EMERGÊNCIA DO GRUPO DE PESTIVÍRUS HOBI-LIKE E IMPACTO NO DIAGNÓSTICO E CONTROLE DO VÍRUS DA DIARRÉIA VIRAL BOVINA

TESE DE DOUTORADO

Fernando Viçosa Bauermann

Santa Maria, RS, Brasil
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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Medicina Veterinária Preventiva, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
Doutor em Medicina Veterinária.

Orientador: Prof. Eduardo F. Flores
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Universidade Federal de Santa Maria
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Programa de Pós-Graduação em Medicina Veterinária
Departamento de Medicina Veterinária Preventiva

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Como requisito parcial para obtenção do grau de
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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
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EMERGÊNCIA DO GRUPO DE PESTIVÍRUS HOBI-LIKE E IMPACTO NO DIAGNÓSTICO E CONTROLE DO VÍRUS DA DIARRÉIA VIRAL BOVINA
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Santa Maria, 17 de Junho de 2013.

O gênero *Pestivirus* é composto por quatro espécies virais: *vírus da diarréia viral bovina tipos 1 e 2* (BVDV-1 e BVDV-2), *vírus da peste suína clássica* (CSFV) e *vírus da doença da fronteira* (BDV). Um novo grupo de pestivírus, provisoriamente denominado de "HoBi-like," "BVDV-3," ou "pestivirus atípicos" foi inicialmente identificado na Europa em uma amostra de soro fetal bovino (SFB) importada do Brasil. O vírus (ou grupo de vírus) *HoBi-like* possui similaridades ao nível genético e antigênico com o BVDV. A presente tese apresenta trabalhos de avaliação do impacto da emergência deste novo grupo de vírus no diagnóstico e controle do BVDV. Utilizando anticorpos monoclonais, verificou-se maior reatividade cruzada entre as espécies de pestivírus (BVDV-1; BVDV-2; e HoBi-like) em epitopos da glicoproteína E^{rns} e proteína não-estrutural NS2/3, comparando com epitopos da glicoproteína E2. Assim, sugere-se que testes panpestivírus devem ser direcionados às proteínas E^{rns} e NS2/3, enquanto testes específicos podem explorar diferenças existentes na glicoproteína E2. Por meio de um teste comercial de ELISA de captura de antígeno, verificou-se que o limiar de detecção do vírus HoBi_D32/00 é estatisticamente similar ao do BVDV. Entretanto, dois kits de ELISA para a detecção de anticorpos contra o BVDV falharam em detectar ao menos 20% dos animais soropositivos para HoBi_D32/00. Sugere-se a necessidade de reformular os testes diagnósticos para BVDV ou desenvolvimento de testes específicos. Da mesma forma, verificou-se que vacinas para BVDV-1 e BVDV-2 contendo antígenos inativados ou atenuados produzem uma proteção cruzada fraca contra o vírus HoBi-like. Os melhores resultados de proteção foram verificados com o uso de vacina viva atenuada, entretanto, o título médio geométrico (GMT) contra vírus HoBi-like foi de 12,9, enquanto para o BVDV-1 e BVDV-2 foram respectivamente, 51,1 e 23,5. Somado aos baixos títulos de anticorpos, a soroconversão verificada para o vírus HoBi-like foi de 68% dos animais, enquanto 100% e 94% para BVDV-1 e BVDV-2,

respectivamente. Relacionado ao diagnóstico e controle dos pestivírus, o comércio de SFB possui papel relevante na disseminação de agentes infecciosos. Composto por um homogeneizado do soro de centenas a milhares de fetos bovinos, lotes de SFB possuem alto potencial para a identificação de pestivírus circulantes na região de origem dos animais abatidos. Assim, foram testados lotes de SFB originados na América do Norte e manufaturados nos EUA ou na Europa. Apesar de vários lotes estarem contaminados com BVDV, não foi evidenciada a presença do vírus HoBi-like ou de anticorpos específicos em lotes produzidos nos EUA. Entretanto, foi detectada a presença do agente em dois lotes manufaturados na Europa, indicando a possibilidade de contaminação dessas amostras durante o processo de "*pooling*" e embalagem na Europa. Demonstra-se imprescindível a criação de regras internacionais relacionadas à certificação de origem do SFB, a implementação de testes padrão para a detecção de agentes, e adoção de medidas que impeçam a contaminação do produto durante o processamento. Verificou-se também a necessidade de incluir testes específicos para os vírus HoBi-like na rotina de testes utilizada para certificação de qualidade do SFB. Em resumo, os resultados demonstraram que os testes diagnósticos para BVDV frequentemente falham em detectar o vírus HoBi-like ou em diferenciar BVDV de HoBi-like vírus. Testes comerciais para detecção de anticorpos contra BVDV não detectam eficientemente animais expostos ao vírus HoBi-like. Da mesma forma, estudos *in-vitro* demonstram que a proteção cruzada contra o vírus HoBi-like conferida a animais vacinados para BVDV é limitada. Assim, medidas de detecção e controle para pestivírus devem incluir testes diagnósticos e vacinas específica para o vírus HoBi-like.

Palavras-chave: Pestivírus atípicos; BVDV-3; controle; diagnóstico; sinais clínicos.

ABSTRACT

Thesis
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

EMERGENCE OF HOBI-LIKE PESTIVIRUS AND IMPACT ON THE DIAGNOSTIC AND CONTROL OF BOVINE VIRAL DIARRHEA VIRUS
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Santa Maria, June, 17th, 2013.

The genus Pestivirus is composed by four important pathogens of livestock: bovine viral diarrhea virus 1 and 2 (BVDV-1 and BVDV-2), classical swine fever virus (CSFV) and border disease virus (BDV) of sheep. An emerging group of pestivirus temptatively called as "HoBi-like," "BVDV-3," or "atypical pestiviruses" was first detected and characterized in Europe from a fetal bovine serum (FBS) lot originated in Brazil. HoBi-like viruses share genetic and antigenic similarities with BVDV species. The present thesis is composed by studies that address the emergence of this virus group and its potential impact on BVDV diagnostic and control. Monoclonal antibody binding demonstrated the existence of conserved regions within epitopes in the glycoprotein E^{rns} and the non-structural protein NS2/3 among the pestiviruses species (BVDV-1; BVDV-2; e HoBi-like), whereas high degree of divergence was detected in glycoprotein E2. These findings suggest that panpestiviruses diagnostic tests may rely on the E^{rns} and NS2/3 proteins, and pestiviruses specific species diagnostic tests should target the E2 glycoprotein. The threshold for detection of HoBi-like virus using commercial BVDV antigen capture ELISA was statistically similar to BVDV detection. On the other hand, two BVDV antibody detection ELISA kits failed to detect at least 20% of animals harboring antibodies against HoBi_D32/00. Thus, it is obvious the need for the development of specific diagnostic tests to HoBi-like viruses. Likewise, it was verified that vaccines containing strains of BVDV-1 and BVDV-2 inactivated or modified live (MLV) virus generate suboptimal protection against HoBi-like viruses. Higher levels of protective antibodies against HoBi-like virus were verified using MLV. Nevertheless the geometric mean titer for HoBi-like was 12.9, while 51.1 and 23.5 for BVDV-1 and BVDV-2, respectively. In addition to low levels of protective antibodies, seroconversion was verified in 68% of animals to HoBi-like virus, although reached 100% and 94% against BVDV-1 and BVDV-2, respectively. Regarding to the diagnostic and control of

pestiviruses, the trade of FBS may poses as an important route for dissemination of agents contaminating this product. Composed by a homogenate of serum from hundreds to thousands of fetuses, pestiviruses screening in FBS lots are potentially an important source of information regarding the presence and/or dissemination of these viruses in the region where samples were originated from. Therefore, FBS lots originated in North American countries and processed in the United States of America (USA) or Europe were tested. While several lots were detected as positive for BVDV, none of the lots manufactured in the USA were detected as positive for HoBi-like viruses or antibodies against it. However, two FBS lots processed in Europe were positive for HoBi-like viruses, suggesting that the contamination did occur during the pooling or package of these samples in Europe. These results strongly indicate the need for international rules regarding certification of FBS origin, employment of certified diagnostic tests and measures to avoid contamination of FBS during the manufacturing process. Was also verified the need to include specific tests to detect HoBi-like viruses in the FBS quality control routine. Briefly, the studies here presented demonstrated that the available BVDV diagnostic tests frequently fail in detecting HoBi-like viruses and/or differentiate between HoBi-like virus and BVDV species. In addition, the detection of animal harboring antibodies against HoBi-like using commercial diagnostic tests is unreliable. Likewise, *in-vitro* studies demonstrated that the cross protection against HoBi-like viruses in serum of animals immunized against BVDV-1 and BVDV-2 is limited. Therefore, the detection and control of this emergent group of viruses require the development of specific diagnostic tests and vaccines.

Key words: Atypical pestiviruses; BVDV-3; clinical signs; control; diagnostic.

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1. INTRODUÇÃO

O vírus da diarréia viral bovina (BVDV) é responsável por perdas econômicas na pecuária mundial, por vezes com manifestações clínicas caracterizadas principalmente por distúrbios respiratórios, gastrointestinais e reprodutivos. Foi demonstrado que o prejuízo decorrente da introdução do vírus no rebanho está relacionado à virulência do isolado. Chi et al. (2002) descreveram que a entrada de cepas causando sinais clínicos brandos em um rebanho leiteiro pode resultar em perdas entre 40-60 USD (dólares norte-americanos) por animal. É também descrito que cepas de alta virulência podem gerar prejuízos entre 40.000-100.000 USD por propriedade (CARMAN et al., 1998). Em rebanhos de corte, dependendo da estrutura e técnicas de manejo adotadas, as perdas com a entrada de um animal persistentemente infectado (PI) no rebanho podem atingir 90 USD por animal (HESSMAN et al., 2009).

Planos de controle e erradicação para o BVDV são práticas comuns, especialmente na Europa, onde o enfoque é a identificação e eliminação de animais persistentemente infectados. Além disso, há o constante monitoramento dos rebanhos e teste dos animais a serem introduzidos na propriedade (HOUE et al., 2006). Eventualmente, vacinas são empregadas, seja como parte integrante de programas de âmbito regional/nacional, ou visando o controle local. O principal método diagnóstico utilizado consiste em testes imunoenzimáticos de ELISA (do inglês: *enzyme linked immunosorbent assay*), tanto para detecção de antígenos virais quanto da resposta sorológica específica (STÅHL & ALENIUS, 2011).

Um grupo emergente de pestivírus em bovinos, detectado principalmente em lotes de soro fetal bovino (SFB) com origem na América do Sul, vem sendo denominado vírus HoBi-like, pestivírus atípico ou BVDV-3 (LIU et al., 2009a; BAUERMANN et al., 2013). A infecção natural de rebanhos por estes vírus foi descrita no Brasil, Itália e Sudeste Asiático (CORTEZ et al., 2006; KAMPA et al., 2009; DECARO et al., 2011). A emergência do vírus na Europa denotou maior importância a enfermidade, em face dos programas de erradicação do BVDV que vários países do continente Europeu conduzem (STÅHL & ALENIUS, 2011).

Tendo em vista a emergência e disseminação de pestivírus atípicos, o presente trabalho investigou as características moleculares e antigênicas dos vírus HoBi-like e suas semelhanças com BVDV. Devido a similaridades encontradas, foram desenvolvidos trabalhos *in-vitro* para

estimar a proteção cruzada conferida ao vírus HoBi-like em animais imunizados para BVDV-1 e BVDV-2. O trabalho incluiu a avaliação das ferramentas diagnósticas existentes para BVDV no diagnóstico de animais infectados por HoBi-like vírus, bem como testes para detecção de anticorpos. Devido ao grande potencial que amostras de SFB possuem em disseminar o agente, testes "panpestivírus" e testes específicos para HoBi-like vírus foram utilizados para avaliar lotes de SFB, além da padronização de um teste de RT-PCR (do inglês: *reverse transciption polymerase chain reaction*) específico para a detecção do vírus HoBi-like.

2. REVISÃO DE LITERATURA

2.1. História e evolução dos pestivírus em bovinos

Na década de 1940 foi publicada a primeira evidência de uma nova enfermidade em bovinos (OLAFSSON et al., 1946). O artigo relatou uma doença com apresentação clínica que incluía leucopenia, febre, desidratação, diarréia, anorexia, erosões gastrointestinais e hemorragias em diversos órgãos. Inicialmente a doença ocorreu em um pequeno rebanho em Ithaca, New York. Os surtos propagaram-se para outras propriedades da região, sendo a morbidade inicial da doença estimada entre 33% a 88%. A leucopenia indicou a etiologia viral da doença. Tentativa terapêutica por meio de transfusão sanguínea demonstrou que animais aparentemente sadios, potencialmente doadores, em alguns casos apresentavam leucopenia mais severa que os animais clinicamente afetados. Verificou-se assim que a infecção também ocorria de forma subclínica. A enfermidade foi então denominada diarréia viral bovina (do inglês: *bovine viral diarrhea*, BVD) (DEREGT, 2005).

Ainda nas décadas de 40 e 50, foram relatados surtos de uma doença com características clínicas semelhantes às anteriormente descritas. Entretanto, com sinais clínicos mais severos, incluindo úlceras no trato gastrointestinal, diarréia profusa e muitas vezes hemorrágica. A morbidade da doença era baixa, porém, invariavelmente letal (DEREGT, 2005). Na década de 1950 a enfermidade foi denominada doença das mucosas (DM) e verificou-se que embora algumas características clínicas fossem semelhantes ao BVD, a primeira não era transmitida experimentalmente (RAMSEY & CHIVERS, 1953). Na década de 1960, por meio de soroneutralização cruzada, verificou-se que agentes envolvidos em casos de BVD e DM eram antigenicamente idênticos. Constatou-se que animais que adoeciam de DM eram soronegativos para BVD e DM, e, com base no caso de um animal que permaneceu com DM e soronegativo por aproximadamente dois meses, foi sugerido que os animais eram incapazes de produzir resposta imune contra a enfermidade (DEREGT, 2005).

No final da década de 1960 tornou-se claro que o vírus possuía habilidade de infectar o feto, e induzir imunotolerância desse ao vírus. Ainda eram inúmeras as questões a respeito da função de cada uma dos biotipos virais, visto que em alguns casos eram detectados animais possuindo o biotipo citopático (CP), outras vezes não citopático (NCP), ou ambos (GILLESPIE

et al., 1961). Somente na década de 1980 a doença das mucosas foi reproduzida experimentalmente (BROWNLIE et al., 1984). Por meio do avanço das técnicas moleculares, foi evidenciado mutações entre vírus CP e NCP, expandindo o entendimento entre a etiologia e patogenia da doença (DONIS & DUBOVI, 1987).

Na década de 1990, uma doença provocando diarréia hemorrágica severa, foi descrita em países da América do Norte. Posteriormente, verificou-se que a doença era causada por isolados do BVDV filogeneticamente distante daqueles até então conhecidos. Os isolados desses surtos foram denominados de BVDV-2 (RIDPATH et al., 1994). Análise retrospectiva de isolados Canadenses, incluindo amostras datando de 1981, identificou a existência de isolados de BVDV-2 desde aquele ano, demonstrando que isolados de BVDV-2 não necessariamente possuíam alta virulência (CARMAN et al., 1998). Assim, os surtos severos de BVDV-2 na América do Norte poderiam ser explicados tanto pela mutação de uma cepa levando ao aumento da virulência da mesma, quanto pela exposição de rebanhos às cepas que até então estavam geograficamente restritas (CARMAN et al., 1998; DEREGT, 2005).

Vacinas contra o BVDV começaram a ser utilizadas em larga escala a partir da década de 1960, sendo que por mais de 30 anos possuíam somente cepas do BVDV-1 em sua composição. Com a descoberta e disseminação do BVDV-2, houve a inclusão desse genótipo na formulação de várias vacinas (VAN-CAMPEN et al., 2000; VAN-CAMPEN, 2010). A proteção contra infecção fetal conferida por vacinas é controversa, e os títulos de anticorpos contra cepas heterólogas ou de diferentes subgenótipos muitas vezes são insuficientes para conferir proteção contra a doença clínica ou prevenir a excreção viral (VAN-CAMPEN, 2010; RIDPATH et al., 2012).

Aliada a toda diversidade encontrada nas espécies de BVDV, no ano de 2004 foi isolado um pestivírus em um lote de SFB originado no Brasil (SCHIRRMEIER et al., 2004). Análise filogenética revelou ser um pestivírus distante daqueles até então descritos. O primeiro isolado recebeu o nome de HoBi_D32/00, sendo que isolados desse grupo constituem uma espécie provisória e são denominados vírus HoBi-like, mas também BVDV-3 ou pestivírus atípicos. Inicialmente o vírus parecia restrito ao subcontinente Sul Americano, contudo casos foram relatados na Tailândia e Itália (STÅHL et al., 2007; KAMPA et al., 2009; DECARO et al., 2011). Apesar do caráter imunossupressor, a infecção por esses vírus geralmente transcorre sem

a presença de sinais clínicos (SCHIRRMEIER et al., 2004), porém devido a carência de métodos diagnósticos específicos para este grupo de vírus, o risco da dispersão destes agentes constitui um desafio ao controle do BVDV (BAUERMANN et al., 2013; LARSKA et al., 2013). Além disso, semelhante ao BVDV-2 (CARMAN et al., 1998), o incremento da virulência por meio de mutações, ou ainda a possibilidade de que este vírus apresentem maior virulência em rebanhos totalmente susceptíveis/soronegativos não devem ser descartados.

2.2. Organização molecular do gênero *Pestivirus*

O gênero *Pestivirus* é membro da família *Flaviviridae,* sendo composto por quatro espécies reconhecidas: *vírus da diarréia viral bovina*, genótipos 1 (BVDV-1) e 2 (BVDV-2), *vírus da doença das fronteiras* (BDV), *vírus da peste suína clássica* (CSFV). Quatro espécies estão em processo de reconhecimento: Pestivírus de Girafa, vírus Pronghorn, vírus Bungowannah e vírus HoBi-like (Figura 1) (THIEL et al., 2005).
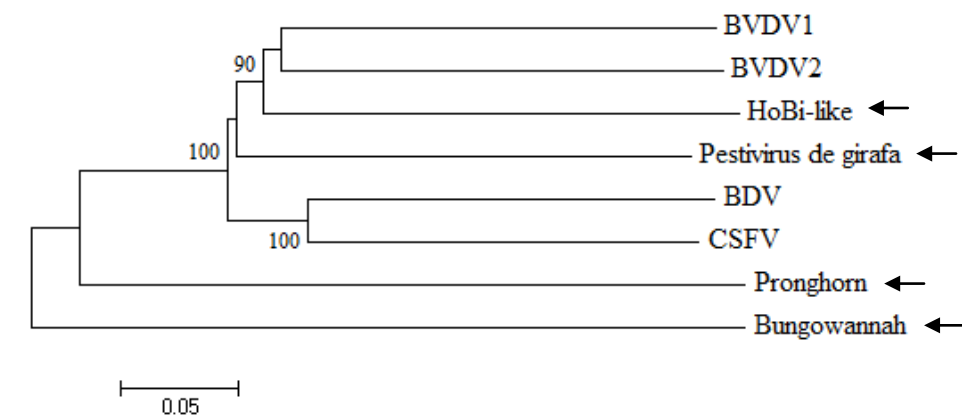
Fig. 1 – Árvore filogenética apresentando os membros do gênero *Pestivirus.* Espécies provisórias indicadas por flechas.

O genoma dos pestivírus é composto por uma fita simples de RNA, polaridade positiva, de aproximadamente 12.3kb. O genoma possui apenas uma ORF (do inglês: *open reading frame*) e as extremidades 5' e 3' contêm regiões não traduzidas (5'UTR e 3'UTR) (BROCK et at., 1992). Os ribossomos reconhecem a região denominada IRES (do inglês: *internal ribosome entry site*) localizada na extremidade 5'. Por meio de análise genética da extremidade 5' -

altamente conservada entre os pestivírus - os isolados de BVDV são divididos em 2 genótipos, BVDV-1 e BVDV-2. O BVDV-1 ainda é subdividido em pelo menos 11 membros (a-k), enquanto o genótipo tipo 2 em ao menos duas subdivisões (a-b) (VILCEK et al., 2001; FLORES et al., 2002). A extremidade 3' é formada por uma pequena cauda poli C (BROCK et at., 1992).

