'UTR showed the least reliable subtyping compared to GC analysis. For BVDV-1, the phylogeny based on C, E^{rns}, E1, E2, p7, NS2, NS3, NS4B, NS5A and NS5B was equivalent to that of GC. Regarding the BVDV-2 coding region, there was at least one non-compliance compared to the GC analysis, in all targets analyzed. After calculating the geodesic distance between GC and the coding region/UTRs trees, NS4B (for BVDV-1) and NS5A (BVDV-2) showed topology and edge lengths closer to the trees generated by GC analysis. In addition, 14 sequences of BVDV-2 could not be classified as subtype a, b or c. A second, more detailed study was then performed to investigate whether these sequences would represent a distinct BVDV-2 subtype. Initially, an "equivalence test" between phylogenetic analyses based on 85 complete/near-complete genomes (CNCGs) and their respective open reading frames (ORFs) proved that ORFs can be reliably used for BVDV-2 phylogeny. This made it possible to increase the data set to 139 sequences. Among these, seven sequences that could not be classified as BVDV-2 a-d formed a distinct cluster in all the phylogenetic trees analyzed. This cluster was suggested as a new subtype, called BVDV-2e. The BVDV-2e sequences also showed 44 amino acid changes compared to BVDV-2a-c. In conclusion, it is suggested that the NS4B and NS5A regions can be used as genomic targets for subtyping BVDV-1 and BVDV-2, respectively. In addition, a cluster of BVDV-2 was identified that may represent a new subtype (BVDV-2e), which could be investigated in future epidemiological and phylogenetic studies of BVDV.

Keywords: NS4B. NS5A. BVDV-2e. Phylogeny.

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LISTA DE ABREVIATURAS E SIGLAS

aa	aminoácido
bp	*base pair*
BVDV	*bovine viral diarrhea virus*
BVDV-1	*bovine viral diarrhea vírus type I*
BVDV-2	*bovine viral diarrhea vírus type II*
CG	genoma completo
CNCG	*complete/near-complete genome*
DAMP	*danger associated molecular pattern*
DNAJ-C14	*J-domain protein interacting with viral protein*
HCV	hepatitis C virus
ICTV	*Comitê Internacional de Taxonomia de Vírus (Internacional Committe on Taxonomy of Viruses)*
IFN-I	interferon tipo I
IRES	*internal ribosomal entry site*
IRF-3	*IFN regulatory factor 3*
kb	kilobases
kDa	kilodaltons
nt	nucleotídeos
NTPase	nucleosídeo trifosfatase
ncp	*non-cytopathic*
ORF	*open reading frame*
RdRp	RNA-dependent RNA polymerase
ssRNA	*single-stranded RNA*
UTR	*untranslated region*

SUMÁRIO

1 INTRODUÇÃO

Os vírus da diarreia viral bovina (*bovine viral diarrhea virus*, BVDV) -1 (BVDV-1, *Pestivirus A*) e BVDV-2 (*Pestivirus B*) pertencem ao gênero *Pestivirus*, família *Flaviviridae* (ICTV, 2023; SMITH et al., 2017). Suas partículas víricas contêm um genoma de RNA de fita simples, de sentido positivo, com capsídeo icosaédrico e envelope lipoprotéico. O genoma possui aproximadamente 12,3 kilobases (kb) e codifica uma poliproteína que é clivada, à medida que vai sendo traduzida, por proteases virais e celulares em 11 ou 12 proteínas: proteína do capsídeo (C), proteínas do envelope (E^{rns}, E1 e E2), proteína 7 (p7), autoprotease N-terminal (N^{pro}) e proteínas não-estruturais [NS2/NS3 (ou NS2 e NS3), NS4A, NS4B, NS5A e NS5B)] (TAUTZ; TEWS; MEYERS, 2015).

A região codificadora do genoma é flanqueada à montante e à jusante por duas regiões não traduzidas (*untranslated regions*, UTRs), 5'UTR e 3'UTR, respectivamente. A 5'UTR apresenta um elevado nível de conservação quando comparada com as demais regiões do genoma e possui uma estrutura secundária conhecida como IRES (*internal ribosomal entry site*), responsável pelo reconhecimento pelos ribossomos no início da tradução (DENG; BROCK, 1993). A 5'UTR também é amplamente utilizada como alvo em diagnóstico molecular, sequenciamento e análises filogenéticas de BVDV (BECHER et al., 2003; FIGUEIREDO et al., 2019; MONTEIRO et al., 2019a; RIDPATH; BOLIN; DUBOVI, 1994).

O genoma dos BVDVs apresenta alta taxa de mutação, o que resulta em grande variabilidade genética e, consequentemente, em grande diversidade (CHERNICK; GODSON; VAN DER MEER, 2014; YEŞILBAĞ; ALPAY; BECHER, 2017). Além disso, eventos de recombinação homóloga e não homóloga também podem contribuir para a diversidade dos BVDVs circulantes (BECHER et al., 1999; DOMINGO et al., 1985). Até o momento, foram descritos pelo menos 21 subtipos de BVDV-1 (a-u) e 4 de BVDV-2 (a-d) (YEŞILBAĞ; ALPAY; BECHER, 2017), embora alguns estudos tenham sugerido novos subtipos do vírus com base principalmente na análise filogenética da 5'UTR (TIAN et al., 2021).

Em geral, a subtipagem de isolados e cepas de BVDV têm sido realizada sobretudo pela análise filogenética de 5'UTR e, secundariamente, pela análise de regiões codificantes, como a N^{pro} ou E2 (NAGAI et al., 2004; WORKMAN et al., 2016). Alguns autores, no entanto, têm descrito que um mesmo isolado/cepa de BVDV pode ser classificado em subtipos diferentes, a depender da região genômica analisada (NAGAI et al., 2001, 2004; NEILL et al., 2019a; SILVEIRA et al., 2017; TAJIMA, 2004; WORKMAN et al., 2016).

Esses achados têm levado à conclusão de que o sequenciamento de genoma completo de BVDV é a estratégia ideal para a classificação do vírus, principalmente quando considerado que genes e genomas podem estar submetidos à diferentes pressões de seleção e, assim, podem apresentar histórias evolutivas diferentes (CHERNICK et al., 2018; KOKKONOS et al., 2020; TANG; ZHANG, 2007). No entanto, muitos laboratórios não têm acesso às plataformas de sequenciamento de genoma completo, o que levanta discussões sobre qual região genômica é a mais adequada para ser analisada, visando a reprodução da análise do genoma viral completo.

Considerando esse contexto, o presente estudo investigou qual região genômica do BVDV poderia levar a uma classificação filogenética semelhante à análise do genoma viral completo. Interessantemente, durante a execução deste trabalho, foram identificadas sequências de BVDV-2 que não puderam ser seguramente classificadas nos subtipos virais previamente descritos. Assim, o presente estudo também descreve um novo subtipo de BVDV-2. Os achados desses dois trabalhos estão descritos nos artigos:

1. “Subtyping bovine viral diarrhea virus (BVDV): which viral gene to choose?” e
2. “A new (old) bovine viral diarrhea virus 2 subtype: BVDV-2e”.

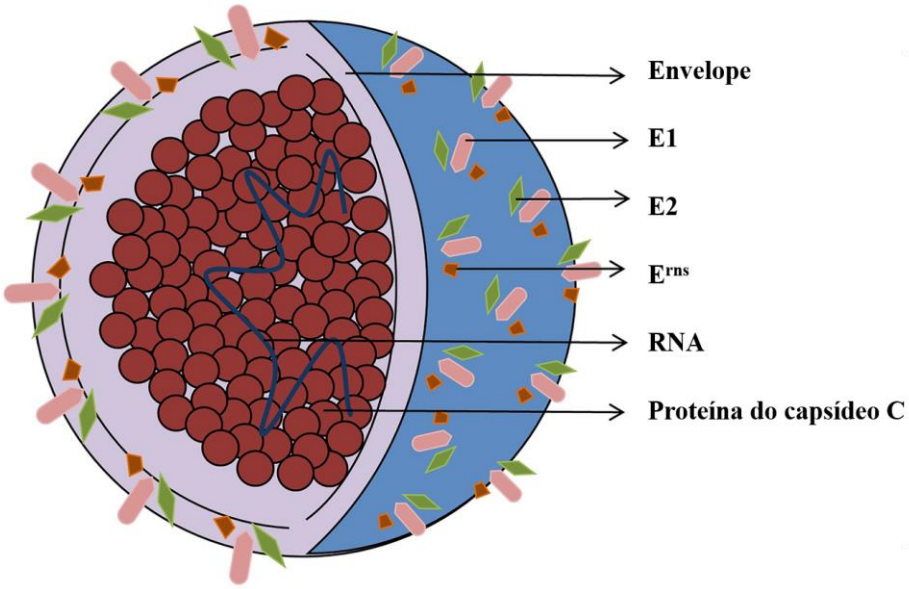
2 REVISÃO BIBLIOGRÁFICA

2.1 VÍRUS DA DIARREIA VIRAL BOVINA

Os vírus da diarreia viral bovina 1 (*bovine viral diarrhea virus*, BVDV-1, *Pestivirus A*) e BVDV-2 (*Pestivirus B*) pertencem ao gênero *Pestivirus*, família *Flaviviridae*. Além dos BVDVs, o gênero também inclui outros vírus de animais: HoBi-PeV (*Pestivirus H*), o vírus da peste suína clássica (*Pestivirus C*), vírus da doença da fronteira de ovinos (*Pestivirus D*), vírus do antílope Pronghorn (*Pestivirus E*), vírus de Bungowannah (*Pestivirus F*), pestivírus de girafa (*Pestivirus G*), pestivirus de carneiro (*Pestivirus I*), pestivirus de roedor (*Pestivirus J*) e pestivirus de suíno (*Pestivirus K*) (ICTV, 2023; SMITH et al., 2017).

Os BVDVs possuem vírions aproximadamente esféricos com diâmetro entre 40 e 60 nm. Os vírions são recobertos externamente por um envelope derivado de membranas celulares composto por uma bicamada lipídica e três glicoproteínas complexadas como homo- e heterodímeros. A presença do envelope torna o BVDV sensível ao calor, solventes orgânicos e detergentes, porém é altamente estável em ambientes ácidos (CALLENS et al., 2016). Esta estabilidade pode ser explicada pelas complexas pontes dissulfeto responsáveis pela ligação das três glicoproteínas do envelope (KREY; THIEL; RÜMENAPF, 2005). O capsídeo dos BVDVs possui simetria icosaédrica, com estudos de microscopia crioeletrônica de alta resolução demonstrando ser uma estrutura pouco organizada (RIEDEL et al., 2017). A estrutura de um vírion do BVDV está representada na figura 1.

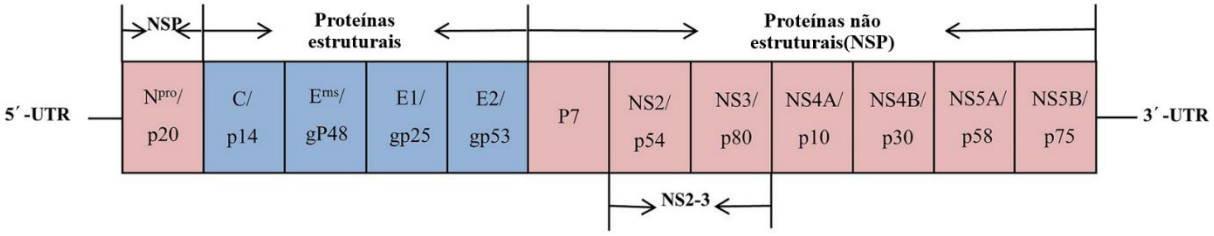
Figura 1 - Representação de vírion do vírus da diarreia viral bovina (BVDV).



Fonte: Adaptado de CHI et al. (2022).

O genoma dos BVDVs é composto por uma molécula de RNA de fita simples (*single-stranded* RNA, ssRNA) de sentido positivo, com aproximadamente 12,3 kb. O genoma possui uma única fase de leitura aberta (*open reading frame*, ORF), flanqueada por duas regiões não traduzidas (5' e 3' UTR), as quais participam da tradução e regulação da replicação do genoma viral. A ORF única codifica uma poliproteína de aproximadamente 4.000 aminoácidos, que é processada por proteases celulares e virais em 11 ou 12 polipeptídeos: autoprotease N-terminal (N^{pro}), proteína do capsídeo (C), proteínas do envelope (E^{rns}, E1 e E2), p7 e proteínas não estruturais (*non-structural*, NS) [NS2/NS3 (ou NS2 e NS3), NS4A, NS4B, NS5A e NS5B)] (Figura 2) (TAUTZ; TEWS; MEYERS, 2015).

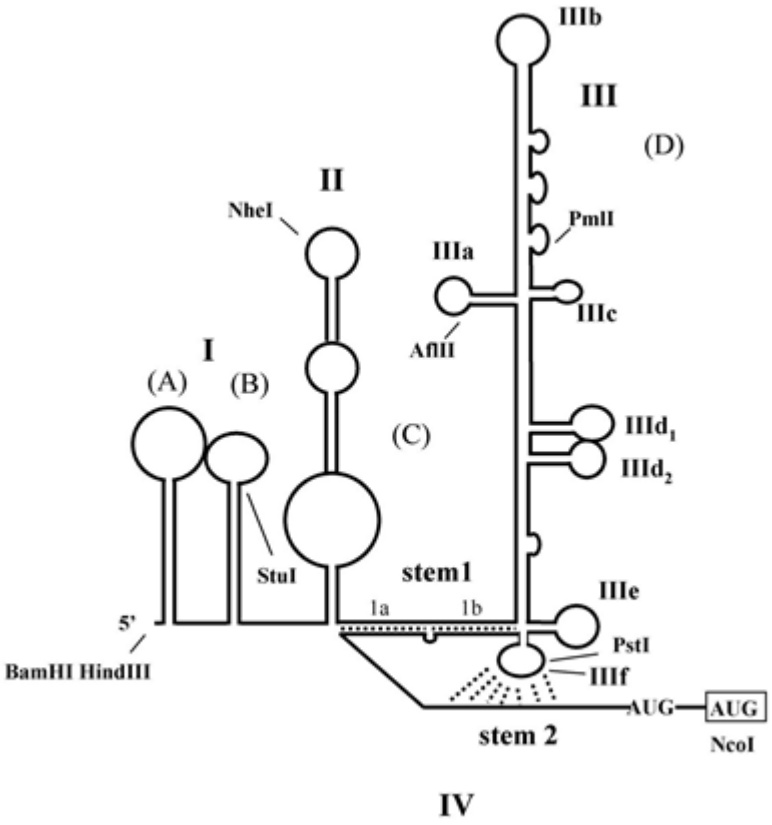
Figura 2 - Organização do genoma do vírus da diarreia viral bovina (BVDV).



