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ALVOS GENÔMICOS PARA SUBTIPAGEM DE VÍRUS DA DIARREIA VIRAL BOVINA 1 (BVDV-1) E BVDV-2

Santa Maria, RS
2023

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Dissertação apresentada ao curso 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 a obtenção do título de **Mestre em Medicina Veterinária**.

Orientador: Prof. Dr. Rudi Weiblen
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Santa Maria, RS
2023

“*A ciência não existiria*
sem pessoas apaixonadas
pelo desejo de descobrir o modo
pelo qual o mundo funciona.” –
Nicola Chalton.

RESUMO

ALVOS GENÔMICOS PARA SUBTIPAGEM DE VÍRUS DA DIARREIA VIRAL BOVINA 1 (BVDV-1) E BVDV-2

AUTORA: Carolina Isabela Mucellini
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A análise filogenética do genoma completo, estratégia mais adequada para a subtipagem dos vírus da diarreia viral bovina 1 (BVDV-1) e BVDV-2, não é viável para muitos laboratórios. Consequentemente, isolados e cepas de BVDV têm sido frequentemente subtipados pela análise de determinadas regiões genômicas, principalmente a região não traduzida 5' (5' *untranslated region*, 5'UTR). Essa abordagem, no entanto, pode levar a classificações equivocadas e/ou com baixo suporte estatístico. Nesse contexto, o presente estudo descreve o desenho e utilização de novos pares de *primers* cujos amplicons podem ser facilmente sequenciados e utilizados para subtipagem de BVDV-1 e BVDV-2. Inicialmente, regiões genômicas do BVDV-1 e BVDV-2, previamente descritas na literatura como os alvos mais adequados para subtipagem dos pestivírus bovinos, foram analisadas para o desenho de *primers* de alta cobertura. Em seguida, as regiões amplificáveis por esses *primers* foram analisadas *in silico* quanto à capacidade de reproduzir a classificação filogenética de 118 (BVDV-1) e 88 (BVDV-2) genomas completos/quase completos (GCQCs) disponíveis no GenBank. Essa análise também foi realizada considerando a região amplificável pelos *primers* HCV90-368, a qual tem sido comumente utilizada para a classificação/subtipagem de pestivírus bovinos. Após comprovar a conformidade entre as análises realizadas com a região amplificável pelos *primers* do presente estudo *versus* as análises de GCQC, seguiu-se com a otimização dos ensaios de RT-PCR e com a avaliação de sua capacidade de amplificar isolados e cepas de BVDV do Brasil, Argentina e Estados Unidos. As regiões de BVDV-1 e BVDV-2 que permitiram o desenho de *primers* de alta cobertura foram a NS3-NS4A (amplicon de 526pb) e NS5B (728pb), respectivamente. A classificação filogenética com base nessas regiões reproduziu integralmente a subtipagem de todos os GCQCs analisados. Por outro lado, a subtipagem baseada na região amplificável pelos *primers* HCV90-368 apresentou quatro (BVDV-1) e doze (BVDV-2) discordâncias em relação à classificação dos GCQCs. Os *primers* de NS3-NS4A e NS5B também permitiram a amplificação de todos os isolados/cepas de BVDV analisados. Por fim, considerando as análises filogenéticas das regiões amplificáveis pelos *primers* descritos aqui, bem como o desempenho dos ensaios de RT-PCR, sugere-se o seu uso em futuros estudos filogenéticos e epidemiológicos de BVDV-1 e BVDV-2.

Palavras-chave: Pestivírus. Filogenia. NS3. NS4A. NS5B.

ABSTRACT

GENOMIC TARGETS FOR SUBTYPING OF BOVINE VIRAL DIARRHEA VIRUS 1 (BVDV-1) AND BVDV-2

AUTHOR: Carolina Isabela Mucellini
ADVISER: Rudi Weiblen

Whole-genome phylogenetic analysis, the most suitable strategy for subtyping bovine viral diarrhea vírus 1 (BVDV) and BVDV-2, is not feasible for many laboratories. Consequently, BVDV isolates/strains have been frequently subtyped by the analysis of single genomic regions, mainly the 5' untranslated region (5'UTR). This approach, however, may lead to inaccurate and/or poorly statistically supported viral classifications. Herein, we describe novel primer sets whose amplicons may be easily sequenced and used for subtyping BVDV-1 and BVDV-2. Initially, genomic regions of BVDV-1 and BVDV-2, previously described as the most suitable targets for bovine pestivirus subtyping, were analyzed for the design of high-coverage primers. Then, the putative amplicons were analyzed *in silico* for their suitability to reproduce the phylogenetic classification of 118 (BVDV-1) and 88 (BVDV-2) complete/near complete genomes (CNCGs) available on GenBank. This analysis was also performed considering the region amplifiable by primers HCV90-368, largely used used for classification/subtyping of bovine pestiviruses. After confirming agreement between the analyses performed with the putative amplicons from our primers *versus* those from the CNCGs, we optimized the RT-PCR assays and evaluated their performance in the amplification of several BVDV isolates/strains from Brazil, Argentina and the United States. Among potential targets for bovine pestivirus subtyping, we designed high-coverage primers for NS3-NS4A (BVDV-1) (526bp amplicon) and NS5B (BVDV-2) (728bp). The phylogenetic classification based on these regions fully reproduced the subtyping of all CNCGs analyzed in the study. On the other hand, subtyping based on the putative amplicon from primers HCV90-368 showed four (BVDV-1) and twelve (BVDV-2) disagreements in relation to the CNCG classification. The NS3-NS4A and NS5B primers also allowed the amplification of all BVDV isolates/strains analyzed here. Finally, considering the phylogenetic analyses from our putative amplicons, as well as the performance of RT-PCR assays, we suggest the use of these primers in future phylogenetic and epidemiological studies of BVDV-1 and BVDV-2.

Keywords: Pestivirus. Phylogeny. NS3. NS4A. NS5B.

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1 INTRODUÇÃO

Os vírus da diarreia viral bovina (BVDV) pertencem à família *Flaviviridae*, gênero *Pestivirus*, e são atualmente classificados em duas espécies virais: *Pestivirus A* (*bovine viral diarrhea virus 1*, BVDV-1) e *Pestivirus B* (*bovine viral diarrhea virus 2*, BVDV-2) (SMITH, 2017; ICTV, 2022). O BVDV-1 e BVDV-2 apresentam distribuição mundial e têm sido associados com uma diversidade de manifestações clínicas em ruminantes, sendo considerados importantes patógenos devido aos efeitos adversos na reprodução e no desempenho produtivo dos rebanhos bovinos (HOUE, 2003; FERNANDES et al., 2016).