A única e longa ORF codifica uma poliproteína que é clivada, durante e após a tradução, podendo gerar 12 polipeptídeos, na seguinte ordem: N^{pro}-C-E^{rns}-E1-E2-p7-NS2/NS3-NS4A-NS4B-NS5A-NS5B (RIDPATH, 2005). Dentre os integrantes da família *Flaviviridae,* as proteínas N^{pro} e E^{rns} são codificadas apenas por membros do gênero *Pestivirus* (Figura 2) (RIDPATH, 2005).
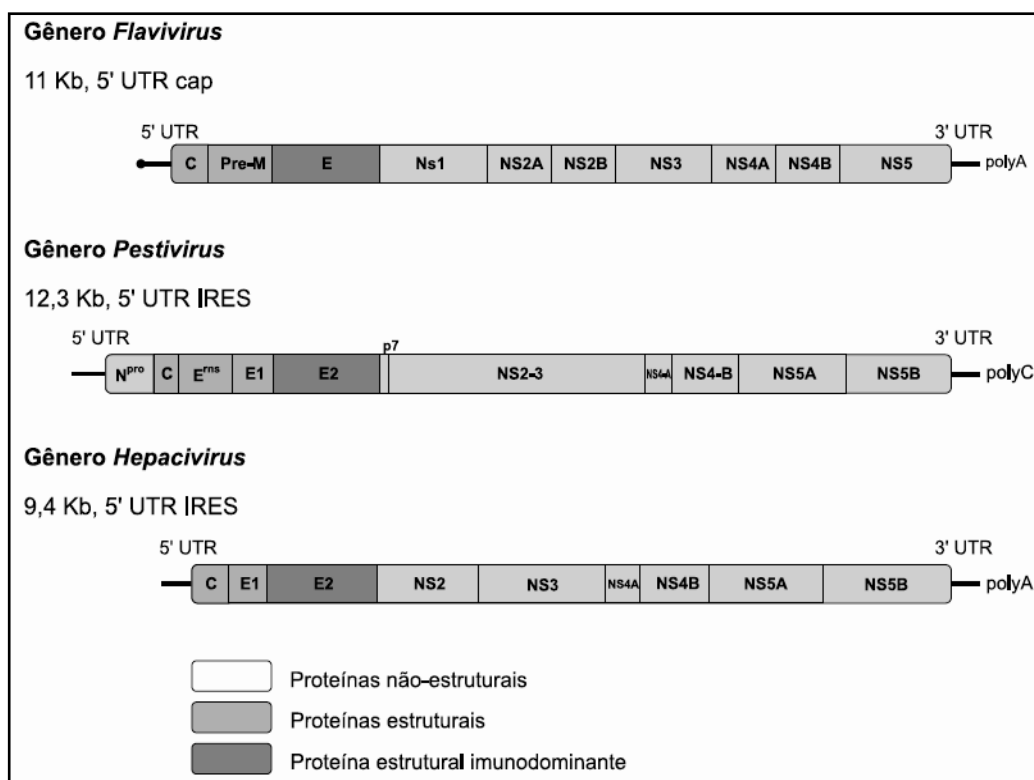
Figura 2 – Esquema representativo da organização genômica dos membros da família *Flaviviridae* (Ridpath et al., 2012).

A N^{pro} é uma protease não essencial *in-vitro*, sendo autoclivada após a tradução. Essa protease interage com fatores do sistema imune inato em células infectadas, conduzindo a degradação de fatores indutores de interferon (CHEN et al., 2007). Constituída por

homodímeros, a proteína estrutural E^{rns} também é secretada em forma solúvel por células infectadas (RUMENAPF et al., 1993). Sua atividade ribonuclease (RNase), sobre RNA fita simples ou dupla, interfere na resposta imune inata contra o vírus. Parte dos anticorpos produzidos contra essa proteína apresenta atividade neutralizante (WEILAND et al., 1992).

O capsídeo viral possui diâmetro entre 40-60 nm, sendo composto por cópias da proteína C. O envelope glicolipídico bilaminar dos pestivírus é originado de membranas das células infectadas, possuindo inclusões das proteínas virais (E^{rns}, E1 e E2) (RIDPATH, 2005). As glicoproteínas E1 e E2 estão relacionadas com a alta eficiência da ligação do vírus aos receptores celulares e podem ser encontradas como homodímeros ou heterodímeros (RONECKER et al., 2008). A E1 parece influenciar positivamente a infectividade viral, sendo que a E2 possui papel predominante na ligação à célula (LI et al., 2008). Parte dos anticorpos contra a E2 possui atividade neutralizante, sendo que a porção amino-terminal da E2 concentra maior parte dos epitopos com capacidade antigênica (LIANG et al., 2003).

Fenotipicamente, os isolados de pestivírus podem ser classificados em CP e NCP, com base na indução de alterações morfológicas e morte celular *in-vitro*. Verifica-se que os vírus NCP expressam a proteína NS2/3 de forma íntregra, e em pequena quantidade de forma clivada, como NS2 e NS3. Entretanto, os vírus CP expressam de forma exacerbada as proteínas NS2 e NS3 (DONIS et al., 1991; PETERHANS et al., 2010). A NS2 possui atividade de protease no sítio entre NS2 e NS3, além de conter peptídeos necessários para a translocação da proteína ao retículo endoplasmático (GUO et al., 2011). A região amino-terminal de NS3 apresenta atividade protease em vários sítios da poliproteína viral. Sua região carboxi-terminal tem atividade de helicase e está relacionada à replicação do RNA. A inativação de uma ou ambas as funções da NS3 inibem a replicação viral (GRASSMANN et al., 1999).

As demais proteínas traduzidas pelos pestivírus são não-estruturais. Entre elas, uma pequena proteína, p7, não crucial para replicação do RNA. Porém, necessária para a produção de vírion infeccioso (HARADA et al., 2000). As proteínas NS4A e NS4B estão envolvidas em processos enzimáticos (protease), replicação do RNA, e rearranjo das membranas celulares na célula infectada. Foi descrito que uma única alteração no aminoácido na posição 15 da proteína NS4B foi capaz de atenuar a citopatogenicidade viral, mesmo mantendo a produção das proteínas NS2 e NS3 de forma clivada (QU et al., 2001). A NS5A parece estar envolvida no

complexo de replicação viral, enquanto a NS5B possuiu função de RNA polimerase viral (ZHONG et al., 1998).

2.3. Espécies provisórias de pestivírus

Diversos são os relatos da emergência de pestivírus atípicos. Na década de 1960, foi identificado um pestivírus (H138) a partir do soro de girafa originado na República do Quênia (THIEL, et al. 2005). Mais de 30 anos depois, um vírus muito semelhante (PG-2) foi isolado de um cultivo celular na África (BECHER et al., 2003; STALDER et al., 2005). Esses isolados são considerados como membros da quinta espécie de pestivírus. O relato de isolados em períodos tão distantes demonstra que o vírus permanece no ambiente, entretanto o conhecimento a respeito da importância e biologia desses vírus é vago (RIDPATH et al., 2012).

O vírus *antelope Pronghorn* foi descrito infectando um antílope nos Estados Unidos da América. A apresentação clínica desse animal se limitava a cegueira. Por meio de isolamento viral e posterior uso da técnica de imunofluorescência, foi detectado a presença de um pestivírus. Sequenciamento e análise filogenética revelaram ser o pestivírus mais divergente detectado até aquele momento (VILCEK et al., 2005). Em um estudo em andamento, 2013, conduzido para detecção de pestivírus em populações de veados nos EUA, um isolado semelhante ao vírus Pronghorn foi identificado, entretanto, não há relato de doença clínica nos animais pesquisados (RIDPATH et al., dados não publicados).

Outro isolado de pestivírus, com distanciamento filogenético ainda maior que o vírus Pronghorn foi detectado na Austrália. No episódio, centenas de suínos foram afetadas em duas granjas. Semanas após o início do surto, foi confirmado como agente etiológico um pestivírus então denominado Bungowannah. As características clínicas da infecção por esse agente incluíam morte súbita em leitões em decorrência de miocardite, além do elevado número de fetos natimortos e mumificações. Em torno de seis meses após o início do surto, os parâmetros reprodutivos das granjas haviam retornado aos padrões anteriores ao surto, e o vírus não era mais detectado nos animais (KIRKLAND et al., 2007). Cerca de cinco anos depois, o vírus novamente foi detectado em animais em uma das granjas, com características clínicas semelhantes ao primeiro surto (PETER KIRKLAND, comunicação pessoal, 2012). Existem evidências

sugerindo que o vírus seja mantido por populações de morcegos naquele país e eventualmente ocorra transmissão a suínos (MARTIN BEER, comunicação pessoal, 2012).

Contrastando com o caráter geográfico restrito das três espécies provisórias descritas, o quarto grupo de pestivírus refere-se a isolados encontrados em pelo menos três continentes, e com origem provável em bovinos e/ou bubalinos. O vírus protótipo do grupo foi originalmente encontrado como contaminante de lotes de SFB originado do Brasil (SCHIRRMEIER et al., 2004). Posteriormente, vários são os relatos da identificação desses vírus nesse produto (STALDER et al., 2005; STÅHL et al., 2007; LIU et al., 2009b; PELETTO et al., 2012). Estudos retrospectivos identificaram vírus HoBi-like em amostras de bubalinos em um surto que incluiu a morte de alguns animais (STALDER et al., 2005; BAUERMANN et al., 2013). Cortez et al. (2006) e Bianchi et al. (2011) descreveram a identificação do vírus HoBi-like, respectivamente, em amostras originados nas regiões Sudeste e Sul do Brasil.

O primeiro isolado com origem Sul Americana não comprovada foi descrito em 2007 (STÅHL et al., 2007; KAMPA et al., 2009). O vírus foi detectado em uma amostra de soro bovino, após o relato de soroconversão ao vírus HoBi-like em alguns rebanhos bovinos na Tailândia. O vírus também foi descrito em 2011 na Itália causando doença respiratória (DECARO et al., 2011). Posteriormente, identificado em um surto de doença entérica e reprodutiva no mesmo país; e durante estudos retrospectivos foi detectada a presença de amostras contendo vírus HoBi-like que datam 2008 (NICOLA DECARO, comunicação pessoal, 2013). Uma ampla descrição dos episódios onde foi detectado o vírus HoBi-like é apresentada no Capítulo 1 da presente Tese.

A comparação da sequência de nucleotídeos da região 5'UTR entre os isolados de HoBi-like mostra similaridade superior a 94%. Por outro lado, quando são comparadas com sequências de isolados de BVDV dos genótipos 1 e 2, esse valor decresce a um patamar de 80% (SCHIRRMEIER et al., 2004). Considerando a identidade de nucleotídeos que codificam a glicoproteína E2 - altamente variável entre os pestivírus - observa-se uma similaridade superior a 92% entre os isolados atípicos HoBi D32/00, SVA/cont-08, e CH-KaHo. Entretanto quando essa região dos vírus HoBi-like é comparada com as cepas NADL e NY93 - pertencente respectivamente aos genótipos 1 e 2 do BVDV - esses valores não ultrapassam 65% (LIU et al., 2009a; LIU et al., 2009b). Essa variabilidade genética possui repercussão antigênica. Schirrmeier

et al. (2004), verificaram que a capacidade de um anti-soro produzido contra cepas de BVDV-1 e BVDV-2 em neutralizar o isolado HoBi_D32/00 pode ser em torno de 100 vezes menor quando comparada à neutralização com anti-soro específico. Resultados semelhantes foram encontrados em outros estudos (STÅHL et al., 2007; BAUERMANN et al., 2012; DECARO et al., 2013), o que mostra uma fraca reatividade sorológica cruzada entre o vírus HoBi-like e as espécies do BVDV.

2.4. Infecção e apresentação clínica dos pestivírus em bovinos

A maioria dos isolados de BVDV possuiu baixa virulência, também denominados BVDV de típica virulência (RIDPATH et al., 2012). A via nasal é a principal rota de infecção viral, ocorrendo replicação primária nas mucosas e linfonodos regionais. A infecção geralmente transcorre de forma assintomática ou com sinais clínicos brandos, incluindo doença respiratória, imunossupressão e hipertermia. Entretanto, a apresentação clínica devido à infecção por BVDV é vasta, e relaciona-se a fatores ligados ao agente, como a virulência e o biótipo; e fatores relacionados ao hospedeiro, como o estado de imunidade e existência/fase da gestação (BAKER, 1995; BOLIN & GROOMS, 2004).

A infecção clínica aguda em animais não gestantes e sem o envolvimento de outros patógenos pode incluir doença respiratória leve (tosse, secreção nasal serosa aumentada), imunossupressão, hipertermia e diarréia. Em casos mais severos, é relatada síndrome hemorrágica devido à trombocitopenia e pneumonia (BAKER, 1995; CARMAN et al., 1998; BOLIN & GROOMS, 2004). Os casos de síndrome hemorrágica podem ser confundidos com os sinais clínicos verificados na doença das mucosas (DM), sendo a última restrita a animais PI. Invariavelmente fatal, a DM culmina em lesões ulcerativas que podem ser visualizadas em órgãos do sistema gastrointestinal (BROWNLIE et al., 1984).

Apesar da variedade de apresentações clínicas, é sugerido que as maiores perdas econômicas em decorrência da infecção pelo BVDV são oriundas dos problemas reprodutivos (RIDPATH, 2010). Nesse contexto inclui-se reabsorção fetal, retorno ao cio, abortos, e mal-formações congênitas. Caso um animal seja infectado por uma cepa NCP durante o terço inicial de gestação, há a possibilidade de que o feto infectado torne-se persistentemente infectado com o

vírus. Esse animal pode nascer aparentemente saudável, porém excreta o vírus constantemente durante o curso de sua vida. Em geral, esses animais morrem nos primeiros meses de vida, mas são de extrema importância epidemiológica para a doença (RIDPATH et al., 2005; HOUE et al., 2006).

O grupo de pestivírus atípicos, vírus HoBi-like, tem demonstrado até o momento que possuí características clínicas indistinguíveis da infecção por cepas de BVDV de típica virulência (RIDPATH et al., 2013). Casos de infecção natural descritos até o momento incluem sinais clínicos respiratórios, reprodutivo, e imunossupressão (DECARO et al., 2011; BAUERMANN et al., 2013). O vírus apresenta alto tropismo fetal, com alta taxa de geração de animais PI (BAUERMANN et al., dados não publicados). Clinicamente semelhante ao BVDV, um vírus HoBi-like também foi encontrado causando DM (MATHEUS WEBER, comunicação pessoal, 2013). Uma ampla e detalhada descrição do quadro clínico em infecções naturais e experimentais relacionados ao vírus HoBi-like é encontrado no Capítulo 1.

2.5. Diagnóstico

Várias são as técnicas disponíveis para a detecção do BVDV ou animais expostos ou vacinados ao vírus, sendo que a maioria não possui a capacidade de diferenciação entre BVDV-1 e BVDV-2. O teste referenciado como prova padrão para detecção do agente é o isolamento viral (SANDVIK, 2005; DUBOVI et al., 2013). Entretanto, a sensibilidade da linhagem de célula usada, método de transporte e processamento da amostra são aspectos com grande influência no teste. Ainda há a possível presença de contaminante viral ou anticorpos no SFB utilizado na amplificação viral. Visto que parte dos isolados de BVDV circulantes são do biotipo NCP, a detecção desses requer um teste subsequente ao isolamento viral. Testes imunoenzimáticos são amplamente utilizados para fazer a confirmação dos testes de isolamento viral (HOUE et al., 2006; STÅHL & ALENIUS, 2011). Entre esses, inclui-se os testes de imunofluorescência, imunohistoquímica, imunoblots e ELISA (CORNISH et al., 2005; DUBOVI et al., 2013). O princípio desses testes é similar, anticorpos específicos são utilizados para a detecção viral, e a revelação do resultado ocorre por meio de uma reação enzimática, sobre substratos específicos ou ainda por meio de anticorpos marcados com fluorocromos (GOYAL, 2005). Testes

moleculares para a detecção do RNA viral também são muito utilizadas. Esses testes geralmente têm como alvo a região 5'UTR, visto ser uma região extremamente conservada entre as diferentes espécies do gênero *Pestivirus* (SHMITT et al., 1994; VILCEK et al., 1994; RIDPATH & BOLIN et al., 1998).

Para a detecção de anticorpos específicos para pestivírus, os testes mais frequentemente utilizados são a SN e ELISA. De forma geral, esses testes não são capazes de diferenciar a resposta sorológica de animais para os diferentes pestivírus (BAUERMANN et al., 2013; LARSKA et al., 2013). Devido às semelhanças entre BVDV-1, BVDV-2, e vírus HoBi-like, alguns testes desenvolvidos para a detecção de antígeno ou anticorpos para o BVDV, em alguns casos, possuem a mesma sensibilidade em detectar vírus HoBi-like (BAUERMANN et al., 2012). Porém, em algumas situações, o número de resultados falso-negativos é elevado (BAUERMANN et al., 2012; BAUERMANN et al., 2013). Mais detalhes a respeito da detecção dos vírus HoBi-like serão descritos no decorrer dos capítulos da presente Tese.

2.6. Controle

Por muito tempo, o controle do BVDV teve como base o uso de vacinas, objetivando reduzir ou evitar o aparecimento de doença clínica nos rebanhos (STÅHL & ALENIUS, 2011). O conhecimento da epidemiologia da doença tem direcionado o foco do controle do agente aos animais PI. O modelo padrão atual para o controle do BVDV tem três fundamentos: 1- a adoção sistemática de medidas de biossegurança possuiu papel central no controle do BVDV a fim de evitar a entrada do vírus em rebanhos livres. Visto que prevalência nos rebanhos tende a decrescer, e na ausência de animais PI, o vírus será naturalmente extinto do rebanho; 2- detecção e eliminação dos animais PI, reduzindo a circulação do vírus no rebanho; 3- monitoramento constante para rápida detecção do vírus em episódios de reinfecção do rebanho (LINDERBERG & ALENIUS, 1999).

Seguindo esses conceitos, países escandinavos e Finlândia obtiveram sucesso no controle e erradicação da enfermidade (MOENNIG et al., 2005; ROSSMANITH et al., 2010). Finlândia e Noruega não reportaram surtos nos últimos anos. Dois casos foram detectados na Dinamarca e três casos na Suécia nos anos de 2010 e 2011 (STÅHL & ALENIUS, 2011). O sucesso desses

países na erradicação do agente incentivou outros países europeus a se engajarem em semelhantes programas. Entre esses, a Áustria iniciou um programa de controle voluntário em 1997, tornando-o compulsório em 2004 (ROSSMANITH et al., 2010). Dados de 2010 estimaram que a porcentagem de rebanho contendo animais PI seja de 0,16% naquele país (ROSSMANITH et al., 2010). Nos últimos cinco anos, programas de controle foram expandidos para outros países europeus. Enquanto a estratégia dos países Escandinavos e da Áustria é testar todos os animais tanto para antígeno quanto para anticorpos contra o BVDV, os programas lançados na Suíça, Alemanha, Escócia e Irlanda testam os animais apenas para antígeno (PRESI et al., 2011; STÅHL & ALENIUS, 2011).

