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**ANÁLISE DO GENOMA DE ISOLADOS
CITOPÁTICOS DO VÍRUS DA DIARRÉIA VIRAL
BOVINA (BVDV) PARA REARRANJOS GENÔMICOS
ASSOCIADOS COM A EXPRESSÃO DA PROTEÍNA
NS3**

DISSERTAÇÃO DE MESTRADO

Valter Leonardo de Quadros

**Santa Maria, RS, Brasil
2005**

**ANÁLISE DO GENOMA DE ISOLADOS CITOPÁTICOS DO
VÍRUS DA DIARRÉIA VIRAL BOVINA (BVDV) PARA
REARRANJOS GENÔMICOS ASSOCIADOS COM A
EXPRESSÃO DA PROTEÍNA NS3.**

por

Valter Leonardo de Quadros

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Orientador: Rudi Weiblen

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DA DIARRÉIA VIRAL BOVINA (BVDV) PARA REARRANJOS
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PROTEÍNA NS3.**

elaborada por
Valter Leonardo de Quadros

como requisito parcial para obtenção do grau de
Mestre em Medicina Veterinária

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Valter e Vera Lúcia pelo incentivo
ao estudo.**

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**Segunda-feira é um dia mais difícil,
porque é sempre a tentativa do começo
de vida nova. Façamos cada domingo
de noite um reveillon modesto,
pois se meia-noite de domingo não é
começo de Ano Novo, é começo
de semana nova, o que significa
fazer planos e fabricar sonhos.**

Clarice Lispector

RESUMO

Dissertação de Mestrado

Programa de Pós-graduação em Medicina Veterinária

Universidade Federal de Santa Maria

ANÁLISE DO GENOMA DE ISOLADOS CITOPÁTICOS DO VÍRUS DA DIARRÉIA VIRAL BOVINA (BVDV) PARA REARRANJOS GENÔMICOS ASSOCIADOS COM A EXPRESSÃO DA PROTEÍNA NS3.

AUTOR: VALTER LEONARDO DE QUADROS

ORIENTADOR: RUDI WEIBLEN

Santa Maria, 8 de agosto de 2005.

Bezerros nascidos persistentemente infectados (PI) com o biótipo não citopático (ncp) do vírus da Diarréia Viral Bovina (BVDV) freqüentemente desenvolvem uma doença gastrointestinal fatal, chamada de Doença das Mucosas (DM). Dos animais afetados pela DM é possível isolar o vírus original não-citopático (BVDVncp) e um vírus antigenicamente idêntico, porém citopático (BVDVcp). Os BVDVcps são gerados a partir do vírus original ncp por diversos mecanismos genéticos, que resultam na expressão da proteína não-estrutural NS3 como uma proteína individual. Em contrapartida, os BVDVncp expressam somente a proteína precursora NS2-3, que contém a seqüência da NS3 no seu terço carboxi-terminal. Este trabalho relata a investigação dos mecanismos genéticos associados com a expressão da NS3 em 41 isolados citopáticos de BVDV. Uma estratégia de RT-PCR foi delineada para detectar inserções no gene da NS2-3 e/ou duplicações no gene da NS3, dois mecanismos freqüentes de expressão da NS3. Amplificação do genoma dos 41 isolados por RT-PCR revelou a presença de inserções no gene da NS2-3 em três isolados, de tamanho similar a inserção presente na cepa de BVDVcp NADL. O seqüenciamento da inserção de um isolado revelou uma seqüência de 296 nucleotídeos, com uma região central de 270 nucleotídeos altamente homóloga (98%) com a inserção da cepa NADL, que corresponde a uma seqüência do gene celular J-Domain. Outro isolado de BVDVcp contém uma duplicação do gene da NS3 na direção 3' da sua posição original. Em 37 isolados cp não foram detectadas inserções na NS2-3 ou duplicações da NS3. Esses resultados demonstram que a clivagem da NS2-3 sem a presença de inserções de RNA ou duplicações do gene da NS3 parecem ser mecanismos frequentes de expressão da proteína NS3 e citopatologia no BVDV.

Palavras-chave: vírus da Diarréia Viral Bovina, BVDV, NS3, citopatologia.

ABSTRACT

Master's Dissertation

Programa de Pós-graduação em Medicina Veterinária

Universidade Federal de Santa Maria

ANALYSIS OF CYTOPATHIC ISOLATES OF BOVINE VIRAL DIARRHEA VIRUS (BVDV) FOR GENOMIC REARRANGEMENTS ASSOCIATED WITH EXPRESSION OF THE PROTEIN NS3.

AUTHOR: VALTER LEONARDO DE QUADROS

ADVISER: RUDI WEIBLEN

Santa Maria, AUGUST 8th, 2005.

Calves born persistently infected (PI) with non-cytopathic bovine viral diarrhea virus (ncpBVDV) frequently develop a fatal gastroenteric illness called mucosal disease (MD). From animals affected by MD, both the original virus (ncpBVDV) and an antigenically identical, yet cytopathic virus (cpBVDV) can be isolated. Cytopathic BVDVs are originated from the ncp counterparts by diverse genetic mechanisms, all leading to the expression of the non-structural polypeptide NS3 as a discrete protein. In contrast, ncpBVDVs express only the large precursor polypeptide, NS2-3, which contains the NS3 sequence within its carboxy-terminal half. The investigation of the mechanism leading to NS3 expression in 41 cpBVDV isolates is reported. An RT-PCR strategy was designed to detect insertions within the NS2-3 gene and/or duplication of the NS3 gene – two common mechanisms of expression of NS3. RT-PCR amplification revealed insertions in the NS2-3 gene of three cp isolates, being the inserts similar in size to that present in the cpBVDV NADL strain. Sequencing of one such insert revealed a 296 nucleotide sequence, with a central core of 270 putative aminoacid sequence highly homologous (98%) to the NADL insert, a sequence corresponding to the cellular J-Domain gene. Another cpBVDV isolate contained a duplication of the NS3 gene downstream from the original locus. In contrast, no detectable NS2-3 insertions nor NS3 gene duplications were observed in the genome of 37 cp isolates. These results demonstrate that cleavage of NS2-3 without bulk RNA insertions nor NS3 gene duplications seems to be a frequent mechanism leading to NS3 expression and BVDV cytopathology.

Key words: bovine viral diarrhea virus, BVDV, NS3, cytopathology.

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1. INTRODUÇÃO

O vírus da Diarréia Viral Bovina (BVDV) é um dos principais patógenos de bovinos, e possui distribuição mundial (BOLIN, 1995). O BVDV pertence a família *Flaviviridae*, gênero *Pestivirus*, juntamente com o vírus da Peste suína Clássica (CSFV) e o vírus da Doença da Fronteira (BDV) (HORZINEK, 1991). O BVDV é um vírus pequeno de 40-60nm de diâmetro, genoma RNA de cadeia simples linear, polaridade positiva, de aproximadamente 12,5 kilobases (kb) (COLLETT et al., 1989). A infecção pelo BVDV em bovinos está associada com enfermidade gastrointestinal, respiratória e distúrbios reprodutivos e variam de infecções subclínicas até enfermidades fatais como a doença das mucosas (DM) (BAKER, 1995).

O BVDV apresenta uma grande variabilidade genética e antigênica sendo dividido em dois genótipos: BVDV-1 e BVDV-2 (FLORES et al., 2002). Pode ainda ser classificado em 2 biótipos: citopático (CP) e não citopático (NCP), de acordo com a sua capacidade de produzir efeito citopático (CPE) em cultivo celular (BOLIN & RIDPATH, 1990). As amostras NCP representam a grande maioria dos isolados de campo; as CP representam a minoria, freqüentemente recuperadas de animais afetados pela Doença das Mucosas (DM) em que vírus CP e NCP estão presentes (BROWNLIE, 1990; DONIS, 1995). Além da diferença na produção de citopatologia as amostras CP e NCP apresentam diferenças moleculares na expressão da proteína NS3, a qual é expressa somente nos isolados CP. Por isso, a NS3 é considerada o marcador molecular dos isolados CP do BVDV (TAUTZ et al., 1996). A sua expressão como um polipeptídeo separado sugere uma associação direta ou indireta com a produção de citopatologia (MEYERS et al., 1989).

A partir da identificação da grande similaridade antigênica entre vírus NCP e CP de um mesmo animal foi sugerida a relação entre os biótipos (DONIS & DUBOVI, 1987). Posteriormente, foi demonstrado que o vírus CP origina-se do NCP por diversos mecanismos genéticos que levam a expressão da NS3 como um polipeptídeo separado. Esses mecanismos incluem a inserção de seqüências celulares, duplicação do gene da NS3, deleções e mutações em ponto (KUMMERER et al., 2000). Variações no tamanho do genoma são decorrentes da presença destas recombinações genéticas (GREISER-WILKE et al., 1992).

O presente trabalho teve como objetivo a análise molecular de 41 isolados citopáticos (CP) do BVDV para a presença de rearranjos genéticos no gene que codifica a proteína NS2-3. Para isso foram utilizados primers que se ligam em regiões específicas da NS2-3 capazes de detectar a presença de inserções e duplicações. Nesses 41 isolados foram identificados

estes dois tipos possíveis de mecanismos de produção da NS3: inserções de RNA e duplicação do gene da NS3.

2. CAPÍTULO 1

Revisão Bibliográfica

O vírus da Diarréia Viral Bovina (*bovine viral diarrhea virus*, BVDV) é um dos patógenos mais importantes para a bovinocultura em todo o mundo (BOLIN, 1995). Nos países livres de Febre Aftosa, o BVDV é considerado o vírus mais importante de bovinos. No Brasil, a infecção pelo BVDV está amplamente difundida e os níveis de prevalência de anticorpos variam entre 14% e 70% (BRITO et al., 2002; MINEO et al., 2002; SERRA et al., 2002; MORAES et al., 2003; NORONHA et al., 2003; POLETTI et al., 2004).