Os efeitos negativos dos pestivírus na reprodução e no desempenho dos animais são as consequências mais importantes da infecção e incluem sinais clínicos inespecíficos de depressão, inapetência, febre e diarreia, além de redução temporária na produção de leite. Quando o vírus atravessa a barreira placentária em vacas prenhes, o quadro se torna mais grave, resultando em danos diretos ao embrião, morte embrionária precoce, mumificação fetal, abortos, natimortalidade e malformações congênitas, ou ainda a geração de bezerros persistentemente infectados (GROOMS, 1998; 2004; BROCK, 2004; GATES et al., 2014).

O genoma dos pestivírus é composto por uma molécula de RNA de fita simples, sentido positivo, de aproximadamente 12,3kb. O genoma contém uma única fase de leitura aberta (*open reading frame*, ORF), flanqueada por duas regiões não traduzidas (5' e 3' *untranslated regions,* UTRs), envolvidas na regulação da tradução e replicação do genoma viral (TAUTZ et al., 2015). A ORF codifica uma única poliproteína que é processada por proteases celulares e virais em 11 ou 12 proteínas: 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] (NEILL, 2013; YESILBAG, et al., 2017).

A N^{pro} é a primeira proteína traduzida e contribui para evasão da resposta imune inata por meio da interação com o fator regulador 3 e 7 do interferon (*interferon regulatory factor* 3, IRF3, e *interferon regulatory factor* 7, IRF7), inibindo a via de indução do interferon tipo I (IFN α e β) (GOTTIPATI et al., 2013). A proteína C é a primeira proteína estrutural a ser traduzida e compõe o capsídeo viral (THIEL et al., 1991). A E^{rns} é uma glicoproteína do envelope viral exclusiva dos pestivírus,

possui atividade de RNAse e também medeia a ligação dos vírions aos glicosaminoglicanos da superfície celular, podendo ser alvos de anticorpos fracamente neutralizantes (IQBAL et al., 2004; TAUTZ et al., 2015). A proteína E1 ainda não apresenta estrutura e função totalmente conhecidas, entretanto é frequentemente encontrada em associação com as outras duas proteínas do envelope (E^{rns} e E2). Com a E2, por exemplo, forma heterodímeros necessários para a penetração do vírus e fusão com a membrana celular (LI et al., 2013; TAUTZ et al., 2015). A proteína E2 atua na ligação aos receptores dos pestivírus, contribuindo para o tropismo viral e sendo o principal alvo de anticorpos neutralizantes (TAUTZ et al., 2015).

O polipeptídeo p7 provavelmente atua como uma viroporina devido às suas características de proteína hidrofóbica integral de membrana, apresentando uma região central carregada, responsável pelo influxo de íons para acidificação de vesículas durante o processo da formação das partículas virais (TAUTZ et al., 2015; ZHAO et al., 2017). As proteínas NS2 e NS3 contribuem para a morfogênese e replicação viral (LACKNER et al., 2004). A NS3 apresenta atividade de helicase de RNA/NTPase e possui um domínio de protease de serina que é essencial para o processamento das proteínas virais (TAMURA et al., 1993; WARRENER et al., 1995; YAMANE et al., 2009). A NS4A representa um cofator para a NS3, enquanto a NS4B, uma proteína integral de membrana, apresenta uma função essencial na replicação do RNA, contribuindo para a formação de membranas onde ocorre a replicação do genoma viral e/ou ancorando o complexo de replicação do vírus (XU et al., 1997; TAUTZ et al., 2015). A NS5A é uma fosfoproteína hidrofílica que possui propriedades associadas à fosforilação de quinases celulares e também apresenta um efeito antagônico na resposta imune inata, a exemplo da inibição do fator de transcrição NF-κB (*factor nuclear kappa* B) (REED et al., 1998; ZAHOOR et al., 2010; SUDA et al., 2018). A NS5B é uma polimerase de RNA dependente de RNA (*RNA-dependent RNA polymerase*, RdRp) responsável pela síntese dos RNAs virais (TAUTZ et al., 2015).

Até o presente, ao menos 23 subtipos de BVDV-1 (a-x) e 4 subtipos de BVDV-2 (a-d) já foram descritos (DENG et al., 2020; GOMEZ-ROMERO et al., 2021). Além desses, um novo subtipo de BVDV, BVDV-2e, também foi sugerido (DE OLIVEIRA et al., 2022). Diferentes regiões genômicas vêm sendo utilizadas para identificação da espécie e subtipagem de isolados e cepas de BVDV: sequências

parciais da 5'UTR são as mais frequentemente utilizadas (RIDPATH et al., 1994; BECHER et al., 1995; NAGAI et al., 2001; VILCEK et al., 2001; CORTEZ et al., 2006; RIDPATH et al., 2010), seguidas pela N^{pro} (BECHER et al, 1997; BECHER et al., 1999; ARIAS et al., 2002; BECHER et al., 2003), E2 (BECHER et al., 1999; COUVREUR et al., 2002; BECHER et al., 2003; TAJIMA et al., 2005; ABE et al., 2017) e NS2-3 (NAGAI et al., 2004; XIA, et al., 2007). Algumas subtipagens também têm sido realizadas pela análise combinada de regiões genômicas próximas, como a 5'UTR-N^{pro} (MIROSLAW; POLAK, 2019; DENG et al., 2020) e NS5B-3'UTR (NAGAI et al., 2004; MISHRA et al., 2007).

Apesar da análise de determinadas regiões genômicas ser útil para a identificação de BVDV-1 e BVDV-2, a subtipagem baseada em apenas uma dessas regiões pode resultar em classificação viral equivocada e/ou com baixo suporte estatístico (YESILBAG et al., 2017; NEILL et al., 2019). Além disso, é possível observar classificações discordantes quando o mesmo vírus é classificado por diferentes regiões genômicas, o que é frequentemente observado quando a subtipagem é inicialmente realizada pela região 5'UTR e, posteriormente, com base em outras regiões, como a E2, NS3 e NS5B-3'UTR (NAGAI et al., 2004; TAJIMA et al., 2004; WORKMAN et al., 2016). Outro aspecto que pode influenciar a classificação filogenética dos pestivírus bovinos é a utilização de diferentes métodos de análise, como *neighbor-joining*, máxima verossimilhança (*maximum likelihood*) e inferência bayesiana (XIA et al., 2007).

Exemplificando as discrepâncias que podem ser observadas na subtipagem dos pestivírus bovinos, Nagai et al. (2004) identificaram que um isolado de BVDV, 190CP, obtido de um caso experimental de doença das mucosas, previamente classificado como BVDV-1e com base nas regiões 5'UTR, N^{pro} e E2, foi classificado como BVDV-1a pela análise da região NS5B-3'UTR. Resultados semelhantes foram obtidos por Tajima (2004) ao analisar dois isolados de BVDV-1 que haviam sido identificados como BVDV-1a com base na região 5'UTR, mas que posteriormente foram classificados como BVDV-1c segundo análise da região E2. Os mesmos autores também observaram que os isolados ILLC e ILLNC, classificados como BVDV-1b após análise das regiões 5'UTR, N^{pro}, E2 e NS5B-3'UTR, poderiam ser classificados como BVDV-1a pela análise da NS3. Aguirre et al. (2014) descreveram alguns isolados de alpacas e lhamas como BVDV-1j com base nas sequências de

5'UTR, entretanto, após análise das sequências de E2, os mesmos isolados foram classificados como BVDV-1e.