Fonte: Adaptado de CHI et al. (2022).

A região 5'UTR possui aproximadamente 385 nucleotídeos (nt) e é composta por quatro domínios, I-IV, sendo IV o maior deles, correspondendo aos dois terços finais da UTR (Figura 3) (DENG; BROCK, 1993; HARASAWA, 1994). A 5'UTR dos BVDVs também se organiza em uma estrutura secundária, formada por regiões palindrômicas, denominada IRES (*internal ribosomal entry site*), que servem de sítio de reconhecimento para os ribossomos durante o início da tradução (DENG; BROCK, 1993). Dessa forma, mutações pontuais na 5'UTR têm uma probabilidade de serem deletérias, no entanto, mutações estáveis podem ser utilizadas para estudos sobre a história evolutiva dos BVDVs (HARASAWA; GIANGASPERO, 1998).

Figura 3 - Representação da estrutura secundária da região 5'UTR do genoma do vírus da diarreia viral bovina (BVDV).



Legenda: As regiões palindrômicas do domínio I (nt 1-60, correspondentes aos *stem-loops* A e B) contribuem pouco para a tradução, sugerindo que possam ser dispensáveis (DENG; BROCK, 1993). Os domínios II e III (nt 61-385, correspondentes aos *stem-loops* C e D); no entanto, são essenciais para uma tradução eficiente.
Fonte: MOES; WIRTH (2007).

O elevado nível de conservação da 5'UTR fez com que essa região fosse escolhida para a confecção de *primers* utilizados para a detecção e/ou análise filogenética de BVDVs, a exemplo dos *primers* HCV 90-368, frequentemente utilizados para identificação e classificação de BVDV, e dos *primers* BP189-389, recomendáveis para detecção molecular de BVDVs (BAUERMANN et al., 2014; GUO et al., 2021; MONTEIRO et al., 2018, 2019a; RIDPATH; BOLIN, 1998). O uso frequente da região 5'UTR para diagnóstico e/ou pesquisa também gerou milhares de sequências parciais dessa região (delimitados pelos pares de *primers* utilizados) disponíveis no GenBank (NATIONAL OF LIBRARY MEDICINE, 2006).

A região 3'UTR do genoma dos BVDVs é composta por aproximadamente 192 nt, e pode ser dividida em uma região conservada, “3'C” (aproximadamente 102 nt terminais), e uma outra variável “3'V” (aproximadamente 90 nt iniciais) (BECHER; ORLICH; THIEL,

1998; YU; GRASSMANN; BEHRENS, 1999). A 3'UTR também é organizada em estruturas secundárias que auxiliam na síntese do RNA antigenômico durante a replicação do genoma viral (ISKEN et al., 2004; NEILL, 2013).

2.2 PROTEÍNAS VIRAIS

A primeira proteína dos BVDVs a ser traduzida é a N^{pro} (p20); uma proteína exclusiva dos pestivírus, composta por 168 aminoácidos (aa) e massa de 20 kilodaltons (kDa). A N^{pro} é uma cisteína protease, que cliva em seu próprio C-terminal (STARK et al., 1993). A N^{pro} também possui uma atividade imunossupressora relacionada à inibição da via de indução do interferon tipo I (IFN-I) por meio da degradação ou redução da expressão do fator regulador 3 do IFN (*IFN regulatory factor 3*, IRF-3) (CHEN et al., 2007; TAMURA et al., 2015). Além disso, a N^{pro} também pode interagir com a proteína celular S100A9, um padrão molecular associado ao dano celular (*danger associated molecular pattern*, DAMP), reduzindo a disponibilidade/atividade desse DAMP e levando a uma diminuição na síntese do IFN-I em células infectadas (ALKHERAIF et al., 2017; DARWEESH et al., 2018).

A proteína C (p14) é a primeira proteína estrutural a ser traduzida, possui 90 aa, sendo clivada na sua região N-terminal pela ação da N^{pro} (HEIMANN et al., 2006). Além de compor o capsídeo viral, a proteína C liga-se ao RNA viral com baixa afinidade e especificidade, com o mínimo de 14 nt por unidade da proteína C (IVANYI-NAGY et al., 2008; MURRAY; MARCOTRIGIANO; RICE, 2008).

Outras três proteínas estruturais (glicoproteínas) estão localizadas no envelope viral: E^{rns} (E0/gp48), E1 (gp25) e E2 (gp53) (NEILL, 2013). A proteína E^{rns} possui 227 aa, 42-48 kDa, sendo um dos alvos para anticorpos fracamente neutralizantes (WEILAND et al., 1992). Possui atividade de RNAse, provavelmente para clivar RNAs virais e evitar a ativação da via de indução de IFN (MÄTZENER et al., 2009; TEWS; MEYERS, 2007). Estudos realizados com BVDVs contendo deleção de E^{rns} demonstraram que os vírus continuavam infecciosos, indicando que E^{rns} é dispensável para penetração em células hospedeiras (RONECKER et al., 2008).

A proteína E1 possui cerca de 27-33 kDa e forma heterodímeros por ligação dissulfeto com a E2 (RONECKER et al., 2008). No entanto, sua estrutura e função na penetração do BVDV ainda não são bem elucidadas. Sugere-se que a E1 do BVDV e do vírus da hepatite C (*hepatitis C virus*, HCV) seja uma proteína de fusão, embora peptídeos (ou sequências)

fusogênicas ainda não tenham sido inequivocamente identificadas em BVDV (QI et al., 2022).

A E2 possui cerca de 53-55 kDa e medeia a ligação do BVDV à célula hospedeira por meio da ligação ao receptor CD46. A E2 forma homodímeros e heterodímeros (E1-E2), os quais são essenciais para o processo de penetração em célula hospedeiras (MAURER et al., 2004; RADTKE; TEWS, 2017). Devido à sua participação na ligação aos receptores, a E2 se constitui no principal alvo para anticorpos neutralizantes (PATON; LOWINGS; BARRETT, 1992; WEILAND et al., 1990).

A p7 é uma proteína hidrofóbica de 6-7 kDa, derivada da E2, e possui dois domínios, um que permanece ligado à região C-terminal de E2 e o outro que é clivado pela ação de uma protease celular (ELBERS et al., 1996). O precursor E2-p7 demonstrou não ser essencial para a replicação do vírus *in vitro* (BECHER; MOENNIG; TAUTZ, 2021). Por ser uma proteína hidrofóbica e integral de membrana, estudos sugeriram que a p7 poderia ser uma viroporina (HARADA; TAUTZ; THIEL, 2000; LARGO et al., 2014), atuando na redução da acidificação da vesículas intracelulares durante o estágio final de montagem da progênie viral (STEINMANN et al., 2007; WOZNIAK et al., 2010).

A NS2-3 (p125), a maior dentre as proteínas não estruturais de BVDV, apresenta cerca de 120 kDa e aproximadamente 1300 aa (MOULIN et al., 2007). A NS2-3 pode atuar na inibição da produção de IFN-I, um dos principais mecanismos da resposta imune inata aos vírus (GAMLEN et al., 2010), contribuindo assim para o sucesso das infecções por BVDV e sua rápida disseminação (PATON; SANDS; ROEHE, 1991). A NS2 (p54) possui 450 aa, tem atividade de autoprotease, sendo responsável pela clivagem de NS2-3. A ativação da NS2 requer interação com uma proteína celular DNAJ-C14 (também descrito como Jiv, *J-domain protein interacting with viral protein*) (RINCK et al., 2001). A NS3 (p80) possui 680 aa e é considerada uma proteína multifuncional, possuindo atividades de helicase, serino-protease e atividade de nucleosídeo trifosfatase (NTPase), regulando a eficiência de replicação do RNA viral (ZHENG et al., 2021). A atividade da NS3 é completa quando presente no complexo NS3/NS4A, quando realiza a clivagem de todas as proteínas a jusante da NS3 (KLEMENS; DUBRAU; TAUTZ, 2015). Além disso, a NS3 é considerada um marcador para o biotipo citopático de BVDV (DONIS, 1995).

A NS4A (p10) é uma pequena proteína com 55 aa e 10 kDa, necessária para a estimulação da atividade de protease de NS3, sendo assim considerada um cofator dessa protease (XU et al., 1997). Possui importante papel na replicação do genoma (LIANG et al., 2009; MOULIN et al., 2007). A NS4B (p32) é uma proteína hidrofóbica de cerca de 35 kDa,

(GRASSMANN et al., 2001), sendo descrita como uma proteína de integral de membrana, podendo ser localizada nas membranas do complexo de Golgi e mitocôndrias (WEISKIRCHER et al., 2009). Essa proteína pode desempenhar funções na replicação do genoma, sendo um componente integral do complexo de replicação de BVDV (WEISKIRCHER et al., 2009).

A NS5A (p58) possui cerca de 450 aa e 58 kDa e é uma fosfoproteína hidrofílica com atividade de ligação ao RNA, que compõe a replicase do BVDV (ISKEN et al., 2014). A NS5B (p75) possui cerca de 77 kDa e é a RNA polimerase dependente de RNA *(RNA-dependent RNA polymerase*, RdRp). Além disso, já foi demonstrada a participação de NS5B na morfogênese das partículas de BVDV (ANSARI et al., 2004; ZHONG; GUTSHALL; DEL VECCHIO, 1998).

2.3 DIVERSIDADE E CLASSIFICAÇÃO GENÉTICA

O genoma dos BVDVs apresenta uma alta taxa de variação relacionada à falta de atividade de revisão de nt (atividade 3'-5' exonuclease) da RpRd (HE et al., 2022). Estima-se que a taxa evolutiva do genoma dos BVDVs seja de 1.4×10^{-3} substituições/sítio/ano (CHERNICK; VAN DER MEER, 2017). Assim quando realiza-se o alinhamento e comparação entre as sequências de genomas de BVDVs disponíveis, encontra-se uma alta taxa de alterações de nucleotídeos. Essas alterações dão origem ao que denominamos de variabilidade, a qual também pode ser relacionada com eventos de recombinação homóloga ou não homóloga entre vírus diferentes (BECHER; TAUTZ, 2011).

A alta taxa evolutiva de BVDV resulta na geração de progênie e, assim, de tipos virais genotipicamente distintos. Já foram descritos 21 subtipos de BVDV-1 (a-u) e 4 de BVDV-2 (a-d) (YEŞILBAĞ; ALPAY; BECHER, 2017). Embora a identificação inicial da maioria desses subtipos tenha sido feita pela análise de seus genomas completos, muitos isolados e cepas de BVDV têm sido subtipados apenas pelo sequenciamento parcial da 5'UTR e/ou pela análise parcial de outras regiões genômicas, a exemplo de N^{pro} e E2 (BIANCHI et al., 2011; CORTEZ et al., 2006; FIGUEIREDO et al., 2019; MERCHIORATTO et al., 2020; MONTEIRO et al., 2018, 2019b; RIDPATH; BOLIN; DUBOVI, 1994). Alguns autores também têm subtipado BVDV pela análise combinada de regiões genômicas vizinhas, como NS5B-3'UTR (MISHRA et al., 2007; NAGAI et al., 2004) e 5'UTR-N^{pro} (BOOTH et al., 2013; DENG et al., 2020; MIROSŁAW; POLAK, 2019).

Alternativamente às análises filogenéticas, Giangaspero et al. (2008) reportaram uma subtipagem de BVDV-2 a partir da análise de estruturas secundárias da 5'UTR. Nesse estudo, os autores identificaram o subtipo BVDV-2d; representado apenas por uma cepa viral isolada na Argentina a partir de um soro fetal bovino. A análise estrutural realizada por Giangaspero et al. (2008) também permitiu subdivisões do BVDV-2a (1.1, 1.2, 1.3, 1.4, 1.5, 1.6 e 2) e -2b (1.1, 1.2, 1.3,1.4 e 2).

As análises filogenéticas baseadas em uma única região genômica têm permitido a identificação correta das espécies de BVDV (FIGUEIREDO et al., 2019; WOLFMEYER et al., 1997). No entanto, em relação às subtipagens, vários autores têm descrito resultados conflitantes dependendo da região do genoma que é analisada. Esses resultados discrepantes podem ser mais frequentemente observados quando isolados e cepas de BVDV, previamente subtipados com base na sequência parcial da 5'UTR, são posteriormente analisados por diferentes regiões codificantes (NAGAI et al., 2001, 2004; NEILL et al., 2019a; SILVEIRA et al., 2017; TAJIMA, 2004; WORKMAN et al., 2016).

No estudo de Tajima (2004), por exemplo, dois isolados de BVDV-1, que haviam sido identificados como BVDV-1a na análise realizada com a 5'UTR, foram classificados como 1c pela análise de E2. De maneira semelhante, Nagai et al. (2004) identificaram três isolados de BVDV, IS7NCP/97, IS8NCP/97 e IS14NCP/99, como 1a na análise baseada na região 5'UTR. No entanto, quando esses isolados foram analisados em relação às regiões de N^{pro} e E2, os autores os classificaram como BVDV-1c. Além de classificações discordantes, análises baseadas na sequência parcial da 5'UTR também podem apresentar baixo suporte estatístico (WORKMAN et al., 2016) ou resultar em isolados e cepas de BVDV sem subtipo definido (SILVEIRA et al., 2017). É possível que a extensão da região da 5'UTR que é analisada, além de seu elevado grau de conservação, seja um dos principais fatores responsáveis por esses resultados conflitantes e/ou equivocados (YEŞILBAĞ; ALPAY; BECHER, 2017).

Assim, muitos autores têm subtipado isolados e cepas de BVDV a partir da análise de regiões codificantes, principalmente N^{pro} e E2. Embora essas análises possam gerar resultados mais consistentes, as subtipagens baseadas em apenas um alvo gênico também podem levar a classificações diferentes a depender do alvo analisado. No estudo de Nagai et al. (2004), os isolados ILLC e ILLNC foram classificados como BVDV-1b nas análises baseadas em 5'UTR, N^{pro}, E2 e NS5B-3'UTR, e como 1a, de acordo com a sequência de NS3 (NAGAI et al., 2004). Workman et al. (2016) observaram que a cepa JZ05-1/China agrupou em um clado muito próximo ao de BVDV-2a após a análise do genoma completo, porém a

mesma cepa foi classificada como 2b quando utilizada somente a região gênica NS2/3. Nesse contexto, uma das soluções para classificações mais seguras de BVDV seria a análise de duas ou mais regiões do genoma viral (LIU et al., 2009; WORKMAN et al., 2016) ou, preferencialmente, o sequenciamento do genoma viral completo (YEŞILBAĞ; ALPAY; BECHER, 2017).

