THAIS NERIS DA SILVA MEDEIROS

DIARREIA NEONATAL BOVINA:

EPIDEMIOLOGIA E CARACTERIZAÇÃO MOLECULAR DOS GENOTIPOS G (VP7) E P (VP4) DE ROTAVÍRUS A, BRASIL, 2006-2015

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Orientador: Prof. Dr. Amauri Alcindo Alfieri

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"Por vezes sentimos que aquilo que fazemos
não é senão uma gota no mar.
Mas o mar seria menor se lhe faltasse uma gota".
Madre Teresa de Calcutá

MEDEIROS, Thais Neris da Silva. **Diarreia neonatal bovina: Epidemiologia e caracterização molecular dos genotipos G (VP7) e P (VP4) de rotavírus A, Brasil, 2006-2015**. 2016. 88f. Tese (Doutorado em Ciência Animal, Área de Concentração: Sanidade Animal) - Universidade Estadual de Londrina, Londrina, 2016.

RESUMO

O rotavírus bovino A (RVA) é uma das principais causas de diarreia neonatal em bezerros de todo o mundo. Os genes VP7 e VP4 de RVA determinam a classificação binária em genotipos G e P, respectivamente. As principais combinações de genotipos G e P descritas em bovinos são: G6P[1], G6P[5], G6P[11], G8P[1] e G10P[11]. A presente pesquisa originou dois estudos temporais, em que foram coletadas amostras de fezes diarreicas de bezerros de corte e de leite de rebanhos brasileiros durante o período de 2006 a 2015. O objetivo do primeiro estudo foi descrever a frequência de RVA presente nas fezes diarreicas avaliadas e o objetivo do segundo estudo foi descrever os genotipos G e P de cepas de RVA circulantes nos rebanhos bovinos brasileiros. No primeiro estudo foram avaliadas 1498 amostras de fezes diarreicas pela técnica de eletroforese em gel de poliacrilamida (ss-PAGE) seguida da coloração pelo nitrato de prata, de 124 rebanhos de corte e 56 rebanhos leiteiros das três principais regiões produtoras de bovinos do Brasil. O RVA foi identificado em 27,4% (410/1498) das amostras fecais avaliadas e a frequência de amostras positivas para RVA em bezerros de corte (31,9%; 328/1.027) foi significativamente maior ($p \leq 0,05$) que a frequência verificada em bezerros leiteiros (17,4%; 82/471). A infecção pelo RVA foi verificada nas três regiões produtoras brasileiras avaliadas, no entanto, a frequência de bezerros com diarreia positivos para RVA na região Centro-Oeste (39,4%), foi significativamente maior ($p \leq 0,05$) que as regiões Sul (19,4%) e Sudeste (17,6%). No segundo estudo, as amostras foram selecionadas a partir de um conjunto que consistia de 1589 amostras fecais previamente avaliadas para a presença de RVA pela técnica de ss-PAGE de cinco regiões geográficas brasileiras e destas, 417 (26,2%) foram positivas para RVA. Foram selecionadas por rebanho bovino uma ou duas amostras fecais positivas para RVA em ss-PAGE, sendo 155 cepas de RVA, 70 pertencentes a rebanhos de corte e 30 de rebanhos leiteiros. Os genes G e P das cepas de RVA foram amplificados por RT-PCR e sequenciados para análise filogenética. Os genotipos G6, G10, P[5] e P[11] foram detectados nas amostras de RVA avaliadas. Uma distribuição diferente de combinações de genotipos G e P foi encontrada de acordo com o tipo de aptidão, sendo o genotipo G6P[5] mais prevalente (68,5%) em rebanhos de corte e os genotipos G10P[11] (40,5%) e G6P[11] (32,4%) mais prevalentes em rebanhos leiteiros ($p<0,05$). Com relação às regiões geográficas analisadas, o maior número de cepas de RVA genotipadas pertenceu à região Centro-Oeste, sendo o genotipo G6 foi único identificado nesta região. As regiões Sul e Sudeste apresentaram maior diversidade de genotipos G e P. A combinação de genotipos G6(IV)P[5](IX) foi predominante em bovinos de corte, e as combinações G6(III)P[11](III) e G10(V)P[11](III) foram prevalentes em bovinos leiteiros. A linhagem P5(II) foi descrita pela primeira vez no Brasil, em rebanho de corte do Rio Grande do Sul. Com os resultados apresentados, conclui-se que o RVA continua sendo um dos principais agentes etiológicos de diarreia neonatal em bezerros nos rebanhos bovinos brasileiros. Em outras regiões do mundo, a diversidade de genotipos G e P é consideravelmente maior do que a encontrada no presente estudo, mesmo utilizando as