O BVDV é um membro da família *Flaviviridae*, gênero *pestivirus*, juntamente com o vírus da Peste Suína Clássica (CSFV) e o vírus da Doença da Fronteira (BDV) dos ovinos (HORZINEK, 1991). O BVDV é um vírus pequeno, de aproximadamente 40-60 nm de diâmetro, que contém um nucleocapsídeo icosaédrico envolto por um envelope lipoprotéico (HORZINEK, 1991). O nucleocapsídeo contém uma molécula de RNA de cadeia simples, polaridade positiva de aproximadamente 12,5 quilobases (kb) (HORZINEK, 1981; COLLETT et al., 1989). O envelope é formado por uma membrana lipídica derivada de membranas celulares e contém pelo menos três glicoproteínas codificadas pelo vírus: a E2 (gp53), E^{rns}(gp48) e E1 (gp25) (DONIS, 1995).

O BVDV apresenta uma grande variabilidade genética e antigênica, evidenciada pela identificação de dois genótipos: BVDV-1 e BVDV-2. O BVDV-1 já foi subdividido em 11 subgenótipos e o BVDV-2 em 2 subgenótipos (FLORES et al., 2002). De acordo com a capacidade de produzir efeito citopático (ECP) em cultivo celular, os isolados de campo do BVDV podem ser alocados nos biótipos citopático (CP) e não-citopático (NCP) (BOLIN & RIDPATH, 1990). A grande maioria dos isolados de campo pertencem ao biótipo NCP; os isolados CP constituem a minoria e são encontrados quase que exclusivamente em animais que desenvolvem a forma clínica denominada de Doença das Mucosas (DM) (BROWNLIE, 1990; DONIS, 1995).

A descrição inicial da enfermidade causada pelo BVDV data de 1946 e referia-se a uma enfermidade gastrointestinal severa com alta mortalidade (OLAFSON et al., 1946). A infecção pelo BVDV pode produzir diversas manifestações clínicas, que incluem desde infecções inaparentes até enfermidades de curso fatal, como a Doença das Mucosas (DM). Enfermidade gastrointestinal ou respiratória, distúrbios reprodutivos com mortalidade embrionária ou fetal, abortos e mumificações fetais, malformações congênitas, natimortos e nascimento de bezerros fracos e inviáveis, além da produção de terneiros persistentemente

infetados (PI), estão entre as consequências da infecção pelo BVDV (BROWNLIE, 1990; BAKER, 1995).

Fetos infectados por amostras NCP entre os dias 40 e 120 de gestação podem se tornar imunotolerantes ao vírus e originar terneiros PI. Esses animais podem ser clinicamente saudáveis, são geralmente soronegativos e excretam o vírus em secreções e excreções. Nos animais PI é comum o desenvolvimento de uma enfermidade fatal, denominada Doença das Mucosas (DM), em que vírus dos biótipos NCP e CP, antigenicamente idênticos, estão presentes (BROWNLIE, 1990). O vírus CP origina-se do vírus original NCP através de mutações e/ou rearranjos genômicos (MEYERS et al., 1991; GREISER-WILKE et al., 1993).

O genoma do BVDV consiste de uma molécula de RNA cadeia simples, linear, de aproximadamente 12,5 kb. Próximo à extremidade 5' existe uma região não codificante (*nontranslated region* – NTR) de 386 nucleotídeos (nt) com vários codons AUG. A região 5'NTR é seguida de uma longa e única seqüência aberta de leitura (*open reading frame*, ORF) que codifica aproximadamente 3900 aminoácidos. A ORF é seguida de uma NTR com 229 nt na extremidade 3' (COLLETT et al., 1988). Variações no tamanho da ORF resultam da presença de seqüências genômicas rearranjadas ou duplicadas, ou ainda devido a inserções de seqüências de RNA mensageiro (RNAm) das células hospedeiras (GREISER-WILKE et al., 1992; MEYERS & THIEL, 1996). A organização do genoma do BVDV encontra-se esquematizada na Figura 1.

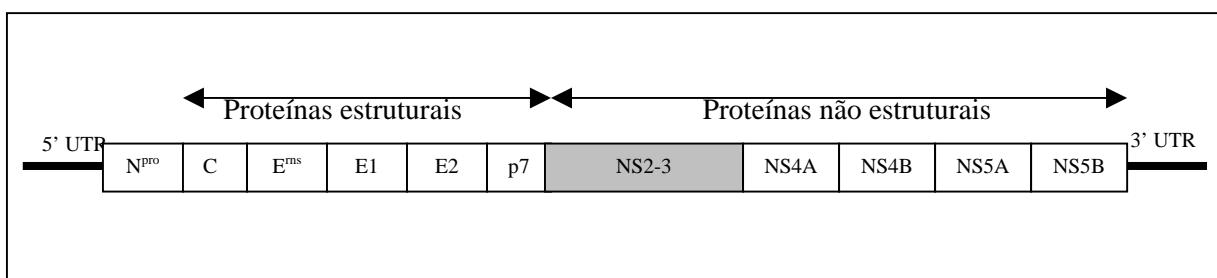


Figura 1. Organização do genoma do vírus da Diarréia Viral Bovina (BVDV).

A poliproteína do BVDV é processada por proteases virais e celulares à medida que vai sendo produzida, para dar origem a 11 a 12 proteínas maduras (COLLETT et al., 1989; DONIS, 1995). Os BVDV-ncp produzem no mínimo 11 proteínas maduras, sendo 4 estruturais e 6 não-estruturais, enquanto os CP expressam uma proteína não-estrutural adicional, a NS3 (p80) (COLLETT et al., 1989). A ordem e nomenclatura das proteínas do BVDV são: N^{pro}(p20), C (p14), E^{rns} (E0 ou gp48), E1 (gp25), E2 (gp53), p7, NS2-3 (p125)

(NS2[p54] e NS3[p80]), NS4A (p10), NS4B (p32), NS5A (p58), e NS5B (p75). A nomenclatura entre parênteses representa o nome original dessas proteínas, agora nomeadas de acordo com a denominação unificada dos membros da família *Flaviviridae* (COLLETT et al., 1992). As proteínas não-estruturais não estão presentes nos vírions, sendo encontradas apenas nas células infectadas e estão envolvidas no processo de replicação viral (DONIS, 1995; POTGIETER, 1995).

A proteína não-estrutural N^{pro} é uma autoprotease e é responsável pela clivagem da sua extremidade carboxi-terminal, resultando na sua liberação da poliproteína e geração da extremidade amino-terminal da proteína C (WISKERCHEN et al., 1991; STARK et al., 1993). A proteína estrutural C tem como função envolver o RNA genômico e proporcionar as interações necessárias entre o nucleocapsídeo e o envelope, para a formação do vírion. A E^{rns} é uma glicoproteína do envelope, com função ainda não esclarecida, mas apresenta atividade de uma ribonuclease (RNase). A função da glicoproteína do envelope E1 é pouco conhecida, sendo uma proteína que apresenta pouca imunogenicidade (DONIS, 1995). A E2 é a principal glicoproteína do envelope do BVDV e parece estar envolvida na ligação do vírus nos receptores celulares. A E2 é muito antigênica e muitos dos anticorpos contra ela possuem atividade neutralizante, reforçando a hipótese de que é responsável pelas interações iniciais do vírion com a célula (BOLIN & RIDPATH, 1990; COLLETT et al., 1991; DONIS, 1995). A p7 é uma proteína hidrofóbica e parece estar envolvida com a reunião, maturação e liberação dos vírions recém-formados (COLLETT, 1996; HARADA et al., 2000). A NS4, NS4B e NS5A são pouco estudadas, porém acredita-se que possuam papel funcional importante na replicação do vírus, devido ao seu nível de conservação entre os *Pestivirus* (DONIS, 1995).

A NS2-3 possui um papel indispensável na replicação viral, estando envolvida na produção dos vírions, bem como na reunião e liberação de partículas víricas (DONIS, 1995; AGAPOV et al., 2004). A NS2-3 juntamente com seu segmento carboxi-terminal (denominado NS3 ou p80) constituem-se nas proteínas mais estudadas do BVDV, correspondendo às seqüências homólogas encontradas nos *Flavivirus* (DONIS, 1995). A NS3 tem recebido maior atenção porque é encontrada exclusivamente nos isolados citopáticos do BVDV (DONIS & DUBOVI, 1987; POCOCK et al., 1987).

A proteína NS2 é um produto proteolítico da NS2-3. Essa proteína apresenta um domínio *zinc finger*, uma região rica em cisteína, localizada entre os códons 1484 e 1512, que acredita-se possuir atividade de ligação ao RNA (DE MOERLOOZE et al., 1990; 1991). A

NS2 é pouco imunogênica e não induz a produção de anticorpos em bovinos infectados (MEYERS et al., 1996).

A NS3 é a proteína mais conservada no gênero *Pestivirus* e é considerada o marcador molecular dos isolados CP do BVDV. A ocorrência da NS3 como um polipeptídeo separado, ao contrário dos vírus NCP onde a NS3 faz parte da NS2-3, sugere que essa proteína está associada direta ou indiretamente com a produção de citopatologia (MEYERS et al., 1989; BOLIN & RIDPATH, 1990). A NS3 apresenta atividades enzimáticas de NTPase, serino-protease e helicase (WISKERCHEN et al., 1991). A atividade protease é caracterizada como uma quimiotripsina, semelhante à proteases da serina, responsável pela clivagem da poliproteína em determinados locais, originando os polipeptídeos não-estruturais através de uma série de clivagens intramoleculares (*in cis*) e intermoleculares (*in trans*) (WISKERCHEN et al., 1991; XU et al., 1997). A NS3 é muito estável em células infectadas e é altamente imunogênica (WISKERCHEN & COLLETT, 1991).