Na América do Norte, os Estados Unidos tem reduzido a intenção de realizar um programa de controle do BVDV em nível nacional. O governo federal, até o momento, não possui iniciativas com objetivo de controlar e eventualmente erradicar o BVDV do país (JULIA RIDPATH, comunicação pessoal, 2013). Vários fatores têm dificultado na escolha e adoção das medidas. Dentre estes, o imenso tamanho territorial e diversidades, que incluem o contato de bovinos com animais silvestres, tipo da unidade produtora e interesse e/ou cooperação dos produtores. Assim, programas existentes são regionais e por iniciativa dos produtores em parceria com empresas privadas (RIDPATH et al., 2012). Quatro estados possuem programas voluntários estabelecidos: Alabama, Montana, Washington, e Michigan. Todos os programas têm como pilar a detecção e a eliminação dos animais PIs. O estado de Michigan é o único com o plano de erradicar o agente em uma área determinada. Por outro lado, os demais estados têm como foco a redução da prevalência da doença (RIDPATH et al., 2012; JULIA RIDPATH, comunicação pessoal, 2013).

Foi demonstrado que o número de rebanhos contendo animais PI pouco alterou entre os estados participantes das campanhas de controle, variando de 13,3% a 13,9% (RIDPATH et al., 2012). A porcentagem de animais PIs dentro do grupo de amostras testadas variou de 0,1% a 0,92% (RIDPATH et al., 2012). Nos últimos anos, os altos custos de alimentação animal devido às secas recorrentes na região do Meio-Oeste dos EUA, e o fraco desempenho da economia Norte Americana conduziram o país ao menor número de bovinos desde que foi iniciado o trabalho de levantamento (1952), número esse estimado em torno de 89.3 milhões de animais (PLAIN, 2013). A situação econômica levou a um progressivo desengajamento dos produtores

em relação aos programas de controle. O único estado onde o programa ainda está completamente ativo é Alabama (RIDPATH et al., 2012; JULIA RIDPATH, comunicação pessoal, 2013). Por outro lado, o único estado que conseguiu reduzir significativamente o número de rebanhos com animais PI foi Michigan, enquanto a redução na prevalência de BVDV nos outros estados não foi expressiva (RIDPATH et al., 2012).

A presente tese é formada por quatro capítulos, o primeiro Capítulo é uma revisão destacando os aspectos clínicos da infecção de bovinos pelo vírus HoBi-like. Também é discutido a respeito de testes diagnósticos para a detecção desse vírus. Os Capítulos 2 e 3 discutem as similaridades e divergências antigênicas encontradas entre BVDV-1, BVDV-2 e o vírus HoBi-like. O quarto Capítulo aborda o diagnóstico de pestívirus em amostras de SFB originadas na América do Norte, sendo um dos primeiros esforços para a detecção do vírus HoBi-like nessa região.

3. CAPÍTULO 1

HoBi-like viruses: an emerging group of pestiviruses

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Abstract. The genus *Pestivirus* is composed by 4 important pathogens of livestock: *Bovine viral diarrhea virus 1* and *2* (BVDV-1 and BVDV-2), *Classical swine fever virus* (CSFV), and *Border*

disease virus of sheep (BDV). BVDV are major pathogens of cattle, and infection results in significant economic loss worldwide. A new putative pestivirus species, tentatively called "HoBi-like," "BVDV-3," or "atypical pestiviruses," was first identified in Europe in fetal bovine serum (FBS) imported from Brazil. HoBi-like viruses are related to BVDV at the genetic and antigenic levels. Further, the disease caused by these new viruses resembles clinical presentations historically associated with BVDV infection, including growth retardation, reduced milk production, respiratory disease, reduced reproductive performance, and increased mortality among young stock. Current BVDV diagnostic tests may fail to detect HoBi-like viruses or to differentiate between BVDV and HoBi-like viruses. Further, commercial tests for BVDV exposure, based on serological response, do not reliably detect HoBi-like virus exposure, and cross protection against HoBi-like viruses conferred by current BVDV vaccines is likely limited. As many HoBi-like viruses, characterized to date, were isolated from FBS originating from Brazil, it is assumed that the agent is probably widespread in Brazilian herds. Nevertheless, reports of natural infection in Southeast Asia and Europe demonstrate that these viruses are not restricted to South America. Increased demand for FBS has led to widespread distribution of FBS originating in HoBi-like virus endemic regions. The contamination of such FBS with HoBi-like viruses may lead to spread of this virus to other regions.

Overview of bovine pestiviruses

Pestivirus is a genus within the family *Flaviviridae*, composed of 4 recognized species, *Bovine viral diarrhea virus 1* and 2 (BVDV-1 and BVDV-2), *Classical swine fever virus* (CSFV), and *Border disease virus* (BDV).[32] Pestivirus infections in cattle, noticeably BVDV-1 and BVDV-2, represent major concerns worldwide, leading to significant economic losses.[15] The disease is associated with a wide range of clinical manifestations. Calves infected with typically virulent BVDV generally display mild upper respiratory signs, a transient decrease in circulating white blood cells, and a low-grade, short-term fever. However, while a minority in nature, BVDV strains with enhanced virulence do exist. Infection with an enhanced virulence BVDV strain may result in severe respiratory disease, gastroenteric disorders, hemorrhagic syndrome, and pneumonia.[1,4-6] Regardless, the most relevant economic losses associated with BVDV infection seem to be due to reproductive disorders.[16] The infection of pregnant cows during the first trimester of gestation with noncytopathic (ncp) BVDV strains, belonging to either BVDV-1 or BVDV-2 species, may lead to failure of fertilization, return to estrus, abortion, congenital malformations, stillbirths, or the birth of persistently infected (PI) animals. While some PI calves may have congenital malformations, others appear clinically normal. Persistently infected animals are negative for BVDV antibodies, prior to ingestion of colostrum, but are positive for virus antigen in nearly all tissues. Such PI animals shed the virus to the environment continuously over the course of their lifetimes.[1,4] Thus, the identification and elimination of PI animals and the adoption of biosecurity measures that prevent the introduction of PI animals into herds are necessary actions in controlling and eradicating BVDV from cattle herds. Inactivated and modified live vaccines are used worldwide to prevent acute disease and fetal infections.[16,21] Vaccination reduces the production of PI animals at the herd level but cannot be relied upon to

prevent all fetal infections. Thus, vaccination alone cannot eliminate BVDV and must be combined with identification and elimination of PI animals and the adoption of biosecurity in control and eradication strategies.

The pestivirus genome is composed of a positive single-stranded RNA molecule approximately 12.3 kb in length, containing a single open reading frame (ORF) flanked by 2 untranslated regions (5'- and 3'-UTR). The long ORF encodes a single polyprotein that is co- and post-translationally processed into 12 viral mature polypeptides in the following order: N^{pro}-C-E^{rns}-E1-E2-p7-NS2/NS3-NS4A-NS4B-NS5A-NS5B.[32] The 5'-UTR region is highly conserved among pestiviruses, and comparison of 5'-UTR sequences is widely accepted for phylogenetic analysis as are comparison of sequences coding for the viral proteins N^{pro} and E2.[14,24,26,28] Due to the nature of the single-stranded genome, these viruses display high mutation rates, which in some cases may lead to the emergence of "new" virus lineages. Whereas the correlation of high mutation rates with emergence of new lineages has not been proven, the rise of new pestiviruses species has been described.[18,28,35]

In addition to the recognized species, 4 additional *Pestivirus* species have been proposed but remain officially unrecognized (Fig. 1). These putative species include Giraffe virus, associated with an outbreak of mucosal-like disease in giraffes in the Nanyuki District of Kenya[32]; Pronghorn virus, isolated from a blind pronghorn antelope in the United States[35]; Bungowannah virus, detected in pigs following an outbreak of stillbirths and neonatal death in Australia[18]; and a group of viruses variously referred to as HoBi-like, BVDV-3, or atypical pestiviruses,[20,24,28] which are the subject of the current review.

The putative species, referred to hereafter as HoBi-like viruses, which was first described by researchers in Europe in 2004, was isolated from a batch of fetal bovine serum (FBS)

imported from Brazil.[30] Since that first description, several reports of genetically similar agents contaminating FBS and cell lines have been published.[25,28,30,36,37] Unpublished data indicates that the more than 30% of FBS batches originated from South America that are tested in Europe are contaminated with HoBi-like viruses (Martin Beer, personal communication, 2012), leading to the hypothesis that these viruses are probably widespread in South American cattle, especially in Brazilian herds. However, HoBi-like viruses have also been detected contaminating FBS that is processed and/or packaged in Europe, originating from Australia, Canada, Mexico, and United States.[37] Detection of HoBi-like viruses was also reported in FBS originating, processed, and packaged in Australia,[36] although this finding remains controversial considering the lack of reports indicating the circulation of these viruses in that country. Natural infection in cattle with HoBi-like viruses has being reported in Southeast Asia,[29] Italy,[8,9,11] and Brazil[3,7,30] (Fig. 2).

In contrast with the apparent widespread distribution of HoBi-like viruses, the other putative pestivirus species mentioned above were isolated from a limited number of animals residing in single geographic regions (Table 1). Therefore, HoBi-like viruses seem to pose the most threat for domestic livestock production of any of the "new" pestiviruses that have emerged recently (between years 2000-2012). The name "HoBi-like viruses" is derived from the first isolate called HoBi_D32/00.[28] Some authors also refer to these viruses as BVDV-3[24] due to the similarity of clinical presentation in cattle following infection with BVDV and HoBi-like viruses. There is resistance to declaring HoBi-like viruses a third species of BVDV because these viruses are genetically distant from BVDV-1 and BVDV-2, and diagnostics and vaccines that work well to detect and control the 2 species of BVDV are less efficacious for HoBi-like viruses.[2,28] The term "atypical pestiviruses" has also been used[8,23]; however, this term could also be applied to any of the 3 other putative pestivirus species. To date, the International Committee

on Viral Taxonomy has not arrived at a decision to recognize HoBi-like viruses as an official species within the pestivirus genus or declared an official name.

The origin/emergence of HoBi-like viruses is unknown. One hypothesis is that the viruses originated in South America and were introduced to other countries and continents through contaminated biological products, such as FBS and vaccines.[29] Another explanation is that the emergence of HoBi-like viruses in cattle is the result of a host species jump in which these viruses crossed from water buffalo to cattle. This hypothesis would explain the presence of the viruses in regions raising significant water buffalo populations, such as Brazil and Thailand. The HoBi-like isolate BrazBuf9[30] was identified in the late 1990s in an outbreak with death of water buffalos in Brazil, yet the outbreak was not officially reported (Claudio Wageck Canal, personal communication, 2011).

Based on phylogenetic analysis, all isolates of HoBi-like viruses identified to date are highly similar and cluster together. Keeping in mind the highly genetic variability of pestiviruses, it could be hypothesized that HoBi-like viruses were originated in South America and the subsequent spread to other regions has been a fairly recent event, in evolutionary terms. On the other hand, the divergence of the isolate from Southeast Asia (Th/04_KhonKaen) from other HoBi-like viruses might also indicate an independent evolution of at least 2 groups of HoBi-like viruses (Fig. 3).

As stated previously, natural HoBi-like virus infections have been reported in South America, Southeast Asia, and Italy. No isolations of these viruses have been reported from cattle residing in the rest of Europe, North America, Africa, India, and Australia, indicating that herds in these regions remain serologically naïve and vulnerable to infection. Spread of HoBi-like viruses into these regions could have profound effects on cattle production worldwide. The

recent introduction of the Schmallenberg virus into Europe illustrates how rapidly an introduced pathogen can spread in naïve herds. The veterinary community was not alerted to the initial introduction of Schmallenberg virus because the clinical presentation following acute infection was mild. However, the Schmallenberg virus, like BVDV and HoBi-like viruses, is able to cross the placenta and infect the fetus. The full impact of the Schmallenberg introduction was not realized until congenitally malformed offspring were born following infection during the early stages of pregnancy. The introduction of HoBi-like viruses would have an even greater impact because HoBi-like viruses can establish persistent infections in offspring following in-utero infection. Further, BVDV diagnostic tests may fail to detect and/or differentiate these agents. Thus, detection of HoBi-like viruses may be overlooked. Improved surveillance for these viruses requires the attention of veterinary diagnostic laboratories, institutions involved with commerce of biological products, and governmental agencies.

In herds in Brazil, Italy, and Thailand, where these viruses may already be endemic, infection of cattle with HoBi-like viruses may contribute to economic losses associated with clinical disease, reduction in productive parameters, and perhaps immunosuppression. HoBi-virus–positive status for endemic countries may also become an issue for international trade of animals and products with countries that consider themselves free of HoBi-like virus.

Clinical presentation of HoBi-like viruses

Natural cases

The first evidence of natural infection with a HoBi-like virus was found in a water buffalo of Brazilian origin[30] in the late 1990s, yet the outbreak was not officially reported (Claudio Wageck Canal, personal communication, 2011) (Table 2). In 2006, HoBi-like virus in 2 aborted fetuses (dated 2002 and 2004) from southeastern Brazil was described.[7] In the same

country, in 2011, the isolation and sequencing of the 5'UTR region of 3 HoBi-like isolates (SV713/09, SV241/10, SV311/10) was reported.[3] The isolate SV713/09 was identified in a commercial semen sample of a bull, following several descriptions of blind newborn calves in herds using semen from this bull. Subsequent diagnostic tests ruling out the presence of other agents evidenced the association of the HoBi-like virus with these events. Isolates SV241/10 and SV311/10 were detected in buffy coats from animals of herds with history of reproductive failure in Rio Grande do Sul State, southern Brazil, although further clinical and epidemiological information of the outbreaks are missing.[3] Also in Brazil, in the midwestern region, a case of acute gastroenteric disease leading to death of a calf, confirmed the presence of a HoBi-like virus in the calf spleen by reverse transcription polymerase chain reaction (RT-PCR) and nucleotide sequencing (Rodrigues WB, Otonel, RAA., Fritzen, JTT, et al.: 2011. Natural infection of calf with an atypical bovine pestivirus (BVDV-3). *In*: Proceedings of XXII National Meeting of Virology & VI Mercosur Meeting of Virology. Virus Rev Res 16(Suppl 1):74. Atibaia, Brazil).

Cases of HoBi-like viruses infecting cattle in countries other than Brazil were reported for the first time in Thailand.[29] Between 2002 and 2004, an epidemiological study focusing on *Bovine herpesvirus 1* and BVDV infections in 186 herds demonstrated that animals in 4 herds seroconverted to HoBi-like viruses, although no clinical signs were observed. Heat-inactivated serum samples were tested using antigen-capture enzyme-linked immunosorbent assay (ACE) for BVDV, and 1 positive calf serum was identified.[17] The virus was segregated to the HoBi-like group by molecular characterization.[29] A subsequent study involving transfection of viral RNA into bovine turbinate cells yielded the isolate called Th/04_Khonkaen.[22]

In southern Italy, in 2009–2010, an outbreak of respiratory disease affecting 26 calves aged 6–7 months old was reported. The clinical signs included fever (39.4–40.1°C), cough,

seromucoid nasal discharge, leukopenia, and accelerated pulse and breath. Most affected animals received support treatment and recovered within 2 weeks; however, the death of 2 animals was reported. Necropsy findings included severe tracheitis and bronchopneumonia involving the apical lung lobes. HoBi-like virus was detected by quantitative RT-PCR (qRT-PCR) in nasal discharge samples of 6 calves and in the lungs of the 2 dead animals. Virus isolation from lung samples was performed in Madin–Darby bovine kidney (MDBK) cells, and the viruses detected were named Italy-1/10-1 and Italy-1/10-2.[8] In the same herd, in June 2011, the occurrence of reproductive disorders was reported, including abortions in 8 cows between the fourth and sixth months of gestation. HoBi-like viruses were isolated from 2 fetuses. The nucleotide similarity of the E2 glycoprotein and the 5'-UTR region was higher than 99% with the strain Italy-1/10-1 that was isolated in the same herd during a previous outbreak of respiratory disease.[8,9] Also in the same herd, the first cytopathic isolate of HoBi-like virus was identified in the lung of a heifer that died with respiratory disease.[11]

Experimental infection

Experimental infections with HoBi-like viruses performed to date are summarized in Table 3. The first controlled study of acute infection used the first reported isolate of HoBi-like virus, HoBi_D32/00, which was involved the infection of only 2 calves and 2 pigs. The pigs seroconvert to the virus, although no shedding or clinical signs were observed. In contrast, in calves, the virus was detected in buffy coats at day post infection (DPI) 5, and virus shedding was observed from DPI 3 to 6 in inoculated calves in addition to seroconversion. Mild increase in body temperature and slight leucopenia was also observed in the calves.[28]

Using the virus strain (Italy-1/10-1) isolated from the outbreak of respiratory disease, a study comprising the inoculation of 3 groups of 7 animals was performed, as follows: six 1-

month-old calves, five 1-month-old lambs, and two 1-month-old piglets. Clinical signs in calves included moderate hyperthermia, mucoserous nasal discharge, and lymphocyte count decreases. No increase in body temperature was observed in lambs, yet moderate to abundant nasal discharge persisted from DPI 4 to 16. Between DPI 5 and 10, occasional depletion in circulating lymphocytes, no higher than 60% compared to baseline values, was observed. Piglets did not display clinical and/or hematological signs, although seroconversion with median antibody titer of 4 was detected. Seroconversion in calves and lambs peaked at DPI 21, with median titer of 512 and 64, respectively.[10]

In a study using the Thai HoBi-like strain, 3–5-month-old calves were inoculated with Th04_Khonkaen or with a highly virulent BVDV strain. The disease observed in animals inoculated with the HoBi-like strain was milder than that produced by the highly virulent BVDV strain. Animals presented limited to bilateral moderate conjunctivitis, watery-mucoid nasal and ocular discharge and cough. Depletion in the lymphocyte counts between DPI 2–5, returning to normal level at DPI 14 was also observed. A decrease in platelet counts was detected at DPI 7. The other hematological parameters remained within normal range.[20]

The severity of clinical signs during the infection with HoBi-like viruses was compared to clinical signs following infection of cattle with strains of BVDV with typical and enhanced virulence. This study demonstrated that HoBi-like strains are transmissible between infected and sentinel calves, resulting in mild respiratory disease, transient pyrexia, and significant white blood cells depletion, which was similar to infection with a typically virulent BVDV strain. In the group of 6 calves infected with HoBi_D32/00 and 6 sentinel animals, 3 infected calves developed pyrexia. Viral RNA was detected in buffy coat and nasal swabs of infected and sentinel calves, and also, seroconversion was detected in all animals. During the experiment, at

DPI 5, 1 animal developed a temperature of 39.4°C and was found dead the next day. At necropsy, no major lesions in the respiratory or gastroenteric tracts were observed. The virus was isolated from the buffy coat obtained from heart blood and RT-PCR. Sequencing confirmed HoBi_D32/00.[27]

In summary, there is no experimental data indicating an adaptation of HoBi-like viruses in hosts other than ruminants, and all field isolations were from cattle or water buffalo. Pathogenesis studies of HoBi-like viruses in water buffalo may address the issue of HoBi-like virus origin. During natural and experimental infections, HoBi-like viruses seem to cause mild disease in cattle. While most descriptions of HoBi-like viruses in natural infections are related to reproductive disorders, most experimental studies center on reproducing acute disease in calves. Both field and experimental exposures resulted in clinical presentations similar to those observed following infection of animals with typical field strains of BVDV. The higher number of HoBi-like isolates detected in reproductive disorders compared to respiratory disease may reflect a greater impact as a reproductive pathogen or may merely reflect a high number of submissions from cow–calf operations. The increased concern and economic losses during outbreaks of abortions rather than in mild respiratory disease may play an important role and bias the detection of HoBi-like viruses under field conditions.

Diagnostic approaches to detecting HoBi-like viruses

Fetal bovine serum testing

Fetal bovine serum is widely used as a supplement in cell culture media and, inadvertently, many batches of this supplement are contaminated with pestiviruses. The production and packaging of FBS usually employs large pools of fetuses. Increasing the number of fetuses contributing to a commercial batch of FBS simultaneously increases the risk of

including an infected fetus. Even low level contamination of FBS is harmful, as use of this supplement to grow cells can result in the infection of the cells and amplification of the virus. Therefore, high sensitivity tests are required. Antigen-capture enzyme-linked immunosorbent assay, RT-PCR, and qRT-PCR have been used for screening FBS for specific pathogens. The need for continuous development and evaluation of tests for broad spectrum pestivirus detection was further highlighted with the detection of HoBi-like viruses in FBS batches originating from regions with no reported case of HoBi-like virus infections in animals.[36,37] It is desirable to have robust, sensitive tests for testing animals and animal products particularly as it applies to international trade.