mesmas metodologias diagnósticas que pode ser devido a eficácia das medidas de controle e profilaxia utilizadas. A importância dos estudos de genotipagem, particularmente com análises retrospectivas realizadas em diferentes regiões geográficas ou tipos de aptidão (corte e leite), contribui para a compreensão da evolução viral e da epidemiologia da infecção de RVA.

Palavras-chave: Bezerros. Diarreia. RVA. Genotipagem. Filogenia. Epidemiologia

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ABSTRACT

Bovine rotavirus A (RVA) is one of the main causes of neonatal diarrhea in calves around the world. The VP7 and VP4 genes of RVA determine the binary classification in G and P genotypes, respectively. The main combinations of G and P genotypes described in cattle are: G6P[1], G6P[5] G6P[11] G8P[1], and G10P[11]. The present research originated two temporal studies, in which diarrheic fecal samples were collected from beef and dairy calves from Brazilian herds during the period from 2006 to 2015. The objective of the first study was to describe the frequency of RVA present in the evaluated diarrheic fecal samples while objective of the second study was to describe the G and P genotypes of RVA strains circulating in Brazilian cattle herds. In the first study, were evaluated 1498 diarrheic fecal samples by silver staining electrophoresis polyacrylamide gel (ss-PAGE), from 124 cattle herds and 56 of dairy herds of the three main cattle producing regions of Brazil. The RVA was identified in 27.4% (410/1498) of the evaluated fecal samples and the frequency of RVA positive samples in beef calves (31.9%; 328 / 1,027) was significantly higher ($p < 0.05$) than the frequency observed in dairy calves (17.4%; 82/471). The RVA infection was verified in the three Brazilian geographic regions evaluated, however, the frequency of calves with diarrhea positive for RVA in the Midwest region (39.4%) was significantly higher ($p < 0.05$) than for South (19.4%) and Southeast (17.6%) regions. In the second study, the samples were selected from a collection consisting of 1589 fecal samples previously evaluated for the presence of RVA by ss-PAGE technique from the five Brazilian geographic regions, in wich, 417 (26.2%) were positive for RVA. One or two fecal samples by bovine herd positive for RVA on ss-PAGE were selected, being 155 strains of RVA, which 70 belonged to cattle herds and 30 to dairy herds. The G and P genes of RVA strains were amplified by RT-PCR and sequenced for phylogenetic analysis. The G6, G10, P[5] and P[11] genotypes were detected in the evaluated RVA strains. A different distribution of combinations of G and P genotypes was found according to the type of exploitation, with the G6P [5] genotype being more prevalent (68.5%) in cattle herds and G10P[11] (40.5 %) and G6P[11] (32.4%) genotypes more prevalent in dairy herds ($p < 0.05$). Regarding the geographic regions analyzed, the largest number of genotyped RVA strains belonged to the Central-West region, but only the G6 genotype was identified in this region. The Southern and Southeastern regions presented greater diversity of G and P genotypes. The combination of G6(IV)P[5](IX) genotypes was predominant in beef cattle herds, and the G6(III)P[11](III) and G10(V)P[11](III) were prevalent in dairy herds. The P5 (II) lineage was described for the first time in Brazil, in a herd of Rio Grande do Sul. With the results presented, it is concluded that RVA remains one of the main etiological agents of neonatal diarrhea in calves in Brazilian herds. Across the world, the diversity of G and P genotypes is considerably greater than that found in the present study, even using the same diagnostic methodologies. The importance of genotyping studies particularly with retrospective analyzes carried out in different geographical regions or

types of exploitation (beef and dairy) contributes to the understanding of viral evolution and epidemiology of RVA infection.