O BVDV pode ser classificado em dois biótipos: CP e NCP, de acordo com sua capacidade de produzir efeito citopático em cultivo celular (BOLIN & RIDPATH, 1990; TAUTZ et al., 1994; RIDPATH, 2003). Além da diferença em produzir ou não citopatologia, os biótipos apresentam diferenças moleculares na proteína não-estrutural NS2-3. Os isolados NCP produzem somente a NS2-3, enquanto que os isolados CP expressam a NS2-3 e também a sua região carboxi-terminal ou NS3 como uma proteína separada (TAUTZ et al., 1996).

Apesar da expressão da NS3 estar associada à infecção pelos vírus CP, o mecanismo responsável pela indução de citopatologia ainda não foi esclarecido. Uma das hipóteses é de que a NS3 poderia induzir apoptose (BAROTH et al., 2000). A localização da NS2-3 e NS3 na membrana do retículo endoplasmático (RE) e o mecanismo de associação com a membrana da célula hospedeira, sugere que possam estar envolvidas com apoptose, devido ao acúmulo de RNA viral ativando a proteína kinase (PK), ou devido ao estresse induzido ao RE. Tem sido sugerido que a presença da NS3 na membrana do RE de células infectadas com cepas CP possa causar o estresse e iniciar um processo apoptótico (ZHANG et al., 2003).

Ativação das caspases e do Fator Nuclear Kappa B (NF κ B) com indução da apoptose por desfragmentação do DNA foi verificada em células infectadas com a cepa CP NADL (VASSILEV & DONIS, 2000). A deleção da inserção de seqüência celular presente na NS2-3 gerou uma amostra NCP. A comparação desta com o vírus CP revelou que não há diferença na cinética de replicação e tamanho das placas. No entanto, detectou-se uma síntese e

acúmulo maior de RNA viral nas células infectadas com o vírus CP (VASSILEV & DONIS, 2000). Entretanto, foi demonstrado que os processos de apoptose e ECP podem ocorrer separadamente. Através do uso de inibidores das caspases observou-se um retardo no início do ECP, mas sem bloqueá-lo por completo. Isto sugere que as caspases desempenham um papel importante, mas não exclusivo na apoptose induzida por BVDV-CP. Em contraste, os BVDV-NCP não induziriam apoptose devido a uma super-expressão da proteína anti-apoptótica Bcl-2 (BENDFELDT et al., 2003).

A grande similaridade antigênica entre os vírus NCP e CP isolados de um mesmo animal foi o primeiro indicador de que esses vírus fossem relacionados entre si (DONIS & DUBOVI, 1987). Posteriormente, foi demonstrado que o vírus CP origina-se do NCP por diversos mecanismos genéticos, dentre eles a inserção de seqüências celulares (MEYERS et al., 1991), duplicação do gene da NS3 (QI et al., 1992; GREISER-WILKE et al., 1993), presença de genomas defectivos que expressam a NS3 (TAUTZ et al., 1994; KUPFERMANN et al., 1996) e mutações em ponto (KUMMERER et al., 1998; KUMMERER et al., 2000). Todos esses mecanismos levam à expressão da NS3 como um polipeptídeo separado. Alguns rearranjos provocam alterações no tamanho do genoma viral (GREISER-WILKE et al., 1992; TOBIAS et al., 2000). Em contrapartida outros rearranjos são de pequenas inserções, deleções e duplicações em que não há alteração detectável no tamanho do genoma (TAUTZ et al., 1996). Os mecanismos já identificados de geração da NS3 encontram-se esquematizados na Figura 2.

A maioria dos rearranjos ocorrem em dois pontos específicos do genoma, denominados de posições A e B. A posição A refere-se ao códon que codifica o aminoácido 1535, e a posição B é localizada na seqüência que codifica o aminoácido 1589 (MEYERS et al., 1998). Rearranjos em cepas de BVDV-1 geralmente consistem da duplicação de seqüências virais e/ou seqüências que codificam a ubiquitina, ocorrendo na posição B (GREISER-WILKE et al., 1993; QI, et al., 1998) e em cepas de BVDV-2 ocorrem na posição A, com a recombinação de uma seqüência celular (RIDPATH & NEILL, 2000). A região do códon que codifica a glicina¹⁵⁹⁰ foi identificada como a posição inicial para a clivagem da proteína NS2-3, devido ao grande número de recombinações *upstream* a esse códon (TAUTZ & THIEL, 2003). Este local de clivagem deve-se a uma seleção funcional do vírus e não a presença de um sítio preferencial de mutação (*hot spot*).

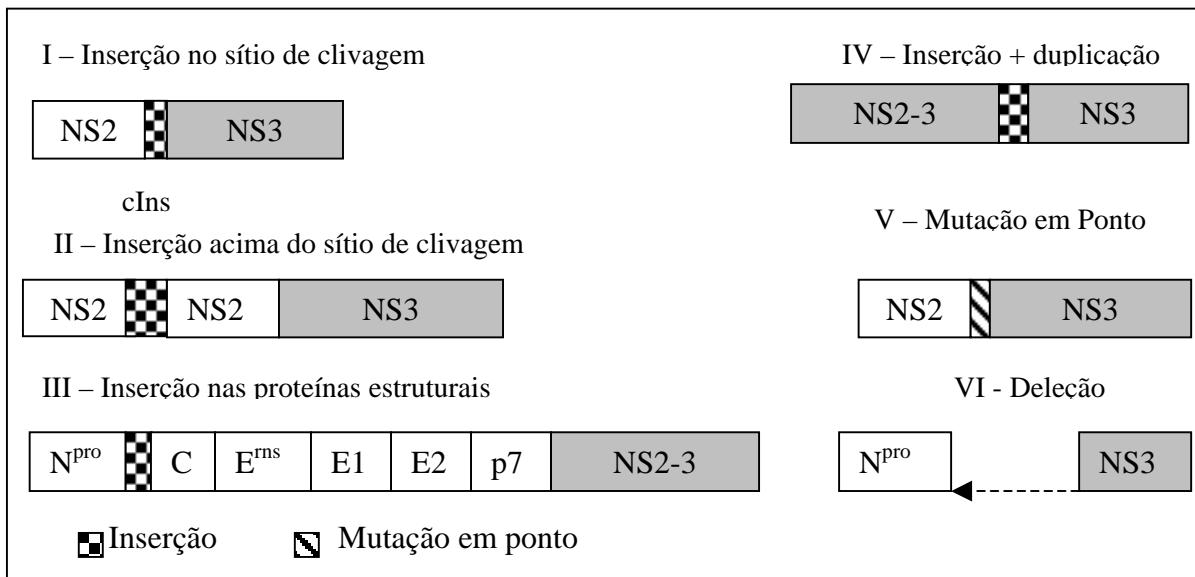


Figura 2. Mecanismos de geração da proteína NS3 em amostras citopáticas do vírus da Diarréia Viral Bovina (BVDV).

A primeira descrição de inserções derivadas de seqüências celulares no genoma de isolados de BVDV-CP foram realizados por MEYERS et al. (1991). Os autores identificaram a inserção de seqüências que codificam a proteína ubiquitina entre os genes das proteínas NS2 e NS3 na amostra Osloss. Posteriormente, TAUTZ et al. (1993) descreveram o mesmo mecanismo e tipo de inserção na amostra CP14.

TAUTZ et al. (1994) descreveram um outro tipo de alteração possível com amostras CP, que é a deleção de grande parte do genoma. A amostra CP9 é composta de duas moléculas de RNA: uma molécula de RNA do BVDV-NCP completo, chamado de vírus “helper” e outra molécula de BVDV-CP defectiva. A molécula defectiva possui uma deleção na região que codifica as proteínas estruturais até a região da NS2, resultando na fusão do gene da N^{pro} com a NS3. A presença do genoma viral defectivo mostrou-se responsável pela expressão da NS3, e em consequência pela produção de efeito citopático.

A caracterização molecular da amostra CP13, que também é composta de uma partícula CP defectiva (DI13) e um vírus NCP “helper”, revelou uma deleção de 1.611 nt e outra de 3.102 nt no genoma. Com exceção de uma pequena porção do gene que codifica a glicoproteína E1, todos os genes que codificam as proteínas estruturais estão ausentes, juntamente com a N^{pro}, p7 e NS2. A exemplo da amostra CP9, o genoma defectivo está relacionado com a produção de ECP (KUPFERMANN et al., 1996).

TAUTZ et al. (1996) identificaram na amostra de BVDV-CP CP7 uma inserção de 27 nucleotídeos na região que codifica a proteína NS2. Essa inserção representa uma duplicação das bases 4064 a 4090 localizadas entre as bases 4353 e 4354, codificando um aminoácido

diferente. Essa inserção demonstrou ser necessária e suficiente para indução da clivagem da proteína NS2-3, gerando a NS3. A cepa CP Oregon apresenta um mecanismo diferente de processamento da NS2-3, pela presença de mutações em ponto na região que codifica a proteína NS2, produzindo a clivagem da NS2-3. Esta foi a primeira descrição da ocorrência de mutações em ponto como mecanismo de produção da NS3 (KÜMMERER et al., 1998).

QI et al. (1998) relataram outra inserção de seqüência celular denominada de “cSNCINS”. Essa inserção está localizada no gene que codifica a proteína NS4B, acompanhada de duplicação do gene da NS3. Essa inserção é homóloga a uma seqüência de aminoácidos da proteína SMT3B de humanos e muito similar à proteína SMT3A. Observou-se ainda que a inserção “cSNCINS” de bovinos é homóloga a SMT3B de humanos, e que a sua inserção de BSMT3B juntamente com a duplicação da NS3 no genoma viral podem conferir citopatogenicidade ao vírus.

Outro tipo de inserção, denominada de LC3, foi descrito por MEYERS et al. (1998) na amostra de BVDV-CP Jasper (JaCP) isolada de um animal com DM. Essa inserção é flanqueada por duplicações truncadas dos genes da NS2 e NS3, gerando um genoma de aproximadamente 14Kb. No vírus Jasper não-citopático (JaNCP) não foram detectados tais rearranjos. Apesar desta inserção induzir a clivagem da proteína NS2-3, a presença da duplicação é necessária para originar um vírus CP autônomo.