2.4 DISTRIBUIÇÃO DOS SUBTIPOS DE BVDV

Infecções por BVDV-1 e BVDV-2 têm sido descritas desde 1946 e 1994, respectivamente (OLAFSON; MACCALLUM; FOX, 1946; PELLERIN et al., 1994; RIDPATH; BOLIN; DUBOVI, 1994). No Brasil, a classificação dos tipos de BVDV se iniciou no final da década de 1990, com os isolados do Rio Grande do Sul que foram identificados como BVDV-1a (cepa BR275) e BVDV-2 (cepa Soldan) (CANAL et al., 1998). Em 2002, também no Brasil, foi descrita a primeira a classificação do BVDV-2 nos subtipos 2a e 2b (FLORES et al., 2002). No país, até o ano de 2017, foram identificados, no mínimo, um total de 106 BVDV-1 (54a, 20b, 4c, 24d, 1e, 1i, além de dois vírus não subtipados) e 55 BVDV-2 (2a, 50b e três vírus não subtipados) (FLORES et al., 2019; YEŞILBAĞ; ALPAY; BECHER, 2017).

Até o ano de 2017, foram identificados geneticamente aproximadamente 1.055 isolados/cepas de BVDV-1 (277a, 738b, 6c, 24d, 1e e 2g, além de sete isolados não classificados) e 337 BVDV-2 (139a, 59b, 1c, 1d e 137 não classificados). No continente asiático, foram identificados 1.411 BVDV-1 (256a, 703b, 251c, 13d, 4j, 117m, 3n, 7o, 9p, 14q, 22u e 12 não classificados) e 359 BVDV-2 (342a, 2b e 15 não classificados). No continente europeu, foram descritos 3.101 BVDV-1 (866a, 732b, 4c, 281d, 376e, 334f, 21g, 309h, 24i, 3j, 79k, 39l, 5r, 1s, 1t, 2u e 24 não classificados) e 119 BVDV-2 (50a, 9b, 28c e 32 não classificados) (YEŞILBAĞ; ALPAY; BECHER, 2017).

3 JUSTIFICATIVA

O conhecimento sobre a diversidade genética das cepas e isolados de BVDV e de sua distribuição geográfica possui importância tanto para o diagnóstico laboratorial quanto para a implementação de programas de controle, principalmente em relação às formulações vacinais, as quais devem ser adequadas aos BVDVs circulantes. Embora a análise da sequência parcial da 5'UTR, assim como dos genes E2 e N^{pro}, frequentemente utilizadas para a classificação de BVDV, permitam a identificação correta das espécies de pestivírus, subtipagens de BVDV baseadas nessas regiões podem gerar resultados equivocados e com fraco suporte estatístico (NAGAI et al., 2001, 2004; NEILL et al., 2019b; WORKMAN et al., 2016). Além disso alguns genomas de BVDV-2 disponíveis no banco de dados *GenBank*, ao serem analisados tanto pelas regiões genômicas ou genoma viral, não apresentam uma subclassificação confiável em 2a-c. Assim pode-se constatar a ausência de uma padronização para a classificação e principalmente subclassificação dos BVDVs. Dessa forma, esta tese apresenta estudos que tiveram como objetivo identificar regiões genômicas de BVDV-1 e BVDV-2 cuja análise filogenética reproduza resultados equivalentes aos obtidos com a análise do genoma viral completo. Também realizou-se uma análise mais detalhada de sequências de BVDV-2 que apresentavam uma subclassificação incoerente e/ou duvidosa, contribuindo, assim, para uma subtipagem viral mais precisa e acessível de BVDV. Essa análise resultou na identificação de um grupo de sequências/vírus filogeneticamente distintos dos demais, sugerindo-se denominar BVDV-2e.

4 ARTIGO 1

SUBTYPING BOVINE VIRAL DIARRHEA VIRUS (BVDV): WHICH VIRAL GENE TO CHOOSE?

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SUBTYPING BOVINE VIRAL DIARRHEA VIRUS (BVDV): WHICH VIRAL GENE TO CHOOSE?

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ABSTRACT**:** Bovine viral diarrhea virus-1 (BVDV-1, Pestivirus A) and BVDV-2 (Pestivirus B) have been clustered into 21 and 4 subtypes, respectively. This genetic diversity, in addition to the lack of consensus on which genomic region to use for BVDV subtyping, has resulted in conflicting classifications depending on the target analyzed. Here, we investigated which genes or UTRs would reproduce the phylogeny obtained by complete genome (CG) analyses. The study was carried out with 91 (BVDV-1) and 85 (BVDV-2) CG available on GenBank database. The viruses were subtyped by analyzing their CG, as well as their individual genes and UTRs (complete 3′ and 5′ UTRs, and partial 5′ UTR); and the phylogeny results were compared to each other. The sequences were aligned using the ClustalW multiple method (BioEdit Alignment Editor software, v.7.0.5.3) and the phylogenetic analyses were performed by the Maximum Likelihood method (MEGA-X software, v.10.2.4), with 1000 bootstrap replicates. The best analysis model for each gene/UTR was defined using the jModelTest software. The geodesic distance between the CG (reference) and individual genes/UTRs trees was also calculated (TreeCmp software, v.2.0). In general, 3′ UTR-based analyses, followed by 5′ UTR, presented the least reliable subtyping results. Regarding BVDV-1, phylogeny based on C, E^{rns}, E1, E2, p7, NS2, NS3, NS4B, NS5A and NS5B was consistent with that of CG. In contrast, analyses performed with individual BVDV-2 genes showed at least one different clustering from the phylogeny based on the CG. After analyzing the geodesic distance between the CG and genes/UTRs trees, we observed that NS4B (for BVDV-1) and NS5A (BVDV-2) presented the closest topology and edge length to the CG analyses. Finally, comparing the phylogeny performed with the CG and the genes/UTRs, as well as the geodesic distance between them, we understand that NS4B and NS5A represent the most suitable targets for BVDV-1 and -2 subtyping, respectively, and may be considered in future phylogenetic studies.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is an important pathogen of cattle associated with considerable economic losses, mainly resulting from reproductive disorders, such as decreased fertility, slow fetal growth, abortion, teratogenesis, embryonic resorption, fetal mummification and stillbirths. In addition, BVDV infections may also cause diarrhea, respiratory disease, immunosuppression and the generation of persistently infected (PI) animals, which are the main sources of infection (Brock, 2004; Houe, 1999).

The BVDVs belong to the family Flaviviridae, genus Pestivirus, which comprises three viral species of cattle, namely Pestivirus A (BVDV-1), Pestivirus B (BVDV-2) and Pestivirus H (HoBi-like virus, HoBi-PeV) (ICTV, 2020; Smith et al., 2017). The pestivirus genome consists of a single-stranded positive RNA of about 12.3 kb, with a single open reading frame (ORF), flanked by two untranslated regions (5′ and 3′UTR). The ORF encodes a long polyprotein subsequently processed by cellular and viral proteases into 11 or 12 polypeptides: N-terminal autoprotease (N^{pro}), capsid protein (C), envelope proteins (E^{rns}, E1 and E2), p7, and non-structural (NS) proteins [NS2/NS3 (or NS2 and NS3), NS4A, NS4B, NS5A, and NS5B] (Tautz et al., 2015).

Currently, 21 BVDV-1 subtypes (a-u), 4 BVDV-2 (a-d) and 4 HoBi-PeV (a-d) have been identified, based mainly on analysis of the partial 5'UTR sequence, followed by analyses of other genomic regions, such as N^{pro} and E2 (Becher et al., 1999; Figueiredo et al., 2019; Nagai et al., 2004; Ridpath et al., 1994; Vilček et al., 1994; Yeşilbağ et al., 2017). Although single-target analysis is useful for identifying bovine pestivirus species, subtyping based on a single genomic region may result in unclassified viruses or in viruses clustered with low statistical support. Furthermore, considering the possibility of recombination between pestiviruses, it is possible that subtyping based on individual targets may generate discrepant clustering according to the genomic region analyzed. The reliable classification of pestivirus subtypes, however, is extremely useful for epidemiological surveys, allowing to monitor the prevalence and distribution of pestiviruses, as well as to identify emerging viruses. Additionally, diagnostic tools and vaccine formulations may need to be adapted according to circulating pestivirus subtypes.

The most appropriate and accurate strategy to solve these issues would be to analyze the complete viral genome. Unfortunately, sequencing of full genomes is still expensive, representing a limitation for many laboratories, and is not feasible for large surveys. In this context, and to find a simple and low-cost alternative to a reliable pestivirus subtyping, we collected complete genomes of BVDV-1 and -2 available on GenBank database and

investigated which genes (or UTRs) would reproduce the phylogeny obtained by CG analyses. The genomic regions with the best results, i.e., with the highest level of compliance with the full genome, represent potential targets for BVDV subtyping and may be considered in future phylogenetic studies.

MATERIALS AND METHODS

Sequence collection. Complete genome sequences of BVDV-1 and BVDV-2 were obtained from the GenBank database, "https://www.ncbi.nlm.nih.gov/search/", using the search terms "Bovine viral diarrhea virus 1" and "Bovine viral diarrhea virus 2", and excluding sequences with less than 12 kb, or sequences that have been manipulated with insertion, deletion or change of nucleotides (nt). The sequences were collected between February 25 and 26, 2021.

Phylogenetic analysis. All complete BVDV-1 or -2 sequences, as well as their individual genes and UTRs, were aligned by the ClustalW multiple method, using the BioEdit Alignment Editor software package, version 7.0.5.3 (Staden, 1996). In addition, we also performed an alignment of the partial 5′UTR sequence, nt 120–408 for BVDV-1 (NADL strain, M31182.1) and nt 114–397 for BVDV-2 (New York 93 strain, AF502399); the largest sequence that may be analyzed with the amplicons obtained with primers 90-368, often used in BVDV phylogeny (Ridpath and Bolin, 1998). In total, 16 alignments were performed for each viral type, BVDV-1 or -2, corresponding to the CG, 5′UTR (complete and partial sequences), 12 individual genes (N^{pro}-NS5B) and 3′UTR. Phylogenetic analyses were performed using the MEGA-X (version 10.2.4) software (Kumar et al., 2018) and the evolutionary history was inferred by the Maximum Likelihood (ML) method, with 1000 bootstrap replicates. The best analysis model for each gene/UTR was defined by the jModelTest software (Posada, 2008). Parameters used in ML phylogeny are listed in Table 1, Table 2.

Subtyping comparison. The BVDV-1 or -2 subtyping based on the CG was compared with the results obtained with the individual genes, as well as from the UTRs analyses. The geodesic distances between the CG (reference) and genes/UTRs trees were also analyzed. This space measure considers both the tree topology and the edge length. In our study, the weighted geodesic distance (Owen and Provan, 2011) was calculated using the TreeCmp software, version 2.0 (https://eti.pg.edu.pl/TreeCmp). Finally, the genes/UTRs that presented the highest conformity with the CG classifications, as well as the shortest geodesic distances from to the CG, were considered the most suitable targets for BVDV subtyping.

RESULTS

BVDV-1 subtyping. A total of 91 BVDV-1 sequences, containing both 5′ and 3′UTRs and the ORF, were obtained from the GenBank database (access numbers are available on Supplementary Data, File S1). After phylogenetic analysis, the full genomes were classified/clustered as BVDV-1a (25 viruses), -1b (31), -1c (4), -1d (5), -1e (4), -1f (3), -1 g (1), -1 h (2), -1i (2), -1j (1), -1 k (2), -1 m (4), -1n (1), -1o (1), -1q (3), -1r (1) and -1u (1) (Fig. 1A). We did not find CG sequences for BVDV-1 l, BVDV-1p, BVDV-1 s and BVDV-1 t.

All sequences were then analyzed according to the UTRs and individual genes, and the results were compared to the CG. Among the genomic regions analyzed, phylogeny based on 3′UTR presented the highest discrepancy in relation to the CG: at least three viruses/genomes were subtyped differently; MH899944 and MH899943 (both BVDV-1f by CG analysis) were identified as 1r by 3′UTR analysis, and KC963967 (1b by CG) was classified as 1a by 3′UTR analysis (File S2). The analyses performed with the 5′UTR (complete and partial sequences) also showed unsatisfactory results. The BVDV-1q sequences were clustered separately in the phylogeny based on the complete 5′UTR sequence (File S2). A similar result was observed with the partial 5′UTR sequence, in which the BVDV-1e clade was divided (File S2).

Regarding the coding region, phylogeny based on N^{pro} and NS4A was unable to classify at least one sequence: AF268278 (BVDV-1a) and MH899941 (BVDV-1b), respectively (File S2). The BVDV-1e clade was also divided in the NS4A analysis (File S2). The analyses performed with C, E^{rns}, E1, E2, p7, NS2, NS3, NS4B, NS5A and NS5B identified all BVDV-1 sequences according to the CG-based phylogeny (Fig. 1B; File S2). However, when we analyzed the geodesic distance between the CG and genes/UTRs trees, we observed that NS4B showed the topology and edge length closest to the CG analysis (Fig. 2; Table 3). These results, combined with those of the subtyping described above, indicate NS4B as the most suitable target for BVDV-1 subtyping.

BVDV-2 subtyping. We collected 85 complete BVDV-2 genome sequences available on GenBank (File S1). After analyzing these sequences, and also considering previous reports, we identified 45, 4 and 22 viruses as BVDV-2a, -2b and 2-c, respectively. However, at least 14 sequences could not be safely clustered according to CG-based phylogeny (GenBank access numbers HQ258810, JF714967, MK599227, KT832818, KX096718, MH231124, MH231132 MH231133, MH806435, MH231148, MH231149, MH231150, MH231151 and MH231152) (Fig. 3A).

When phylogeny was performed with the UTRs or genes, we observed different levels of compliance in relation to CG analysis. The statistical support of 3′UTR-based phylogeny was very low, making it difficult to classify most sequences (File S3). The analyses carried out with the complete and partial 5′UTR sequences were unable to classify at least four sequences of BVDV-2a (KT832817, MH231134, MH231131 and MH806437) (File S3).

Regarding the coding region, p7 and NS3 presented the least satisfactory results, leading to at least eight and five different classifications from the CG, respectively (File S3). Analyses based on C and E2 resulted in at least three conflicting classifications. The NS2 phylogeny presented at least two non-conformities in relation to the CG (File S3). Analyses performed with N^{pro}, C, E^{rns}, E1, NS4A, NS4B, NS5A and NS5B showed similar results among them. These genes presented at least one non-conformity in relation to phylogeny based on CG (Fig. 3B; File S3).