Keywords: Calves. Diarrhea. RNA. Genotyping. Phylogeny. Epidemiology

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1.1 INTRODUÇÃO

O Brasil é o quinto maior país do mundo em território, com aproximadamente 20% (174 milhões de hectares) da extensão territorial ocupada por pastagens (ABIEC, 2016). O rebanho bovino brasileiro proporciona o desenvolvimento de dois segmentos lucrativos, as cadeias produtivas da carne e leite. O valor bruto da produção desses dois segmentos, estimado em 20,90 bilhões de dólares, aliado a presença da atividade em todos os estados brasileiros, evidenciam a importância econômica e social da bovinocultura no país. O clima tropical e a extensão territorial do Brasil contribuem para esse resultado, uma vez que permitem a criação da maioria dos bovinos em pastagens, ou seja, sistema de criação extensivo (ABIEC, 2016).

O Brasil possui o maior rebanho bovino comercial do mundo com cerca de 213 milhões de cabeças criadas a pasto (MAPA, 2016). Estima-se que apenas 3% do rebanho é terminado em sistema intensivo. Em 2004, o Brasil se consolidou como potência na produção e exportação de carne bovina, e assumiu a primeira colocação dentre os exportadores com 1/5 da carne comercializada internacionalmente e vendas para mais de 180 países (ABIEC, 2016).

O agronegócio do leite e seus derivados, onde o Brasil se posiciona como o sexto produtor mundial, desempenha um papel relevante no suprimento de alimentos e na geração de empregos e renda para a população. Os maiores países produtores de leite bovino no mundo são EUA, Índia, China, Rússia, Alemanha, Brasil e Nova Zelândia que, juntos, produzem 48% do leite mundial. A partir do ano 2000, o Brasil iniciou acesso ao mercado internacional, alcançando, em 2007 e 2008, saldo positivo na balança comercial de lácteos (IICA, 2010).

Com a finalidade de aumentar a eficiência e a produtividade da bovinocultura, algumas estratégias estão sendo adotadas por criadores de todo o país. O confinamento para terminação, o semi confinamento, e a suplementação para o período seco são algumas delas e contribuem para a redução do ciclo de produção, para obtenção de melhor acabamento de carcaça e, consequentemente, para uso mais sustentável da terra e dos recursos naturais. A tecnologia aplicada à bovinocultura, aliada ao desenvolvimento de pesquisa nacional e de

técnicas específicas aos sistemas produtivos, está impulsionando os índices de produtividade dos animais e colaborando para uma bovinocultura cada dia mais eficiente e sustentável (ABIEC, 2016).

O crescimento da produção bovina de corte e leite somente foi possível com o aprimoramento em técnicas de criação, manejo zootécnico e sanitário, nutrição e melhoramento genético. No entanto, com a intensificação da produção houve aumento na ocorrência de doenças infecto-parasitárias e contagiosas já existentes e o surgimento de novos variantes de agentes etiológicos já conhecidos que acometem os bovinos (PALOMBO et al., 2002; ALFIERI, 2007; MARTELLA et al., 2010).

Levando em consideração a produção intensiva de bovinos, os principais problemas sanitários que acometem os bezerros com até 1 ano de idade são de ordem entérica e respiratória. Dentre as causas de ordem entérica, as diarreias neonatais são as que determinam as maiores perdas econômicas na bovinocultura brasileira (ALFIERI et al., 2007).

1.2 DIARREIAS NEONATAIS

Enfermidades tais como a diarreia durante o primeiro mês de idade podem apresentar consequências econômicas diretas e indiretas devido às taxas de mortalidade e morbidade, aumento nos custos com o tratamento dos animais acometidos, perda de peso e aumento da suscetibilidade a outras infecções, principalmente doenças respiratórias (BENDALI et al., 1999; ALFIERI et al., 2007).