Na cepa NADL, MEYERS et al. (1991) identificaram uma inserção celular (cIns) de 270 nucleotídeos na região que codifica a proteína NS2. MENDEZ et al. (1998) realizaram a deleção desta inserção e observaram que não houve a clivagem da NS2-3. Também não houve reflexos na produção de progênie viral nem na produção das proteínas virais, porém houve uma redução na síntese e acúmulo de RNA. Através destes dados os autores concluíram que a cIns não somente modula a produção de NS3 ,mas também regula a replicação do RNA no seu isogênico NCP.

Isolados de um surto da DM permitiram a análise de quatro pares de vírus de BVDV - 2 (BECHER et al., 1999) em que foram identificados RNAs subgenômicos com seqüências da ubiquitina, a qual induz a produção da NS3. Esta foi a primeira descrição da ocorrência natural de RNAs subgenômicos carreando inserções celulares. A comparação das seqüências de nucleotídeos revelou que os diferentes subgenomas do BVDV-2CP surgiram do respectivo BVDV-2 NCP.

BAROTH et al. (2000) identificaram uma duplicação de parte da NS2, NS3, NS4A e parte da NS4B juntamente com uma inserção celular no genoma da amostra de BVDV citopático CP821. Esta inserção corresponde à proteína celular NEDD8, a qual apresenta

58% de homologia com a proteína ubiquitina. A inserção da NEDD8 juntamente com a seqüência viral duplicada representam a base genética para a citopatogenicidade da cepa CP821.

Recombinação molecular entre um vírus NCP persistente e uma cepa vacinal (RIT) na cepa 1741 foi identificada por BECHER et al. (2001). Diferentes RNAs subgenômicos foram identificados, cada um contendo deleções juntamente com uma seqüência codificando parte da proteína ribossomal S27a fusionada com um monômero de ubiquitina. Esta inserção, juntamente com a seqüência viral flanqueando parte da NS3 e NS4B foram 99% idênticas à seqüência da cepa vacinal RIT. Interessantemente, outro isolado de BVDV, CP4584, originário de um caso de DM também apresentou a inserção RIT, derivada de cepa vacinal, junto com a duplicação da região da NS3 e parte da NS4A e NA4B. Tanto na cepa 1741 quanto na cepa 4584, a presença de deleção, inserção de seqüência da cepa RIT e duplicação, são responsáveis pela geração de citopatogenicidade.

A formação de um complexo entre a proteína celular “J-domain” um membro da “DnaJ-chaperone family” com a proteína NS2, foram descritos por RINCK et al. (2001). Este complexo foi denominado de Jiv, em função da interação da proteína J-domain com proteínas virais. Jiv, por sua vez, tem o potencial de induzir a clivagem da NS2-3 em “*trans*”. NEILL & RIDPATH (2001) analisando amostras de BVDV-2 também descreveram a inserção celular bDnaJ1, como sendo responsável pela maioria dos rearranjos entre os biótipos CP e NCP em amostras de BVDV-2, BDV e em alguns BVDV-1.

A identificação de um novo tipo de inserção na amostra de BVDV CP8 foi realizada por MÜLLER et al. (2003). Essa inserção compreende um complexo de seqüências virais e celulares, incluindo dois fragmentos do gene da proteína celular “J-domain” (Jiv), localizada na região N-terminal da poliproteína, entre a proteína N^{pro} e a proteína C. NAGAI et al. (2003) em estudos com a cepa KS86-1cp, um vírus quimérico originado de um animal PI com a cepa KS86-1NCP após ser exposto a cepa CP NOSE, identificaram a inserção de uma seqüência celular (cINS) e uma duplicação da proteína C e parte da N^{pro} localizada na região que codifica para a proteína C das cepas NOSE e KS86-1cp. Estas descrições, juntamente com dados anteriormente reportados por QI et al. (1998), representam os primeiros relatos de rearranjos fora do gene NS2-3.

QI et al. (1992) analisaram quatro pares de vírus e observaram a presença de duplicações e inserções em três deles. Além disso, observaram a presença de seqüências altamente conservadas entre a região 3' da inserção celular (ubiquitina) e 5' da NS3 e uma grande variação na região 5' da inserção. VILCEK et al. (2000) analisaram 33 amostras

citopáticas para a presença de inserções celulares. Nestas, detectou-se a presença de inserções do tipo cIns em 13. Estas inserções apresentaram um alto nível de conservação nas suas seqüências, diferindo apenas em poucos nucleotídeos nas extremidades 5' e 3'. GREISER-WILKE et al., (1993) realizaram a análise molecular de 16 amostras de vírus CP, encontrando três vírus com inserções, três com duplicações, uma com inserção mais duplicação e em nove amostras não foram detectadas inserções, deleções ou duplicações. Os pares NCP também estavam livres de inserções e duplicações. Estes estudos sugerem a existência de mecanismos adicionais, ainda não descritos de geração da NS3 e produção de vírus CP.

O presente trabalho teve como objetivo investigar o mecanismo genético de geração da NS3 em 41 isolados citopáticos de BVDV. Para tal, foram utilizados primers que permitem a detecção de inserções no gene da NS2-3 e duplicações do gene da NS3.

3. CAPÍTULO 2

A search for RNA insertions and NS3 gene duplication in the genome of cytopathic isolates of bovine viral diarrhea virus (BVDV).¹

Running title: Insertions and gene duplications in the BVDV genome.

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Abstract

Calves born persistently infected (PI) with non-cytopathic bovine viral diarrhea virus (ncpBVDV) frequently develop a fatal gastroenteric illness called mucosal disease (MD). From animals affected by MD, both the original virus (ncpBVDV) and an antigenically identical, yet cytopathic virus (cpBVDV) can be isolated. Cytopathic BVDVs are originated from the ncp counterparts by diverse genetic mechanisms, all leading to the expression of the non-structural polypeptide NS3 as a discrete protein. In contrast, ncpBVDVs express only the large precursor polypeptide, NS2-3, which contains the NS3 sequence within its carboxy-terminal half. We herein report the investigation of the mechanism leading to NS3 expression in 41 cpBVDV isolates. An RT-PCR strategy was designed to detect RNA insertions within the NS2-3 gene and/or duplication of the NS3 gene – two common mechanisms of expression of NS3. RT-PCR amplification revealed insertions in the NS2-3 gene of three cp isolates, being the inserts similar in size to that present in the cpBVDV NADL strain. Sequencing of one such insert revealed a 296 nucleotide (nt) sequence, with a central core of 270nt, coding for an aminoacid sequence highly homologous (98%) to the NADL insert, a sequence corresponding to part of cellular J-Domain gene. Another cpBVDV isolate contained a duplication of the NS3 gene downstream from the original locus. In contrast, no detectable NS2-3 insertions nor NS3 gene duplications were observed in the genome of 37 cp isolates. These results demonstrate that processing of NS2-3 without bulk mRNA insertions nor NS3 gene duplications seems to be a frequent mechanism leading to NS3 expression and BVDV cytopathology.

Key words: bovine viral diarrhea virus, BVDV, NS3, cytopathology.

Introduction

Bovine viral diarrhea virus (BVDV) is an important pathogen of cattle and causes significant economic losses to the livestock industry around the world (1). BVDV is a small enveloped RNA virus belonging to the *Flaviviridae*, genus *Pestivirus*, along with classical swine fever virus (CSFV) and border disease virus (BDV) of sheep (1, 2). The BVDV genome is a linear, single stranded, positive sense RNA molecule of approximately 12.5 kb in length (3). The genome contains a single large open reading frame (ORF) encoding a protein of around 3900 aminoacids. The ORF is flanked by a 5' 381-386 nucleotides (nt) long untranslated region (5'UTR) and by a 3' 229 nt long UTR (3). The 5' UTR contains a secondary structure believed to act as an internal ribosomal entry site (IRES) to direct the translation of the ORF upon internalization of the viral genome into the host cell (4). Translation of the ORF produces a long polyprotein, which is co- and post-translationally processed by viral and cellular proteases giving rise to 11 to 12 mature viral proteins (3, 5). The structural proteins (the exception is N^{pro}, a non-structural protein) are encoded by the 5' third of the genome, as follows: N^{pro}, C, E^{rns}, E1, E2 and p7. Non-structural proteins are encoded downstream: NS2-3, NS4A, NS4B, NS5A and NS5B (3, 5, 6, Figure 1). The vast majority of BVDV field isolates do not induce cytopathology in cell culture and are called non-cytopathic (*ncp*); cytopathic (*cp*) isolates are found almost exclusively in cattle suffering from MD (6, 7).

Infection of seronegative cattle with BVDV may result in a variety of clinical manifestations, ranging from inapparent infections to gastroenteric, respiratory, hemorrhagic syndrome to the severe acute BVD and the fatal mucosal disease (MD) (7, 8, 9). Infection of pregnant cows is often associated with reproductive losses, including early or late embryonic or fetal deaths, abortion or mummification, congenital malformations, stillbirths and the birth of weak, unthrifty calves (8, 10). Fetuses infected between 40 and 120 days of gestation with *ncp* isolates may survive the infection and be born as immunotolerant, persistently infected (PI)

calves (8, 10). Most PI animals develop and die of mucosal disease (MD) within the first 6 to 24 months of life; *cp* and *ncp* BVDV biotypes can usually be isolated from sick animals (7, 8, 10). Cytopathic and noncytopathic viruses isolated from cases of MD are antigenically identical to each other and constitute what has been called a *virus pair* (7, 11). Molecular analyses of *cp-ncp* pairs indicated that the *cp* virus is originated from the original *ncp* counterpart by diverse genetic mechanisms (12-17).