Em relação ao BVDV-2, Workman et al. (2016) descreveram alguns isolados de BVDV-2 que não puderam ser seguramente subtipados com base na análise filogenética da região 5'UTR, sendo necessário sequenciar o genoma completo para uma classificação viral precisa. De Oliveira et al. (2021) também demonstraram que algumas sequências de BVDV-2 disponíveis no GenBank não puderam ser consistentemente classificadas pela análise de 5'UTR. Nesse mesmo estudo, os autores identificaram que a classificação baseada em 5'UTR ainda pode levar a resultados discordantes daqueles obtidos pela análise do genoma viral completo. Além desses resultados conflitantes, Neill et al. (2019) demonstraram que a análise da 5'UTR de BVDV-2 pode apresentar baixo suporte estatístico, dificultando a classificação dos vírus.

Umas das estratégias para assegurar uma classificação mais adequada dos pestivírus bovinos é a análise filogenética do genoma viral completo (YESILBAG, et al., 2017; DE OLIVEIRA et al., 2021). No entanto, o sequenciamento de genomas completos apresenta um elevado custo, sendo inviável quando se trabalha com um grande número de amostras, tornando-se uma realidade distante para muitos laboratórios. Para contornar essas dificuldades, De Oliveira et al. (2021) sugeriram que a classificação de BVDV-1 e BVDV-2 por meio de análise das regiões de NS4B e NS5A, respectivamente, pode levar a resultados semelhantes aos obtidos pela análise do genoma viral completo (DE OLIVEIRA et al., 2021).

Aprofundando a discussão acima e objetivando contribuir para os estudos dos pestivírus bovinos, o presente trabalho descreve o desenho e a utilização de *primers* cujo amplicons podem ser facilmente sequenciados e utilizados para a subtipagem adequada do BVDV-1 e BVDV-2, a saber, reproduzindo a classificação obtida pela análise filogenética do genoma viral completo. Essa estratégia pode contribuir para os estudos epidemiológicos, de evolução e de distribuição dos pestivírus bovinos, podendo auxiliar também nas abordagens de diagnóstico e formulação de vacinas.

2 ARTIGO

Novel genomic targets for proper subtyping of bovine viral diarrhea virus 1 (BVDV-1) and BVDV-2

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Novel genomic targets for proper subtyping of bovine viral diarrhea virus 1 (BVDV-1) and BVDV-2

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Abstract

Whole-genome phylogenetic analysis, the most suitable strategy for subtyping bovine viral diarrhea virus 1 (BVDV-1) and BVDV-2, is not feasible for many laboratories. Consequently, BVDV isolates/strains have been frequently subtyped based on analysis of single genomic regions, mainly the 5' untranslated region (UTR). This approach, however, may lead to inaccurate and/or poorly statistically supported viral

classification. Herein, we describe novel primer sets whose amplicons may be easily sequenced and used for BVDV subtyping. Initially, genomic regions previously described as the most suitable targets for BVDV subtyping were analyzed for design of high-coverage primers. The putative amplicons were analyzed *in silico* for their suitability to reproduce the phylogenetic classification of 118 (BVDV-1) and 88 (BVDV-2) complete/near-complete genomes (CNCGs) (GenBank). This analysis was also performed considering the region amplifiable by primers HCV90-368 (5'UTR), widely used for BVDV classification/subtyping. After confirming the agreement between the analyses of our primers' amplicons *versus* the CNCGs, we optimized the RT-PCRs and evaluated their performance for amplification of BVDV isolates/strains. Among the potential targets for BVDV subtyping, we designed high-coverage primers for NS3-NS4A (BVDV-1) (526bp amplicon) and NS5B (BVDV-2) (728bp). The classification based on these regions fully reproduced the subtyping of all CNCGs. On the other hand, subtyping based on the putative amplicon from primers HCV90-368 showed four (BVDV-1) and twelve (BVDV-2) disagreements in relation to CNCG. The NS3-NS4A and NS5B primers also allowed the amplification of all BVDV isolates/strains tested. Finally, we suggest the use of these primers in future phylogenetic and epidemiological studies of BVDVs.

Keywords: Pestivirus. Phylogeny. NS3. NS4A. NS5B.

1 Introduction

Bovine viral diarrhea viruses (BVDV) belong to the family *Flaviviridae*, genus *Pestivirus*, and have been classified into two viral species: *Pestivirus A* (bovine viral diarrhea virus 1, BVDV-1) and *Pestivirus B* (bovine viral diarrhea virus 2, BVDV-2) [1, 2]. BVDV-1 and BVDV-2 have a worldwide distribution and have been associated with several clinical manifestations in cattle, resulting in adverse effects on reproduction, animal health and performance [3, 4].

The pestivirus genome is composed by a single-stranded, positive-sense RNA molecule of approximately 12.3kb, containing a single open reading frame (ORF), flanked by two untranslated regions (5' and 3' UTRs). The ORF encodes a single polyprotein that is co- and post-translationally processed by cellular and viral proteases into 11 or 12 mature proteins: N-terminal autoprotease (N^{pro}), capsid

protein (C), envelope glycoproteins (E^{rns}, E1, and E2), p7, and non-structural proteins (NS2/NS3 [or NS2 and NS3], NS4A, NS4B, NS5A, and NS5B) [5].

At least 23 BVDV-1 (a-x) and 4 BVDV-2 (a-d) subtypes have been described to date [6, 7] and an additional BVDV-2 subtype, BVDV-2e, has been recently proposed [8]. In general, different genomic regions have been used for species identification and subtyping of BVDV isolates/strains, mainly partial 5'UTR [9-14], followed by coding regions, such as N^{pro} [14-17], E2 [16-17, 19-21] and NS2-3 [22-23]. Although single-target analysis allows for correct identification of pestivirus species, subtyping based on a single genomic region may result in inaccurate classifications or clusterings with low statistical support [22, 24-27]. A proper BVDV classification, in turn, would be useful for epidemiological, evolutionary and vaccine studies [14, 28-29].

The ideal strategy to circumvent these biases is the phylogenetic analysis of the complete viral genome. However, sequencing whole genomes may be expensive, especially when analyzing a high number of isolates, making it unfeasible for many laboratories [27]. In this sense, de Oliveira et al. [27] described that phylogenetic analyses based on NS4B (BVDV-1) and NS5A (BVDV-2) would lead to subtyping equivalent to complete genome analysis. Although this is an interesting approach, these targets are unlikely to be reliably sequenced in a single Sanger sequencing round due to their lenghts (about 1,041 nt for NS4B and 1,491 nt for NS5A). In addition, it would be necessary to identify conserved sequences within these regions to design high-coverage primers.