There are several commercial tests available for detection of BVDV. Studies have been conducted to see if these tests can be used to detect HoBi-like viruses. A commercial kit was able to detect the isolate HoBi_D32/00 in supernatant of infected cells[2] and the isolate Th/04_Khonkaen in samples of experimentally infected animals and individual serum samples.[29] Interestingly, it was reported that the isolate IZSPLV_To was not detected by an ACE kit in a FBS batch.[25] In the current authors' experience, more than 90% of FBS batches positive for pestivirus by molecular tests (RT-PCR) are not detected by ACE (personal observation). Therefore, FBS testing should not rely only on the use of ACE. However, ACE may be used with other diagnostic assays. The widely used pairs of primers HCV90-HCV368[26] and 324-326[34] were designed to detect a broad range of pestiviruses. The selection of the primer pair 324-326 for quality control of FBS has been described.[19] However, whereas this pair of primers is highly sensitive for BVDV and BDV strains, failure to detect HoBi-like viruses may occur. In order to address this issue, the current authors performed ten-fold dilutions of supernatant of bovine turbinate cells infected separately with pestivirus isolates, including HoBi_D32/00 and Italy-

280/11A with titer from $10^{3.7}$ to $10^{-0.7}$ TCID/50 µl. Supernatant of cells infected with the Brazilian HoBi-like strain SV713/09 were irradiated, and RNA was also tested. Reverse transcription PCR using the primer pairs HCV90-HCV368 and 324-326 was performed following conditions described previously.[26,34] Both pan-pestivirus primer pairs tested displayed sensitivity of approximately $10^{0.7}$ TCID/50 µl to BVDV strains while the sensitivity for HoBi-like strains was approximately $10^{3.7}$ TCID/50 µl (Fig. 4). Tests were unable to detect virus RNA from irradiated supernatant of cells infected with the HoBi-like strain SV713/09 using these pan-pestiviruses primers.

To date, 2 specific RT-PCR reactions for HoBi-virus detection have been published. However, neither was validated under field diagnostic conditions. A qRT-PCR was successful in detection of HoBi-like viruses in FBS and in samples from calves acutely infected under experimental conditions.[20,23,36] Cross-reaction with samples harboring high titers of BVDV-2 was observed.[12] The second reaction was a nested-RT-PCR.[12] Its application in screening large numbers of samples may be labor- and time consuming. Further, the increased number of steps in the reaction could lead to cross contamination. To date, there is no test fully validated to detect all bovine pestivirus species and/or to specifically detect HoBi-like viruses. Again, the high variability among pestiviruses indicates that laboratory diagnosis of these viruses should not rely on the use of a single test.

Virus detection in acutely and persistently infected animals

Commercial ACE kits are available and are designed to detect PI animals. Viral antigen may be detected in acutely infected animal samples such as buffy coats, nasal swabs, and tissues. These tests may also detect PI animals harboring HoBi-like viruses. However, studies validating

the use of commercial BVDV tests to identify PI animals infected with HoBi-like viruses are still needed. These tests cannot differentiate between BVDV and HoBi-like viral infection.

The isolation and amplification of HoBi-like viruses in cell cultures seems to be easily performed using bovine turbinate cells and MDBK cells following standard protocols for BVDV isolation.[2,9] Because most pestiviruses detected during acute and persistent infection are noncytopathic, virus identification requires the use of a post-test, usually RT-PCR, fluorescent antibody testing, or immunoperoxidase. The commercially available monoclonal antibody (mAb) 15C5 was shown to detect most BVDV isolates in immunoassays[13] and was also shown to detect HoBi-like viruses[2] (see reactivity of mAb with HoBi-like viruses and other pestivirus species in Table 4). Differentiation of animals acutely or persistently infected with HoBi-like viruses or BVDV can be performed by virus isolation followed by RT-PCR.[2,12,23] Nucleotide sequencing of target sequences are required to confirm the agent identity. The use of commercial BVDV tests may be successful in some situations, although the differentiation between BVDV and HoBi-like viruses will require further testing such as RT-PCR and sequencing.

Identification of exposed animals

Commercially available ELISA kits to detect BVDV antibodies (Ab-ELISA) were recently reported to yield false-negative results upon testing of serum samples of calves harboring moderate to high antibody titers against HoBi-like viruses.[2,20] An additional concern is the time frame necessary for the development of detectable titers of antibodies by infected animals (i.e., up to 3-4 weeks).[20,27] Also, differentiation as to whether the animal is harboring antibodies to BVDV, HoBi-like virus, or both is not possible with the current Ab-ELISA kits. Recently, 2012, a novel assay, called microsphere immunoassay, was used to detect antibodies against BVDV-1 and HoBi-like viruses based on binding to the glycoprotein Erns of a HoBi-like

isolate.[33] Differentiation between BVDV-1 and HoBi-like immune response is not possible with this test in its present format.

Virus neutralization test (VNT) using HoBi-like strains can be used to detect exposure,[2,20] and seems to be promising for differentiation between animals exposed to BVDV or HoBi-like viruses through the comparison of antibody titers against these viruses (unpublished data). It seems that cross-reaction between HoBi-like viruses and BVDV is limited. Therefore, serum samples harboring antibody titers to HoBi-virus 2- or more-fold higher than to BVDV strongly suggest exposure to HoBi-like viruses. Although VNT is time consuming and requires trained staff and laboratory structure for cell culture, to date, it seems the most suitable approach for detection and/or differentiation of animals exposed to BVDV and/or to HoBi-like viruses. Nonetheless, validation of this method is still required. At the herd level, comparative viral neutralizing titers between HoBi-like viruses and BVDV strains may indicate whether a herd was exposed to HoBi-like viruses. The detection of high titers to BVDV and/or HoBi-like viruses in nonvaccinated herds may suggest the presence of PI animals. Also, the presence of higher titers to HoBi-like viruses than BVDV species in herds vaccinated for BVDV is also an indicative factor of exposure to the emerging viruses because inactivated and modified live BVDV vaccines induce only limited cross-reaction against HoBi-like viruses. Regardless, the best diagnostic approach seems to be the search for PI animals by virus isolation followed by molecular characterization.

Conclusion

Although the origin remains unknown, data gathered to date indicates the widespread distribution of HoBi-like viruses in Brazilian cattle, and to a lesser extent, in other selected countries. The extent of economic losses due to HoBi-like virus infection and the prevalence of

exposed animals are as yet unknown and, thus, are goals for future investigations. Further, there is a need for improved diagnostics, as the lack of accurate diagnostic tools for virus or antibody detection and the scarce information regarding the clinical presentation of the disease may lead to misdiagnosis at both the field and laboratory levels. To summarize, the clinical signs associated with HoBi-like infection may be undistinguishable from those observed in typical BVDV infections and, therefore, the impact of the disease may be underestimated due to misdiagnosis. The genetic and antigenic similarities among HoBi-like viruses and BVDV species may also promote a false idea that BVDV diagnostic tests are accurate enough to be used for HoBi-like virus detection. Veterinary diagnostic laboratories and biological product companies need to be aware that, whereas the diagnosis may be successfully performed in certain situations using BVDV tests, the percentage of misdiagnosis may be high, resulting in uncontrolled spread of the viruses and potentially leading to significant economic losses.

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Table 1. Worldwide distribution and control of pestiviruses.

Pestivirus species	Status*	Target host	Distribution	Eradication efforts	Vaccine available
Classical swine fever virus	Recognized by ICTV	Swine	All continents	Eradicated from Canada and United States	Yes
Bovine viral diarrhea virus 1	Recognized by ICTV	All even-toed ungulates	All continents	Eradication nearing completion in Scandinavia, in process in several other regions in Europe	Yes
Bovine viral diarrhea virus 2	Recognized by ICTV	All even-toed ungulates	All continents	Eradication nearing completion in Scandinavia, in process in several other regions in Europe	Yes
Border disease virus	Recognized by ICTV	Primarily small ruminants	All continents	None	No
Giraffe pestivirus	Putative	Giraffe	Africa	None	No
Pronghorn pestivirus	Putative	Pronghorn antelope	North America	None	No
Bungowannah pestivirus	Putative	Swine	Australia	None	No
HoBi-like viruses	Putative	Cattle and water buffalo	South America, Southeast Asia, and Europe	None	No

* ICTV = International Committee on the Taxonomy of Viruses (http://www.ictvonline.org).

Table 2. Brief description of HoBi-like viruses detected to date (by sampling date).*

Place of detection	Origin	Sample	Sampling date	Reference
Switzerland	Brazil–NI	Water buffalo–NI/Cell-line contaminant	Late 1990†–NI	30
Sweden	Thailand	Calf serum	Between 2000–2004	29
Brazil	Brazil	Aborted bovine fetus	2002 and 2004	7
Brazil	Brazil	Semen	2009	3
		Buffy coat	2010	
Italy	Italy	Nasal swab and lung	Between 2009–2010	8
Italy	Italy	Aborted fetus	2011	9
Italy	Italy	Lung	2011	11
Switzerland	Brazil	Fetal bovine serum	NI	28
Sweden	South America	Fetal bovine serum	NI	24
Italy	Brazil	Fetal bovine serum	NI	25
Brazil	Brazil	Calf spleen	NI	Rodrigues et al. 2011‡
Sweden	Australia, Brazil, Canada, Mexico, and United States	Fetal bovine serum	NI	37
Sweden	Australia	Fetal bovine serum	NI	36

* NI = not informed.

† Claudio Wageck Canal, personal communication, 2012.

‡ Rodrigues WB, Otonel, RAA., Fritzen, JTT, et al.: 2011. Natural infection of calf with an atypical bovine pestivirus (BVDV-3). *In*: Proceedings of XXII National Meeting of Virology & VI Mercosur Meeting of Virology. Virus Rev Res 16(Suppl 1):74. Atibaia, Brazil.

Table 3. Concise description of clinical manifestation following experimental infection of calves with HoBi-like strains.*

Strain/Reference	Temperature	Respiratory signs	Decrease in WBC	Viremia	Shedding	Seroconversion
HoBi_D32/00[28]	Mild increase	No clinical signs	Slight leucopenia	At DPI 5	Nasal shedding from DPI 3–6	Verified
Italy-1/10-1[10]	Approx. 40°C at DPI 3 and 7	Mucoserous nasal discharge from DPI 3–10	Reaching approx. 50% from DPI 3–10	From DPI 5–24	Nasal and fecal shedding from DPI 5–21 and 7–21, respectively	Verified at DPI 14; median titer of 512 at DPI 21–28
Th04_Khonkaen[20]	Approx. 39°C at DPI 7–10	Conjunctivitis, nasal and ocular discharge, and moderate cough	Approx. 40% from DPI 5–7	Virus isolation from DPI 5–9	Nasal shedding from DPI 5–7 in nasal swabs	Mean titer at DPI 14 of 54 and 1763 at DPI 42
HoBi_D32/00[27]	Mild increase	No clinical signs	Reaching 65% with peak around day 9	Intermittent from DPI 3–9	Intermittent virus detection in nasal secretion from DPI 3–9	Verified at DPI 20; VNT titer ranging from 4–512 at DPI 42

* WBC = white blood cell count; DPI = day post infection; VNT = virus neutralization test.

Table 4. Reactivity of monoclonal antibodies with different pestivirus species virus isolates.*

Monoclonal antibodies	BVDV-1a			BVDV-1b			BVDV-1c		BVDV-2a			HoBi-like		BDV
	VM	7443	C24V	NY-1	NE	TGAN	AusB675	AusB843	890	1373	296nc	HoBi_ D32/00	Italy- 280/11A	BD31
Erns														
15C5	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E2														
N2	+	+	+	+	+	+	+	+	+	+	+	+	+	+
19F7	+	+	+	+	+	+	+	+					+	
10.11.2	+	+	+	+	+	+	+	+					+	
BZ25									+	+	+			
BZ81									+	+	+	+		
BZ82									+	+	+	+	+	
CA36	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CA80			+	+	+	+	+	+						
CA82		+	+	+	+	+	+	+					+	
NS2/3														
24.8	+		+	+	+	+	+	+		+	+	+	+	+
1.11.3	+	+	+	+	+	+	+	+				+		
8.12.7	+	+	+	+	+	+	+	+		+	+	+	+	+
20.10.6	+	+	+	+	+	+	+	+	+	+	+	+	+	+

* BVDV = *Bovine viral diarrhea virus*; BDV = *Border disease virus*. Table was adapted from Bauermann et al., 2012. Shading indicates positive binding.
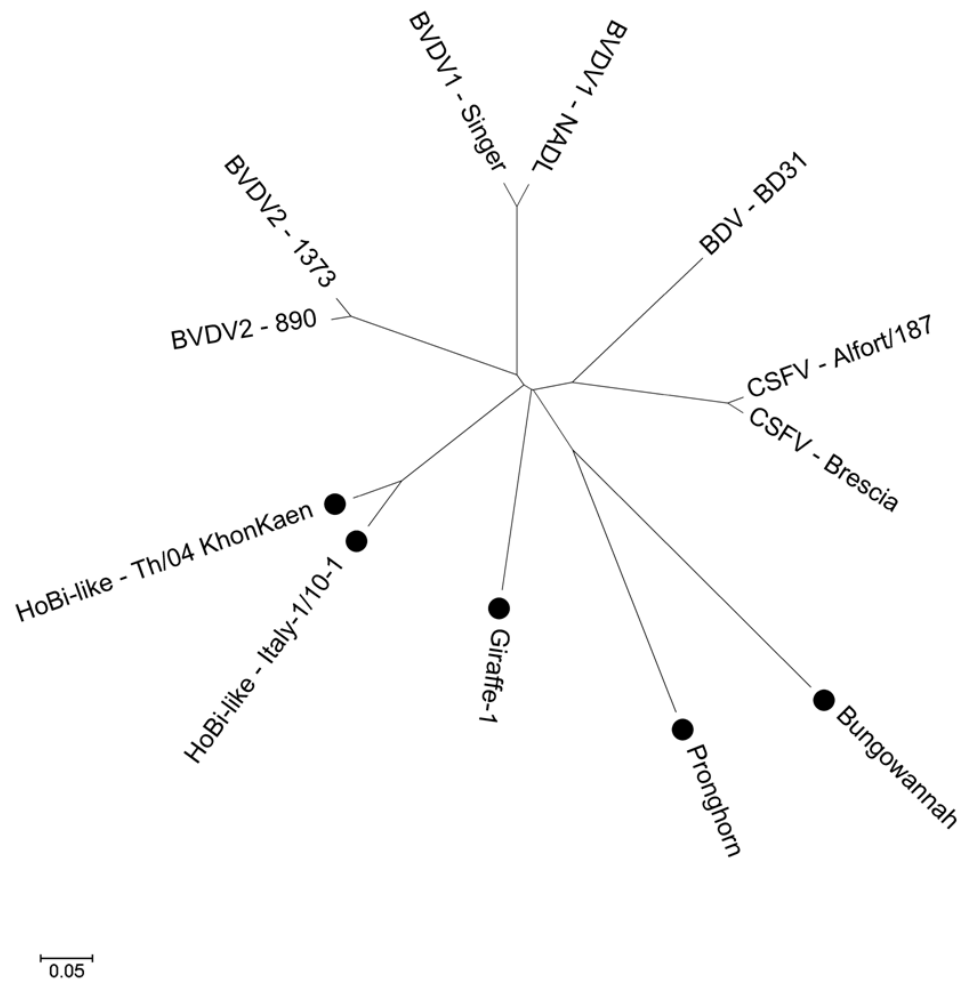
Figure 1. Phylogenetic tree of *Pestivirus* genus. Official and putative species represented. The isolates in the 4 putative species are market with "●". Analyses were conducted in MEGA5,[31] using the neighbor-joining method; distances were computed using the Poisson correction method. Region analyzed consisted of the amino acids from the polypeptides N^{pro}; C; E^{rns}; E1 and E2.
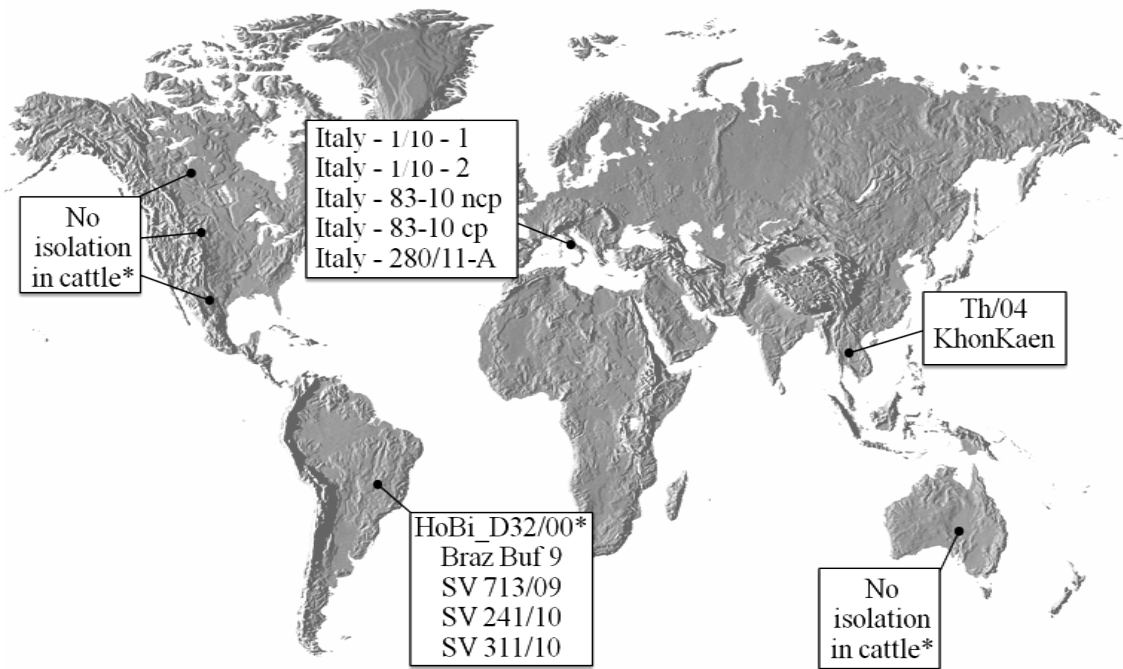
Figure 2. Distribution of HoBi-like viruses isolated to date. *Identification of HoBi-like viruses in fetal bovine serum batches claimed to be originated in these countries. cp = cytopathic; ncp = noncytopathic
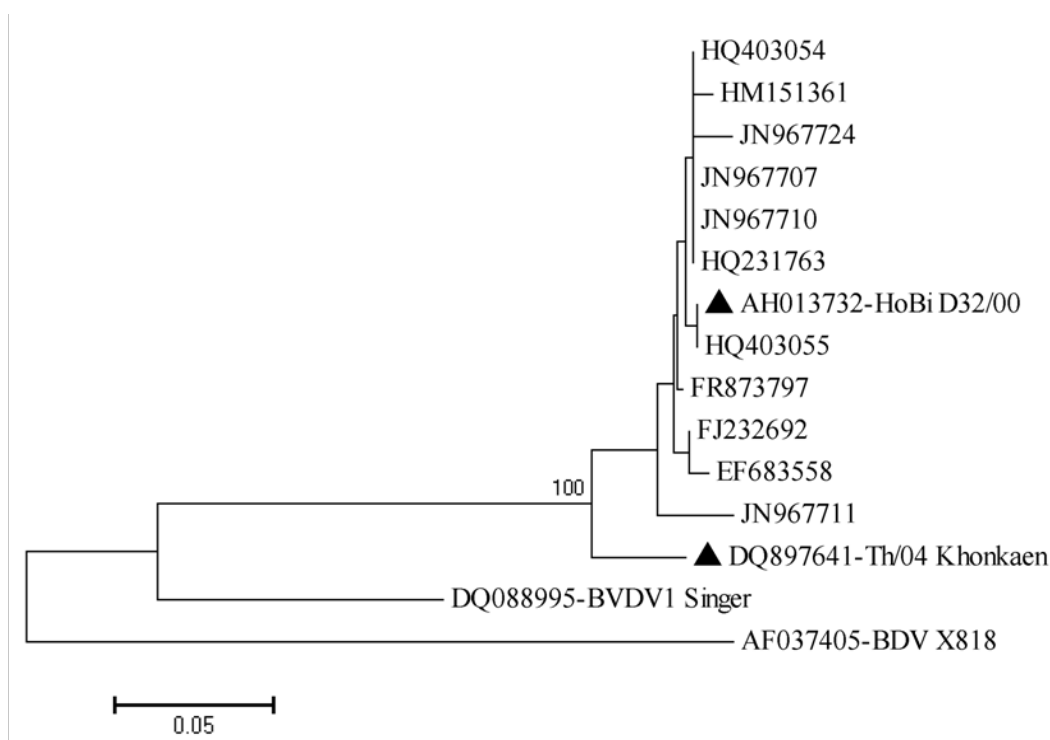
Figure 3. Representation of several HoBi-like pestiviruses based on the 5'–untranslated region (5'-UTR). Sequences are identified by GenBank accession number (available at http://www.ncbi.nlm.nih.gov/pubmed/). "▲" was used to mark the prototypes of 2 possible HoBi-like virus lineages (HoBi_D32/00 and Th/04_Khonkaen). Evolutionary analyses were conducted in MEGA5,[31] using the neighbor-joining method. Distances were computed using the Kimura 2-parameter method.
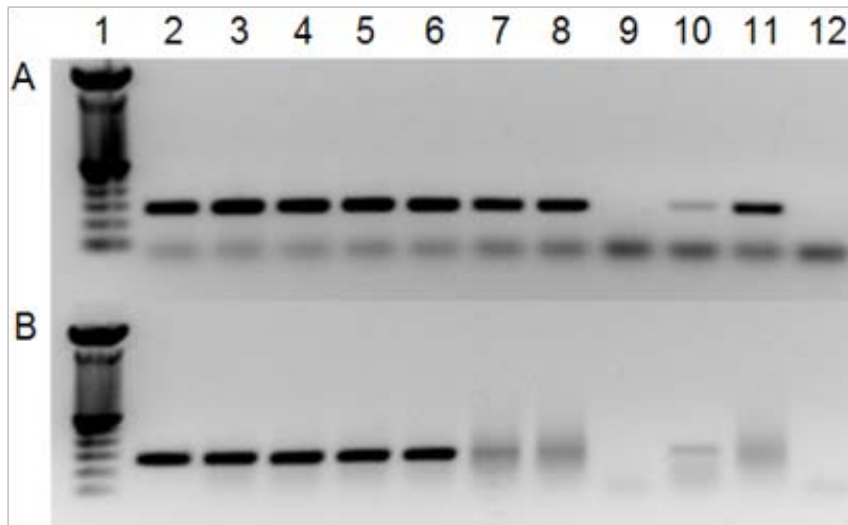
Figure 4. Reverse transcription polymerase chain reaction (RT-PCR) using the pan-pestivirus primers 324-326 (**A**) and HCV90-HCV368 (**B**). Total RNA extracted from the supernatant of cells infected with virus titer of $10^{3.7}$ TCID/50 µl were used as template. Lane 1: 100-bp marker; lane 2: *Bovine viral diarrhea virus 1* (BVDV-1)-Van Meter; lane 3: BVDV-1-7443; lane 4: BVDV-1-Nebraska; lane 5: BVDV-2-296nc; lane 6: BVDV-2-1373; lane 7: BDV-BD31; lane 8: BDV-WABD; lane 9: HoBi-like-SV713/10 (irradiated sample); lane 10: HoBi-like-HoBi_D32/00; lane 11: Pronghorn virus; lane 12: negative control.