A diarreia neonatal apresenta etiologia multifatorial e multietiológica, em que falhas no manejo zootécnico e nutricional, bem como vírus, bactérias, protozoários e outros agentes, desempenham papel crucial em seu desenvolvimento (BENDALI et al., 1999). Dentre os principais fatores de falha de manejo que aumentam o risco para o aparecimento de diarreia neonatal estão más condições de higiene, privação do colostro aos bezerros, fatores individuais de suscetibilidade (idade e raça), uso de antibióticos e a presença ou histórico de enteropátogenos no rebanho (BARTELS et al., 2010).

Entre os agentes infecciosos mais frequentes que ocasionam diarreia neonatal nas espécies bovinas destacam-se bactérias (*Escherichia coli* K99, *Clostridium perfringens* tipo C), protozoários (*Cryptosporidium parvum*) e vírus (rotavírus e coronavírus) (BENDALI et al., 1999; ALFIERI et al., 2007). Para o tratamento e prevenção adequada, é necessário saber a causa da diarreia. O exame clínico é insuficiente para diferenciar entre as possíveis causas

de diarreia, por isso é necessária aplicação de técnicas de diagnóstico laboratoriais em amostras do rebanho (BARTELS et al., 2010).

1.3 ROTAVÍRUS

Rotavírus (RV) pertence ao gênero *Rotavirus* da família *Reoviridae*. O vírion, desprovido de envelope lipídico, apresenta simetria icosaédrica com aproximadamente 70-100 nm de diâmetro e o capsídeo é composto por três camadas proteicas concêntricas (Figura 1). O genoma é constituído por 11 segmentos de RNA fita dupla (*ds*RNA), que codificam seis proteínas estruturais (VP1 a VP4, VP6-VP7) e seis proteínas não estruturais (NSP1 a NSP6) que são encontradas em partículas virais maduras. Cada segmento genômico codifica apenas uma proteína, com exceção do segmento 11 que codifica duas proteínas (Figura 2) (ALFIERI et al., 2007; ESTES; KAPIKIAN, 2007).