The most important molecular difference between the *cp* and *ncp* biotypes of BVDV is the expression of the non-structural polypeptide NS3 (formerly known as p80). Whereas *ncp*BVDVs express a single polypeptide of approximately 125kd called NS2-3 (or p125), *cp*BVDVs express both the entire NS2-3 and NS3 as separate protein, which corresponds to the carboxy-terminal two thirds of NS2-3 (5, 11, Figure 1). NS2-3 is a multifunctional protein believed to be involved in several steps of viral replication. It contains a zinc finger motif and an hydrophobic domain in its amino-terminal third (NS2); and helicase, NTPase and protease motifs in its carboxy-terminal half (NS3)(5, 6). Although the expression of NS3 as a single polypeptide is the molecular marker of *cp*BVDV isolates, its exact role in the production of cytopathology is still unclear.

Several mechanisms of expression of NS3 by *cp* viruses have been described, including insertions of cellular RNA sequences in NS2-3, near the boundary between NS2 and NS3 (12, 13), downstream duplication of the NS3 gene (16); expression of NS3 from a defective RNA genome (15), point mutations in the NS2-3 gene (14) and insertions of cellular sequences plus viral gene duplications in the N-terminus of the polyprotein (17).

The differential expression of NS3 and its contribution for BVDV cytopathology *in vitro* and the pathogenesis of MD *in vivo* constitutes a major issue in pestivirus biology and pathogenesis and has been a matter of intensive investigations in the last two decades. The number of possible mechanisms of expression of NS3 – and therefore, the generation of *cp*

viruses – increases as more cpBVDV isolates are characterized. Herein the molecular mechanism of expression of NS3 through searching for insertions and/or gene duplications in the genome of 41 cpBVDV isolates was investigated.

Material and methods

Forty one cytopathic isolates of BVDV were biologically cloned to obtain pure cpBVDV. Individual *cp* clones were then propagated and their genomic RNA was extracted and submitted to RT-PCR analysis for the presence of insertions in NS2-3, close to the boundary between the NS2 and NS3 genes, and for duplications of the NS3 gene. The viral genomes containing insertions and/or duplications were partially sequenced to determine the nature of the genomic rearrangements.

Cells, viruses and monoclonal antibodies (mabs)

All procedures of virus multiplication, quantitation and plaque assays for biological cloning were performed in *pestivirus*-free Madin-Darby bovine kidney cells (MDBK, American Type Culture Collection, Rockville, MD, USA). Cells were maintained in minimal essential medium (MEM) containing penicillin (1.6mg/l), streptomycin (0.4mg/l) and fungizone (0.0025mg/l), supplemented with 5% horse serum. Sixty cytopathic BVDV isolates obtained from cases of mucosal disease (MD) were kindly provided by Dr. Fernando A. Osorio (Department of Veterinary and Biomedical Sciences, University of Nebraska at Lincoln/UNL/USA). After isolation in primary bovine lung cells (BL) and identified by fluorescent antibody assay (FA), the viruses were further propagated in MDBK cells. The standard BVDV strains cpSinger, cpNADL cpOregon and cpTGAC were provided by Dr Ruben O. Donis (DVBS/UNL/USA). The monoclonal antibodies used in FA assays to distinguish/identify between the two viruses giving rise to an homologous inserts of similar size (cpNADL, cpBVDV-26, cpBVDV-44 and cpBVDV-63) were provided by Dr Ruben Donis. The antigenic profile of these viruses was performed by FA in MDBK cells infected with each virus

and incubated with each one of the 24 Mabs as primary antibody, according to standard FA protocols described elsewhere (11).

Biological cloning of cytopathic (cp) viruses

After propagation in MDBK cells, the isolates were biologically cloned for the obtention of pure *cp* virus clones and further propagated. Biological cloning for *cp* viruses was performed by standard plaque assays. Purified *cp* viruses were propagated and submitted to RT-PCR analysis.

RNA preparation

MDBK cells grown in T-25cm² flasks were infected with each cloned of *cp* virus at an multiplicity of infection (m.o.i.) of 0.1 to 1. When the cytopathic effect (CPE) reached approximately 70% of the monolayer (typically 36 to 48h post-inoculation), cells were harvested by trypsinization followed by resuspension in culture medium and centrifugation (3.000 x g for 10 min). The cell pellet was submitted to total RNA extraction using TRIZOL Reagent, according to the manufacturer's protocol (GIBCO, BRL). The RNA pellet was then rinsed with 70% ethanol and resuspended in 50µl of DEPC treated water. All procedures of RNA manipulation were performed using disposable pipettes and tubes and diethylpyrocarbamate (DEPC)-treated water.

Primers

Oligonucleotide primers corresponding to conserved sequences of the published cpNADL and cpOsloss genomes were used (17). The sequences of the primers are listed below: primer 11 (cDNA primer) 5'- CTGTTGTTGCTTGCAA -3'(position 5703-5686); primer 10 (PCR primer) 5'- GGACTTTATGTACTAC - 3'(4546-4564); primer 14 (PCR primer) 5'- TCCCAATGATAAACAGACATA - 3' (7545-7564). The strategy used for detection of insertions or gene duplication was based on previous reports on the location of insertions of cellular mRNA in the viral genome (17, 18). Primers 10 and 11 were used for detection of insertions in the NS2-

NS3 gene boundary; primers 11 and 14 were used for detection of downstream duplication of NS3 gene (17; Figure 2).

RT-PCR amplification

RNAs from all cp clones were initially submitted to RT-PCR using primers 10 and 11 (for insertions). RNA of standard cpBVDVs Singer and NADL were used as positive controls. Total RNA extracted from mock-infected MDBK cells were used as negative control. Subsequently, the RNAs were submitted to RT-PCR using primers 11 and 14 (for NS3 gene duplication). RNAs from TGAC, a virus containing a NS3 gene duplication downstream from the original gene (17) was used as control. RT-PCR was performed in a 50 μ l reaction, using the Ready-to-Go RT-PCR Beads kit (Amersham Biosciences, Piscataway, NJ, USA), according to the manufacturer's recommendations. Briefly, 2 μ l of the RNA suspension (approximately 1 μ g of total RNA) and 2 μ l of each primer (100ng each) were added of 44 μ l of DEPC-treated water and mixed with the RT-PCR beads preparation. Each 50 μ l reaction contained 2 units of Taq DNA polymerase, 10 mM Tris-HCl (pH 9.0), 60 mM Kcl, 1.5 mM MgCl₂, 200 μ M of each dNTP, MuLV reverse transcriptase, RNase inhibitor and stabilizers. The tubes were incubated 20 min at 42⁰C for RT, 5 min at 95⁰C and then submitted to 35 cycles of 95⁰C-1min, 50⁰C-1min and 72⁰C-1min; followed by an extension of 5 min at 72⁰C. RT-PCR products were visualized under UV light in a ethidium bromide-stained 1% agarose gel after electrophoresis.

Nucleotide sequencing and analysis

The 4 amplicons, were directly purified through polyethyleneglycol (PEG) precipitation (19). DNA sequencing was then performed directly from the purified amplicons in a MegaBACE 500 automatic sequencer. The dideoxi chain-termination reaction was implemented with the use of the DYEnamic ET® (Amersham) kit and the 10 and 11 primers. Sequence analysis and sequence alignments were done with Clone Manager Professional Suite, Align plus 5, version 5.10, Sci Ed. Central.

Results

Sixty isolates of BVDV which produced cytopathology upon virus isolation in BL cells were initially propagated in MDBK cells. As field cpBVDV isolates usually contain a mixture of related *cp* and *ncp* viruses – called a virus pair (11), the isolates were biologically cloned by plaque assay to yield pure populations of each *cp* counterpart. For some isolates, plaque purification was performed twice or even three times to ascertain the purity of the *cp* cloned viruses. Besides the characteristic cytopathology, the identification of each isolate was confirmed by IFA (data not shown). After plaque purification, *cp* cloned viruses were propagated in MDBK cells to produce viral stocks and subsequently used to infect cells for RNA extraction. For some isolates, biological cloning of the *ncp* counterparts was performed by limiting dilution. Forty one pure clones of cpBVDV were then further used in the study. In the remainders 19 *cp* isolates, biological cloning of pure *cp* viruses could not be achieved.

Total RNA extracted from cells infected with each of the 41 cloned cpBVDVs was initially submitted to RT-PCR amplification using primers 10 and 11. In viral genomes lacking insertions within the NS23 gene, the PCR product obtained by using these primers should be a 900bp fragment (17; Figures 2 and 3). RNA extracted from cells infected with cpBVDV Singer strain – a virus known to lack cellular insertions in the NS2-3 gene (14) – and the RNA from cpBVDV NADL strain – whose genome contains a 270 nt insert (12) – were used as controls. RT-PCR amplification of RNA of thirty eight cpBVDV with primers 10 and 11 produced amplicons undistinguishable in size from that of Singer strain (Table 1; Figure 3). Further separation of DNA fragments by extending the time of electrophoresis did not allow detection of size differences among these amplicons (data not shown). Therefore, the genome of 38 cpBVDV isolates examined does not contain insertions within the NS2-3 in the region comprised between primers 10 and 11, as ascertained by agar-gel electrophoresis of PCR products. The possible mechanisms of expression of NS3 by these virus will be discussed later.

As the RT-PCR amplification of NS2-3 gene of isolates 26, 44 and 63 yielded a product undistinguishable in size to that of NADL, one could ask whether these amplicons did correspond to amplification of the genome of different viruses or were products of laboratory contamination with NADL. To discard the later hypothesis, a monoclonal antibody profile of these viruses was performed. Cells infected with NADL, cpBVDVs 26, 44 and 63 were submitted to IFA tests, using 24 different Mabs as primary antibody (11). The profile of reactivity of the Mabs with these viruses is depicted in Table 2 and clearly shows that they are indeed different viruses. Thus, cpBVDVs 26, 44 and 63 harbor insertions in the NS2-3 gene which are undistinguishable in size to that present in NADL strain, as ascertained by agar gel electrophoresis analysis.