4. CAPÍTULO 2

Antigenic relationships between *Bovine viral diarrhea virus* and HoBi virus:

possible impacts on diagnosis and control

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Abstract

The emergence of a newly recognized group of pestiviruses in cattle, the HoBi-like viruses, requires an evaluation of the available diagnostic tools and vaccines. The present study compared antigenic characteristics of *Bovine viral diarrhea virus* (BVDV) strains and HoBi virus. This comparison was based on detection of a HoBi virus and antibodies against it by commercial enzyme-linked immunosorbent assays (ELISAs) and the level of cross neutralizing antibodies present in sera from animals vaccinated with BVDV. Reactivity with a panel of monoclonal antibodies (mAbs) revealed greater cross reactivity between BVDV species (BVDV-1, BVDV-2) and HoBi epitopes within E^{rns} and NS2/3 proteins than between epitopes located in the E2 glycoprotein. These results suggest that a diagnostic test designed to detect both BVDV species and HoBi could be based on E^{rns} or NS2/3 epitopes, while variation among E2 epitopes could be exploited in tests for differentiation of pestivirus species. The threshold of detection of HoBi virus by an antigen capture ELISA kit based on detection of E^{rns}, was statistically similar to that for BVDV. In contrast, two commercial ELISA kits designed to detect antibodies against BVDV, missed 22.2% and 77.7% of serum samples harboring HoBi-virus neutralizing antibodies. In addition, sera of calves vaccinated with BVDV-1 and BVDV-2 presented low neutralizing activity against HoBi-virus. These results demonstrate that, in spite of antigenic similarities, HoBi is antigenically distinct from both BVDV species. Detection and control of HoBi infections in cattle would, thus, require the development of new diagnostic reagents and reformulation of current vaccines.

Key words: Enzyme-linked immunosorbent assay, HoBi-like, vaccines, virus neutralization.

Introduction

Pestiviruses are enveloped, positive sense RNA viruses, members of the family *Flaviviridae*. Currently, this genus is comprised by four recognized virus species: *Bovine viral diarrhea virus* 1 (BVDV-1) and 2 (BVDV-2), *Border disease virus* (BDV), and *Classical swine fever virus* (CSFV).[27] Pestiviruses of bovine origin, mainly represented by the two species of BVDV, are globally distributed and lead to major losses in dairy and beef herds. Respiratory, gastroenteric, and reproductive diseases are among the list of clinical consequences of BVDV infection in cattle.[2] Economic analysis revealed that economic losses might reach around $88/animal.[12]

In addition to the recognized pestivirus species, several other putative species have been proposed. These include giraffe, based on the H138 virus, which was isolated from giraffe in Kenya,[4] Pronghorn virus, isolated from a blind pronghorn antelope in the USA,[29] the Bungowannah virus, isolated from an Australian outbreak of myocarditis syndrome in swine,[14] and the HoBi-like viruses. HoBi-like viruses are unique among the putative pestivirus species since they have been found in more than one continent, have been isolated from multiple animal species and infection results in clinical presentations that cannot be distinguished from those associated with BVDV infection.[7,8,22,25,26]

The first isolate of HoBi (D32/00_HoBi) was described in Switzerland, contaminating a batch of fetal bovine serum (FBS) imported from Brazil.[22] Subsequently, other HoBi-like viruses were identified, including two isolates present in FBS batches from South America,[16,17] the isolate CH-Kaho/cont, a cell culture contaminant,[26] the isolate Brz buf 9 found in buffaloes in Brazil,[26] Th/04 Khonkaen, found in serum of calves in Thailand,[25] and two isolates identified in aborted fetuses in Brazil.[7] In Italy, 2010, a

HoBi-like virus was isolated during an outbreak of severe respiratory disease in calves, being the first description of this virus in Europe.[8]

The genome of pestiviruses is a single stranded RNA molecule around 12.3kb in length, containing a long open reading frame (ORF) flanked by two untranslated regions (5' and 3' UTRs). The long ORF encodes a single polyprotein that is co- and post-translationally processed into 12 viral mature polypeptides in the following order: N^{pro}-C-E^{rns}-E1-E2-p7-NS2/NS3-NS4A-NS4B-NS5A-NS5B. Based on the phylogenetic analysis of the 5' UTR sequences, which is widely used for classification of genus members, [3,11,19,22] viruses within the HoBi-like group share a high level of homology (higher than 93%). In contrast, the similarity between these viruses and BVDV genotypes 1 and 2 is around 75% and 80%, respectively.[15,17,25] Although this new pestivirus group has not been definitively classified, it has been proposed to compose a third BVDV genotype (BVDV-3),[15] or a fifth pestivirus species (HoBi-like),[22,25] the last is used is the present article.

Control and/or eradication programs for BVDV developed by several countries are based on the identification and elimination of persistently infected animals (PI), biosecurity measures to prevent the introduction of infected animals, and surveillance strategies to monitor for BVDV exposure. These programs may also incorporate vaccination, depending on the incidence of BVDV exposure in the region.[13] Reliable diagnostic tests, to monitor both for the detection of persistently infected animals and exposure to BVDV, are crucial for the success of control programs and for the maintenance of BVDV-free status. In this sense, several diagnostic tests, for detection of virus or specific antibodies, are available for BVDV.[13] Virus isolation (VI) is considered the most accurate virus detection technique.[9] However, if the samples are inappropriately conserved or autolysed, enzyme-linked immunosorbent assays (ELISA)

for antigen detection, or RNA detection by reverse transcription-polymerase chain reaction (RT-PCR) may yield higher sensitivity.[6,21,23] For antibody detection, virus neutralization test (VNT) and ELISAs are the most commonly used techniques. Reports of the isolation of HoBi-like viruses contaminating cells and FBS batches from South America have caused concern. Fetal bovine serum is widely used to propagate tissue culture cells and for propagation of viral strains for vaccine production. As HoBi-like viruses have been isolated from FBS, the contamination of vaccines could result in the introduction of HoBi-like strains into new regions. Further, these uncharacterized pestiviruses may be a threat for BVDV control and eradication programs worldwide. As infection with HoBi-like viruses has been linked to reproductive and respiratory disease, the introduction of HoBi-like viruses into naïve cattle could have serious economic impact.[24] Thus, surveying for the presence of HoBi-like strains in animals, animal products, and biologics is important.

The goal of the present study was to investigate the suitability of monoclonal antibodies (mAbs) and routinely used BVDV tests for the diagnosis of HoBi virus. The antigenic cross-reactivity between HoBi virus and other pestivirus species was also investigated in order to identify proteins with conserved epitopes and to evaluate levels of cross protection resulting from vaccination with a commercial vaccine against BVDV.

Materials and methods

Viruses and cells

Eighteen non-cytopathic isolates of pestiviruses[a] were used: BVDV-1a (VM; C24V; 7443), BVDV-1b (TGAN; NE; NY-1), BVDV-1c (AusB675; AusB730; AusB843), BVDV-2a (296nc; 890; 1373), BDV (Idaho207; CB5; BD31) and three isolates from putative new pestivirus species (HoBi; Pronghorn; Bungowannah).[14,22,29]

Viruses were propagated in bovine turbinate cells [a] (BT), typically used between passages seven and twelve. Cells were grown in minimal essential medium (F15 Eagle medium[b]), supplemented with l-glutamine (final concentration, 1.4mM), gentamicin (final concentration, 50 mg/l), and 10% FBS, tested free of BVDV and antibodies against BVDV, by RT-PCR and virus neutralization test (VNT), respectively.[19,20] Cells were tested by RT-PCR and were found free of BVDV.[19] Cells and serum were also tested and found to be free of HoBi by RT-PCR.[19] The primers forward 5'-GGGTAGTCGTCAATGGTTCGA-3' and the reverse 5'-TAGCAGGTCTCTGCAACACCC-3', predicted to amplify a fragment of the 5'UTR of HoBi virus were used. Cell culture monolayers were inoculated with the respective viruses when they were approximately 70% confluent. After inoculation, cultures were incubated at 37°C for 72-96 h. Cultures were harvested by freezing at - 20°C. After freeze–thaw cycle followed by centrifugation for 10 min at $1,000 \times g$, supernatants were collected, aliquoted and stored at - 80°C until use. The virus stocks were titrated in 96-well microtiter plates and the titers were expressed as 50% tissue culture infective doses ($TCID_{50}$).[18]

Monoclonal antibody (mAb) binding

A panel of 16 monoclonal[a,c] antibodies (mAbs) with specificity for the BVDV proteins, E^{rns}, N2, or NS2/3 was used to compare antigenic similarity among pestivirus species. For this purpose, 18 pestivirus isolates (Table 1) were individually inoculated (final concentration of 10^4 $TCID_{50}$/ml) in BT cells grown in 96-well plates, and left to adsorb for 90 min. The first two columns of the plate were used as positive controls, being inoculated with BVDV-1a (7443) and BVDV-2a (890), respectively. The last column was kept as negative control. Culture medium with 10% fetal bovine serum was added and plates were kept at 37°C for 96 h. Cells were then fixed and subjected to an

immunoperoxidase test (IMPT) executed as previously described.[1,20] MAbs with availability of ascites fluids were diluted 1:1000 in PBS with bovine albumin (PBS-BSA 0.01%) and used as primary antibody. In the absence of ascites (Table 1), the supernatants of hybridoma cell cultures were diluted 1:2 in PBS-BSA 0.01%. Goat anti-mouse IgG conjugated with horsehardish peroxidase[d] was used as secondary antibody and 3-amino 9-ethylcarbazole was used as the substrate. IMPT for all mAbs was performed for each virus in two sets of plates in duplicate wells.

Bovine viral diarrhea virus antigen capture ELISA (ACE)

To determine whether a commercially available BVDV ACE test would be suitable for detection of genetically divergent pestiviruses, ten-fold dilutions of viral stocks in MEM were prepared. Three strains from each of the five genotypic groups (BVDV-1a-c, BVDV-2, BDV) from recognized pestivirus species and HoBi virus were diluted (10^6-10^4 $TCID_{50}$). Due the low initial virus titers, dilutions from 10^5 to 10^3 $TCID_{50}$ were used for Bungowannah and Pronghorn viruses (Table 2). Detection of pestiviruses was performed using a commercially available BVDV ACE kit,[e] following the manufacturer's instructions. The optical density (OD) value was used to calculate the sample to positive (S/P) ratio, where samples were considered positive with this value above 0.39. S/P ratios for dilutions of each virus were used to calculate the R-square and logarithmic regression, using a statistical software.[f] Based on the regression equation, the lowest titer with positive result for each virus was calculated, and the average of the lowest titer detected for each BVDV sub-genotypes and BDV species was determined. Then, means were submitted to Tukey-Kramer test, using statistical analysis software.[g]

Neutralizing activity of HoBi antisera against other pestiviruses

Sera from two groups of calves infected with HoBi virus were used (Ridpath et al., manuscript in preparation). Briefly, the first group was composed by one calf inoculated intranasally with 2 ml of HoBi virus (10^5 $TCID_{50}$) and three calves kept in contact. In the second group, two calves were inoculated in the same conditions and three animals were kept in contact (Table 3). The sera of animal #504 (group 1) and a pool from animals from group 2 (#509, #510 and #513) from the day 42 post inoculation (pi) were selected. These sera were tested by VNT against viruses listed (Table 3) and the results were visualized through IMPT as previously described.[1,20] Briefly, sera dilutions from 1:4 to 1:256 were incubated, individually, with 10^4 $TCID_{50}$ of each virus for 90 min at 37°C in 5% with CO_2. Suspensions of BT cells were added and after incubation of four days at 37°C in 5% CO_2, monolayers were fixed and subjected to IMPT. The mAb used for detection of Bungowannah antigens was 15C5[c]; the other viruses, with the exception of Pronghorn, were detected using antibody N2[a]. Neutralization of Pronghorn virus was verified by RT-PCR, due the lack of specific mAbs. For RT-PCR testing, RNA was extracted from VNT dilutions of each serum, individually, using a kit[h]. RT-PCR was performed using panpestivirus primers, following conditions described previously.[28] In the presence of negative result, the serum dilution was considered positive for neutralizing activity. VN titers were expressed as the reciprocal of the highest dilution that neutralized viral infectivity.

Detection of HoBi serologic response with BVDV antibody detection ELISA kits

To determine whether the ELISA kits designed to detect BVDV antibodies are adequate to detect antibodies to HoBi viruses, two groups of animals were inoculated with HoBi, keeping some animals as sentinels, for a total of nine animals (Table 4). Sera obtained at the day of birth, and days 9, 18, 42 post inoculation were tested for antibodies by

ELISA kits from two companies, ab-ELISA-1[i] and ab-ELISA-2,[j] according to the manufacturers' instructions. In parallel, virus neutralization test using HoBi virus was performed on these samples (Table 4). The titers were expressed as the reciprocal of the highest dilution that neutralized viral infectivity.

Neutralizing antibody response to pestiviruses following BVDV vaccination

In order to analyze the cross-serological response between BVDV and uncharacterized pestiviruses, fifty calves were immunized twice, 30 days apart, with an inactivated vaccine[k] containing BVDV-1 and 2. After 8 weeks, sera was collected and tested by VNT and IMPT against 13 pestiviruses (Table 5). The presence/absence of viral antigens in cells was used as indicator of presence/absence of neutralizing antibodies. In this way, IMPT was performed using mAbs 15C5 for Bungowannah and N2 mAb for the other viruses. Pronghorn virus was detected by RT-PCR. The titers were expressed as the reciprocal of the highest dilution that neutralized viral infectivity.

<div align="center">

Results

</div>

Monoclonal antibody (mAb) binding

The use of a panel of mAbs demonstrated conserved epitopes shared among HoBi, BVDV-1 and BVDV-2 isolates (Table 1). MAb 15C5, specific for the pestivirus E^{rns} glycoprotein, reacted with the highest number of isolates, failing to detect only the Pronghorn virus. On the other hand, mAbs against E2 displayed a variable range of reactivity. In fact, only two E2 mAbs (N2 and CA36) reacted with both BVDV genotypes and HoBi virus. Seven mAbs bound only to isolates within BVDV genotypes and two mAbs (BZ81 and BZ82) recognized BVDV-2 isolates and HoBi. However, mAbs against E2 failed to react with Bungowannah and Pronghorn pestiviruses. The four NS2/3 mAbs used (20.10.6; 1.11.3; 8.12.7; 24.8) were able to identify most pestiviruses isolates, but among the uncharacterized pestiviruses used in

this study, only HoBi was detected by these mAbs. MAb 1.11.3 bound to BVDV-1a-c

isolated and to HoBi virus. According to these results, an IMPT test designed to detect

a wide range of pestiviruses should include mAbs N2, CA36, 8.12.7, 20.10.6 and 15C5,

which are able to recognize a wide range of isolates. In addition, differential binding by

mAbs 1.11.3 and BZs 25, 81 and 82 may allow differentiation between HoBi and the

two BVDV genotypes.

BVDV antigen capture ELISA (ACE)

The BVDV antigen capture ELISA kit was able to detect HoBi virus, but failed to detect

Pronghorn and yielded inconclusive results with Bungowannah virus. All strains of

BVDV and BDV used in the test yielded positive results. Using logarithmic regression,

the lowest HoBi titer detected by ACE was determined to be $10^{4.5}$TCID$_{50}$ (Table 2).

Analyses with Tukey-Kramer test using a statistics software[g] showed no significant

difference between the threshold of HoBi detection and the average of the lowest titer

detection of other BVDV genotypes. For these viruses, the threshold varied from $10^{4.3}$ to

$10^{4.8}$ TCID$_{50}$. The only significant difference in the threshold of detection was verified

with BDV, where the minimum titer detected was $10^{5.4}$ TCID$_{50}$. These results show that

ACE test is adequate to detect HoBi virus, but could not be used to differentiate

between HoBi and BVDV-1 or BVDV-2 isolates.