After calculating the geodesic distance between the CG and genes/UTRs trees, we found that the analysis based on NS5A presented the closest topology and edge length to the CG (Fig. 4; Table 3). Therefore, the spatial analysis of the trees, in association with the results previously described, indicate NS5A as the most suitable target for BVDV-2 subtyping.

DISCUSSION

Numerous studies have demonstrated the high genetic diversity of bovine pestiviruses, consequently several viral subtypes have been identified, mainly for BVDV-1 (Yeşilbağ et al., 2017). In addition, several studies have also reported that pestivirus subtyping may present conflicting results depending on the genomic region analyzed (Nagai et al., 2004; Neill et al., 2019; Tajima, 2004; Workman et al., 2016). Taking into account this genetic variability and the need for reliable subtyping, monitoring viral diversity, as well as the emergence of new pestiviruses, represent a real challenge. Considering these issues, we investigated which genes/UTRs would be the most suitable targets for a BVDV subtype classification. We believe that this single-target analysis may be very useful when CG sequencing is not feasible.

Initially, we investigated the reliability of BVDV typing based on individual genes or UTRs. Although the partial 5′UTR sequence, easily amplified with internal primers, is often used for the BVDV classification into species (Monteiro et al., 2019; Ridpath and Bolin, 1998), we noticed that any of the individual genes/UTRs may also be used to differentiate BVDV-1 and -2 (data not shown). However, regarding BVDV subtyping, we observed conflicting results when comparing analyses of different genes/UTRs with the CG.

BVDV-1 subtyping. In our study, the UTR analyses showed the least satisfactory results for BVDV-1 subtyping. Phylogeny based on 3′UTR, for example, was unable to subtype three BVDV-1 sequences. In addition, the BVDV-1q and 1e clusters were divided into distant "subclades" after the analysis of the complete and partial 5′UTR sequences, respectively. These results are probably associated with the high degree of conservation, as well as the length of the analyzed region. Indeed, several authors have reported inconsistent subtyping after 5′UTR analysis (Nagai et al., 2004; Tajima, 2004; Yeşilbağ et al., 2017).

In this context, the coding region, which may generate more reliable results, has often been chosen to confirm the subtyping performed with partial 5′UTR. For example, several BVDV-1 subtyping based on partial 5′UTR has been successfully confirmed after analysis of gene regions, such as N^{pro} and E2 (Deng et al., 2015; Giammarioli et al., 2015; Mao et al., 2016; Mirosław and Polak, 2019; Robesova et al., 2009). However, conflicting classifications have also been reported when comparing analyses with 5′UTR and N^{pro}, or E2 (Nagai et al., 2004; Tajima, 2004; Yeşilbağ et al., 2017). Some authors have described that the same virus may be classified into different subtypes depending on the analyzed gene/region (Nagai et al., 2004; Workman et al., 2016). In general, these discrepant results may be influenced by several factors, including possible recombination events.

Herein, although we also observed that BVDV-1 genes are more reliable than UTRs for viral subtyping, we found misclassifications in the analyses performed with N^{pro} and NS4A. On the other hand, phylogeny based on C, E^{rns}, E1, E2, p7, NS2, NS3, NS4B, NS5A and NS5B did not present inconsistent classifications in relation to CG analysis. To resolve this issue and find the best target for BVDV-1 subtyping, we calculated the geodesic distance between the CG and genes/UTRs trees. This measure considers the tree topology and the edge length, and allowed us to identify NS4B as the best target for BVDV-1 subtyping.

Interestingly, to the best of our knowledge, NS4B has not been used for BVDV-1 subtyping. The lack of NS4B-subtyped BVDV-1 or NS4B-based reference sequences could represent a limitation for the use of this region as target for BVDV-1 subtyping. A potential solution to this issue would be the approach adopted in our study. It is possible to analyze the NS4B of previously sequenced genomes, compare its result with that of the CG and, thus, find NS4B reference sequences for each subtype. However, despite its considerable viability, this strategy would not allow us to classify BVDV-1 into 1l, 1p, 1s or 1t subtypes. To our knowledge, these subtypes have been identified based on the analysis of partial 5′UTR and/or N^{pro} (Decaro et al., 2017).

Taken these results together, we encourage: i) the CG analysis of the BVDV-1 reference strains, which is also important to confirm the identity of BVDV-1l, 1p, 1s or 1t; and ii) NS4B analysis in future phylogenetic studies, in order to investigate the suitability of this target for subtyping BVDV-1 strains and isolates.

BVDV-2 subtyping. Similar to that observed with the BVDV-1 subtyping, BVDV-2 was better classified by the coding region than by the UTRs. However, unlike the analysis of most BVDV-1 genes, which allowed the sequences to be classified according to the CG, all BVDV-2 genes failed to fully reproduce the results obtained by the CG analysis. The analyses carried out with individual BVDV-2 genes subtyped at least one virus that was not classified by the CG, and/or had at least one classification different from the CG.

Also, in contrast to that observed in the BVDV-1 analyses, in which all viruses were subtyped, at least fourteen BVDV-2 isolates/strains could not be classified by CG analysis. Among these, eleven viruses are described in published articles: HLJ-10 strain (JF714967.1) (Liu et al., 2012) and SH-28 strain (HQ258810.1) (Tao et al., 2013), and nine sequences reported by Neill et al. (2019), 1786c strain (MH231124), AzSpl strain (MH231132), B69519c strain (MH231133), 1336H strain (MH806435), 12-149,150 strain (MH231148), Short strain (MH231149), 12–151,955-317 strain (MH231150), 14,622 strain (MH231151) and 2412 strain (MH231152). In agreement with our analysis, Tao et al. (2013) were also unable to subtype the SH-28 strain. Regarding the HLJ-10 strain, although the authors did not address its classification, they pointed out its high similarity with the non-subtyped SH-28 strain (Liu et al., 2012).

On the other hand, nine sequences, which were unable to be subtyped by our CG analysis, were classified by Neil et al. (2019) after 5′UTR, N^{pro}, E2 and ORF analyses. The authors identified the viruses as 2a (MH231132 and MH806435) or 2c (MH231124, MH231133, MH231148, MH231149, MH231150, MH231151 and MH231152). Regarding the sequence MH806435, although it was not classified by our CG, N^{pro}, E2 and ORF (data not shown) analyses, it was identified as 2c in our analyses performed with 5′UTR (complete and partial sequences). For the sequence MH231132, we found different results in the phylogeny based on 5′UTR, N^{pro}, E2 and ORF (data not shown): the sequence was classified as 2a in the analysis carried out with partial 5′UTR; and as 2c (or remaining unclassified) in the analyses performed with complete 5′UTR, N^{pro}, E2 and ORF (data not shown).

Despite the MH231132 and MH806435 classifications have shown conflicting results when comparing our CG and single target analyses, all sequences identified as 2c by Neil et al. (2019), mentioned above, could not be safely classified both in our CG analysis and in

those of genes/UTRs. Interestingly, in the study by Neil et al. (2019), the authors used a virus previously identified as 2b, by analyzing the 5′UTR (Flores et al., 2002), as a reference sequence for 2c. Although the reason for this was not clear for us, we believe that it would be interesting to repeat the analyses using well-consolidated sequences as reference for 2c. In addition, the authors did not analyze the CG of these sequences, containing the UTRs and ORF; this is also an issue to be considered.

Importantly, we do not believe that the different findings between our study and that of Neil et al. (2019) is due to the methodology. Similar to our study, the authors performed the analyses using the ML method. The analysis models of Neil et al. (2019) for 5′UTR were the same used here, Kimura 2-parameter. Regarding the N^{pro} analysis, the best model for our data was the Kimura 2-parameter. Neil et al. (2019) performed the phylogeny of N^{pro} by Tamura-Nei. For the ORF and E2 analyses, we and Neil et al. (2019) used the General Time Reversible model.

Considering this context, we believe to be timely (and useful) to discuss new classifications/subtypes of BVDV-2 in order to accommodate viral isolates/strains that cannot be safely clustered into subtypes a-c. It is important to consider that an additional BVDV-2 subtype, named BVDV-2d, was reported by Giangaspero et al. (2008). However, this classification was based on the 5′UTR secondary structure and included a single isolate from Argentina (Giangaspero et al., 2008), previously reported as a contaminant of fetal bovine serum (Jones et al., 2001).

In addition, in the perspective discussed here, and based on our results, we believe that subtyping performed with non-ideal targets may lead to at least two worrisome results: the report of BVDV-2 a-c as unclassified viruses, or the classification of BVDV-2 into a different subtype than the one it belongs to. Our findings indicate that NS5A-based BVDV-2 subtyping may minimize these misunderstandings. The only non-conformity between the CG and NS5A analyses was related to the sequence KX096718. This sequence was not subtyped by CG analysis, but it was classified as 2a by NS5A. In addition, the geodesic distance between the CG and the NS5A trees was the shortest among the analyzed genes/UTRs.

Interestingly, similar to that observed with NS4B for BVDV-1 subtyping, few studies have focused on NS5 to subtype BVDV-2. When comparing the analysis of nine complete BVDV-2 genomes with their respective individual genes, Workman et al. (2016) identified that N^{pro}, E2, NS5A and NS5B provided the highest resolution and statistical support, including the tree topology most similar to the CG. These findings are in line with the results observed herein. After NS5A, the genes with the least geodesic distances from CG were E2,

NS5B and N^{pro}. Specifically on E2, the region had a geodesic distance of 0.082 from the CG; value very close to the distance between NS5A and CG, 0.08. However, while NS5A presented only one non-conformity in relation to the CG, three sequences not classified by the GC (HQ258810, KX096718 and MH231132) were identified as 2a or 2c by E2.

CONCLUSION

Our findings suggest NS4B and NS5A as the most suitable targets for BVDV-1 and -2 subtyping, respectively, and encourage the analysis of these gene regions in future studies, including to investigate their real phylogenetic usefulness. We also raised the discussion about the need to organize new BVDV-2 subtypes, in order to cluster viruses that are not be consistently identified as 2a-d. The results presented in our study are supported by the comparative phylogenetic analysis between CG and genes/UTRs, as well as by the geodesic distance between them.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no competing interests.

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Table 1 - Parameters used in Maximum Likelihood phylogeny of BVDV-1 sequences.

Genomic region	Substitution model[a]	Gamma shape parameter	Proportion invariant sites	BIC[b]	Log likelihood
Complete Genome	GTR+G+I	0.7135	0.29	486,581.242	-241,957.05
5'UTR[c]	K2+G	0.4278	NA[e]	12,390.822	-5,211.35
5'UTR 90-368[d]	GTR+G	0.2496	NA	5,701.630	-1,931.64
N^{pro}	T92+G	0.4039	NA	22,238.752	-10,113.01
C	TN93+G+I	1.0451	0.19	13,163.549	-5,628.48
E^{rns}	GTR+G+I	0.6433	0.24	25,435.177	-11,692.01
E1	T92+G+I	1.5847	0.21	24,956.118	-11,478.09
E2	GTR+G+I	1.0256	0.16	59,299.849	-26,899.31
p7	T92+G+I	1.5588	0.16	10,784.884	-4,493.82
NS2	GTR+G+I	0.8675	0.23	55,983.883	-28,359.89
NS3	GTR+G+I	0.6336	0.26	124,804.580	-61,271.95
NS4A	K2+G	0.1638	NA	7,951.153	-3,047.47
NS4B	GTR+G+I	0.6216	0.44	48,071.531	-22,949.46
NS5A	GTR+G+I	0.8844	0.18	63,697.946	-30,768.16
NS5B	GTR+G+I	0.6782	0.22	76,315.575	-3,7012.94
3'UTR	T92+G	1.4289	NA	10,104.130	-4,105.67

[a] GTR: general time reversible; G: gamma distribution; I: invariant sites; K2: Kimura 2-parameter; T92: Tamura 3-parameter; TN93: Tamura-Nei;
[b] Bayesian information criterion;
[c] Untranslated region;
[d] nt 120-408 (NADL strain, M31182.1);
[e] Not applicable

Table 2 - Parameters used in Maximum Likelihood phylogeny of BVDV-2 sequences.

Genomic region	Substitution model[a]	Gamma shape parameter	Proportion invariant sites	BIC[b]	Log likelihood
Complete Genome	GTR+G+I	0.5107	0.27	220,578.245	-109,037.91
5'UTR[c]	K2+G	0.2486	NA[e]	7,433.017	-2,820.03
5'UTR 90-368[d]	K2+G	0.2156	NA	4,584.590	-1,440.08
N^{pro}	K2+G+I	1.4671	0.25	10,510.868	-4,349.34
C	TN93+G	0.3486	NA	6,702.183	-2,455.32
E^{rns}	TN93+G+I	1.9052	0.28	12,796.495	-5,445.88
E1	T92+G	0.3468	NA	11,597.047	-4,869.11
E2	GTR+G+I	0.6437	0.24	23,430.692	-10,702.25
p7	K2+G+I	1.9608	0,36	6,272.556	-2,303.07
NS2	GTR+G+I	0.6102	0.22	35,492.272	-16,688.04
NS3	TN93+G+I	2.2114	0.31	31,218.572	-14,552.82
NS4A	K2+I	NA	0.30	4,483.409	-1,424.45
NS4B	TN93+G+I	2.7608	0.29	18,039.630	-8,028.01
NS5A	TN93+G+I	2.1407	0.25	26,974.515	-1,2450.77
NS5B	TN93+G+I	1.7571	0.27	36,867.203	-17,389.62
3'UTR	T92+G	0.6587	NA	5,712.214	-2,266.72

[a] GTR: general time reversible; G: gamma distribution; I: invariant sites; K2: Kimura 2-parameter; TN93: Tamura-Nei; T92: Tamura 3-parameter;
[b] Bayesian information criterion;
[c] Untranslated region;
[d] nt 114-397 (New York 93 strain, AF502399);
[e] Not applicable

Table 3 - Geodesic distance between the complete genomes and genes/UTRs trees.

Genomic region	BVDV-1	BVDV-2
5'UTR[a]	0.485	0.267
5'UTR 90-368[b]	0.455	0.267
N^{pro}	31.357	0.089
C	0.568	0.106
E^{rns}	0.358	0.101
E1	0.292	0.100
E2	0.388	0.082
p7	1.524	0.128
NS2	0.526	0.465
NS3	0.356	0.189
NS4A	0.470	0.235
NS4B	**0.209**[c]	0.098
NS5A	0.499	**0.080**
NS5B	0.474	0.086
3'UTR	0.782	0.491

[a] Untranslated region;
[b] BVDV-1: nt 120-408 (NADL strain, M31182.1). BVDV-2: nt 114-397 (New York 93 strain, AF502399);
[c] The values in bold correspond to the genes with the shortest geodesic distance from the complete genome.