Considering the above issues and recognizing the current demand for a simple approach to accurately classify/subtype bovine pestiviruses, we carefully analyzed genomic targets previously suggested as most suitable for BVDV classification [27] and designed novel primer sets whose amplicons may be easily amplified, sequenced and used for the accurate subtyping of BVDV-1 and BVDV-2.

2 Methodology

2.1 Study design

Initially, we analyzed the most suitable genomic regions for subtyping BVDV-1 and BVDV-2 described by De Oliveira et al. (2021) in order to identify conserved regions for the design of high-coverage primers. Then, an *in silico* analysis was performed to assess whether the subtyping based on the putative amplicons from

these primers would reproduce the classification from the BVDV complete/near-complete genome (CNCG). We also performed this *in silico* comparison with putative amplicons from the primers HCV90-368, which are often used for BVDV classification. Finally, after confirming that the classification based on our primers was more accurate than that obtained from primers HCV90-368, we analyzed the performance of the novel primer sets in amplifying several BVDV isolates/strains, including viruses of unknown sequences and from different countries.

2.2 Primer design

The most suitable genomic regions for subtyping BVDV-1 and BVDV-2 described by De Oliveira et al. [27] were analyzed for the presence of conserved regions for primer design. This screening was performed with the dataset previously reported by the authors [27], which includes 91 BVDV-1 (subtypes BVDV-1a, -1b, -1c, -1d, -1e, -1f, -1g, -1h, -1i, -1j, -1k, -1m, -1n, -1o, -1q, -1r and -1u) and 85 BVDV-2 CGs (BVDV-2 a-c) (Online Resource 1). Primer sequences were analyzed for dimer formation (https://idtdna.com/pages/tools/oligoanalyzer) and non-specific annealing [Basic Local Alignment Search Tool (BLAST), https://blast.ncbi.nlm.nih .gov/Blast.cgi].

2.3 In silico analyses

2.3.1 Sequence collection

The BVDV-1 and BVDV-2 CNCG sequences were obtained from the Genbank database (https://ncbi.nih.gov/search), using the search terms “bovine viral diarrhea virus 1” and “bovine viral diarrhea virus 2”, filtered to the 11-13kb range. After collection, sequences that did not present the annealing sites for the primers HCV90-368, as well as those from infectious clones or patents, were excluded from the analyses. The BVDV-1 and BVDV-2 sequences were collected on March 15, 2022.

2.3.2 Phylogenetic analyses

The BVDV-1 and BVDV-2 CNCGs were aligned by the Multiple Alignment using Fast Fourier Transform (MAFFT) (https://mafft.cbrc.jp/alignment/software, version 7), trimmed and analyzed using the MEGA-X software (version 10.2.6) [37]. The evolutionary history was inferred by the Maximum Likelihood (ML) method, with 1,000 *bootstrap* replicates. Analyses of the putative amplicons from our primers, as

well as primers HCV90-368, were performed as described above. The best analysis model for each alignment was defined by the MEGA-X (version 10.2.6) (Table 1). Finally, CNCG-based subtyping was compared with results obtained by analyzing our primers and HCV90-368 as well.

2.4 RT-PCR assays

2.4.1 Viruses

Singer (BVDV-1) and VS-253 (BVDV-2) strains were used for RT-PCR optimization and as positive controls throughout the study. Thirty-five BVDV-1 and 33 BVDV-2 isolates/strains were used to evaluate the performance of the RT-PCR assays. The BVDV isolates were from the virus bank of the Virology Section (*Universidade Federal de Santa Maria*, UFSM, Brazil). Details about the viruses used are listed in Table 2.

2.4.2 RNA extraction

Supernatant of MDBK cell cultures (Madin-Darby bovine kidney, ATCC CCL-22) inoculated with the viruses described above were subjected to total RNA extraction using the TRIzol® Reagent (Thermo Fisher Scientific™), according to the manufacturer. RNA quality/integrity was assessed by NanoDrop spectrophotometry (Thermo Fisher Scientific™).

2.4.3 Reaction optimization

Complementary DNA (cDNA) was synthesized using the GoScript™ Reverse Transcription Mix kit, Random Primers (Promega). PCR reactions were performed using the Taq DNA Polymerase, Recombinant (5U/µL) (Thermo Fisher Scientific™). The BVDV-1 and BVDV-2 reactions were optimized for annealing temperature (46 to 60°C, with a 2°C interval), $MgCl_2$ concentration (1.5, 2, 2.5 and 3mM), and Taq DNA Polymerase (1, 2.5, 5 and 10U). Amplicons were visualized by electrophoresis on a 1% agarose gel stained with GelRed® (Biotium).

2.4.4. Nucleotide sequencing

After the RT-PCR reactions, the identity of the amplicons obtained during the optimization of the assay was confirmed by nucleotide sequencing. Sequencing was

performed by the Sanger method, using the BigDye™ Terminator v3.1 Cycle Sequencing kit, performed by *ACTGene Análises Moleculares Ltda* (Brazil).

3 Results

3.1 Primer design

For BVDV-1, primers NS3-F (5'-ARATGAAYTAYGAYTGGAG-3') and NS4A-R (5'-TGGGTDGTGTCYTC-3') were designed: NS3-F anneals to nt 7,077-7,095 (NS3) and NS4A-R to nt 7,589-7,602 (NS4A), of the NADL strain sequence (GenBank accession number M31182.1). The NS3-F and NS4A-R primer set generates a 526bp amplicon (nt 7077-7602).

For BVDV-2, primers NS5B-F (5'-CATATGGTGTCAGCATACCA-3') and NS5B-R (5'-TCTCCRGCTTCCCAGTC-3') were designed: NS5B-F anneals to nt 10,250-10,269 and NS5B-R to nt 10,961-10,977 (strain 890, GenBank accession number U18059.1). The NS5B-F and NS5B-R primer set generates a 728bp amplicon (nt 10250-10977).

3.2 In silico analyses

3.2.1 BVDV-1

One hundred and sixty-seven BVDV-1 CNCGs were initially collected from GenBank. After selection analysis, 49 sequences of infectious clones, patent or lacking the annealing site for the primers HCV90-368 were excluded. The 118 CNCGs in the final dataset were classified as BVDV-1a (28), -1b (31), -1c (4), -1d (7), -1e (9), -1f (6), -1g (2), -1h (5), -1i (5), -1j (2), -1k (7), -1m (5), -1n (1), -1o (1), -1q (3), -1r (1) and -1u (1) (Fig. 1a). No CNCGs of BVDV-1l, BVDV-1p, BVDV-1s, BVDV-1t, BVDV-1v and BVDV-1x were found.