Neutralizing activity of HoBi antisera against pestiviruses

The neutralizing activity of HoBi antisera against BVDV-2a by VNT was higher than

against other pestiviruses (Table 3). The two serum samples tested (#504 and pool)

displayed, respectively, the same neutralizing titer (32 and 512) against HoBi and the

isolate 890 (BVDV-2a). The VN titers against BVDV-2a 296nc were 4 and 128,

respectively. For isolates from sub-genotypes of BVDV-1a-b, the titers ranged from < 4

to 16 for calf #504; and from 4 to 32 for the pool. No neutralization was observed against BVDV-1c, BDV, and the other uncharacterized pestiviruses.

Detection of HoBi seroconversion using BVDV antibody detection ELISA kits

Positive reaction was only detected in sera collected at day 42 pi, by both kits. Ab-ELISA-1 detected 3 out of 9 positive samples; ab-ELISA-2 detected 7 out of 9 positive samples (Table 4). By VNT, at day 42 pi, all samples were positive, with titers ranging from 64 to 512. Two samples (animals #512 and #514) were positive earlier, at day 18 pi, with VN titer of 4.

Neutralizing antibodies to pestiviruses in sera of BVDV vaccinated cattle

Eight weeks after vaccination of cattle with two doses of a vaccine containing inactivated BVDV 1 and 2 strains, sera from 50 animals were collected and tested by VNT against 12 pestiviruses (BVDV-1-a-c, BVDV-2, BDV and atypical isolates). As expected, the highest neutralizing titers were observed against viruses from the homologous genotypes (Table 5). GMT reached 141 and 111 for BVDV-2 strains 296nc and 890, respectively. For isolates of BVDV-1a-b, GMT ranged from 46 to 105. Lower GMT was found for BVDV-1c, with values of 34 and 13 for isolates AusB843 and AusB675, respectively. For BVD strain BD31, the GMT was 3.8 and 5.0 for HoBi. The neutralization for Pronghorn was 1.5; no neutralization was observed against Bungowannah.

Discussion

The emergence of novel bovine pestiviruses, including the HoBi-like viruses, and their potential introduction into naïve cattle populations represents a major concern for beef and dairy producers. The contamination of FBS with HoBi-like viruses may also pose a threat for the safety of biological products. Therefore, the availability of adequate reagents and suitable techniques to promptly detect these agents is important for BVDV

control programs, and for surveillance of animals and animal products subject to international trade. In particular, HoBi virus poses the greatest threat among the uncharacterized pestiviruses, based on reports of persistent infection, apparent dissemination in different continents and its detection in commercial FBS batches.[16,17,22,25,26]

A panel of mAbs showed that HoBi virus was recognized by four mAbs directed to the glycoprotein E2, the major and most variable envelope BVDV glycoprotein[5], with greater similarity with BVDV-2 (Table 1). A closer relationship between HoBi and BVDV-2 (than between Hobi and BVDV-1) was also observed in VN tests performed in the course of this study, in which serum against HoBi displayed higher neutralizing activity against BVDV-2 isolates than against BVDV-1 isolates (Table 3).

The antigen capture ELISA detected HoBi virus in culture supernatants with the same sensitivity as it detected both BVDV genotypes. On the other hand, inconclusive and negative results were obtained for Bungowannah and Pronghorn viruses, respectively (Table 2). These findings are not completely unexpected since phylogenetic analysis has shown that these viruses are more distant from the recognized pestivirus species than HoBi-like virus.[14,29] MAb 15C5, directed to a well conserved epitope within E^{rns},[5] was able to recognize Bungowannah in IMPT (Table 1). In contrast, an ACE test based on this mAb yielded inconclusive results. Probably, technical adjustments in the test would be able to increase the sensitivity. On the other hand, none of the mAbs tested bound to Pronghorn antigens. Analysis of Pronghorn E^{rns} revealed an amino acid mutation at position 315 (data not shown), a change in this site has already been described as responsible for the failure of mAb 15c5 to recognize BVDV-2 isolate AU501.[10] In addition, mutations in the adjacent sites 314 and 316 were also found, which might explain the failure of mAb biding. A new version of this kit is now

commercially available, in which a second mAb was added for detection of isolates with mutations similar to AU501. Thus, the new kit may be able to detect Pronghorn and Bungowannah viruses. Nevertheless, further studies are required to confirm this information. Although genetically more distantly related to BVDV than Pronghorn virus[14,15,29] Bungowannah virus was recognized by mAb 15C5 in IMPT. Interestingly, the E^{rns} amino acid residue at position 315 is conserved in this virus (data not shown).

The high sensitivity of the ACE test, in which HoBi detection titer was comparable to those for BVDV genotypes, indicates that the ACE test may be adequate for HoBi detection. This finding agrees with another study that reported the detection of the HoBi-like Th/04 Khonkaen, using this kit.[25] In contrast, the HoBi-like – isolate IZSPLV_To was not detected using the same test.[17] Further studies including a broader selection of HoBi-like isolates are required to determine whether this kit would be suitable for surveillance of HoBi-like viruses.

The evaluation of ELISA kits for BVDV antibodies demonstrated the need for specific diagnostic tests for HoBi-like viruses, since they failed to detect a considerable number of samples harboring HoBi antibodies (Table 4). The VNT against HoBi virus showed that the samples missed by these kits had VN titers ranging from 64 to 256. None of the kits was able to detect the two Ab positive samples collected on day 18 pi, while VNT detected 2 positive samples with titer of 4. Despite the fact that these tests rely on proteins, NS3 and E^{rns}, that show relatively high homology among pestiviruses, the tests are not sensitive enough to detect antibody levels below 256. Following natural infections with BVDV strains, antibody titers are frequently below 256. Thus, this lack of sensitivity could lead to failure in detecting animals natural exposed to HoBi viruses. Surveillance for exposure to HoBi-like viruses could begin by including HoBi-like viruses in routine VN tests. However, a comparison of VNT results using other isolates

of HoBi-like viruses needs to be completed to determine the degree of antigenic variability among HoBi-like isolates.

Sera from animals receiving an inactivated BVDV-1 and -2 vaccine presented low neutralizing GMT (geometric mean titer) against HoBi virus, comparable to GMTs to BDV strains (Table 5). These findings demonstrated that the serological cross reactivity between these viruses is low, in spite of antigenic similarities with BVDV. Furthermore, neutralization against Pronghorn was even lower; and no neutralization was detected against Bungowannah. The relative antigenic divergence observed correlates with the phylogenetic distance between these groups of viruses. The sequence similarity between E2 protein of the reference strains NADL (BVDV-1) and 890 (BVDV-2) is around 70%, while the similarity of NADL with Bungowannah and Pronghorn viruses is around 48% and 56%, respectively.[14] Interestingly, animals infected with HoBi virus, and with high antibody titers to HoBi, showed moderate to high neutralizing titers to BVDV-2 isolates. In contrast, animals with high titers to BVDV-2 showed low to moderate neutralizing activity against HoBi. These findings agree, in part, with another study in which anti-BVDV-2 sera showed low neutralizing titer against HoBi, although higher than anti-BVDV-1 sera.[22] Modified live (MLV) BVDV vaccines tend to induce broader cross reactivity with BVDV isolates, which may also be observed with HoBi. Further studies using sera of animals immunized with inactivated and MLV vaccines would be useful to compare the degree of cross-neutralizing activity HoBi virus.

The emergence and spread of HoBi-like viruses in commercial dairy operations in Southeast Asia raised concerns about the efficacy of detection of HoBi-like viruses with diagnostic tests used for BVDV. One concern is determining whether current surveillance programs for BVDV can be adapted for surveillance for HoBi-like viruses.

The other concern is that HoBi virus outbreaks may be misdiagnosed as BVDV outbreaks. MAb binding in the present study demonstrated that, while HoBi virus shares epitopes with BVDV isolates, the binding pattern is distinct for the pestiviruses species. A conserved epitope in the E^{rns} viral protein was confirmed by the ACE test, where HoBi was detected with the same efficiency as BVDV-1 and BVDV-2. Nevertheless, this region seems to be variable within HoBi-like viruses since the isolate IZSPLV_To was not detected in another study.[17] Although similarities were identified with mAb binding at NS2/3 protein, ab-ELISA-1 based on the NS3 only detected 33.3% of HoBi antibody sera. These results indicate the need to develop appropriate ELISAs for surveillance for exposure to this new virus group. Similarly, VNT routine should include HoBi, since moderate levels of HoBi antibodies were missed in VNT using the strains 7443 (BVDV-1a) and TGAN (BVDV-1b) (Table 3). Also, specific vaccines for these viruses are needed, because antibodies generated by animals vaccinated against BVDV 1 and/or 2 have displayed low cross reactivity against HoBi.

The evolution/emergence of "new" pestiviruses is an unpredictable event. Therefore, the adoption of control/eradication programs for pestiviruses and the safety testing for biological products must be in constant evolution and should not rely on a single test. As the present study was performed using a single isolate of HoBi-like virus, additional studies involving a higher number of isolates are obviously required. Regardless, it is possible to conclude that the emergence and spread of HoBi-like viruses do represent a justifiable concern since the traditional commercial serological diagnostic tests for BVDV may fail to detected animals harboring HoBi antibodies. Comparative quantitative VN using HoBi-like and BVDV strains may be the best option for increasing the sensitivity of pestivirus antibody detection, and may contribute to the knowledge about the presence/absence of BVDV and HoBi-like virus in tested herds.

Further studies are necessary to access the diagnostic impact in populations where both viruses are present. On the other hand, the antigenic similarities observed between HoBi and BVDV may pose difficulties for discrimination among pestivirus species.

Declaration of conflicting interests

Disclaimer: Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Sources and manufacturers

a. National Veterinary Service Laboratories/APHIS/USDA, Ames, IA.

b. GIBCO, Life Technologies Corp., Carlsbad, CA.

c. Cornell University, Ithaca, NY.

d. Cappel Laboratories Inc., Cochranville, PA.

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Table 1 – Reactivity of monoclonal antibodies with different pestivirus species.

Monoclonal antibody		BVDV[*]-1a			BVDV-1b			BVDV-1c			BVDV-2a			BDV[†]			Uncharacterized pestiviruses		
		VM	7443	C24V	NY-1	NE	TGAN	AusB 675	AusB 843	AusB 730	890	1373	296nc	BD31	CB5	Idaho 207	HoBi	PH[‡]	BW[§]
E[rns]	15C5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+
E2	N2[i]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
	4E1	+	+	+	+	+	+	+	+										
	19F7	+	+	+	+	+	+	+	+	+									
	39A7	+		+															
	10.11.2	+	+	+	+	+	+	+	+										
	BZ25[i]										+	+	+						
	BZ81[i]										+	+	+				+		
	BZ82[i]										+	+	+				+		
	CA36[i]	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+		
	CA80[i]			+	+	+	+	+	+										
	CA82[i]		+	+	+	+	+	+	+										
NS2/3	24.8	+		+	+	+	+	+	+	+	+	+	+	+		+	+		
	1.11.3	+	+	+	+	+	+	+	+	+							+		
	8.12.7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
	20.10.6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		

*Bovine viral diarrhea virus. †Border disease virus. ‡ Pronghorn virus. § Bungowannah virus. ⁞ Hybridoma culture supernatants; otherwise are mAbs from mouse ascites fluid.

Table 2 – Sensitivity of a *Bovine viral diarrhea virus* antigen capture enzyme-linked immunosorbent assay kit for detection of pestiviruses in supernatant of infected bovine turbinate cells

Specie/ subgenotype	Virus	S/P* ratios for viruses titers (TCID$_{50}$) [†]			Logarithmic correlation	Lowest titer detected[‡]	Lowest titer detected/genotype[‡]
		10^6	10^5	10^4			
BVDV[§]-1a	VM	1.38	0.58	0.1	0.95	4.5	
	7443	1.45	0.8	0.15	0.99	4.3	4.3
	C24V	1.53	1.25	0.26	0.93	4.1	
BVDV-1b	NY-1	1.38	0.6	0.09	0.96	4.5	
	NE	1.35	0.85	0.16	0.99	4.3	4.4
	TGAN	0.87	0.68	0.18	0.96	4.5	
BVDV-1c	AusB675	1.07	0.29	0.04	0.88	4.8	
	AusB843	1.02	0.27	0.04	0.88	4.8	4.8
	AusB730	1.19	0.36	0.04	0.9	4.7	
BVDV-2a	890	1.31	0.66	0.11	0.98	4.5	
	1373	1.51	1.08	0.25	0.98	4.1	4.4
	296nc	1.42	0.31	0.03	0.85	4.7	
BDV[¦]	BD31	0.62	0.17	0.02	0.88	5.4	
	CB5	0.44	0.08	0.02	0.81	6.1	5.5[¶]
	Idaho207	0.93	0.34	0.06	0.83	4.8	
Uncharacterized pestivirus[#]	HoBi	0.94	0.72	0.12	0.95	4.5	4.5

* Sample to positive ratio. † Mean tissue culture infective dose. ‡ Using sample to positive ratio values from virus dilutions, the logarithmic regression equation was used to calculate the lowest titer detected. Titers are expressed as $log_{10}TCID_{50}$/ml.
§ Bovine viral diarrhea virus. ¦ Border disease virus. # Bungowannah and Pronghorn viruses are not represented due to inconclusive and negative results, respectively. ¶ Significant statistical difference against others species/genotypes lowest titer detection, using Tukey-Kramer test.

Table 3 – Reactivity of positive HoBi sera with different pestiviruses species.

Sample / VN[*] titer	BVDV[†]-1a		BVDV-1b		BVDV-1c		BVDV-2a		BDV[‡]	Uncharacterized pestiviruses		
	7443	C24V	NY-1	TGAN	AusB 675	AusB 843	890	296nc	BD31	HoBi	PH[§]	BW[ǀ]
504	<4	4	16	<4	<4	<4	32	4	<4	32	<4	<4
510/512/514	4	8	32	8	<4	<4	512	128	<4	512	<4	<4

* Virus neutralization. VN titers were expressed as the reciprocal of the highest dilution that neutralized viral infectivity. The absence of virus neutralization is expressed as <4. † Bovine viral diarrhea virus. ‡ Border disease virus. § Pronghorn virus. ǀ Bungowannah virus.

Table 4 – Comparison of antibody detection enzyme-linked immunosorbent assay kits and virus neutralization test for HoBi antibodies in sera of experimentally infected calves.

Calf		ab-ELISA-1[*]	ab-ELISA-2[*]	VNT[†] with HoBi	
		D42[‡]	D42[‡]	D18[‡]	D42[‡]
Group 1	504	-	+	<4	64
	505[§]	-	+	<4	256
	506	-	+	<4	64
	508	+	+	<4	128
Group 2	509[§]	-	-	<4	64
	510	-	+	<4	256
	512	+	+	4	256
	513[§]	-	-	<4	128
	514	+	+	4	512

* Antibody detection enzyme-linked immunosorbent assay. Absence of reaction, or reaction with value above threshold, indicating negative result is represent with "-". Reaction with values over the threshold, indicative of positive result is represented by "+". ELISA results for all calves for samples from day of birth, and days 9 and 18 post infection are negative and not represented in the table. † Virus neutralization test. VN titers were expressed as the reciprocal of the highest dilution that neutralized viral infectivity. The absence of virus neutralization is expressed as <4. All samples from day of birth and day 9 post infection are negative and not represented in the table. ‡ Representation of days 18 and 42 post inoculation. § Calves inoculated with HoBi, the others are sentinel animals.

Table 5 - Virus neutralization test of sera from 50 calves vaccinated with BVDV-1 and 2 strains, against different pestivirus species

Specie/ subgenotype	Virus	Number of animals for each VN[*] titer (<4 to ≥256)								GMT[†]
		<4	4	8	16	32	64	128	≥256	
BVDV[‡]-1a	7443	4	2	2	1	2	4	5	30	88
	C24V	8	-	6	4	7	2	5	18	46
BVDV-1b	NY-1	4	-	3	1	1	4	2	35	105
	TGAN	6	3	-	1	8	5	5	22	56
BVDV-1c	AusB675	10	5	7	6	6	11	5	-	13
	AusB843	6	-	7	3	9	8	7	10	34
BVDV-2a	890	4	-	1	2	2	3	3	35	111
	296nc	4	-	-	-	-	3	4	39	141
BDV[§]	BD31	20	9	10	7	1	3	-	-	3.8
Uncharacterized pestiviruses	HoBi	14	13	9	7	3	4	-	-	5
	PH[¦]	35	15	-	-	-	-	-	-	1.5
	BW[#]	50	-	-	-	-	-	-	-	1

* Virus neutralization. † Geometric mean titer. ‡ Bovine viral diarrhea virus. § Border disease virus.

¦ Pronghorn virus. # Bungowannah virus.

5. CAPÍTULO 3

In- vitro neutralization of HoBi-like viruses by antibodies in serum of cattle immunized with inactivated or modified live vaccines of *bovine viral diarrhea virus 1* and 2

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Abstract

HoBi-like viruses are an emerging species of pestiviruses with genetic and antigenic similarities to *bovine viral diarrhea viruses 1* and 2 (BVDV-1 and BVDV-2). Vaccines for HoBi-like viruses are not yet available. However, both modified live virus (MLV) and killed virus (KV) vaccines against BVDV are widely used worldwide. This study evaluated the cross reactive antibody response against HoBi-like pestiviruses in sera of cattle immunized with BVDV-1 and BVDV-2 vaccines. Groups "KV" and "MLV", with 25 calves each, received killed or modified live vaccines, respectively, containing both BVDV-1 and BVDV-2 antigens. The antibody response was evaluated by virus neutralization test. The average of geometric mean titers (GMT) of neutralizing antibodies in serum against HoBi-like viruses in the MLV group was 12.9, whereas GMTs to BVDV-1, BVDV-2 and border disease virus (BDV) were 51.1, 23.5, and 12.4 respectively. In this group, neutralizing antibodies against BVDV-1, BVDV-2, HoBi-like viruses and BDV were detected in 100%, 94%, 68% and 68% of calves, respectively. The GMT of neutralizing antibodies in serum against BVDV-1, BVDV-2, HoBi-like viruses and BDV in the KV group were 24.7, 14.5, 10.4 and 11 respectively. Similarly, the percentage of animals with neutralizing antibodies against BVDV-1, BVDV-2, HoBi-like viruses and BDV were 84%, 56%, 34% and 44% respectively. These results indicate that MLV or Killed BVDV-1 and BVDV-2 vaccines induce a cross reactive antibody response comparatively weak to HoBi-like viruses, and this response would likely not suffice to confer protection.

Key words: atypical pestivirus; BVDV-3; neutralizing antibodies, vaccination.

Introduction

A group of viruses called variously HoBi-like viruses, BVDV-3 or atypical pestiviruses, which make up a putative new pestivirus species, have been isolated from FBS (Schirrmeier et al., 2004) and from natural cases of infection of bovines in several continents (Bauermann et al., 2013). The disease developed by calves infected with HoBi-like strains resembles that historically associated with infection by typical virulence BVDV strains (Ridpath et al, 2013).

A variable level of serological cross reactivity among pestiviruses is a well-known phenomenon. Whereas cross reactivity may represent a concern for diagnosis, it suggests some level of cross protection *in vivo*, which is a desirable aspect for immunization strategies. Killed (KV) and modified live (MLV) vaccines are frequently used in BVDV control programs (Ståhl and Alenius, 2011). Compared to KV vaccines, MLV vaccines usually induce higher levels of heterologous protection (Kelling et al., 2007). *In vitro* cross neutralization between BVDV and HoBi-like viruses has been demonstrated (Bauermann et al., 2012; Decaro et al., 2012). While no specific vaccine for HoBi-like viruses is currently available, BVDV vaccines are frequently used in the field. The purpose of the present study is to evaluate the neutralizing antibody response against BVDV-1, BVDV-2, HoBi-like and BDV pestiviruses developed in cattle immunized with either MLV or KV BVDV vaccines.