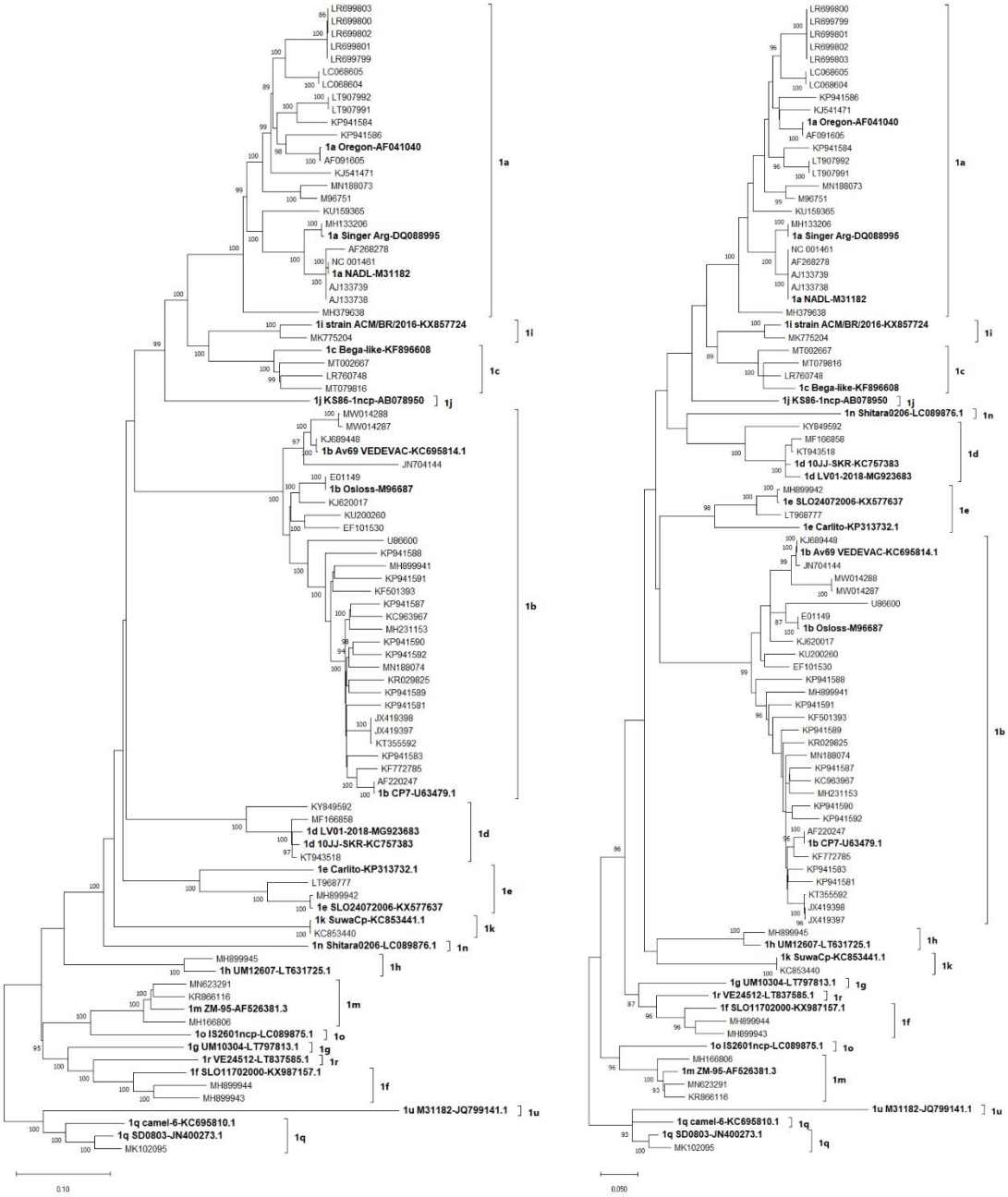

Fig. 1. Phylogenetic trees of BVDV-1 subtypes. Phylogeny was performed with the complete genomes (A) and NS4B (B) of BVDV-1. The analyses were performed using the MEGA-X (v.10.2.4) software and Maximum Likelihood method, according to the parameters listed in Table 1. The evolutionary distances were calculated using p-distance method. The bootstrap values were calculated in 1000 replicates. Branches supported by >85% of bootstrap replicates are indicated. Reference sequences for each subtype are in bold.

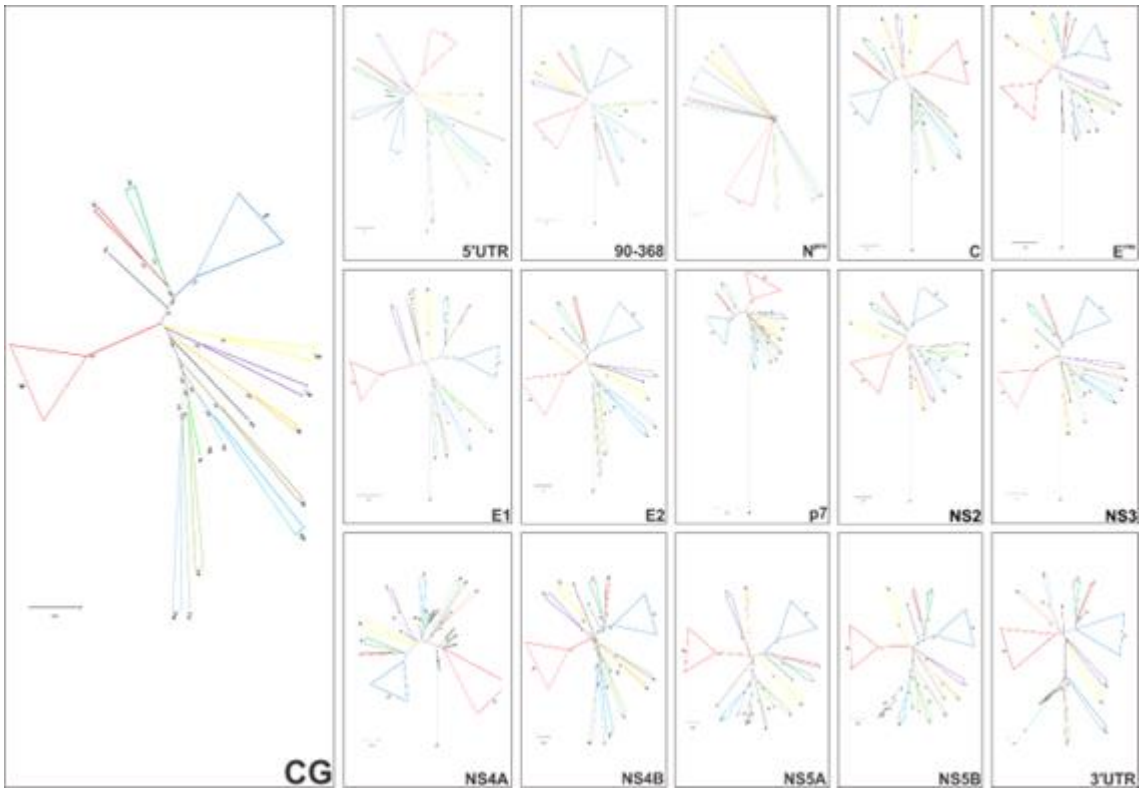

Fig. 2. Tree topology of BVDV-1 subtypes. The analyses were carried out with the complete genomes (CG) of BVDV-1 and their respective UTRs and individual genes. We also performed a tree of the partial 5´UTR sequence, nt 120-408 (NADL strain, M31182.1), which may be amplified with primers 90-368. The analyses were performed using the MEGA-X (v.10.2.4) software, the Maximum Likelihood method and the parameters listed in Table 1. The evolutionary distances were calculated using p-distance method. The bootstrap values were calculated in 1000 replicates.

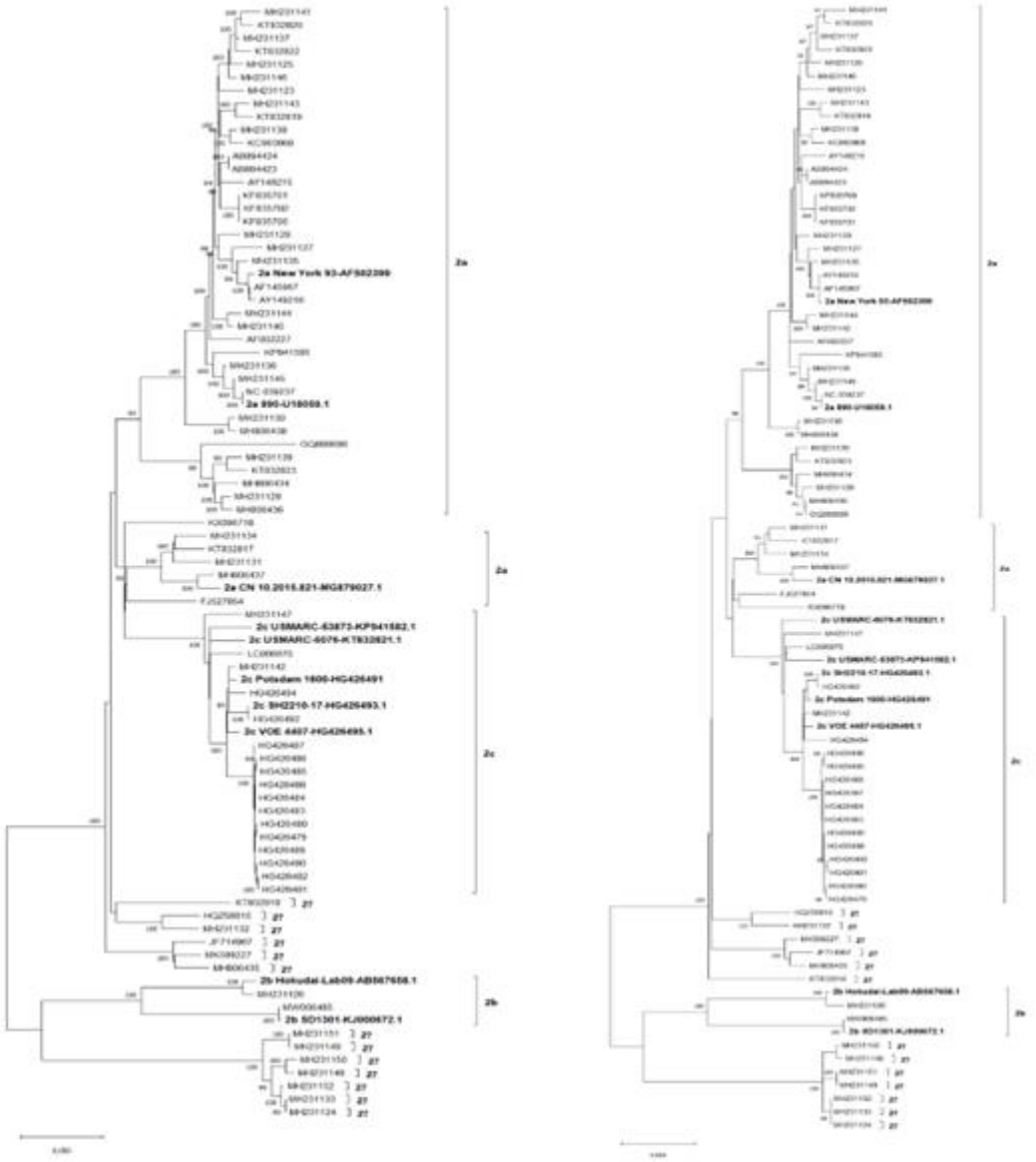

Fig. 3. Phylogenetic trees of BVDV-2 subtypes. Phylogeny was performed with the complete genomes of BVDV-2 (A) and their respective NS5A (B). Non-subtyped BVDV-2 sequences were identified as "BVDV-2?". The analyses were performed using the MEGA-X (v.10.2.4) software and Maximum Likelihood method, according to the parameters listed in Table 2. The evolutionary distances were calculated using p-distance method. The bootstrap values were calculated in 1000 replicates. Branches supported by >85% of bootstrap replicates are indicated. Reference sequences for each subtype are in bold.

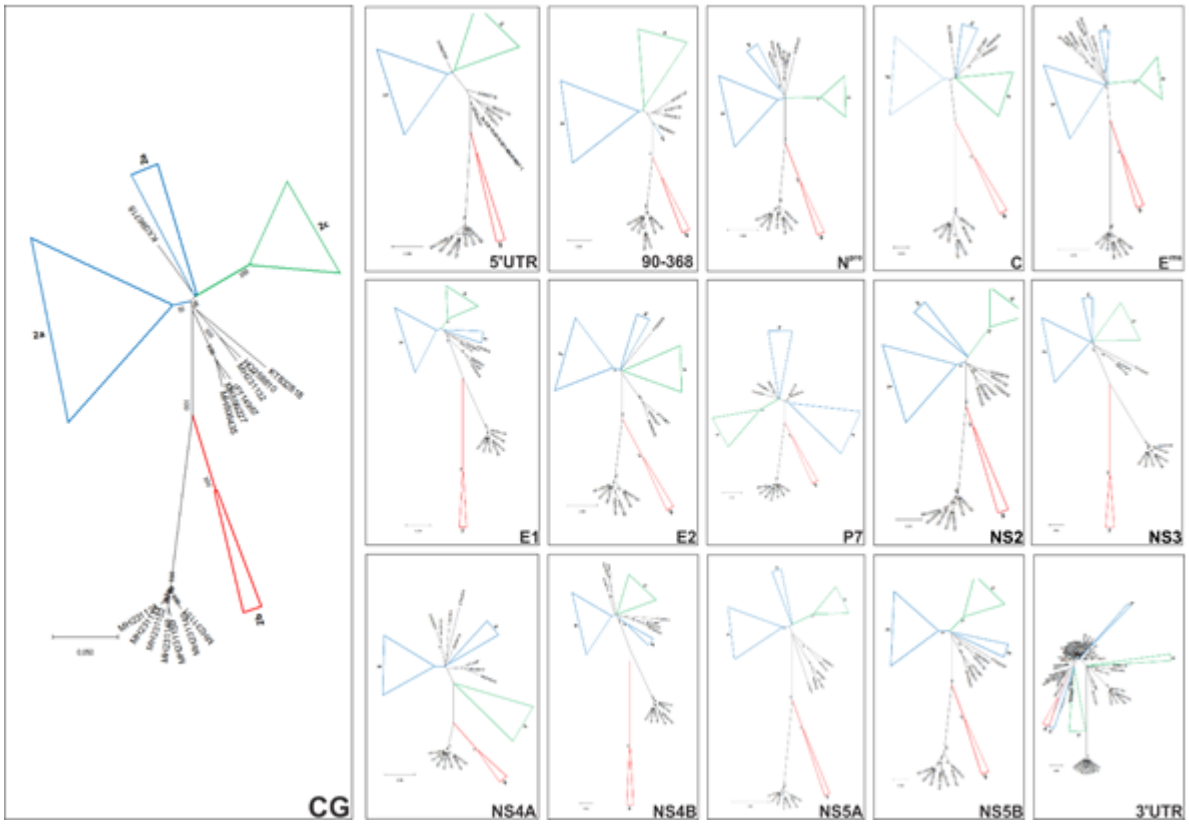

Fig. 4. Tree topology of BVDV-2 subtypes. The analyses were carried out with the complete genomes (CG) of BVDV-1 and their respective UTRs and individual genes. We also performed a tree of the partial 5′UTR sequence, nt 114-397 (New York 93 strain, AF502399), which may be amplified with the primers 90-368. The analyses were performed using the MEGA-X (v.10.2.4) software, the Maximum Likelihood method and the parameters listed in Table 2. The evolutionary distances were calculated using p-distance method. The bootstrap values were calculated in 1000 replicates.