The amplicons obtained by PCR amplification of genomes of cpBVDVs 26, 44 and 63 were then submitted to nucleotide sequencing to determine the nature of the insertion present in these genomes. After several sequencing attempts, only the PCR product of isolate 44 yielded a reliable sequence that could be edited and analysed. The insert is 296 nucleotides long and contains a core sequence (270 nucleotides) which is 98% identical and whose predicted aminoacid sequence is 100% identical to cIns insert present in the genome of NADL strain (Figure 5). This insert is highly homologous to a sequence within the bovine gene coding for the J-Domain and is likely the result of recombination between the viral and cellular RNAs during genome replication (20). In the sequences of the other amplicons (cpBVDVs 26 and 63) stretches of mixed nucleotides were frequently observed, probably reflecting co-amplification of different products. Attempts to obtain these sequences are still in course.

After the first round of RT-PCR with primers 10 and 11, the RNAs of all 41 cpBVDV clones were submitted to another amplification reaction, using primers 11 and 14. These primers were designed to detect possible duplications of the NS3 gene (Figure 2). RNA from TGAC strain, whose genome contains downstream insertion of ubiquitin RNA plus a duplication of NS3 gene (17) was used as positive control. The RNA of Singer strain, with no contain any genome

rearrangement was used as negative control. The amplicon produced by primers 11 and 14 in TGAC genome strain is about 900 bp (17). Using primers 11 and 14 resulted in RT-PCR amplification in only one cpBVDV isolate (Table 1, Figure 4). The amplicon generated for cpBVDV 14 was smaller than the TGAC amplicon, with an estimated size of about 800-850 bp (Figure 4). No other cpBVDV isolate originated amplicons in the reactions using primers 11 and 14. The obtention of amplicons from these RNAs by using primers 10 and 11 discarded any possible problems with RNA quality or problems in the PCR reaction. This result indicates that cpBVDV isolate 14 does possess a duplication of the NS3 gene downstream of the original site that would be amplified by primers 11 and 14. Attempts to determine the complete nucleotide sequence of such genome rearrangement are in course.

Discussion

Analysis of genomes of 41 cpBVDV isolates by RT-PCR revealed that three isolates contain RNA insertions in the NS2-3 gene and one virus contains a NS3 gene duplication downstream from the original locus. All three insertions have an undistinguishable size, when examined by agar-gel electrophoresis, a size correspondent to the NADL insert. In fact, one of such inserts was shown to be of the cIns type, being highly homologous to the insert found in the genome of cp BVDV NADL. The NADL insert is homologous to a sequence found within the cellular J-Domain gene. Expression of NS3 from these genomes likely occurs either by protease cleavage within the inserted sequences (for the viruses harboring inserts) or through direct translation of NS3 polypeptide out of the duplicated gene (21). In contrast, the genomes of 37 *cp* isolates do not contain detectable insertions within the region examined nor NS3 gene duplication. The most likely mechanism of NS3 generation in these viruses – which lack large genomic rearrangements – appears to be the presence of point mutations within the NS2 gene leading to cleavage of NS2-3 (14). Nonetheless, as the PCR analysis only focused in two sites of genomic rearrangements, other possible rearrangements as being responsible for NS3 expression

in these viruses should not be discarded.

The identification of BVDV isolates capable of inducing cytopathic effect in cultured cells dates from the late 50's (18). However, data concerning differences of the viral genomes (*cp/ncp*) were not available for a long time. As a consequence, the origin of cpBVDVs and their role in the pathogenesis of mucosal disease were only elucidated three decades later (7, 22). Once the origin of cpBVDV was determined, molecular markers of cpBVDV at genomic and polypeptide levels were shortly identified (11, 12). Thereafter, expression of NS3 as a discrete polypeptide – and not as part of NS2-3 as in ncpBVDVs - has been universally considered the molecular marker of *cpBVDV* (11-17). As the cytopathic phenotype of BVDV is strictly correlated with the expression of NS3, and no exceptions have been reported to date, we did not investigated the expression of NS3 in all isolate. Only a few isolates were examined by western blot to confirm the expression of NS3 as a discrete polypeptide (data not shown).

Molecular analyses of many cytopathic field isolates revealed that cpBVD viruses are originated from the ncp counterparts by diverse genetic mechanisms, usually as a result of RNA recombination (12, 13, 15, 16). Insertions of cellular sequences – accompanied or not by duplication of viral sequences - and duplications and rearrangements of viral sequences have been identified in the genomes of cpBVDVs (12, 13, 15-18, 23). These genomes alterations have been shown to be responsible for the expression of NS3, the molecular marker of cytopathology. Two widely used cpBVDV strains (Osloss and NADL) were the first to have the mechanism of NS3 generation elucidated (12, 24). The genome of cpBVDV Osloss strain contains an integration of a cellular sequence – an ubiquitin-coding sequence (12). The cellular homologue inserted into the NADL genome, termed cIns, has been recently identified and corresponds to sequences of the cellular J-Domain protein, a chaperonin (25). The genome rearrangement observed in these isolates is probably result of nonhomologous recombination by template switching during RNA replication (21). Further analysis of cpBVDV genomes demonstrated that

integration of ubiquitin-coding sequences and cIns-like sequences within the NS2-3 gene represents a frequently means of generating cpBVD viruses (12, 16-18, 21, 23, 26). In addition, other cellular insertions have been detected in this genomic region of cpBVDV isolates, namely part of light chain 3 microtubule-associated proteins (LC3) (13), SMT3B (27), ribosomal S27a coding sequence (28), NEDD8 cellular sequences (29), ubiquitin-like proteins and part of a chaperon called Jiv (25). The expression of NS3 from genomes containing cellular insertions within NS2-3 probably occurs by proteolytic cleavage of NS2-3. Inserted sequences likely contains additional cleavage sites for cellular or viral proteases, resulting in release of the N-terminus of NS3 (26, 30). Alternatively, the Jiv insert appears to induce cleavage of NS2-3 *in trans*, through a stable interaction with the viral NS2 (25).

In the present study, three *cp* viruses had insertions within the NS2-3 gene. One such insert has been sequenced and showed a high nucleotide homology with the NADL cIns insert, which corresponds to sequences of a cellular gene called J-Domain protein (25). Insertions of the cIns type have been shown to be very frequent among cpBVDV isolates and are usually present in very similar position within the genome (20). Many of these sequences have been shown to code for a 89 highly conserved aminoacid sequence with only minor differences between isolates. The high conservation of this core sequence suggests a relevant biological function (20). Indeed, the cellular insert present in the NADL genome is essential for generation of NS3 by proteolytic cleavage of NS2-3 and production of cytopathology (24). In the present study, the insert in the genome of two isolates could not be yet determined. The size of the amplified products, taken together with its frequent occurrence suggests that they might also be of the cIns type. Likewise, the profile of Mab binding performed with these viruses discarded any possible cross-contamination of viruses.

Although insertions leading to proteolytic cleavage of NS2-3 seems to be the most common mechanism of generation of NS3 in cpBVDV isolates (21), other mechanisms

responsible for expression of NS3 have been identified. NS3 can be expressed from a duplicated gene, associated or no with other sequence rearrangements, including duplication of N^{pro} right upstream of the duplicated NS3 gene (30), or from a defective genome with deletion of all structural genes (15). These findings also demonstrated that the NS2 itself is not required for induction of cytopathology (15, 30). Recently, a novel mechanism of NS3 generation has been described. BVDV CP8 contains cellular insertions and viral sequence duplications in the N-terminal region of the polyprotein – and not in the NS2-3 boundary as most characterized isolates. Two fragments of the cellular J-domain Jiv (J-domain protein interacting with viral protein) and viral gene sequence duplications (partial C protein gene and N^{pro}) are located right downstream of N^{pro}. Expression of a Jiv fusion protein out of the viral genome leads to NS2-3 cleavage and generation of NS3 (20). Thus, it is tempting to speculate that the possible mechanisms of generation of NS3 will increase as more cpBVDV isolates are characterized. In the present study, one isolate presented a NS3 gene duplication downstream of the original locus, yielding an amplicon of approximately 800-850 nt. This amplicon is shorter than the amplicon generated by amplification of the genome of TGAC, a virus shown to contain an insertion of ubiquitin sequences and a NS3 gene duplication downstream of the original locus (17). Attempts to determine the nucleotide sequence of this region are currently ongoing. In genomes contained duplicated NS3 gene, the expression of NS3 polypeptide is believed to occur by direct translation of the duplicated gene (17, 21). Thus, production of cytopathology appears to depend exclusively upon NS3 expression rather than upon the expression of both NS2 and NS3 as separate polypeptides (21).

Although the initial focus given to cellular insertions and gene duplications as a molecular mechanism of generation of NS3, examination of a large number of isolates failed in detecting such genomic alterations (17, 18, 30). In other words, many cpBVDV isolates do not contain any detectable RNA insertion within the NS2-3 gene nor NS3 gene duplication as to

explain the molecular basis for cytopathogenicity (17, 18, 30). These results indicated that, although gene duplication and/or insertions are important genomic alterations leading to cytopathology, other yet unidentified mechanism may also contribute to NS3 expression.

Analyses of the genome of cpBVDV Oregon – a *cp* strain whose genome contains no NS2-3 insertion nor NS3 duplication - revealed a novel mechanism of NS3 generation (14). In the genome of BVDV Oregon and other *cp* isolates including Singer strain, the information necessary for NS2-3 processing resides within the NS2 gene and probably involves a set of point mutations that somehow affects the cleavability of NS2-3 (14). It has been suggested that these point mutations may induce specific conformation change in NS2 which would be important for proteolytic cleavage (14). Regardless the molecular mechanism leading to NS3 generation, these findings demonstrated that in addition to RNA recombination, a different genetic mechanism – point mutations within the NS2 gene - may be responsible for the production of cpBVDV. This mechanism may explain – even partially - the generation of NS3 in *cp* viruses containing no insertions in the NS2-3, NS3 duplication or expression of NS3 from defective genomes (14, 15). Indeed, examination of a large number of cpBVDV isolates have indicated that this mechanism seems to be more frequent than expected before (17, 18, 30).