All sequences were also analyzed considering the putative amplicons amplifiable by primers NS3-F/NS4A-R and HCV90-368, and the results were compared with those described above. The NS3-F/NS4A-R classification showed 100% agreement with CNCG-based subtyping (Fig. 1a and Fig. 1b). On the other hand, the classification based on the amplicon obtained by primers HCV90-368 had four mismatches in relation to CNCG: i) the sequences MW250799.1 and MW250798.1 were classified as 1a by CNCG, but could not be safely classified by HCV90-368; MH379638.1 was subtyped as 1a by CNCG, but classified as 1c by

HCV90-368; iii) and AB078952.1 was identified as 1j by CNCG, but 1a based on HCV90-368 amplicon (Fig. 1a and Fig. 1c).

3.2.2 BVDV-2

One hundred and fourteen BVDV-2 CNCGs were collected from GenBank. After the exclusion criteria, 26 sequences of infectious clones, patent or lacking the annealing region for the primers HCV90-368 were removed. Among the remaining 88 CNCGs, 48 were subtyped as BVDV-2a, 4 as BVDV-2b, 23 as BVDV-2c and 7 as BVDV-2e. Six sequences could not be classified: KT832810.1, HQ258810, MH231132.1, JF714967.1, MK599227.1 and MH806435.1 (Fig. 2a).

All classifications, as well as non-classifications, obtained by CNCG were maintained in the phylogenetic analysis performed with the putative amplicon from the primers NS5B-F and NS5B-R (Fig. 2a and Fig. 2b). Concerning the putative amplicon obtained by primers HCV90-368, we observed 12 disagreements regarding the CNCG results: KT832817.1, KX096718.1, MH231134.1, MH231131.4, MW168422.1, MH806437.1, MG879027.1, MN527354.1, JF14967.1, MH806435.1, MK599227.1 and HQ258810.1. Sequences KT832817.1, KX096718.1, MH231134.1, MH231131.4, MW168422.1, MH806437.1, MG879027.1 and MN527354.1 were classified as BVDV-2a by CNCG, but could not be reliably classified by analysis of the HCV90-368 amplicon (Fig. 2a and Fig. 2c); JF14967.1, MH806435.1, MK599227.1 and HQ258810.1 could not be classified by CNCG, but were identified as BVDV-2c by HCV90-368 (Fig. 2a and Fig. 2c).

3.2.3 RT-PCR standardization

PCR reactions were optimized to a final volume of 10µL. For BVDV-1, 1µL of cDNA, 0.5µL of each primer (NS3-F and NS4A-R) (both at 10µM), 0.5µL of $MgCl_2$ (50mM), 0.2µL of dNTPs (10mM), 1µL of buffer (10X) and 1µL of Taq DNA Polymerase (5U/µL) were used. The conditions of the reactions were: initial denaturation (94°C for 5min), 35 cycles of 94°C for 45s, annealing at 48°C for 30s and extension at 72°C for 45s, followed by a final extension at 72°C for 5min.

For BVDV-2 reaction, 1µL of cDNA, 0.5µL of each primer (NS5B-F and NS5B-R) (10µM), 0.4µL of $MgCl_2$ (50mM), 0.2µL of dNTPs (10mM), 1µL of buffer (10X) and 0.25µL of Taq DNA Polymerase (5U/µL) were used. The conditions were: initial denaturation (94°C for 5min), 35 cycles of 94°C for 45s, annealing at 52°C for 30s

and extension at 72°C for 1min, followed by a final extension at 72°C for 5min. The identity of the amplicons obtained during assay optimization was confirmed by nucleotide sequencing.

Finally, after optimization, the RT-PCR assays using the novel sets of primers amplified all BVDV-1 and BVDV-2 isolates/strains described in Table 2. Figure 3 illustrates the amplification pattern for BVDV-1 and BVDV-2.

4 Discussion

4.1 BVDV-1

We initally analyzed the most suitable targets for BVDV-1 subtyping, according to the ranking described by De Oliveira et al. [27]. Although NS4B was suggested as the best candidate for this purpose, the current study did not identify conserved sequences within NS4B coding region for primer design. Likewise, it was not possible to design high-coverage primers for the E1 coding region, the second candidate for BVDV-1 classification [27]. Herein, RT-PCR was designed for amplification of the NS3-NS4A cassette (395 nt of NS3 and 131 nt of NS4A). The NS3 coding region would be the third-best candidate for BVDV-1 subtyping, also allowing for a classification equivalent to that of the whole viral genome [27]. Interestingly, NS4A was not among the regions/sequences suggested by De Oliveira et al. [27], however our NS3-NS4A combination showed satisfactory results, as discussed below.

We performed a careful *in silico* analysis of the suitability of NS3-NS4A for BVDV-1 subtyping before optimizing its amplification. For this, we compared the classification of 118 BVDV-1 (GenBank) by CNCG *vs*. NS3-NS4A amplicon. Comparative analysis showed that subtyping based on NS3-NS4A fully reproduced the classification obtained by CNCG. The analysis covered at least one representative of BVDV-1a, -1b, -1c, -1d, -1e, -1f, -1g, -1h, -1i, -1j, -1k, -1m, -1n, -1o, -1q, -1r and -1u. Unfortunately, the suitability of NS3-NS4A for the classification of BVDV-1l, BVDV-1p, BVDV-1s, BVDV-1t, BVDV-1v and BVDV-1x could not be assessed since CNCG representatives of these subtypes were not available in GenBank.

Despite the high compatibility between classification by NS3-NS4A and CNCG, few studies have used NS3 for BVDV-1 subtyping [22, 38]. Furthermore, we found no reports of BVDV-1 subtyping by NS4A analysis. Nagai et al. [22] subtyped 47 Japanese BVDV-1 isolates by 5'UTR, N^{pro}, E2, NS3 and NS5B-3'UTR analysis.

Irianingsih et al. [38] subtyped two BVDV-1 isolates by analyzing 5'UTR, N^{pro}, E2, NS3 and NS5B, using next-generation sequencing. Importantly, considering our dataset (Online Resource 1), the primers used in these classifications have low coverage and may fail in amplifying some BVDV-1 isolates/strains (data not shown). In addition, no analyses were performed comparing subtyping by these amplicons *vs.* CNCG.

After demonstrating the equivalence between CNCG and NS3-NS4A amplicon, a comparative analysis between CNCG subtyping *vs.* primers HCV90-368 was performed. Primers HCV90-368 were chosen because they are often used to classify several BVDVs, regardless of virus sequences [31-36] - a proposal similar to our primer set. The subtyping based on the putative amplicon obtained by primers HCV90-368 showed four mismatches in relation to CNCG analysis, mainly in isolates/strains identified as BVDV-1a. In general, this result is in agreement with previous studies, which discuss the "fragility" of the 5'UTR-based subtyping [26-27]. In addition, the NS3-NS4A phylogenetic analysis was more statistically supported than the HCV90-368 amplicon-based analysis. The low *bootstraps* in the 5'UTR analyses are also in line with previous reports [26-27].