5 ARTIGO 2

A NEW (OLD) BOVINE VIRAL DIARRHEA VIRUS 2 SUBTYPE: BVDV-2E

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A NEW (OLD) BOVINE VIRAL DIARRHEA VIRUS 2 SUBTYPE: BVDV-2E

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ABSTRACT**.** Bovine pestiviruses are members of the species Pestivirus A (bovine viral diarrhea virus 1, BVDV-1), Pestivirus B (BVDV-2) or Pestivirus H (HoBiPeV). To date, BVDV-2 isolates/strains have been classified into three subtypes (a-c) by phylogenetic analysis, and an additional subtype (d) has been proposed based on 5' untranslated region (UTR) secondary structures. In a previous study, we identified some BVDV-2 sequences in the GenBank database that could not be classified as subtype a, b or c by phylogenetic analysis of their genomes, UTRs or individual genes. Here, we performed a detailed study of these sequences and assessed whether they might represent a distinct BVDV-2 subtype. Initially, we collected 85 BVDV-2 complete/near-complete genomes (CNCGs) from GenBank and performed a "proof of equivalence" between phylogenetic analyses based on CNCGs and open reading frames (ORFs), which showed that ORFs may be reliably used as a reference target for BVDV-2 phylogeny, allowing us to increase our dataset to 139 sequences. Among these, we found seven sequences that could not be classified as BVDV-2a-c. The same was observed in the phylogenetic analysis of CNCGs and viral genes. In addition, the seven non-BVDV-2a-c sequences formed a distinct cluster in all phylogenetic trees, which we propose to term BVDV-2e. BVDV-2e also showed 44 amino acid changes compared to BVDV-2a-c, 20 of which are in well-defined positions. Importantly, an additional phylogenetic analysis including BVDV-2d and a pairwise comparison of BVDV-2e and BVDV-2d sequences also supported the difference between these subtypes. Finally, we propose the recognition of BVDV-2e as a distinct BVDV-2 subtype and encourage its inclusion in future phylogenetic analyses to understand its distribution and evolution.

INTRODUCTION

Bovine pestiviruses are positive-sense single-stranded RNA viruses with a genome of approximately 12.3 kb in length, composed of two terminal untranslated regions, the 5' and 3'UTR, flanking a single open reading frame (ORF) [1]. The ORF encodes a polyprotein of ~3900 amino acids (aa), which is processed by cellular and viral proteases to generate the structural and non-structural proteins N^{pro}, C, E^{rns}, E1, E2, p7, NS2/NS3 (or NS2 and NS3), NS4A, NS4B, NS5A and NS5B [2].

Bovine pestiviruses belong to the genus Pestivirus, family Flaviviridae, and have been assigned to the species Pestivirus A (bovine viral diarrhea virus 1, BVDV-1), Pestivirus B (BVDV-2) or Pestivirus H (HoBiPeV), which have so far been divided into 21 (a-u), 4 (a-d) and 4 (a-d) subtypes, respectively [3, 4]. These subtypings have been based mainly on analysis of partial 5'UTR sequences, followed by N^{pro} and E2 analyses [3,5,6,7,8,9,10].

Pestivirus infections have been reported over the years in cattle herds worldwide and have been associated with important economic losses [11,12,13]. Concerning BVDV-2, the first infections were described in 1994 in North America [8, 14]. Shortly thereafter, between 1999 and 2002, the first BVDV-2 subtypes were reported, initially with the identification of BVDV-2b in isolates from Panama, Argentina, Brazil and North America through the analysis of 5'UTR, N^{pro}, E2 and NS2/3 [5, 15]. BVDV-2c was first reported in 2001 (in isolates from 1996 and 1999 in Germany) based on E2 analysis. Since then, this subtype has only been identified on the European and American continents [3, 16]. The last BVDV-2 subtype described was 2d. However, unlike subtypes a-c, which were identified based on phylogenetic analysis of different genomic regions, BVDV-2d was classified by secondary structure analysis of the 5'UTR and includes only one isolate (non-cytopathic biotype, ncp) found contaminating fetal bovine serum in Argentina in 1995 (accession number AF244959) [17, 18].

Despite the consolidated classification of BVDV-2 into three (or four) subtypes, we have observed that some BVDV-2 genomes available in the GenBank database cannot be reliably clustered into 2a-c, regardless of the genomic region analyzed, i.e., UTRs, individual genes or viral genome [19]. Considering these findings, we have deepened our previous study, and now we propose the establishment of a distinct BVDV-2 subtype, termed BVDV-2e. As detailed below, our suggestion is mainly based on the analysis of 85 complete/near-complete genome (CNCG) sequences and 139 ORFs, as well as the identification of several unique aa residues in the proteins encoded by the putative BVDV-2e.

MATERIALS AND METHODS

Study design

In a previous report [19], we identified seven BVDV-2 sequences that could not be reliably classified as 2a-c: MH231124, MH231133, MH231148, MH231149, MH231150, MH231151 and MH231152. This was a secondary result from an analysis performed with BVDV-2 sequences available in GenBank. Here, we focus on these sequences and analyze whether they would represent a distinct BVDV-2 subtype.

Initially, we collected BVDV-2 CNCGs from GenBank and compared the CNCGs-based classification with that of their respective ORFs. This "proof of equivalence", in turn, allowed us to use the ORF as a reference target for our study, increasing our dataset and making our analysis more robust. The additional ORFs also enabled a more-detailed aa sequence analysis. Finally, we performed an additional phylogenetic analysis including BVDV-2d and also generated a pairwise identity matrix between non-subtyped BVDV-2 and -2d sequences to assess the difference between these subtypes.

Sequence collection

Complete/near-complete genomes and ORFs of BVDV-2 were obtained from GenBank, using the search terms "Bovine viral diarrhea virus 2" and "Bovine viral diarrhea virus 2 CDS", respectively. Manipulated/cloned sequences were excluded from our dataset. All sequences were collected on June 28 and 29, 2021.

Complete/near-complete genomes vs. open reading frames

The CNCG sequences were aligned using MUSCLE [20], trimmed and their phylogenetic tree was compared with that of their respective ORFs. The phylogenetic analysis was performed using MEGA X software (version 10.2.4) [21], and the evolutionary history was inferred by the maximum-likelihood method with 1,000 bootstrap replicates. The best model for analysis of each dataset (CNCG and ORF sequences) was defined using jModelTest software [22]. Parameters used in the phylogeny are listed in Table 1. In addition, we calculated the weighted geodesic distance [23] between the CNCG- and ORF-based phylogenetic trees using TreeCmp software, version 2.0 (https://eti.pg.edu.pl/TreeCmp) [24].

Open reading frames vs. individual genes

The phylogeny based on ORF sequences was compared to those obtained using individual genes. In total, 12 phylogenetic comparisons were performed: ORF vs. 12 individual genes (N^{pro}-NS5B). Alignment and sequence analysis were performed as described above.

Amino acid sequence analysis

Amino acid sequences of the seven BVDV-2 genomes previously identified as non-subtyped [19] were predicted using MEGA X software (version 10.2.4) and then compared with those of BVDV-2a-c from our ORF dataset. Importantly, BVDV-2 insertions or duplications (both related and unrelated to cytopathogenicity) were removed for correct aa sequence comparisons without changing the viral reading frame.

Non-subtyped BVDV-2 vs. BVDV-2d

Considering that BVDV-2d was initially classified based on structural analysis of the 5'UTR [18], this subtype is not frequently included in phylogenetic studies. However, to assess whether non-subtyped BVDV-2 sequences would cluster with BVDV-2d, we added this to the phylogenetic analysis. Importantly, as only the partial 5'UTR sequence of BVDV-2d is available (AF244959), only this region was analyzed (Table 1). In addition, we also calculated pairwise identity between non-subtyped BVDV-2 and BVDV-2d sequences, and between non-subtyped BVDV-2 sequences, using the Sequence Demarcation Tool (SDT) software (version 1.2) [25].

RESULTS

Analysis of complete/near-complete genomes vs. open reading frames

We collected 85 BVDV-2 CNCGs from GenBank (accession numbers are available in Supplementary Data, File S1). Based on phylogenetic analysis, 45, 4 and 22 sequences were subtyped as BVDV-2a, -2b and -2c, respectively. Furthermore, 14 sequences could not be reliably subtyped by CNCG analysis (HQ258810, JF714967, MK599227, KT832818, KX096718, MH231124, MH231132, MH231133, MH806435, MH231148, MH231149, MH231150, MH231151 and MH231152). All classifications (or “non-classifications”) were observed in the phylogeny of the respective ORFs, except KX096718, which was subtyped as BVDV-2a (Fig. 1). The geodesic distance between the CNCG and ORF trees was 0.0082.

Analysis of open reading frames vs. individual genes

After demonstrating the equivalence between CNCG and ORF analysis, we collected 54 additional ORFs from GenBank (Supplementary File S2), resulting in a dataset of 139 sequences. Among the 139 ORFs, 93, 4 and 29 were identified as BVDV-2a, -2b and 2-c, respectively. In addition, at least 13 sequences could not be reliably classified by ORF analysis: KT832818, JF714967, MK599227, MH806435, HQ258810, MH231132, MH231124, MH231133, MH231148, MH231149, MH231150, MH231151 and MH231152. Although the first six sequences described above were not subtyped by ORF analysis, they were classified as 2a or 2c depending on the gene analyzed (Supplementary File S3).

On the other hand, the last seven sequences (MH231124, MH231133, MH231148, MH231149, MH231150, MH231151 and MH231152) remained unclassified in the phylogenetic analysis of all genes (N^{pro}-NS5B). Furthermore, in all analyses (CNCG, ORF and N^{pro}-NS5B), these sequences were grouped in a distinct cluster, supported by high bootstrap values. We refer to this group as BVDV-2e (Fig. 2). Additional information about BVDV-2e is shown in Table 2.

Amino acid sequence analysis

Out of the 139 BVDV-2 ORFs analyzed here, 19 contained insertions or duplications (either related or unrelated to cytopathogenicity), which were removed for aa analysis. In detail: (a) Eight sequences were from cytopathic (cp) BVDV with an insertion of the J-domain protein interacting with viral protein (Jiv): BVDV-2a (5) (MH231129, MH231127, MH806438, MH806436 and MH806434), BVDV-2c (1) (LC006970) and BVDV-2e (2) (MH231124 and MH231133); (b) Eight sequences were from ncp BVDV-2, but with a p7/NS2 duplication: BVDV-2a (2) (890-U18059.1 and NC_039237) and BVDV-2c (6) (HG426480, HG426482, HG426484, HG426486, HG426488 and HG426490);and (c) Three ncp BVDV-2a sequences contained insertions of unknown origin (AF145967, HQ174303 and HQ174302).

Comparing the aa sequences of BVDV-2e and BVDV-2a-c, we identified 44 unique aa residues in 2e, 20 of which are in conserved positions in other subtypes. Arranging the proteins in descending order of aa differences, we observed the following: NS5A (10 differences) > E1, E2, NS2 and NS5B (5 each) > N^{pro}, C and E^{rns} (3 each) > p7 and NS3 (2 each) > NS4B (1). Only NS4A showed no aa differences when comparing BVDV-2e and 2a-c. Details of aa positions and changes are shown in Table 3.

BVDV-2e vs. BVDV-2d

Phylogenetic analysis of the partial 5'UTR sequence of all BVDV-2 subtypes, including 2d, showed that BVDV-2e also clustered separately from BVDV-2d (Fig. 3). A pairwise identity matrix showed a range of 98.31-100% between BVDV-2e sequences and 92.79-93.64% between BVDV-2e and -2d (Table 4).

DISCUSSION

Several studies have demonstrated that a given bovine pestivirus isolate/strain may sometimes be classified into different subtypes depending on the genomic region analyzed [7, 26,27,28]. The conflicting results may be influenced by genetic recombination between different bovine pestiviruses and by different levels of conservation or length of the analyzed

genomic targets [3, 29]. Considering this context, complete viral genome analysis is the most reliable approach to classifying bovine pestiviruses into subtypes [3]. In a previous study [19], we identified seven BVDV-2 genomes in GenBank that could not be classified as either 2a-c. Interestingly, these sequences also remained unclassified in the analyses of UTRs and individual genes, including NS5A and NS5B, which we have identified as the best targets for BVDV-2 subtyping [19]. The consistency of these findings led us to analyze these genomes further and assess whether they might represent a distinct BVDV-2 subtype.

Initially, we increased our dataset through a “proof of equivalence” between the phylogeny obtained using CNCGs and that we obtained using ORF sequences. Herein, we demonstrated that CNCGs-based subtyping was maintained when their ORFs were analyzed, except for KX096718, which cannot be classified as 2a-c by the CNCG, but was clustered with 2a in the ORF analysis. Interestingly, this sequence showed inconsistent classification, also being subtyped as 2a by analysis of some individual genes, such as E2 (Supplementary File S3). In addition to the equivalence between the CNCG and ORF subtyping, we observed a considerably small geodesic distance between the CNCG and ORF phylogenetic trees (0.0082), indicating that the topology and stem length of the trees were very similar. Together, these results allowed us to use the ORFs as reference targets for our study and thus increased our dataset from 85 (CNCG) to 139 (ORF) sequences.