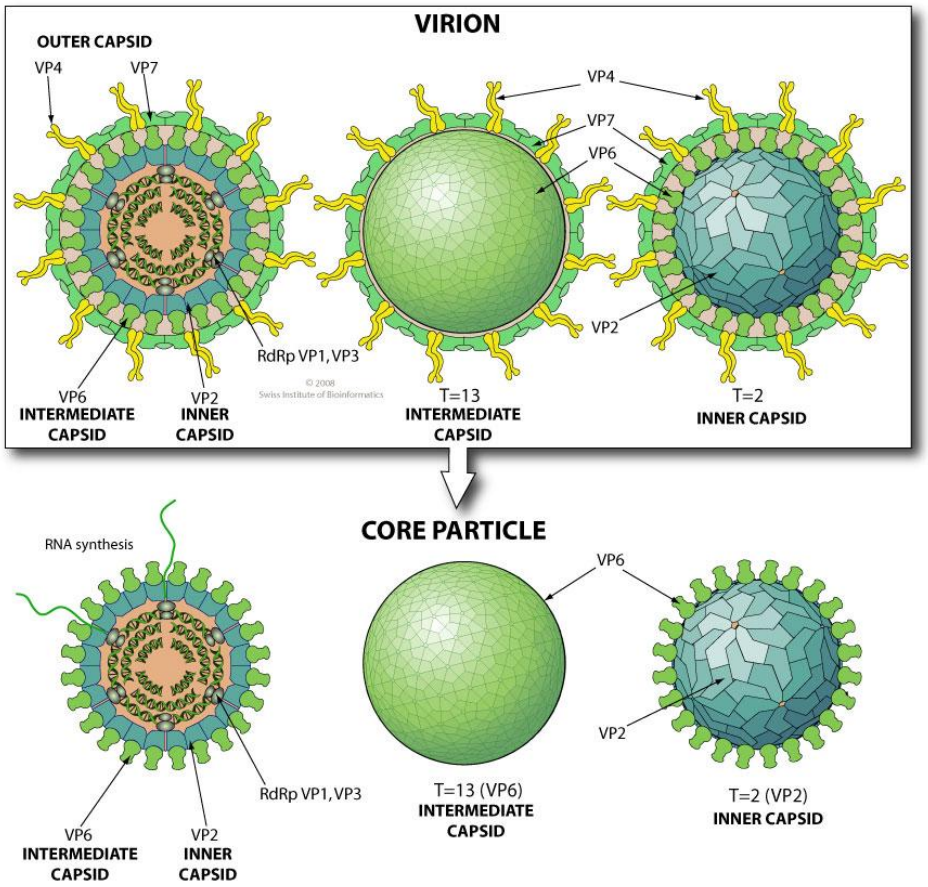

Figura 1 - Esquema da partícula viral do rotavírus. Três camadas protéicas concêntricas de simetria icosaédrica constituem o capsídeo viral (camada externa, intermediária e interna), evidenciando a localização das proteínas estruturais (VP).
Fonte: www.bibliotecadigital.ufmg.br. **Acessado** em 11.10.2016.

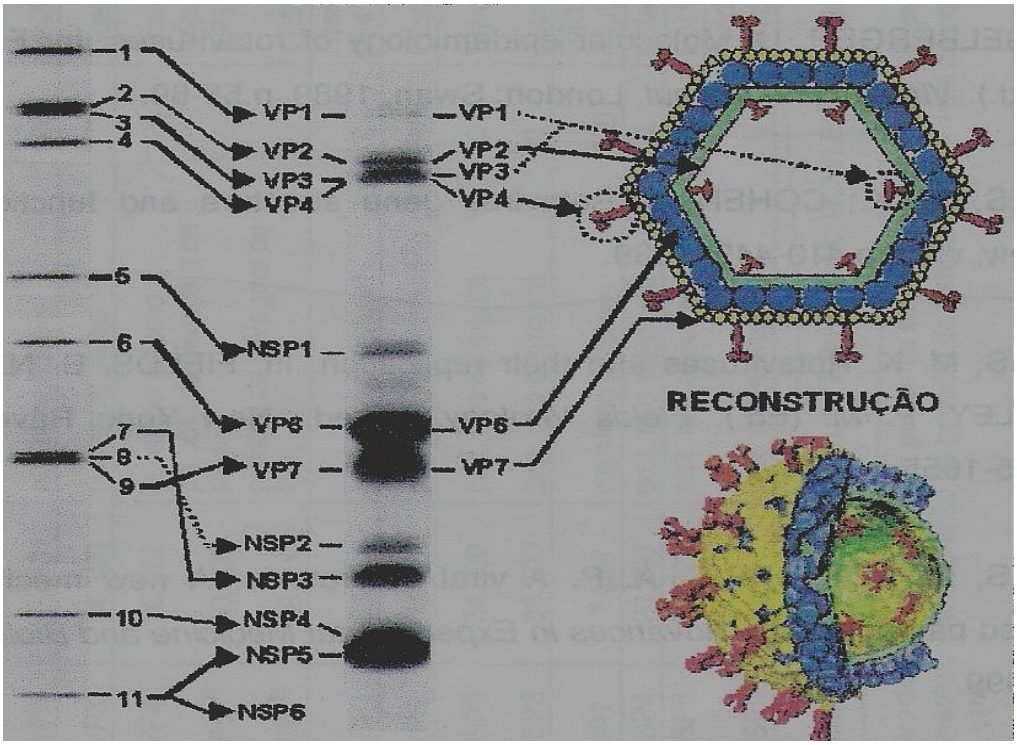

Figura 2 - Eletroforese em gel de poliacrilamida do genoma e das proteínas do rotavírus. RNA fita dupla segmentado do rotavírus, e as proteínas codificadas por cada segmento genômico. Partícula de rotavírus representada esquematicamente.

Fonte: ESTES (2001) (adaptado).