Following *in silico* analyses, when the suitability of the NS3-NS4A amplicon was demonstrated, the RT-PCR assay was standardized. The reaction was optimized for annealing temperature, $MgCl_2$ and Taq DNA polymerase and was able to amplify all BVDV-1 strains and isolates analyzed, including viruses not previously sequenced from Brazil, Argentina and the United States (US). Overall, phylogenetic analyses and RT-PCR performance encourage the use of our primer set for molecular epidemiological studies of BVDV-1.

4.2 BVDV-2

Similarly, we initially looked at the possibility of designing primers for the best candidate for BVDV-2 subtyping [27]. Again, it was not possible to design high-coverage primers for the most suitable target for subtyping BVDV-2, *i.e.*, NS5A. Therefore, RT-PCR was directed to NS5B amplification, since its *in silico* analysis was comparable with that of NS5A, with minimal differences in the geodesic distance [27].

We used 88 CNCGs available on GenBank to assess the suitability of the NS5B region for BVDV-2 subtyping. Among the 88 sequences, 82 were subtyped by

CNCG (48 as BVDV-2a, 4 as BVDV-2b, 23 as BVDV-2c and 7 as BVDV-2e). In addition, six sequences could not be safely classified based on CNCG and were identified as “BVDV-2?”. All these classifications, as well as the non-classifications, were maintained in the phylogenetic analysis of the NS5B putative amplicon. Importantly, we included the recently proposed BVDV-2e subtype, whose subtyping has been well supported by high *bootstrap* values in phylogenetic analyses of the viral genome, ORF and individual regions encoding BVDV proteins [8]. Interestingly, the BVDV-2e classification was also strongly supported in our study.

Interestingly, few studies have used NS5B for BVDV-2 subtyping. Mishra et al. [39] identified one goat BVDV isolate as BVDV-2a based on partial NS5B analysis. One sheep BVDV isolate was identified as BVDV-2b after partial NS5B analysis [40]. Spetter et al. [41] subtyped BVDV-2 from tissue samples and cell supernatant by partial NS5B. All these reports, however, were performed using low-coverage primers and/or nested PCR reactions. Similar to what was discussed for BVDV-1, no analyses were performed comparing subtyping by these amplicons and CNCG.

We also performed an *in silico* analysis to assess the agreement between the CNCG subtyping and primers HCV90-368. Here, eight sequences that were subtyped as BVDV-2a by CNCG could not be classified by HCV90-368 and were identified as “BVDV-2?”. Furthermore, four sequences that could not be safely classified based on CNCG were subtyped as BVDV-2c by HCV90-368. These findings are in agreement with those of other studies. Workman et al. [25], for example, also reported difficulties in subtyping some BVDV-2 by the 5'UTR analysis. Neill et al. [42] described that BVDV-2 analysis based on 5'UTR may have low *bootstrap*, which may hinder viral classification.

After confirming the suitability of the NS5B amplicon for BVDV-2 subtyping, we optimized the RT-PCR for its amplification, adjusting the annealing temperature, $MgCl_2$ concentration and Taq DNA polymerase. Similar to the BVDV-1 RT-PCR, the reaction amplified all analyzed BVDV-2, including viruses from Brazil and the US of unknown sequence. Finally, our *in silico* analyses, as well as the RT-PCR assay, support the use of the primers NS5B-F/NS5B-R for future BVDV-2 studies.

Conclusion

We describe novel primers sets for accurate subtyping of BVDV-1 and BVDV-2. The primers described here have high coverage and may be useful for the

classification/subtyping of BVDVs. The primers HCV90-368 have been largely used for this purpose, however, unlike their amplicon, which has often been questioned as to the suitability for subtyping BVDV, the analysis based on our primers fully reproduced the CNCG classification. RT-PCR assays based on our primers also amplified all analyzed BVDV isolates/strains, regardless of sequence and country of origin. Importantly, considering their length, the amplicons generated may be easily analyzed in a single Sanger sequencing. Based on these findings, we encourage the use of our primer sets in future phylogenetic and epidemiological studies of BVDV-1 and BVDV-2.

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Disclosure of potential conflicts of interest

The authors declare that they have no competing interests.

Financial interests

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Data availability

Datasets generated and/or analyzed during the current study will be available on GenBank and may also be made available by the corresponding author upon reasonable request.

Authors contributions

CIM, JVJSJr, PSBO, EFF and RW conceived the study design; CIM, JVJSJr and PSBO designed the primer sets; CIM and JVJSJr performed data collection; CIM, JVJSJr and PSBO analyzed the data; CIM and JVJSJr prepared the draft manuscript; EFF and RW critically reviewed the manuscript. All authors read and approved the final manuscript.

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Fig.1 Bovine viral diarrhea virus 1 phylogenetic trees

Phylogenetic analyses were performed considering complete/near-complete genomes (CNCGs) (A) and putative amplicons from primers NS3-NS4A (B) and HCV90-368 (C), using the MEGA-X software (version 10.2.4) and the Maximum Likelihood method (see Table 1). The *bootstrap* values were calculated in 1,000 replicates. Branches supported by ≥70% of the bootstrap replicates are indicated.

Non-subtyped BVDV-1 sequences were identified as “BVDV-1?”. *Mismatch between HCV90-398 and CNCG trees.

Fig.2 Bovine viral diarrhea virus 2 phylogenetic trees

Phylogenetic analyses were performed considering complete/near-complete genomes (CNCGs) (A) and putative amplicons from primers NS5B (B) and HCV90-368 (C), using the MEGA-X software (version 10.2.4) and the Maximum Likelihood method (Table 1). The *bootstrap* values were calculated in 1,000 replicates. Branches supported by ≥70% of the bootstrap replicates are indicated. Non-subtyped BVDV-2 sequences were identified as “BVDV-2?”. *Mismatch between HCV90-398 and CNCG trees.

Fig.3 Amplification of genomic targets for bovine viral diarrhea virus (BVDV) sequencing and subtyping

1: Singer strain (BVDV-1) amplified with primers NS3-F and NS4A-R (526bp amplicon); 2: BVDV-1 RT-PCR negative control (water). 3: VS-253 strain (BVDV-2) amplified with primers NS5B-F and NS5B-R (728bp amplicon); 4: BVDV-2 RT-PCR negative control (water). M: 100bp DNA Ladder (marker) (Ludwig, RS, Brazil). Amplicon migrations were slightly influenced by the GelRed® stain (Biotium).