In our study, 37 isolates apparently do not contain NS2-3 insertions nor NS3 gene duplication. However, it can not be discarded that some of these viruses may contain genome alterations not detected by the PCR strategy employed, like the genome rearrangements recently described (20). An important pitfall of the PCR strategy described herein is that only small regions of the genome were analysed and the choice of the primers restricted the possible detectable genomic changes. Likewise, the limitation of resolution of agar-gel electrophoresis analysis of RT-PCR products would not allow to detect small insertions that would not alter significantly the size of the amplified product. Summarizing, it can not be discarded that some of the isolates found that seem not to contain insertions in the NS2-3 gene by the PCR strategy

employed might harbor small insertions that would not be detected in the gel. Likewise, the choice of primers allowed for detection of insertions in NS2-3 and NS3 gene duplication only in the regions more frequently affected, while missing other possible genomic rearrangements outside of the PCR-targetet region.

In summary, the results presented herein demonstrate that expression of NS3 in cytopathic BVDV isolates – although frequently associated with NS2-3 insertions and/or NS3 gene duplication – appears to be more frequently associated with cleavage of NS2-3 polypeptide without the presence of bulk genome rearrangements. It is tempting to speculate that additional mechanisms of generation of NS3 may arise as more cpBVDV isolates are examined.

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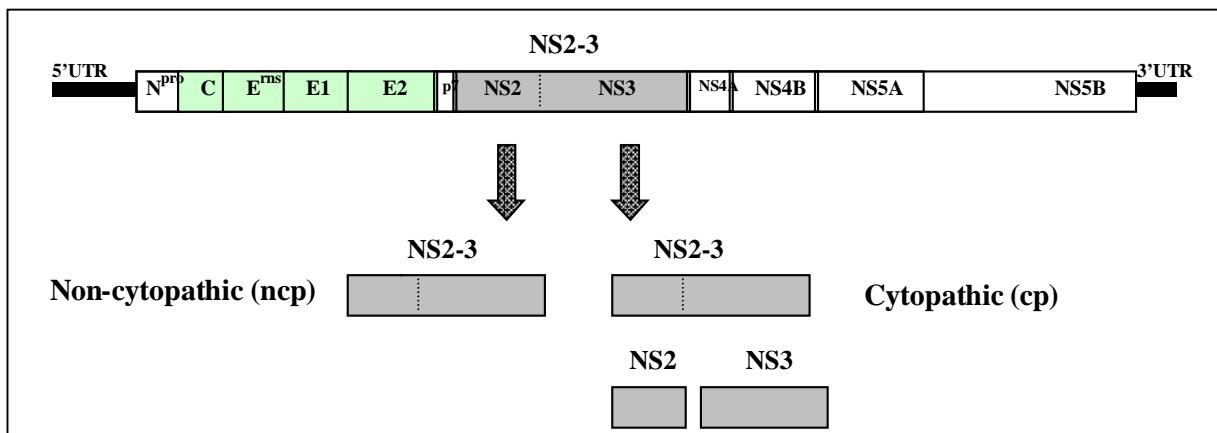


Figure 1. Organization of the bovine viral diarrhea virus (BVDV) genome and processing of the NS2-3 polypeptide in cytopathic (cp) and non-cytopathic (ncp) isolates.

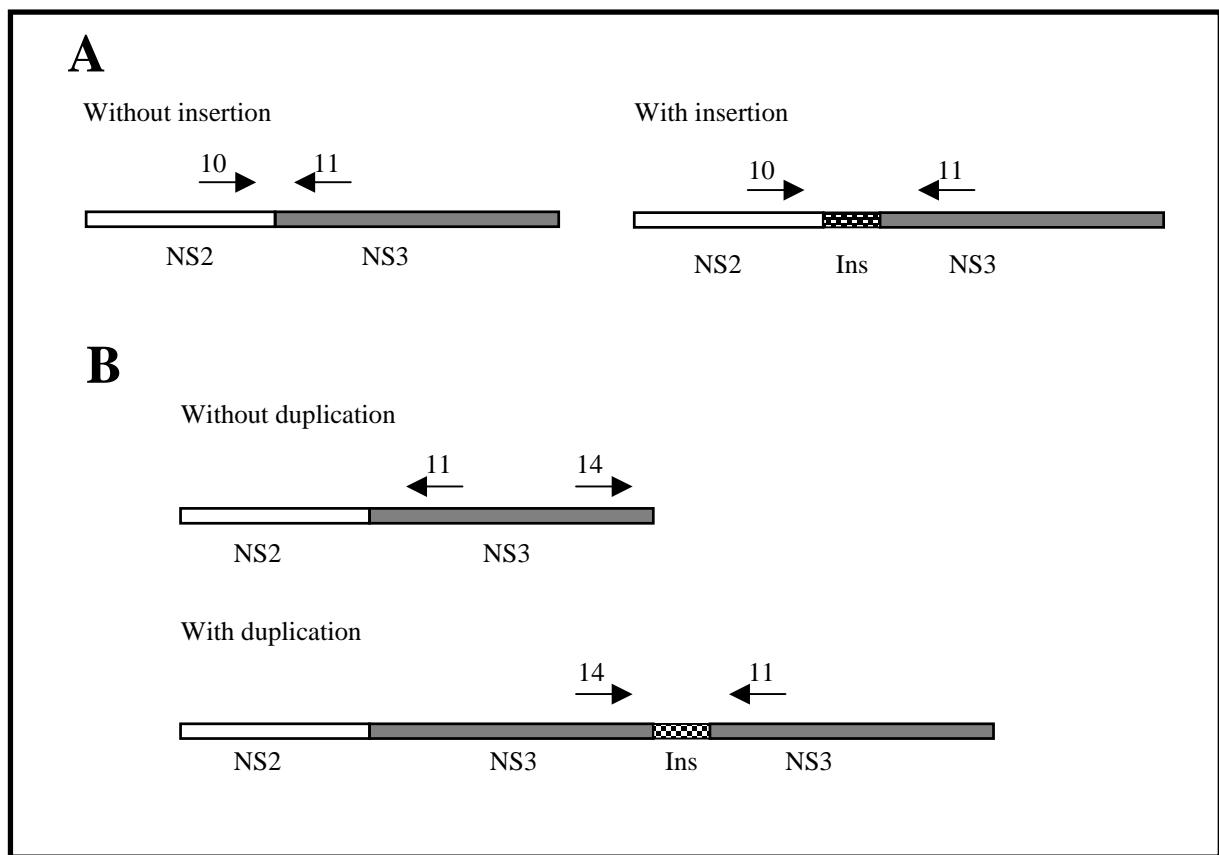


Figure 2. RT-PCR strategy used for detection of rearrangements in the bovine viral diarrhea virus (BVDV) genome. (A) Detection of possible RNA insertions at the junction between NS2 and NS3. Primer 11 (5686-5703) is 286bp downstream of the ubiquitin insertion site in Osloss and is complementary to the viral genome. Primer 10 (4546-4564) is 450bp upstream of the cellular insertion site in NADL. (B) Detection of possible duplications of the NS3 gene. Primer 14 (7545-7564) is 450bp upstream of the proposed 3' end of NS3. PCR using the first-strand BVDV cDNA as template and primers 11 and 14 should not generate any product since the two primers go in opposite directions. A product would be obtained only when the NS3 gene is duplicated.

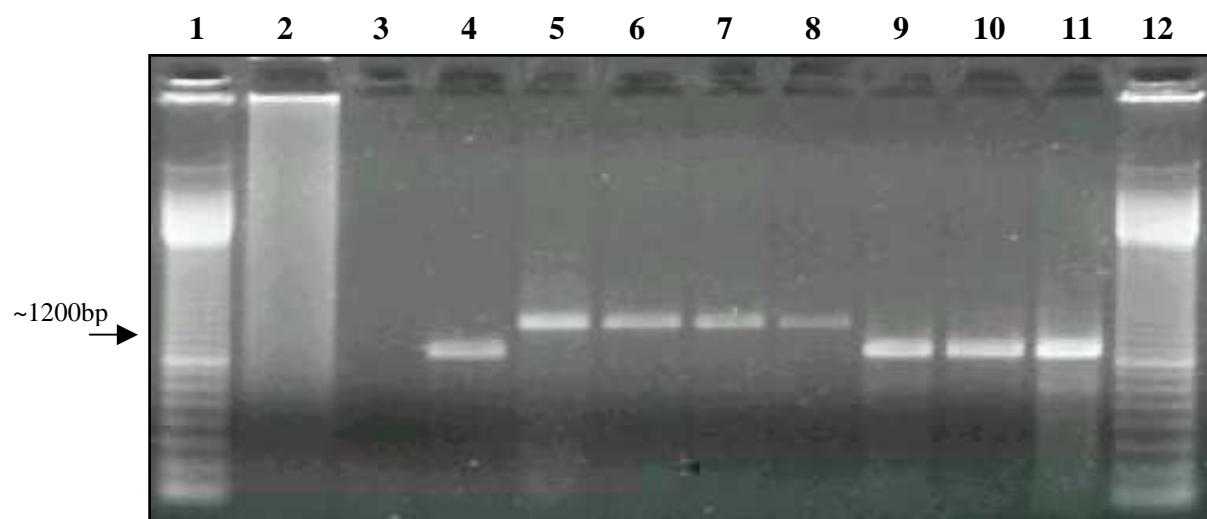


Figure 3. RT-PCR analysis for detection of insertions in the NS23 gene in the cytopathic Bovine Viral Diarrhea virus (BVDV) isolates. The lanes of panel show: lane 1, molecular weight marker (100bp ladder); lane 2, negative control; lane 3, no template; lane 4, Singer strain; lane 5, NADL strain; lane 6, isolate 26; lane 7, isolate 44; lane 8, isolate 63; lane 9, isolate 13; lane 10, isolate 33; lane 11, isolate 73; lane 12, molecular weight marker. RT-PCR products were electrophoresed in a 1% agarose gel, stained with ethidium bromide and visualized under UV light. The size of amplified products from isolates containing inserts is indicated by arrow.