Among the 139 sequences analyzed, we observed that some were inconsistent in their classification, not being subtyped by the ORF analysis, but clustering as 2a or 2c depending on the analyzed gene. On the other hand, the sequences MH231124, MH231133, MH231148, MH231149, MH231150, MH231151 and MH231152 were not grouped with any of the subtypes in the phylogenetic analyses performed here, i.e., CNCG, ORF and 12 genes (N^{pro}-NS5B). In addition, these sequences formed a cluster in all phylogenetic trees, which was supported by high bootstrap values. Considering these findings, together with the identification of unique aa residues in their predicted polyprotein sequences (discussed below), we believe that these viruses represent a non-BVDV-2 a-c subtype, which we propose to name "BVDV-2e". Bovine pestiviruses belonging to this new (old) subtype were collected between 1989 and 2012 in the United States of America (US) and were initially classified as BVDV-2c [28]. Of the seven putative BVDV-2e sequences, five are of the ncp biotype (MH231148, MH231149, MH231150, MH231151 and MH231152) and two are cp BVDV with a Jiv insertion (MH231124 and MH231133).

As mentioned above, we also identified several aa differences in the BVDV-2e ORF compared to BVDV-2a-c; comprising 44 unique aa in the 2e subtype. Of these, 20 are located

in highly conserved positions in BVDV-2a-c. The E2 glycoprotein, the most immunoprotective antigen of bovine pestiviruses [30,31,32], had the second highest number of aa differences (together with E1, NS2 and NS5B). Out of the five aa differences observed in E2, one is located in the DA domain (773, I→V), two in the DB domain (822, Q/L→T and 842, V/I→T) and two in the DD domain (1021, D→N and 1061 I/M/G/V→A). Considering that (i) the DA domain, the most immunogenic domain of E2 [33, 34], showed the lowest number of aa differences; (ii) none of the differences observed in BVDV-2e E2 are in previously identified bovine pestivirus epitopes; and (iii) the NS5A protein, a critical component of the viral replicase [32, 35], was the protein with the highest number of aa differences (10 aa); we suggest that BVDV-2e evolution might be driven by constraints other than immune-mediated selection.

Finally, we evaluated whether BVDV-2e would cluster separately from BVDV-2d. Despite the low bootstrap value obtained, which is often observed in 5'UTR-based phylogenetic trees of bovine pestiviruses [9], BVDV-2d was phylogenetically distant from other BVDV-2 subtypes, including BVDV-2e. This is an interesting issue, as BVDV-2d was initially classified based on 5'UTR secondary structures [18]. The paired identity matrix also showed that the BVDV-2e sequences had a high degree of similarity to each other (98.31-100% identity) and less similarity to BVDV-2d (92.79-93.64% identity), even in a conserved genomic region such as the 5'UTR [9]. Overall, these data support the conclusion that the sequences grouped as BVDV-2e may indeed represent a new subtype of bovine pestivirus.

CONCLUSION

After detailed phylogenetic analysis of BVDV-2 CNCGs available in GenBank, we observed that some sequences could not be reliably classified into subtypes 2a-c. Among these, we found seven sequences that grouped in a cluster distinct from that of BVDV-2a-c – a result observed in the 14 phylogenetic trees constructed in our study (based on CNCG, ORF and 12 individual genes). Furthermore, aa sequence prediction of these sequences showed several aa differences compared to BVDV-2a-c subtypes, including changes at highly conserved positions. Phylogenetic analysis based on partial 5'UTR sequences and pairwise comparisons also supported the difference between BVDV-2e and BVDV-2d. Given the consistency of these findings, we suggest including these bovine pestiviruses in a non-BVDV-2a-d subtype, referred to herein as "BVDV-2e". Interestingly, this new (old) subtype comprises bovine pestiviruses found in samples collected since 1989 in the US, so far classified into another subtype (2c). Finally, we suggest that BVDV-2e sequences should be

considered in future phylogenetic analyses in order to understand their epidemiology and evolution.

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CONTRIBUTIONS

PBSO performed the amino acid and phylogeny analyses and assisted in the experimental design, interpretation of results and manuscript preparation; JVJr assisted in the analysis of amino acids and phylogeny, experimental design, and interpretation of results and prepared the final version of the manuscript; RW assisted in the interpretation of results and manuscript review; and EFF assisted in the experimental design, interpretation of results, and elaboration and final review of the manuscript and guided the study.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ETHICAL APPROVAL

Not applicable. This article does not contain any studies with human participants or animals performed by any of the authors.

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Table 1 - Parameters used in the phylogenetic trees of BVDV-2.

Dataset (sequences)	Genomic region	Substitution model[a]	Gamma shape parameter	Proportion invariant sites	BIC[b]	Log likelihood
85	Complete/near-complete genome	GTR+G+I	0.5106	0.27	105,937.295	-106,208.33
	Open reading frame	GTR+G+I	0.5162	0.27	209,719.625	-103,621.23
139	Open reading frame	GTR+G+I	0.5299	0.27	222,753.659	-110,208.72
	N^{pro}	K2+G+I	1.4011	0.25	12,480.253	-4,688.15
	C	TN93+G+I	2.9424	0.27	8,428.316	-2,697.22
	E^{rns}	TN93+G+I	1.9593	0.27	15,001.349	-5,884.27
	E1	T92+G	0.3499	NA[c]	13,818.371	-5,296.14
	E2	GTR+G+I	0.6756	0.23	27,221.635	-11,918.81
	p7	K2+G	0.5738	NA	5,113.907	-1,186.90
	NS2	GTR+G+I	0.6331	0.27	35,593.930	-16,039.37
	NS3	TN93+G+I	2.2212	0.31	34,876.657	-15,684.40
	NS4A	K2+G	0.3340	NA	5,896.296	-1,530.14
	NS4B	TN93+G+I	2.2728	0.28	20,913.141	-8,796.72
	NS5A	TN93+G+I	1.8073	0.25	30,330.056	-13,437.80
	NS5B	GTR+G+I	0.5186	0.27	41,424.952	-18,913.33
86	Partial 5'UTR	TN93+G	0.5133	NA	1,240.445	-1,244.51

[a]GTR: general time reversible; G: gamma distribution; I: invariant sites; K2: Kimura 2-parameter; T92: Tamura 3-parameter; TN93: Tamura-Nei;
[b]Bayesian information criterion;
[c]Not applicable

Table 2 – Additional information on BVDV-2e isolates

Isolate	Country	Collection date	Sample	Biotype	Genome (bp)	Access number (GenBank)
1786c	USA[a]	1989	NA[b]	Cytopathic	12,643	MH231124
Short	USA	1989	NA	Non-cytopathic	12,254	MH231149
2412	USA	1989	NA	Non-cytopathic	12,254	MH231152
14622	USA	2005	NA	Non-cytopathic	12,255	MH231151
B69519c	USA	2006	FBS[c]	Cytopathic	12,629	MH231133
12-149150	USA	2012	NA	Non-cytopathic	12,260	MH231148
12-151955-317	USA	2012	NA	Non-cytopathic	12,260	MH231150

[a]United States of America;
[b]Not available;
[c]Fetal bovine serum

Table 3 – Amino acid changes between BVDV-2a/b/c and BVDV-2e proteins

Protein	Amino acid[a]	Changes
N^{pro}	1-167 1..168	18 (A;E;V/A/A[b]→T), 109 (A/A/A;V→S), 110 (S;I/S/S→N)
C	168-270 169..270	178 (S;N/S/S → K), 244[c] (L; P/P/P→Q), 267 (L;V/L/L→P)
E^{rns}	271-497	401 (R;S/R;S/R→G), **458** (N/N/N→D), 465 (A;V/A;Q;V;T/Q→K)
E1	498-692	**539** (E/E/E→D), 589 (D/D;T/T→A), **678** (I/I/I→V), **687** (I/I/I→L), **688** (T/T/T→S)
E2	693-1064	**773** (I/I/I→V), 822 (Q;L/Q/Q→T), 842 (V/V;I/V→T), **1021** (D/D/D→N), 1061 (I;M/G/I;G;M;V→A)
p7	1065-1134	1101 (L;Q/Q/P;Q;S→R), 1115 (A;S;V/S/A;S→C)
NS2	1135-1588 1135..1587	1256 (F/F/F;L→S), **1319** (K/K/K→R), **1440** (N/N/N→K), 1474 (N;S/N;S/S → G), **1515** (A/A/A→S)
NS3	1589-2271 1588..2270	**1901** (E/E/E→G), **1905** (I/I/I→V)
NS4A	2272-2335 2271..2334	No change
NS4B	2336-2682 2335..2681	**2373** (G/G/G→S)
NS5A	2683-3179 2682..3178	**2696** (K/K/K→R), 2942 (E;M;P;T/T/E;T→V), 3008 (D;N;S/N;S/N;S→Y), 3019 (D/D;E/D;E→N), 3030 (G;S/G/GS→D), **3046** (K/K/K→R), **3075** (K/K/K→Q), **3076** (E/E/E→K), 3114 (P/P;S/P→Q), **3177** (K/K/K→R)
NS5B	3180-3968 3179..3897	3182 (N;S/S/S→G), 3207 (K;S/N/G;N;S→D), 3273 (D;E/E/E→K), **3298** (K/K/K→R), **3513** (S/S/S→L)

[a]Position according to CN10.2015.821 strain (access number MG879027.1);

[b]Amino acid residue according to the BVDV-2 subtype: BVDV-2a/BVDV-2b/BVDV-2c;

[c]In bold: consensus amino acid residues in BVDV-2a-c.

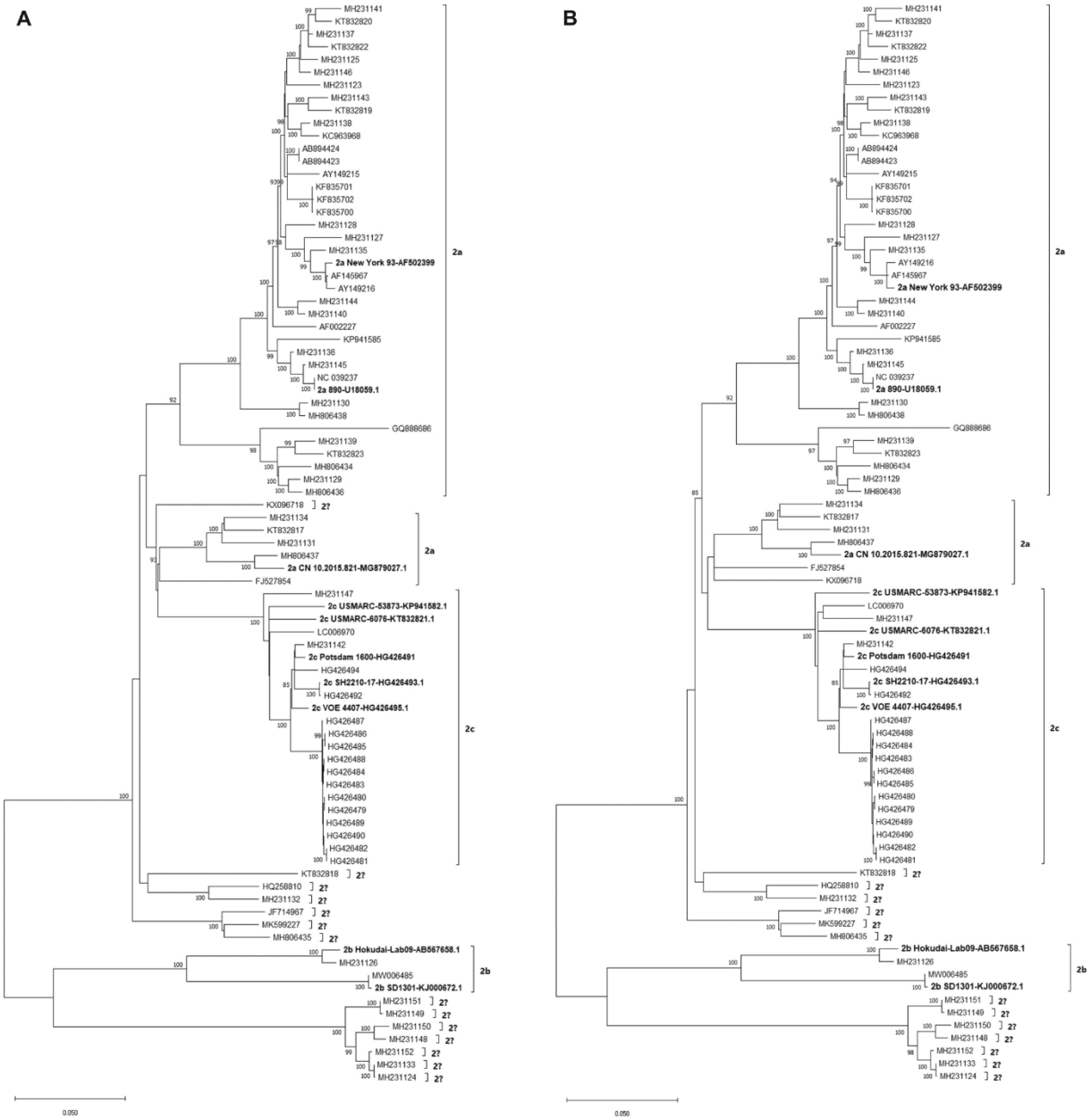

Fig. 1. Phylogenetic trees based on complete/near-complete genomes (CNCGs) and open reading frames (ORFs) of BVDV-2. Eighty-five BVDV-2 CNCGs were collected from GenBank. Phylogenetic analysis was performed with the CNCG sequences (A) and their respective ORFs (B), using MEGA X software (version 10.2.4) and the maximum-likelihood method (see Table 1). The bootstrap values were calculated from 1,000 replicates. Branches supported by >85% of the bootstrap replicates are indicated. Reference sequences are in bold. Non-subtyped BVDV-2 sequences were initially identified as "BVDV-2?".