A camada interna do capsídeo (*core* ou núcleo) é constituída pelas proteínas VP2, a mais abundante do núcleo, e pelas proteínas VP1 e VP3, que estão associadas diretamente ao genoma viral. As proteínas estruturais VP4 e VP7 formam a camada externa do capsídeo. A proteína VP6 encontra-se na camada intermediária do capsídeo (ESTES; KAPIKIAN, 2007). As proteínas NSPs são sintetizadas em células infectadas e apresentam funções em etapas do ciclo replicativo ou interação com proteínas do hospedeiro, que influenciam a patogênese ou a resposta imune na infecção (GREENBERG; ESTES, 2009).

A proteína VP6 representa cerca de 50% a 60% da massa viral, é segregada independentemente das proteínas VP7 e VP4 e é altamente antigênica e imunogênica, representa ainda os antígenos de grupo e sorogrupo dos RV. De acordo com as características antigênicas e moleculares da proteína VP6, os RV são classificados em oito grupos/espécies (A-H) (ATTOUI et al., 2012; MATTHIJNSSENS et al., 2012). Recentemente, um novo grupo/espécie de rotavírus denominado I, encontrado em cães foi proposto por Mihalov-Kovács et al. (2015). Os grupos A, B, C e H podem infectar animais e seres humanos e os

sorogrupos D-G e I causam infecção apenas em animais (ESTES; COHEN, 1989; ESTES; KAPIKIAN, 2007; ABE et al., 2009).

As proteínas VP4 e VP7 são responsáveis pela indução de anticorpos neutralizantes. A VP4 realiza a ligação dos RV aos enterócitos, possui um sítio de clivagem pela tripsina que origina duas proteínas, VP5* e VP8*, que são responsáveis por aumentar a infectividade viral. Os antígenos neutralizantes que apresentam reatividade cruzada da proteína VP4 estão localizados na subunidade VP5* e os epítopos tipo específicos (GORZIGLIA et al., 1990) e o local de domínio de maior variabilidade, estão principalmente na VP8* que correlaciona a especificidade genotípica da proteína VP4 (LARRALDE; GORZIGLIA, 1992; LARRALDE et al., 1991). A proteína VP7 é uma glicoproteína altamente imunogênica que está envolvida no processo de penetração do vírus na célula (ESTES; COHEN, 1989; ESTES; KAPIKIAN, 2007).

1.4 ROTAVIRUS A (RVA)

A rotavirose representa um problema de saúde pública mundial, pois o RV é um dos agentes etiológicos mais comuns de gastroenterite aguda em crianças. Em animais, os RV também determinam diarreia em mamíferos domésticos, silvestres e aves (ALFIERI et al., 2004, 2006; ELSCHNER et al., 2005; BARMAN et al., 2006).

RVA é o grupo de RV mais frequentemente detectado e o de maior importância em humanos e animais, sendo verificada a presença do vírus e o quadro clínico de diarreia (ALFIERI et al., 2006; ESTES; KAPIKIAN, 2007; ABE et al., 2009; BUZINARO et al., 2009). A ampla disseminação dos RV é facilitada por sua excreção prolongada e em altos títulos (cerca de 10^{10} a 10^{11} partículas virais por grama de fezes) (MURPHY et al., 1999; ESTES, 2001); pela existência de animais infectados assintomáticos; e pela grande resistência do vírus (ALFIERI et al., 2007), o que facilita a contaminação ambiental e a transmissão (ESTES, 2001).

1.5 ROTAVIROSE BOVINA

RVA causa diarreia principalmente em bezerros, tendo sido relatado alguns casos em bovinos adultos (SATO et al., 1997), e mais raramente em infecções assintomáticas (FUKAI et al., 2007; ABE et al., 2009). As infecções assintomáticas e/ou subclínicas podem afetar a

manutenção e a evolução dos RV, podendo ser encontrados novos genotipos ou genotipos menos relatados (FUKAI et al., 2007; ABE et al., 2009) servindo de fonte de infecção para outros bezerros e animais suscetíveis (LUCCHELLI et al., 1992).

Na bovinocultura, a rotavirose apresenta grande importância epidemiológica e sanitária devido às taxas de morbidade e mortalidade que variam de 8 a 36% e 3 a 6%, respectivamente em animais jovens (ALFIERI et al., 2007). A rotavirose causa alteração na taxa de conversão alimentar, no ganho de peso aumentando custo com reposição de animais e na produção (GARAICOECHEA et al., 2006; ALFIERI et al., 2007).