Material and Methods

Viral strains used in virus neutralizing tests (VNT) were BVDV-1 (BVDV-1b-NE, BVDV-1c-AusB675), BVDV-2 (BVDV-2a-890, BVDV-2a-296nc), HoBi-like (HoBi_D32/00, Italy-1/10-1) and border disease virus (BD31). Border disease virus (BDV), another species in the pestivirus genus that previously was shown to harbor

cross reactive epitopes with BVDV (Ridpath et al., 2000), was included for comparison purposes. All viruses were noncytopathic strains and were genotyped based on the 5' UTR.

Sera used in this study were collected from cattle immunized using either an experimental killed (KV) or an experimental modified live virus (MLV) vaccine. Prior to immunization, cattle were tested and found negative for BVDV and antibodies against BVDV. The experimental killed and MLV vaccines used to generate the antisera contained the minimum efficacious dose of antigen needed to generate an immune response and are not reflective of any commercial product. Both the KV and MLV vaccines contain one noncytopathic (NCP) strain of BVDV-1, and one strain of BVDV-2. Both of these NCP strains were the same in both vaccines. In addition KV vaccine included a cytopathic strain of BVDV-1 and an oil base adjuvant. In the KV group, 25 calves, 11 month old, were immunized twice, 28 days apart, with an inactivated vaccine. The MLV group was composed by 25 calves, 5 month old, that received a single dose of a BVDV MLV vaccine. Sera were collected approximately 50 days after the first dose (KV group) or sole vaccination (MLV group). Serum neutralizing antibody titers were measured by VNT as described below. Heterologous noncytopathic viruses were used in VNT and dilution endpoints were determined by immunoperoxidase test (IMPT). Antibody detection was performed as described previously (Bauermann et al., 2012), with minor changes as detailed below. Briefly, twofold dilutions (1:5 to 1:640) of serum samples were run in a triplicate in 96 well plates. The titer of each of the viruses used for VNT was 200 TCID/ml, in accordance with the OEI standards (30-421TCID/ml). Convalescent sera from animals infected either with a BVDV or a HoBi-like virus were used as positive controls. IMPT was done using a monoclonal antibody (N-2) as described (Ridpath et al., 2000; Bauermann

et al., 2012). VNT were calculated and expressed as geometric mean titers (GMT) using standard methods (Thrusfield, 1986), and the statistical analyses were performed with Friedman-Dunns test using the software GraphPad Prism®. Differences were considered significant when the *p* value was below 0.05.

The initial phase of the study used serum from animals immunized with experimental vaccines formulated with minimal efficacious dose. To determine the vaccination response of conventional vaccines delivered to animals raised under production conditions, sera from a third set of calves was evaluated. These calves were vaccinated twice, 20 days apart, with commercially available MLV BVDV vaccine containing strains of BVDV-1 and BVDV-2 (Bovishield Gold 5®, Pfizer Animal Health). Twenty days after the second dose (day 40), the serum was collected and submitted to VNT against HoBi_D32/00 and the cytophatic strains of BVDV-1 (NADL) and BVDV-2 (296c), following the protocol described above.

Results

The antibody titers developed by animals in the KV and MLV groups are presented in Fig.1a. In the KV group, the GMTs were statistically higher (*p* <0.05) for BVDV-1 strains (GMT=24.7) than to the other pestiviruses tested. While the titers were higher against BVDV-2 (GMT=14.5) than to HoBi-like strains (GMT=10.4) and BDV-BD31 (GMT=11), the differences were not statistically significant (*p* <0.05). Among animals in the MLV group, GMT values for BVDV-1, BVDV-2, BDV and HoBi-like viruses were respectively, 51.1, 23.5, 12.9 and 12.9 (Fig. 1a). The average number of animals that developed serum antibodies that neutralized BVDV-1, BVDV-2, HoBi-like virus and BDV in the KV group was 84%, 56%, 34% and 44%, respectively (Fig. 1b).

In the MLV group antibodies were detected in all animals to BVDV-1, 94% for BVDV-2, 68% for HoBi-like and 68% for BDV (Fig. 1c).

Serum of animals immunized with the commercial vaccine had GMT values of 25, 30.5 and 13.3, respectively to BVDV-1-NADL, BVDV-2-296c and HoBi_D32/00. The number of animals with measurable level of neutralizing antibodies in this group was 96% against both BVDV-1 and BVDV-2 and 60% against HoBi_D32/00.

Discussion

These results demonstrate that the antigenic divergence of HoBi-like virus from BVDV-1 and BVDV-2 is higher than that observed between BVDV-1 and BVDV-2. Although HoBi-like virus share similarities with BVDV-1 and BVDV-2 at the glycoprotein E2 (Bauermann et al., 2012), the seroconversion rate against either BVDV species following vaccination with KV or MLV vaccines was 25% higher than the seroconversion rate against HoBi-like virus strains.

Immunization with either MLV or KV vaccines elicits both B and T cell responses (Sandbulte and Roth, 2003; Stevens et al., 2009). While protective T cell responses may be present even in the absence of a detectable humoral response (Endsley et al., 2003; Ridpath et al., 2003), the level of antibody response is widely used to measure the efficiency of vaccines (Houe, 1995; Ridpath et al., 2003). Previous studies have shown that in conventionally vaccinated animals, virus neutralizing titers and lymphocyte proliferation have moderate correlation (Reber et al., 2006). The failure of 66%, 32% and 40% of cattle vaccinated respectively with the KV, experimental and commercial MLV vaccine, to develop detectable neutralizing titers against HoBi-like viruses suggests that a substantial proportion of animals vaccinated with a BVDV vaccine would be susceptible to HoBi-like virus infection. Further, when cross reactive

neutralizing antibodies were detected they were at low levels. The level of neutralizing antibodies required to prevent disease is unknown. It has been demonstrated that passively acquired immunity with antibody titers of 1:16 would protect animals against BVDV clinical disease but not viral shedding (Bolin and Ridpath, 1995). In the present study, percentage of serum from vaccinated calves that had a titer <1:20 (GMT<2) against HoBi-like virus were respectively 84%, 42% and 54% to the experimental KV, experimental and commercial MLV.

Significantly higher neutralizing antibodies titers were detected against HoBi-like viruses in animals vaccinated with the MLV vaccines tested compared to the KV vaccine, suggesting that MLV vaccines against BVDV may be more effective in preventing infection with HoBi-like viruses. However, while challenge studies are required for a definitive answer, the level of protection based on seroconversion rates, suggests MLV BVDV vaccines would fail to protect a substantial proportion of animals from clinical disease, virus spread and generation of PI animals. In addition, failure of BVDV-1 based vaccines to protect against BVDV-2 infections has led to the inclusion of both BVDV-1 and BVDV-2 strains in current vaccines. Thus, the increasing number of reports of these viruses worldwide urgently require the production of specific vaccines for HoBi-like viruses.

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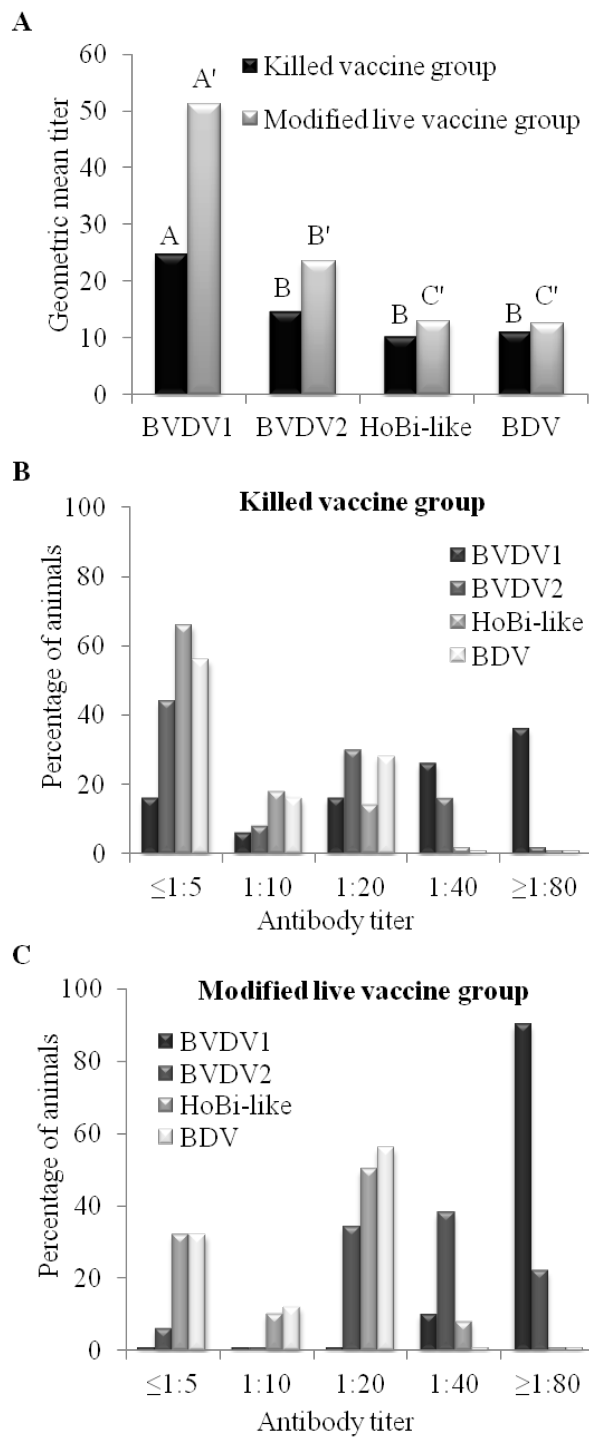
Fig. 1.
Representation of the geometric mean titer (GMT) for antibodies detected using virus neutralizing test against several pestiviruses in serum of animals vaccinated either with a BVDV killed (KV) or modified live vaccines (MLV) (A). Distribution of animals according to the antibody titer detected for each pestivirus species in KV (B) and MLV (C) groups. With exception of BDV, results for BVDV-1, BVDV-2 and HoBi-like viruses are composed by the average of the two isolates used for each species. Serum samples harboring neutralizing antibodies titers ≥1:10 were considered positive.

6. CAPÍTULO 4

Lack of evidence for the emerging HoBi-like viruses in fetal bovine serum lots originated in North America and packaged in the United States of America

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ABSTRACT

The detection of HoBi-like virus in fetal bovine serum (FBS) labeled as United States of America (USA) origin, but packaged in Europe, raised concerns that this emerging pestivirus may have entered the USA. The present study screened 90 lots of FBS originating in North America. Samples in group-1and group-2 (G1-samples 1 to72 and G2-samples 73 to 81) originated in North America and were packaged in the USA. Group 3 (G3) was composed of nine samples ("A" to "I") collected in North America and processed in Europe. Samples in G1 tested negative for bovine viral diarrhea virus (BVDV) by the processor, while samples in G2 and G3 tested positive. Samples were screened using two panpestivirus and two specific HoBi-like primer pairs, a BVDV antigen capture enzyme linked immunosorbent assay (ELISA-ACE), and virus isolation (VI). Pestivirus antibodies detection was done by BVDV antibody detection ELISA (ab-ELISA), and virus neutralization test (VNT) using strains of BVDV1, BVDV2 and HoBi-like virus. All lots in G1 and G2 tested negative by RT-PCR using HoBi specific primers. G1 lots #24 and #48 tested positive by RT-PCR using both sets of panpestivirus primers. Lot #48 was also VI positive. All G2 lots (G2-#73 to G2-#81) were positive by both panpestiviruses RT-PCR tests. In addition lots G2-#76; G2-#77; G2-#78; G2-#80 were VI positive and lot G2-#73 was ACE positive. Lots G3 "C" and "I" were positive using both HoBi-like virus specific RT-PCR tests. All lots were negative for antibodies against HoBi_D32/00. Seven lots (G1-#41; G1-#42; G1-#50; G1-#51; G2-#80; G3-"F" and G3-"H") had antibodies against BVDV by VNT and/or Ab-ELISA. While this data finds no evidence of HoBi-like viruses in the USA, further studies are required to validate HoBi virus free status and provide measure to prevent the introduction and/or dissemination of these agents in the USA.

Key words: atypical pestiviruses; BVDV3; diagnostic; North America; serology.

1. Introduction

Fetal bovine serum (FBS) is widely used as a growth supplement in cell culture media and as a component of many biologicals used in animal and human health. The use of FBS and its international trade pose a potential risk of introducing extraneous agents, that are contaminants of FBS, into countries. While regulation of FBS, used in the production of veterinary medicinal products, comes under the Code of Federal Regulation Title 9 (CRF9) in the United States and the European Medicines Agency (EMEA) – Committee for Medicinal Products for Veterinary EMEA/CVMP/743/00 in Europe, to date there is no body of enforced international standards regarding testing of FBS for extraneous agents and no international certification of country of origin. Each distributor of FBS, without outside verification, develops testing protocols and screens their own product. Labeling of FBS for country of origin is not regulated and is at the discretion of the distributer. There is considerable variation in the extent and efficacy of testing performed by distributers.

Natural infection by the emerging HoBi-like viruses in cattle was reported in Thailand, Brazil and Italy (Bauermann et al., 2013; Decaro et al., 2011; Stahl et al., 2007). Clinical presentation in cattle following exposure to HoBi-like viruses is similar to that observed following infection with typical field BVDV strains (Ridpath et al., 2013). Similar to bovine viral diarrhea virus 1 and 2 (BVDV1 and 2), HoBi-like viruses have been found as contaminants of FBS (Mao et al., 2012; Peletto et al., 2012; Schirrmeier et al., 2004; Xia et al., 2011). Reports of pestivirus contamination of animal and human vaccines have been published, and in many cases the source of contamination was the FBS (Falcone et al., 1999; Makoschey et al., 2003; Studer et al., 2002). In addition, the presence of antibodies against bovine viruses in FBS may interfere with virus detection (Kozasa et al., 2011). Although no outbreaks of pestivirus

related disease has been reported in humans, prolonged cultivation of human derived cells in FBS contaminated with pestiviruses poses an opportunity for adaption of these viruses to humans. While this may be a low risk scenario, it is an easily avoided if FBS supplies are appropriately screened.

In 2011, a detection of HoBi-like viruses in FBS lots assembled and packaged in Europe from FBS collected in North America raised the concern that HoBi-like viruses had been introduced into the USA (Xia et al., 2011). While no HoBi virus PI animals have been detected in monitoring efforts for BVDV PI animals in the USA, the presence of HoBi-like viruses in the country cannot be ruled out, since the current BVDV diagnostic tests my fail in detecting HoBi-like virus infection or in differentiating from BVDV infections (Bauermann et al., 2012). RT-PCR tests using panpestivirus primers may fail to detect and/or differentiate HoBi-like viruses (Bauermann et al., 2013). While a commercial antigen capture ELISA (ACE) widely used in the USA to screen for persistent BVDV infection will detect calves infected with HoBi-like it does not differentiate between BVDV and HoBi-like virus infections (Bauermann et al., 2012; Larska et al., 2012).

The detection of HoBi-like virus in FBS lots labeled as USA origin opens the possibility that HoBi-like viruses are present in the USA but have not been detected because clinical signs are similar to BVDV infections and diagnostic tests currently in use do not discriminate between BVDV and HoBi-like virus infections (Larska et al., 2013; Ridpath et al., 2013). On the other hand, contamination of these samples during the pooling and processing in a facility, outside the USA, that was handling HoBi virus contaminated sera collected from other continents cannot be discounted. Indeed, the procedures adopted during FBS manufacturing such as pooling and/or processing of lots from different sources in the same equipment provide opportunity for cross

contamination. As the identification of infectious agents in regions claimed to be free may pose barriers for biological products trade, and documentation of chain of custody, country of processing as well as country of origin must be conducted.

The efficient detection of contaminating agents in FBS is crucial to avoiding the use of contaminated products and the potential risk of pathogens introduction into free regions. The screen for bovine pestivirus antigen and antibodies in North America FBS, with special interest on HoBi-like viruses, represents one of the first efforts to look for HoBi-like viruses in the region.

2. Material and methods

Study design

This study included 90 samples of FBS lots packaged between 2009 and 2011 in the USA. While labeled as USA origin, the lots are composed of pools of FBS collected from abattoirs in North America (including the USA, Canada and Mexico) FBS pools (personal communication with processor). Samples belonged to one of three groups. Samples in group-1and group-2 (G1 and G2) were filtered and non gamma-irradiated lots of FBS that originated in North America and were packaged in the USA. These samples are numbered from 1 to 81. G1 is composed of 72 samples (numbered from 1 to 72) tested negative for BVDV by the commercial processor. G2 is composed of 9 samples (numbered from 73 to 82) from FBS lots that were tested positive for BVDV by the commercial processor. A third group (G3) was composed by nine filtered and gamma-irradiated samples, identified from "A" to "I". These samples were previously tested positive for BVDV by the processor. While the serum making up the FBS lots in G3 was collected in North America, pooling and processing of the commercial lots was done in Europe (lot numbers on the samples tested corresponded to FBS that was

packaged in Europe). G3 included samples of two USA origin lots previously described as positive to HoBi-like virus (Xia et al., 2011) (Personal communication with the manufacturers). Pestivirus screening included RT-PCR using two widely used pairs of panpestivirus primers (HCV90-368 and 324-326 primers) (Ridpath et al., 1994; Vilcek et al., 1994), a HoBi-like specific real time RT-PCR (Liu et al., 2008) and a HoBi-like specific RT-PCR described below. Samples were also tested for the presence of pestivirus antigen using a commercial antigen capture ELISA (ACE) (HerdChek® - IDEXX) and the presence of live virus by isolation (VI) as described below. Detection of pestivirus antibodies was done using a commercial ELISA (PrioCHECK® BVDV Ab - Prionics) and a comparative virus neutralizing test (VNT) (Bauermann et al., 2012).

Viruses and cells

Antigen detection assays used eight noncytopathic (NCP) pestivirus isolates as positive controls: two isolates of BVDV1 (NY-1), two isolates of BVDV2a (296nc; 1373); two isolates of border disease virus (BDV) (BD31; WABD), and one cytopathic BVDV1 (C24V) isolate from the putative pestivirus species Pronghorn, and two isolates of HoBi-like viruses (HoBi_D32/00 and Italy-1/10-1). The cytophatic (CP) viruses BVDV1-NADL and BVDV2-296c were used for VNT as well as the NCP strain HoBi_D32/00. The BVDV isolates and HoBi-like virus were propagated and titered in bovine turbinate cells (BTu). Pronghorn virus and BDV isolates were amplified and titered in ovine turbinate cells (OFTu). Cells were grown in minimal essential medium (MEM), supplemented with l-glutamine (final concentration, 1.4mM), gentamicin (final concentration, 50mg/l), and 10% FBS; tested free for pestivirus antigen and antibodies, by PCR and VNT, respectively. Cells were tested by PCR and found free of both BVDV and HoBi-like viruses (Bauermann et al., 2012).

RNA samples

An aliquot of 140µl of each FBS batch was used for RNA extraction using a Qiacube® (Qiagen) and the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacture's recommendations. The nine positive controls were diluted in FBS to a final titer of 10^2 TCID/ml (tissue culture infective dose). Aliquots of FBS, MEM, and supernatant of mock infected cells (BTu and OFTu) were used as negative controls. The FBS was tested and free for pestivirus antigen and antibodies.

Panpestivirus RT-PCR

The panpestivirus primer pairs HCV90-368 and 324-326 were used as previous described (Ridpath et al., 1994; Vilcek et al., 1994). Primers 324-326 detected Pronghorn virus while HCV90-368 did not. Primers 324-326 also appear to detect a higher number of BDV isolates than the pair 90-368. Both primer pairs amplify sequences from the HoBi-like isolates tested in this study, yet with lower sensitivity than for BVDV and BDV isolates. PCR products were not cloned but sequenced directly in both directions and all samples were done in duplicate. Sequencing templates were labeled according to manufacturer's recommendations using Invitrogen BigDye® v3.1 terminator chemistries and sequenced using an Invitrogen 3130xl genetic analyzer. Phylogenetic analyses were performed using Mega5® software (Tamura et al., 2011).