Table 1 – Parameters used in bovine viral diarrhea virus (BVDV) phylogenetic analyses

Viral species	Genomic region	Substitution model[b]	Gamma shape parameter	Proportion invariant sites	BIC[c]	Log likelihood
	CNCG[a]	GTR + G + I	0.7174	0.33	599,594.560	-298,288.60
BVDV-1	5'UTR (HCV90-368)[d]	K2 + G + I	0.3602	0.2385	9,229.023	-3,424.94
	NS3-NS4A[e]	TN93 + G + I	1.4198	0.2576	22,517.155	-9,941.47
	CNCG	GTR + G + I	0.5119	0.3056	215,962.629	-106,713.36
BVDV-2	5'UTR (HCV90-368)[f]	K2 + G	0.2256	NA[h]	4,500.149	-1,361.85
	NS5B[g]	TN93 + G	0.4406	NA	13,730.005	-5,861.35

[a] Complete/near-complete genome;

[b] GTR: general time reversible; G: gamma distribution; I: invariant sites; K2: Kimura 2-parameter; TN93: Tamura-Nei;

[c] Bayesian information criterion;

[d] Putative region analyzable by primers HCV90-368 (nt 124-372, according to strain NADL, M31182.1);

[e] Putative region analyzable by primers NS3-F and NS4A-R (nt 7,096-7,588, according to strain NADL, M31182.1);

[f] Putative region analyzable by primers HCV90-368 (nt 124-372, according to strain New York 93, AF502399.1);

[g] Putative region analyzable by primers NS5B-F and NS5B-R (nt 10,270-10,960, according to strain New York 93, AF502399.1);

[h] Not applicable.

Table 2 – Bovine viral diarrhea virus (BVDV) isolates and strains used to evaluate the performance of RT-PCR assays

BVDV-1		BVDV-2	
ID	**Country[a]**	**ID**	**Country**
1463	US[b]	13875	US
11740	US	17513	US
13443	US	17530	US
13619	US	21693	US
14180.94	US	8056-9	US
14859	US	VS 26	US
15027.94	US	VS 61	US
17415	US	SV 11/03	Brazil
19271	US	SV 125/17-1182	Brazil
19729	US	SV 125/17-2139.1	Brazil
20593	US	SV 125/17-2139.2	Brazil
VS 67	US	SV 125/17-2140	Brazil
M35	US	SV 125/17-3267	Brazil
SV 125/17-1368[c]	Brazil	SV 125/17-382	Brazil
SV 125/17-2370	Brazil	SV 125/17-4271	Brazil
SV 125/17-2526	Brazil	SV 125/17 430	Brazil
SV 125/17-3656	Brazil	SV 154/08	Brazil
SV 256/17-1568	Brazil	SV 193/08	Brazil
SV 357/13	Brazil	SV 256/17-426	Brazil
SV 508/17-2920	Brazil	SV 275/08	Brazil
SV 508/17-3036	Brazil	SV 323/04	Brazil

SV 605/17-3377	Brazil	SV 432/05	Brazil
EMP 2	Brazil	SV 508/17-1832	Brazil
IBSP-2	Brazil	SV 508/17-4106	Brazil
IBSP-4	Brazil	SV 508/17-4437	Brazil
IBSP-5	Brazil	SV 508/17-4446	Brazil
M53	Brazil	SV 508/17-4896	Brazil
UFSM 2	Brazil	SV 56/03	Brazil
126.1	Brazil	SV 605/17-1150	Brazil
126.8	Brazil	SV 605/17-2368	Brazil
153.1	Brazil	SV 605/17-3499	Brazil
156.15	Brazil	SV 605/17-42	Brazil
INTA 1	Argentina	SV 778/09	Brazil
INTA 3	Argentina	-	-
INTA 4	Argentina	-	-

[a] Origin;

[b] The United States;

[c] SV: protocol record of the Virology Section (*Universidade Federal de Santa Maria*, UFSM).