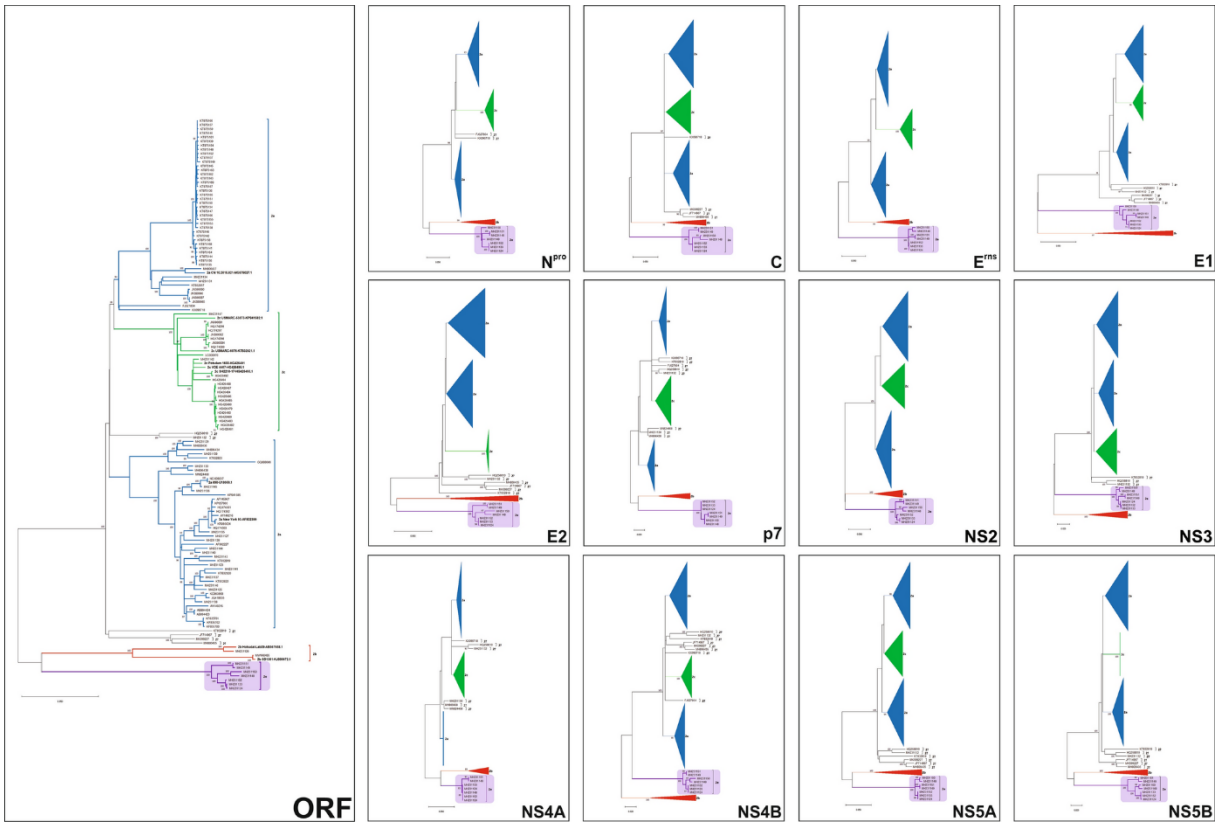

Fig. 2. Phylogenetic trees based on open reading frames (ORFs) and genes of BVDV-2. One hundred thirty-nine BVDV-2 ORFs were obtained from GenBank. The ORFs and individual genes were phylogenetically analyzed using the MEGA X software (version 10.2.4) and the maximum-likelihood method. The bootstrap values were calculated in 1,000 replicates. Branches supported by >85% of the bootstrap replicates are indicated. The BVDV-2e cluster is highlighted in all phylogenetic trees. Inaccurate BVDV-2 classifications remained identified with "BVDV-2?". Uncompressed trees are shown in Supplementary Data (Supplementary File S3).

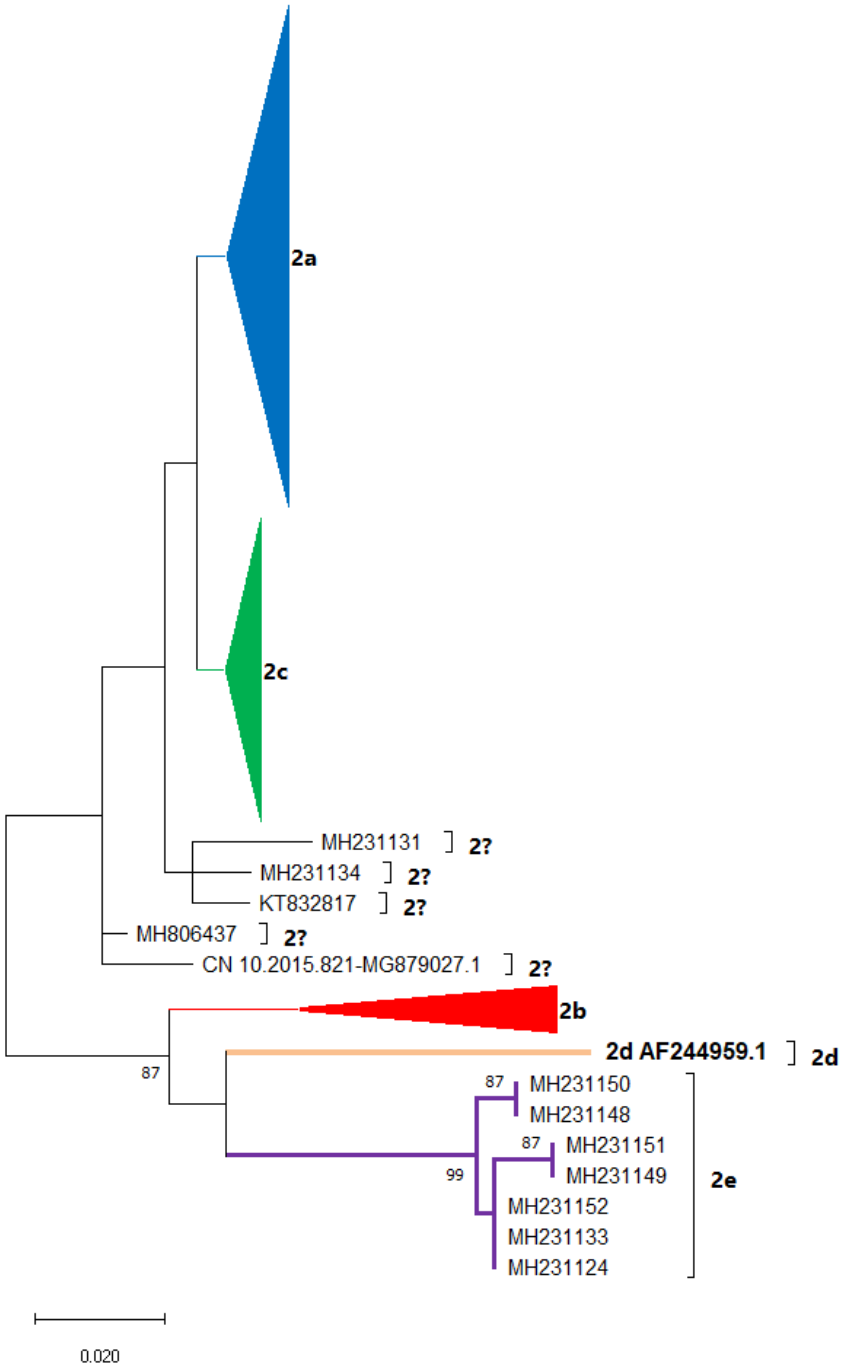

Fig. 3. Phylogenetic tree based on partial 5'UTR sequences of BVDV-2a-e. The partial 5'UTR sequences of all BVDV-2 subtypes, including BVDV-2d, were phylogenetically analyzed using the MEGA X software (version 10.2.4) and the maximum-likelihood method (Table 1). The bootstrap values were calculated from 1,000 replicates. Branches supported by >85% of the bootstrap replicates are indicated. Reference sequence is in bold.

6 DISCUSSÃO

Os BVDVs, assim como outros vírus de genoma de RNA, apresentam elevadas taxas de mutações, o que têm resultado em uma alta diversidade viral, sendo, até o momento, descritos pelo menos 21 subtipos de BVDV-1 (a-u) e 4 de BVDV-2 (a-d) (YEŞILBAĞ; ALPAY; BECHER, 2017). Classificações realizadas com base em análises filogenéticas de uma única região genômica são consideradas confiáveis para a determinação da espécie de BVDV (FIGUEIREDO et al., 2019; WOLFMEYER et al., 1997). No entanto, estudos baseados na análise de apenas uma região do genoma viral podem resultar em subtipagens imprecisas ou em classificações discordantes daquelas apresentadas pela análise do genoma viral completo (FIGUEIREDO et al., 2019; WOLFMEYER et al., 1997).

Com o objetivo de definir um alvo gênico para uma subtipagem adequada de BVDV, o primeiro estudo descreve a análise filogenética de 91 e 85 sequências de genomas completos de BVDV-1 e BVDV-2, respectivamente, disponíveis no *GenBank*. A análise dessas sequências demonstrou que é possível diferenciar BVDV-1 e BVDV-2 a partir da análise filogenética da região codificante ou das UTRs (dados não mostrados). No entanto, observou-se resultados conflitantes nas subtipagens de BVDV quando diferentes regiões genômicas ou UTRs foram comparadas com análise do genoma completo.

Para as sequências de BVDV-1, as regiões de 5'UTR e 3'UTR apresentaram resultados menos precisos para a subtipagem, provavelmente devido à elevada conservação das sequências de nucleotídeos dessas regiões, assim como ao tamanho reduzido da região analisada. Essas características da subtipagem baseada em UTR também já foram observadas por outros autores (NAGAI et al., 2004; TAJIMA, 2004; YEŞILBAĞ; ALPAY; BECHER, 2017). Na tentativa de se obter resultados mais semelhantes à análise do genoma completo de BVDV, regiões genômicas adicionais, principalmente N^{pro} e E2, têm sido empregadas para confirmação da subtipagem de BVDV. Porém, alguns estudos também já descreveram classificações imprecisas com base na análise dessas regiões (NAGAI et al., 2004; WORKMAN et al., 2016)

No presente estudo, as análises realizadas com N^{pro} e NS4A levaram a classificações diferentes daquelas observadas pela análise do genoma viral completo. Por outro lado, a análise das regiões C, E^{rns}, E1, E2, p7, NS2, NS3, NS4B, NS5A e NS5B reproduziram a subtipagem do genoma viral. Assim, para selecionar, dentre essas regiões, o melhor alvo para a subtipagem de BVDV-1, calculou-se a distância geodésica entre a árvore de genoma

completo e a das regiões citadas acima. Após essa análise, a região de NS4B foi indicada como o melhor alvo para a subtipagem do BVDV-1.

Em relação às análises das regiões de BVDV-2, a 5'UTR e 3'UTR também se apresentaram como as escolhas menos adequadas para a subtipagem viral. Além disso, nenhuma das regiões genômicas analisadas aqui reproduziu os resultados obtidos pela análise do genoma viral completo. Nesse contexto, considerou-se a região de NS5A como o alvo mais indicado para a subtipagem de BVDV-2, devido ao fato de apresentar apenas uma não conformidade quando comparada à análise baseada no genoma completo e por possuir a menor distância geodésica da árvore do genoma viral. Em estudo anterior, Workman et al (2016) demonstraram que as árvores filogenéticas de BVDV-2 com base nas sequências de N^{pro}, E2, NS5A e NS5B podem fornecer elevado suporte estatístico, bem como apresentar uma topologia semelhante àquela da árvore de genoma completo.

No primeiro estudo, também foi observado que pelo menos 14 isolados/cepas de BVDV-2 não puderam ser classificados pela análise do genoma viral completo (LIU et al., 2012; NEILL et al., 2019a; TAO et al., 2013). Dessas, sete sequências tinham sido previamente classificadas como BVDV-2c por Neil et al (2019): MH231124, MH231133, MH231148, MH231149, MH231150, MH231151 e MH231152.

No segundo artigo, após confirmar a segurança da aplicação das ORFs como um alvo de referência para a filogenia do BVDV-2, realizou-se uma análise mais robusta com 139 sequências de genomas completos/quase completos (CNCGs) de BVDV-2 obtidos no *GenBank*. Observou-se que algumas destas sequências apresentavam inconsistências em sua classificação, não sendo subtipadas pela análise de ORF, mas agrupando-se como 2a ou 2c em algumas regiões gênicas.

No segundo artigo, as sete sequências descritas acima foram analisadas quanto à possibilidade de representarem um novo subtipo de BVDV-2. Inicialmente, para aumentar o número de sequências a serem analisadas e fortalecer as análises filogenéticas, foi realizada uma prova de equivalência entre a classificação baseada nos genomas completos/quase completos de BVDV-2 *versus* suas respectivas ORFs. Após confirmar a adequação das ORFs para a classificação filogenética de BVDV-2, mais sequências de regiões codificantes foram coletadas do *Genbank*, resultando em um banco de dados de 139 ORFs.

Nas análises filogenéticas baseadas na região codificante das 12 proteínas virais, todas as sete sequências não classificadas de BVDV-2 mantiveram-se agrupadas com forte suporte estatístico e permaneceram distantes evolutivamente dos BVDV-2, -2b e -2c. Para descartar a possibilidade que essas sequências pudessem ser de BVDV-2d, foi feita uma análise

filogenética adicional com base na sequência parcial da 5'UTR. É importante destacar que a análise foi realizada apenas com essa região porque ela é a única disponível para o subtipo BVDV-2d. Além disso, é importante também considerar que o BVDV-2d foi inicialmente identificado com base na análise da estrutura secundária da 5'UTR (GIANGASPERO et al., 2008) e que por isso não tem sido considerado em análises filogenéticas de BVDV. Na análise realizada aqui, todas as sete sequências de BVDV-2 não identificadas como BVDV-2a-c também permaneceram distantes da sequência de BVDV-2d, o que foi reforçado pela matriz de identidade de BVDV-2e *versus* BVDV-2d. Esses achados levaram à conclusão que as sete sequências aqui citadas compõem um subtipo de BVDV-2 ainda não identificado, que sugere-se denominar BVDV-2e. Interessantemente, todas essas sequências são de vírus oriundos dos Estados Unidos (NEILL et al., 2019a) .

Além da análise filogenética, as sequências de BVDV-2e também apresentaram 44 resíduos de aminoácidos diferentes daqueles encontrados em sequências de BVDV-2a, -2b e -2c. Grande parte das diferenças de aminoácidos foi encontrada na proteína NS5A, que participa da replicação do genoma viral (ISKEN et al., 2014), sugerindo que a emergência do BVDV-2e possa estar relacionada a outros fatores de seleção além da resposta imune do hospedeiro.

Em conclusão, os resultados apresentados nesta tese podem contribuir para futuras análise filogenéticas de BVDV-1 e BVDV-2 e também alertam para a circulação de BVDVs ainda não classificados.

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