HoBi-like virus specific detection

HoBi-like viruses specific qRT-PCR was performed using Quantitect@ Probe RT-PCR kit (Qiagen) as previously described (Liu et al., 2008). Reactions were run in a DNA Engine Opticon2@ cycler (Bio-Rad, MJ Research). Samples were tested in quadruplicate (2 runs with 2 duplicates per run). An in-house HoBi-like specific RT-

PCR was run in parallel. This reaction employs the primers forward N2 (TCGACGCATCAAGGAATGCCT) and reverse R5 (TAGCAGGTCTCTGCAACACCCTAT) which amplify an approximately 150 nucleotide fragment from the untranslated 5' end (5'UTR). Reaction mix (25 µl total) included 3µl of total RNA and SuperScript® III one-step RT-PCR system with Platinum® Taq high fidelity (Life Technologies, Invitrogen) following manufacture's recommendations. Reaction conditions included reverse transcription at 55°C for 25min, followed by 2min at 94°C, 35 cycles of 94°C for 30s, 55°C for 30s, 68°C for 25s, with a final extension at 68°C for 5min. The PCR products were detected by electrophoresis in a 1.0% agarose gel stained with GelRed® and visualized under UV light. PCR products were sequenced and phylogenetic analyses performed as described above.

Virus isolation

VI was done on BTu cells grown in 24-well plates using protocols described previously for the isolation of BVDV (Ridpath et al., 2002). Three passages of 4 days each were performed using 70% confluent, 24h fresh cell monolayer. Plates were kept at 37ºC with 5% CO_2 and monitored for cytophatic effect during the period. RNA was extracted from the supernatant following the last passage and cells were fixed. Immunoperoxidase test (IMPT) was used to detect antigens of noncytopathic pestiviruses in fixed cells, using the monoclonal antibody (mAb) N-2 as previously described (Bauermann et al., 2012). The N-2 mAb reacts with a wide range of pestivirus species, including BVDV, BDV and HoBi-like virus isolates (Bauermann et al., 2012; Ridpath et al., 2000).

Antigen capture ELISA and BVDV antibody detection ELISA

Lots of FBS were individually tested with ACE (HerdChek® - IDEXX) and ab-ELISA (PrioCHECK® BVDV Ab - Prionics), following the manufacture's recommendations. Samples were run in quadruplicates (duplicates in two separate runs).

Virus neutralizing test

A comparative VNT was performed using the following cytopathic (CP) strains BVDV1-NADL; BVDV2-296c and the NCP strain HoBi_D32/00 (Bauermann et al., 2012). Briefly, serum was diluted in MEM from 1:2 to 1:256 in 96 wells plates, and 200 $TCID_{50}$ of each virus were added individually in accordance with the OEI standards (30-421TCID/ml). BTu cells were included after incubation of 90min at 37ºC with 5% CO_2. After four days of incubation, results were obtained for strains NADL and 296c by observing cytophatic effect (CPE) in cell monolayer and for HoBi_D32/00, readings by IMPT as described (Bauermann et al., 2012). Sera, collected from convalescent animals infected with BVDV or HoBi-like viruses under controlled conditions, with known neutralizing antibody titer were used as positive controls. Samples with titer ≥ 4 were considered positive.

3. Results

Detection of pestivirus contamination

None of the lots in G1 tested positive by PCR using HoBi specific primers while two lots (G1-#24 and G1-#48) tested positive by PCR using both sets of panpestivirus primers. Lot G1-#48 was also positive by VI and IMPX test. Phylogenetic analysis of amplicons demonstrated that the amplified viral sequences were derived from BVDV1b isolates.

None of FBS lots composing G2 were detected as positive for HoBi-like viruses using HoBi specific primers. All nine lots were positive using the panpestiviruses primers. Phylogentic analyses revealed the amplified sequences came from isolates from BVDV1b subgenotype. Lots G2-#76; G2-#77; G2-#78; G2-#80 were also positive by VI and IMPX test. The sequence of the isolated viruses matched the sequence directly amplified from these lots. Sample G2-#73 was also detected as positive by ACE.

The G3 group included samples from the two lots (G3-"C" and G3-"I") previously identified as HoBi-like virus positive (Xia et al., 2011). Nine of the eight samples in this group tested positive for BVDV with both panpestivirus reactions. Sample G3-C was detected as positive using the primers 324-326 but not primers HCV90-368. G3-"C" and G3-"I" tested positive using both HoBi-like virus specific tests. Phylogenetic analyses of amplified sequences demonstrated that eight samples were contaminated with BVDV1 and one (G3-G) with BVDV2. All were negative by ACE and VI and IMPX test.

Antibody detection

None of the tested lots contained antibodies that neutralized HoBi_D32/00. Seven lots had antibodies against BVDV by VNT and /or Ab-ELISA (Table 2). Four were from G1 (#41, #42, #50, #51), one from G2 (#80) and two from G3 ("F" and "H"). Three positive samples in G1 (#41, #50, #51) were detected with Ab-ELISA and displayed higher antibody titer to BVDV1 than to BVDV2. Sample #42 was negative using Ab-ELISA, VNT against BVDV2 and HoBi_D32/00, whereas it was positive for VN antibodies against BVDV1 (titer 1:4). The single antibody positive sample in G2 (#80) was detected using Ab-ELISA, while no antibodies to BVDV1, BVDV2 or

HoBi_D32/00 were detected by VNT. Two samples in G3 ("F" and "H") were detected as antibody positive using the ELISA and VNT. The neutralizing antibodies titers of both samples were similar against BVDV1 and BVDV2.

4. Discussion

The international FBS trade may provide a route for dissemination of infectious agents such bovine pestiviruses, which are common contaminants of this supplement. The emergence of new pestivirus strains and cross species transmissions represent rewly recognized risk factors for BVDV control programs. Despite the absence of reports of HoBi-like viruses infecting North American cattle, the report of HoBi-like viruses in FBS processed in Europe and labeled as originating in the USA raised a great deal of concern. Thus, the present study was conducted to gather further evidences about the possible presence of these viruses in the USA.

The presence of HoBi-like viruses in two samples previously described as positive for these viruses was confirmed here. Nucleotide sequencing demonstrated that these samples correspond to the sequences available at genbank (available at http://www.ncbi.nlm.nih.gov/pubmed/, identification JN967714.1 and JN967748 for samples "C" and "I" respectively). Both samples were also contaminated with BVDV1. An important point is that only nucleotide sequence of BVDV1 was obtained from these samples by using the panpestivirus primers pairs.

The sequencing results led to further investigation regarding the detection and identification of pestiviruses in samples containing more than one pestivirus species. To this end, FBS was contaminated with known concentrations (TCID) of BVDV1 (C24V) and/or HoBi_D32/00. The RNA was extracted and samples were submitted to the RT-PCR and sequencing using primers HCV90-368, 324-326, and N2-R5, as

described above. It was verified that RNA extracted from FBS containing virus load 100 to10.000 higher to HoBi_D32/00 than to BVDV1 yielded clear nucleotide sequence of BVDV1 (Table 3). This might be partially explained by mismatches detected in primers sequence (Figure 1). Therefore, the detection and identification of HoBi-like viruses required the application of specific HoBi-like primers, reinforcing the need for use of multiple tests for bovine pestivirus identification. In addition, the genetic diverse of HoBi-like viruses is not fully known, and their detection might not rely on a single test.

The detection of BVDV contaminated sample in all three groups, including G1 (tested negative for pestiviruses by commercial processor) was not surprising. Reports have shown the presence of BVDV in commercial FBS lots claimed free for BVDV (Kozasa et al., 2011). Phylogenetic analyses revealed that, from a total of 20 positive samples for BVDV, 19 belong to BVDV1 species and one to BVDV2. This finding likely reflects the higher distribution and prevalence of BVDV1b in North America (Ridpath et al., 2011). It is important to note that the recovery of viable viruses was successful only from non gamma-irradiated samples, lots G1-#48; and G2-#76, G2-#77, G2-#78 and G2-#80, and therefore no assumption about the safety of sterilizing process can be taken.

The ACE test is highly indicated to detect BVDV persistently infected animals, and has been demonstrated effectiveness in detecting HoBi-like acutely infected calves and supernatants of HoBi virus infected cells (Bauermann et al., 2012; Larska et al., 2012). The high number of false negative results using ACE is probably due to pooling of samples to compose a FBS batch, leading to excessive antigen dilution. These findings reinforce the need for a constant update in pestivirus diagnostic and the use of multiple tests. It also demonstrated the need for further regulations in FBS trade,

including the development and validation of internationally accepted tests and protocols.

Using the Ab-ELISA and VNT, 7 samples (7.8%) were detected by Ab-ELISA and/or VNT, confirming the circulation of BVDV in North American herds. These antibodies are likely results of *in uterus* infection. VNT and Ab-ELISA demonstrated similar sensitivity to detected antibodies in FBS, although both assays have drawbacks and an accurate diagnostic may require more than a single test as some discordant results were observed. Lot G2-#80 was positive by Ab-ELISA, but negative by VNT. One possible reason for this discrepancy is that viable virus (BVDV1) present in G2-#80 might have interfered with VNT test. In contrast, sample G1-#42 from G1, was negative by Ab-ELISA but positive by VNT when tested against BVDV1 (titer of 4). The low VN titer may explain the lack of recognition using by Ab-ELISA.

No evidence of HoBi-like virus (antigen or antibody) in FBS originated in North America and packaged in USA was detected in this study. This is in agreement with a comparative serology study using serum from cattle herds from Texas (manuscript in preparation). If HoBi-like viruses spread north out of South America, Texas herds may represent a sentinel population. To date, HoBi-like virus antigen or antibodies have not been detected in Texas herds surveyed in preliminary studies.

The information described here suggests that the regulation of FBS international trade needs to be reviewed. Pooling of FBS lots originated from different parts of the world should be avoided. The use of the equipment to manufacturing FBS with different origins may also be review, in order to avoid contamination among lots. Another indication supporting the possibility for contamination during the manufacture process is found by Xia et al. (2011), who reported the presence of BVDV1d in a sample claimed to be originated in the USA. As well as for HoBi-like viruses, there is no

diagnostic of BVDV1d in USA herds to date (Evermann and Ridpath, 2002; Fulton et al., 2005; Ridpath et al., 2010; Ridpath et al., 2011). The data presented here suggest the absence of HoBi-like virus in the USA, although further efforts and constant monitoring are obviously required to validate the status and avoid introduction and/or dissemination of these agents in the country.

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Table-1. Pestivirus detection in fetal bovine serum lots using panpestivirus and HoBi-like virus specific diagnostic assays[1].

Sample identification		Panpestivirus detection			HoBi-like detection		Virus isolation	Sequencing
		324-326	HCV90-368	ACE	RT-PCR	qRT-PCR		
Group-1	24	+	+					BVDV1
	48	+	+				+	BVDV1
Group-2	73	+	+	+				BVDV1
	74	+	+					BVDV1
	75	+	+					BVDV1
	76	+	+				+	BVDV1
	77	+	+				+	BVDV1
	78	+	+				+	BVDV1
	79	+	+					BVDV1
	80	+	+				+	BVDV1
	81	+	+					BVDV1
Group-3	A	+	+					BVDV1
	B	+	+					BVDV1
	C	+			+	+		BVDV1/ HoBi-like[2]
	D	+	+					BVDV1
	E	+	+					BVDV1
	F	+	+					BVDV2
	G	+	+					BVDV1
	H	+	+					BVDV1
	I	+	+		+	+		BVDV1/ HoBi-like[2]

[1] Samples with negative results in performed tests are not displayed.

[2] Detection and sequencing of BVDV1 were performed using panpestivirus primers.

HoBi-like detection and sequencing was performed using primers N2-R5.

Table-2. Detection of antibodies against pestiviruses in fetal bovine serum lots[1]

Sample		Virus neutralizing test			Ab-ELISA
		BVDV1 NADL	BVDV2 296nc	HoBi_ D32/00	
Group-1	41	1:8	1:4	-	+
	42	1:4	-	-	-
	50	1:256	1:8	-	+
	51	128	-	-	+
Group-2	80	-	-	-	+
Group-3	F	1:4	1:8	-	+
	H	1:8	1:8	-	+

[1] Samples with negative results in performed tests are not displayed.

Table-3. Fetal bovine serum contaminated with known concentration of BVDV1 and/or HoBi-like virus and results following RT-PCR and sequencing using primer pairs 324-326, HCV90-368, and N2-R5.

BVDV1 + HoBi-like (TCID)/ml		Sequencing-primers 324-326	Sequencing-primers 90-368	Sequencing-primers N2-R5
10^5	10^5	BVDV1	BVDV1	HoBi-like
10^5	10^3	BVDV1	BVDV1	HoBi-like
10^5	10^1	BVDV1	BVDV1	HoBi-like
10^3	10^5	BVDV1	BVDV1	HoBi-like
10^3	10^3	BVDV1	BVDV1	HoBi-like
10^3	10^1	BVDV1	BVDV1	HoBi-like
10^1	10^5	BVDV1	-	HoBi-like
10^1	10^3	BVDV1	-	HoBi-like
10^1	10^1	BVDV1	-	HoBi-like
10^5	-	BVDV1	BVDV1	-
10^3	-	BVDV1	BVDV1	-
10^1	-	BVDV1	-	-
-	10^5	HoBi-like	-	HoBi-like
-	10^3	-	-	HoBi-like
-	10^1	-	-	HoBi-like

Primer 324

```
                         ATGCCCWTAGTAGGACTAGCA
HoBi_D32/00              ..A..T.C........G....
HoBi-like Italy-1/10-1   .....T.........G....
HoBi-like Th/04_KhonKaen ..A..T.........G....
BVDV1-NADL              .....................
BVDV1-Singer           .....................
BVDV1-C24V             .....................
BVDV2-890              ....................C
BVDV2-NY93             .....................
BDV-BD31               ..A.A.G..............
BDV-Gifhorn            ..A.........G....
BDV-Reindeer-1         ..A...G.............
BDV-X818               ..A.A.G..............
```

Primer 326*

```
                         GTACATGGCACATGGAGTTGA
HoBi_D32/00              ................A...T
HoBi-like Italy-1/10-1   ....................T
HoBi-like Th/04_KhonKaen ....................T
BVDV1-NADL              .....................
BVDV1-Singer           .....................
BVDV1-C24V             ...............A.....
BVDV2-890              ....................T
BVDV2-NY93             ....................T
BDV-BD31               .....................
BDV-Gifhorn            .....................
BDV-Reindeer-1         ...............AG....
BDV-X818               .....................
```

Primer HCV90

```
                         CATGCCCATAGTAGGAC
HoBi_D32/00              ...A..TTC........
HoBi-like Italy-1/10-1   ......TTC........
HoBi-like Th/04_KhonKaen ...A..TT.........
BVDV1-NADL              ......T..........
BVDV1-Singer           ......T..........
BVDV1-C24V             ......T..........
BVDV2-890              ......T..........
BVDV2-NY93             ......T..........
BDV-BD31               ...A.A.G.........
BDV-Gifhorn            ...A...T.........
BDV-Reindeer-1         ...A...G.........
BDV-X818               ...A.A.G.........
```

Primer HCV368*

```
                         CTGTACATGGCACATGG
HoBi_D32/00              .................
HoBi-like Italy-1/10-1   .................
HoBi-like Th/04_KhonKaen .................
BVDV1-NADL              .................
BVDV1-Singer           .................
BVDV1-C24V             .................
BVDV2-890              .................
BVDV2-NY93             .................
BDV-BD31               .................
BDV-Gifhorn            .................
BDV-Reindeer-1         .................
BDV-X818               .................
```

Primer N2

```
                         TCGACGC-----ATCAAGGAATGCCT
HoBi_D32/00              .......-----..............
HoBi-like Italy-1/10-1   .......-----..............
HoBi-like Th/04_KhonKaen .......-----.....
BVDV1-NADL              .......---CTTGG..TA..G.T..
BVDV1-Singer           .......---CTTGG..TA..G.T..
BVDV1-C24V             .......---CTTAAC.T...G.T..
BVDV2-890              .....A.TCCTTTAGTC.AGGA.T..
BVDV2-NY93             .....A.TCCATTAGTC.AGGA.T..
BDV-BD31               ..A....--AA.CATG-CTC.....
BDV-Gifhorn            .......---GGTCATC.C.TC.T..
BDV-Reindeer-1         ..A....--AA-.GTG.AGT.....
BDV-X818               ..A....--AGGCACG.TTC.....
```

Primer R5*

```
                         ATAGGGTGTTGCAGAGACCTGCTA
HoBi_D32/00              ........................
HoBi-like Italy-1/10-1   ........................
HoBi-like Th/04_KhonKaen ........................
BVDV1-NADL              .........C.......G..CA..G
BVDV1-Singer           .........C.......G..CA..G
BVDV1-C24V             .........C.......G..CA..G
BVDV2-890              ............A...........
BVDV2-NY93             ............A...........
BDV-BD31               ......C.C.....-G..CA.GT
BDV-Gifhorn            .........C.......G..CA.GC
BDV-Reindeer-1         .........C.......G..CA.GC
BDV-X818               .........C.......G..CA.G.
```

Fig.1. Alignment of pestivirus species and panpestiviruses primer pairs (324-326 and HCV90-368) and the HoBi-like specific primer pair (N2-R5).

* Reverse primers are presented as the reverse complement of the original nucleotide sequence.

7. CONCLUSÃO

Apesar das evidências de que vírus do grupo HoBi-like se encontram disseminados no rebanho bovino brasileiro, ainda é imensurável o impacto econômico desse agente. Isolados do grupo HoBi-like possuem semelhanças genéticas e antigênicas com isolados de BVDV, além da apresentação clínica indistinguível daquela observada em animais infectados com isolados de BVDV de típica virulência. Esses fatores podem induzir profissionais relacionados ao diagnóstico e controle desses agentes, a utilizar testes diagnósticos (para detecção de antígenos ou anticorpos) desenvolvidos para BVDV na identificação do vírus HoBi-like ou resposta sorológica específica. No presente trabalho foi verificado que em determinadas situações, os testes para a detecção de BVDV identificaram com sucesso amostras contendo o vírus HoBi-like. Entretanto, em outras circunstâncias, os testes possuem altas taxas de falhas. Embora alguns testes eficientemente detectem o vírus HoBi-like, a diferenciação entre as espécies de pestivírus geralmente não pode ser realizada. Foi demonstrada homologia entre BVDV-1, BVDV-2 e vírus HoBi-like na glicoproteína E^{rns}, podendo essa servir de base para testes diagnósticos panpestivírus, enquanto a variabilidade antigênica encontrada entre epitopos da glicoproteína E2 pode ser empregada em testes diagnósticos diferenciais. Constatou-se novamente a importância do SFB como disseminador dos pestivírus, sendo que várias amostras previamente testadas e negativas para BVDV (pelo fornecedor comercial) foram detectadas como positivas. Verificou-se a importância do uso de múltiplos testes para a segura detecção desses agentes, bem como o uso de diagnóstico específico para o vírus HoBi-like. Os resultados também demonstram a necessidade de regulação do comércio do SFB, e revisão/padronização dos procedimentos de coleta e processamento do produto. O trabalho foi conduzido à esfera de controle do agente em nível de campo pela marcante característica de reação sorológica cruzada entre os pestivírus. Somado ao fato de que vacinas vivas atenuadas induzem resposta humoral de maior intensidade que imunógenos inativados. A hipótese de que o soro de animais imunizados com vacinas vivas atenuadas contra BVDV-1 e BVDV-2 teria maior atividade neutralizante contra o vírus HoBi-like foi testada. Verificou-se, entretanto, que a indução de títulos de anticorpos contra o vírus HoBi-like foi baixa, e similar entre animais que receberam vacinas vivas ou inativadas. Uma significativa parcela dos animais (cerca de 35%) imunizados com vacina viva atenuada

permaneceram soronegativos contra o vírus HoBi-like. Demonstrou-se claramente a necessidade da inclusão de cepas de HoBi-like nas vacinas existentes para BVDV-1 e BVDV-2, ou ainda a produção de vacinas específicas.

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