Figure 1 – Bovine viral diarrhea virus 1 phylogenetic trees

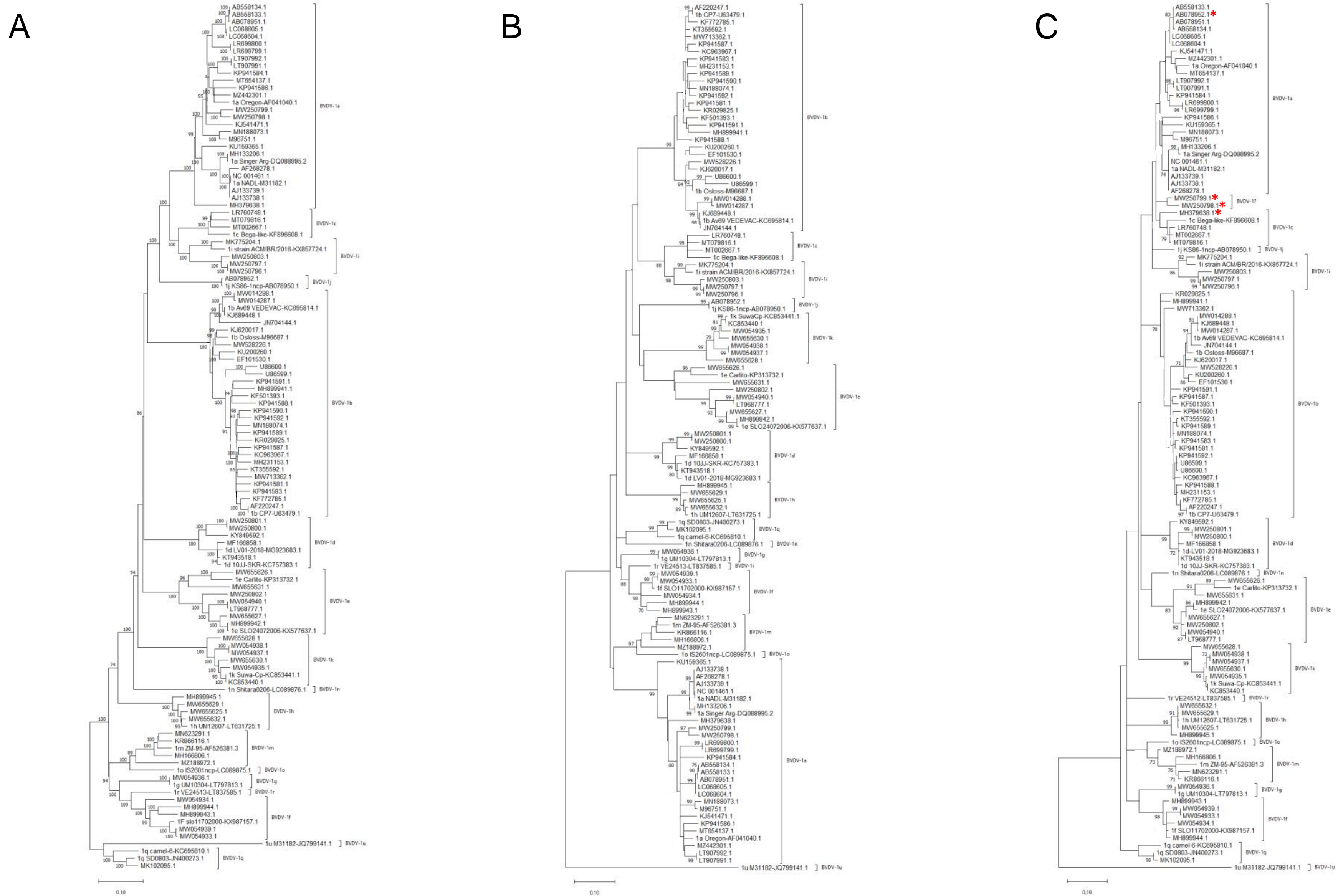


Figure 2 – Bovine viral diarrhea virus 2 phylogenetic trees

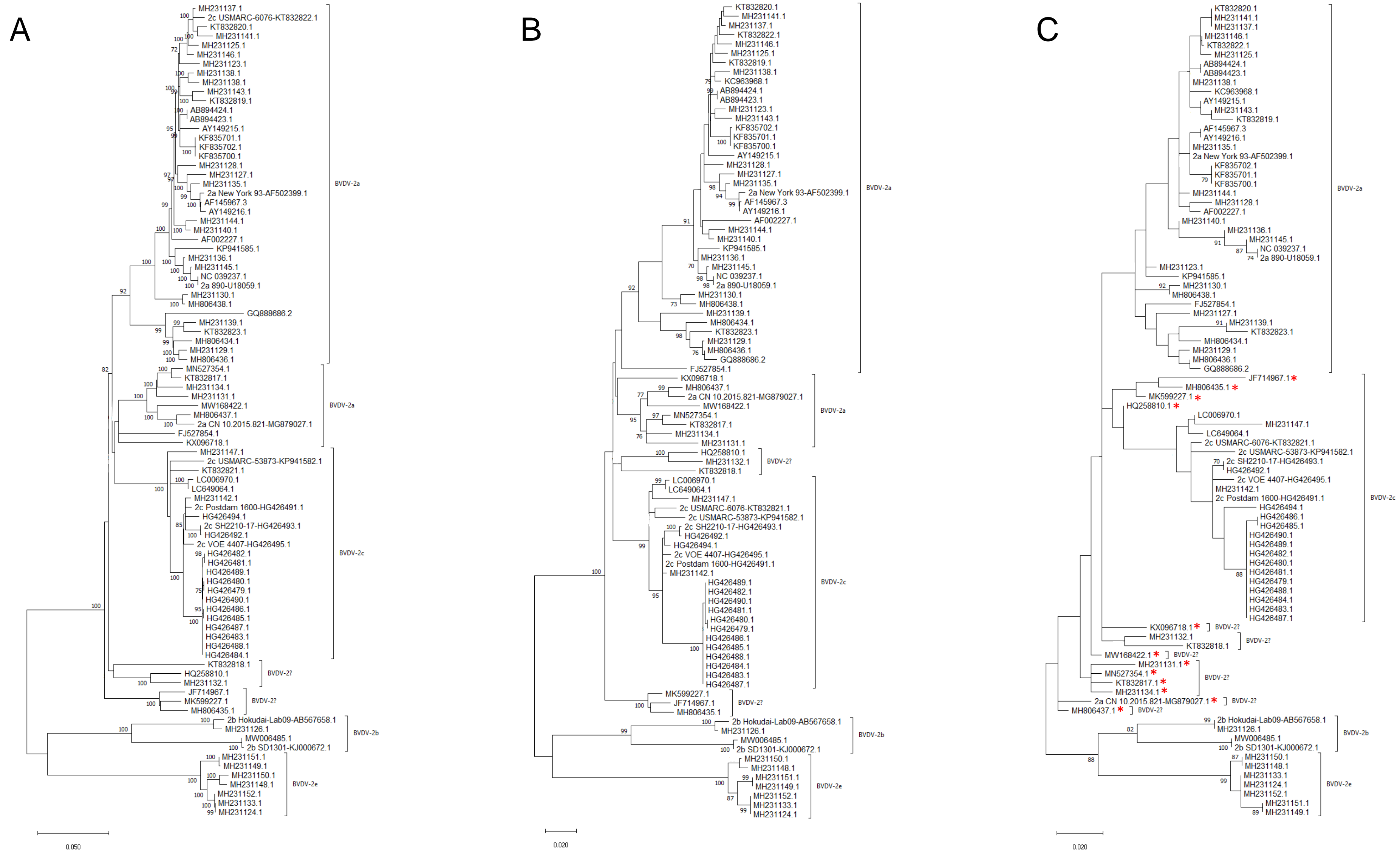
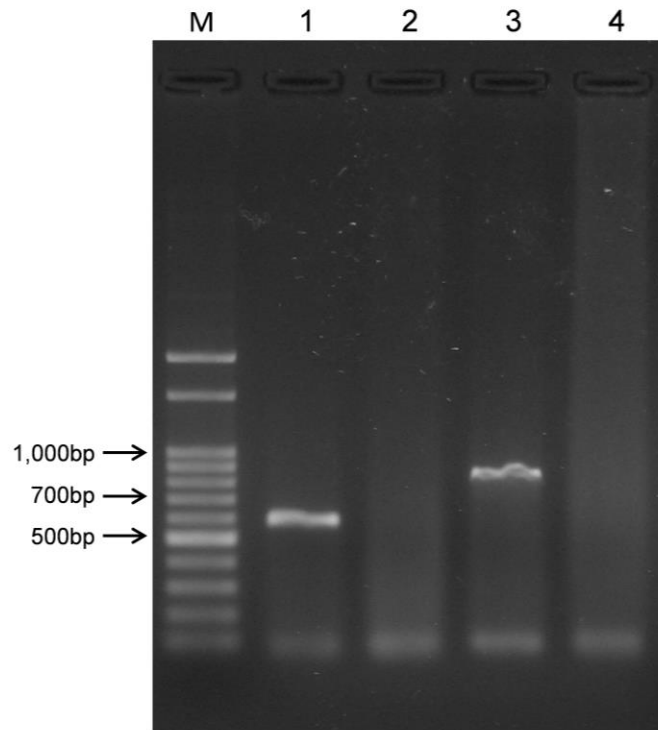


Figure 3 – Amplification of genomic targets for bovine viral diarrhea virus (BVDV) sequencing and subtyping



3 CONCLUSÃO

Esse estudo descreve novos pares de *primers*, cujos amplicons podem ser usados para a subtipagem de BVDV-1 e BVDV-2. Os *primers* aqui descritos apresentam alta cobertura e podem ser úteis para a classificação/subtipagem de BVDVs. Os *primers* HCV90-368 têm sido frequentemente usados com essa finalidade, no entanto, ao contrário da região amplificada pelo HCV90-368, que tem sido frequentemente questionada quanto à precisão da subtipagem do BVDV, a análise baseada nos *primers* do nosso estudo reproduziu integralmente a classificação obtida pela análise do genoma completo/quase completo de BVDV. Os ensaios de RT-PCR baseados nesses *primers* também amplificaram todos os isolados/cepas analisados, independentemente da sequência e origem. Por fim, é importante ressaltar que, considerando o tamanho dos alvos gerados com esses *primers*, os amplicons podem ser facilmente sequenciados pelo método Sanger, facilitando seu uso em futuros estudos filogenéticos e epidemiológicos de BVDV-1 e BVDV-2.

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