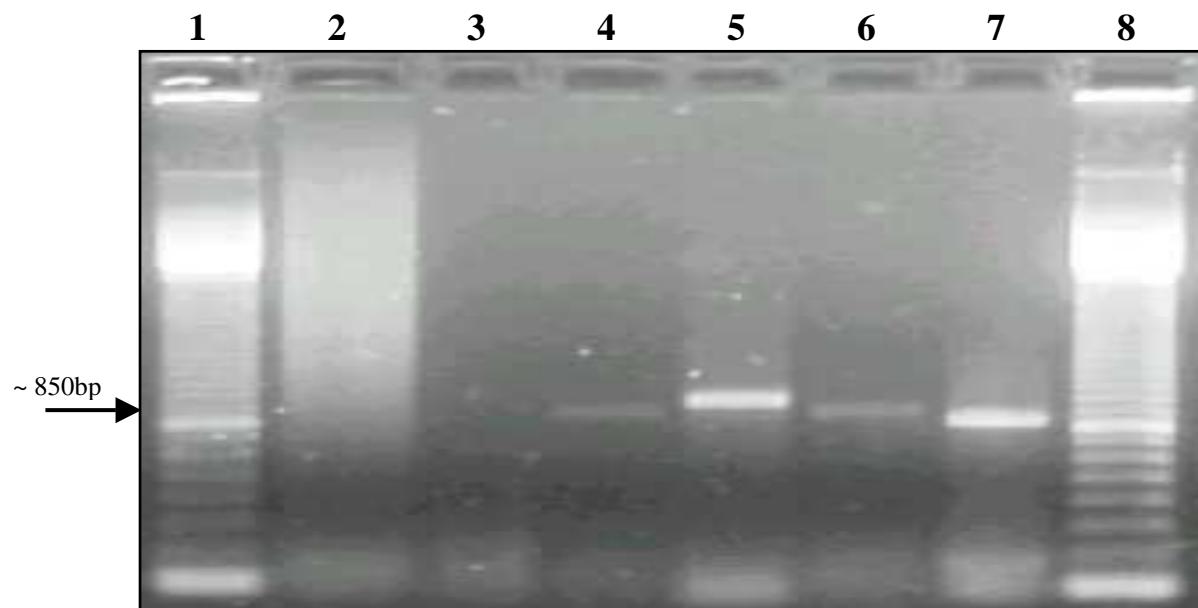
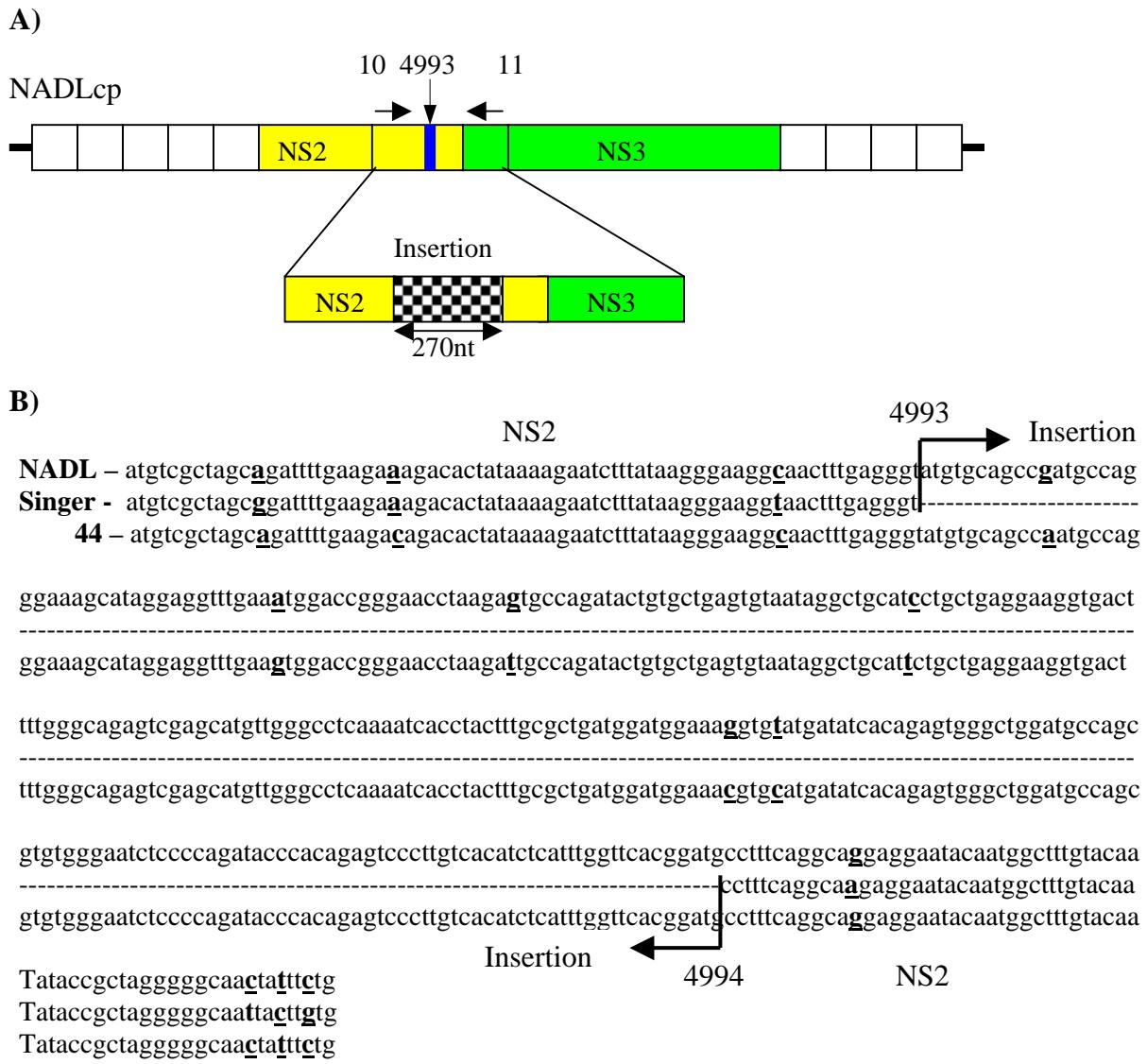


Figure 4. RT-PCR analysis for detection of NS3 gene duplication in the genome of cytopathic Bovine Viral Diarrhea virus (BVDV) isolates. The lanes of panel show: lane 1, molecular weight marker (100bp ladder); lane 2, negative control; lane 3, no template; lane 4, TGAC strain; lane 5, TGAC strain; lane 6, isolate 14; lane 7, isolate 14; lane 8, molecular weight marker. Lanes 4 and 6 show amplified products of isolates TGAC and 14 using primers 10 and 11. Lanes 5 and 7 show the amplified products of isolates TGAC and 14 using primers 11 and 14. RT-PCR products were electrophoresed in a 1% agarose gel, stained with ethidium bromide, and visualized under UV light. The size of amplified products from isolate containing duplication is indicated by arrow.



C)

J-Domain – atgtgcagccgatgccaggaaagcataggaggttgaaatggaccggAACCTAAGAGtgccagatactgtgctgagtg

Insertion 44 -a.....g.....t.....

taataggctgcatcctgctgaggaagggtgactttggcagagtcaagcatgtggcctaaaatcacctacttgcgctgatggatggaaaggtg
.....t.....g.....c....c

atgatatcacagagtggctggatgccagcgtgtggaatctccccagatacccacagagtccctatcacatctcattggtcacggatg
.....g.....

Figure 5. Sequence alignment of the amplicons obtained with primers 10 and 11 of Bovine Viral Diarrhea virus (BVDV) isolates NADL, Singer, and 44 in the NS23 region. The number corresponds to the published NADL sequence. (A) Diagram of the genome of BVDV NADL strain showing the location of the insert within the NS23 gene. (B) Alignment of sequences of NADL, Singer and isolate 44 in the region corresponding to the insert. (C) Alignment of sequences of insertion of isolate 44 and the cellular J-Domain protein. The difference sequences is in bold and underline.

Table 1. Summary of RT-PCR analysis of the genome of cytopathic bovine viral diarrhea virus (BVDV) isolates for insertions in the NS23 gene and duplications of the NS3 gene.

Genetic mechanism	Primers used	Isolates (n)	Amplicon size (bp)	Negative isolates (n)	Amplicon size (bp)
Insertion in NS23 gene	10 and 11	3	~1200	38	~900
Duplication of NS3 gene	11 and 14	1	~850	40	None
Undetermined	10 and 11	37	Primers 10-11: ~900	na ^a	Na
	11 and 14	40	Primers 11-14: - ^b		

^a Not applicable.

^b No amplification.

Table 2 – Reactivity of a panel of monoclonal antibodies with antigens of cytopathic bovine viral diarrhea virus (BVDV) isolates harboring insertions in the NS23 gene^a

Clone	Protein specificity (mAb) ^b																						
	NS23			E0			E1			E2										Not determined			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
NADL	•			•		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
44	•		•			•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
63	•		•			•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
26										•											•	•	

^a Reactivity was assayed by indirect immunofluorescence (IFA) using each Mab as primary antibody. Dots (•) indicate positive reaction.

^b Monoclonal antibodies produced to BVDV Singer strain (Donis & Dubovi 1987). 1 – 15.14.5; 2 – 3D8; 3 – 6F11C3; 4 – 6F11E6; 5 – 19F7; 6 – 4D1; 7 – 26C6; 8 – F11/4D8; 9 – 31C4F7; 10 – 12G4; 11 – 20G7; 12 – 6D11; 13 – 19F9; 14 – 10F9; 15 – 27B3; 16 – 18D4; 17 – 6C5; 18 – 3C6; 19 – 2D5; 20 – 32B3; 21 – CA34; 22 – N2; 23 – BZ73; 24 – BZ81.

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