Os enterócitos apicais das vilosidades do intestino delgado do hospedeiro possuem função digestiva e absortiva, e a replicação do RV ocorre nestas células, acarretando lise e descamação intestinal (LUNDGREN; SVENSSON, 2001). Em bezerros privados de colostro, os enterócitos são substituídos por células cuboides imaturas, provenientes da cripta intestinal que apresentam pouca capacidade de absorção e digestão da lactose presente no leite (LUNDGREN; SVENSSON, 2001). Os bezerros recém-nascidos apresentam reposição de enterócitos mais lenta que os animais mais velhos, nos quais existe uma competição entre a taxa de reposição celular e a replicação vírica, e apenas as cepas virais muito virulentas são capazes de causar diarreia nos animais adultos. No caso dos animais mais jovens, o vírus é capaz de fazer um ciclo completo de replicação e produzir novas progênies virais que podem infectar outras células, o que favorece a duração da infecção e a lesão nas vilosidades intestinais, causando a diarreia (VARSHNEY et al., 1995). Os principais sinais clínicos ocasionados pelo RV em bezerros jovens incluem diarreia, depressão, anorexia e febre baixa (ALFIERI et al., 2007).

1.6 ROTAVIRUS A BOVINO

O RVA é um dos principais agentes etiológicos do “Complexo Diarreia Neonatal Bovina” e acomete, principalmente, animais com até quatro semanas de idade, tanto em criações extensivas quanto intensivas. Os animais são mais frequentemente acometidos entre 16 e 30 dias de idade, diminuindo consideravelmente em animais entre 31 e 45 dias (BARTELS et al., 2010). A maior suscetibilidade dos animais jovens é ocasionada pela deficiência transferência de imunidade passiva aos recém-nascidos, dependente da passagem de imunoglobulinas (IgA) presentes no colostro (ALFIERI et al., 2006; BUZINARO et al., 2009).

Na bovinocultura de corte, o manejo reprodutivo utilizando estação de monta de 90 a 120 dias resulta em concentração dos nascimentos, o que facilita a transmissão viral entre os bezerros. Isto não ocorre com tanta frequência na bovinocultura de leite, uma vez que os nascimentos são distribuídos durante o ano (ALFIERI et al., 2006). A presença de carga viral baixa de RVA circulantes durante o ano todo em bezerros de rebanhos leiteiros e a constante presença de hospedeiros suscetíveis contribui para a diversidade antigênica e molecular do RV (GARAICOECHEA et al., 2006).

1.7 GENOTIPOS G (VP7) E P (VP4) DE RVA EM BOVINOS

As proteínas estruturais VP4 e VP7 de RVA são responsáveis pelas interações iniciais do vírus com a célula hospedeira, pela indução da produção de Acs neutralizantes e são utilizadas na classificação viral em sorotipos e genotipos (ESTES, 1996; HOSHINO et al., 2002). Devido dificuldade de disponibilidade de Acs monoclonais para diferenciação de forma eficiente os RV em sorotipos, a genotipagem é utilizada como alternativa à sorotipagem (ESTES, 1996; HOSHINO et al., 2002).

De acordo com o segmento genômico que codifica as proteínas VP4 e VP7 de RVA são relatados 47 diferentes genotipos P (VP4) e 32 genotipos G (VP7) (RWCG, 2016) em mamíferos domésticos, silvestres e aves. Como o genoma viral é segmentado, pode ocorrer o ressortimento, que é a troca de segmentos genômicos entre cepas virais distintas quando estas infectam a mesma célula, o que pode favorecer o surgimento de diferentes genotipos (PALOMBO, 2002; MARTELLA et al., 2003; ALFIERI et al., 2007; ABE et al., 2009).

Pelo menos 14 genotipos G (G1–G8, G10, G11, G15, G18, G21 e G24) e 11 genotipos P (P[1], P[3], P[5], P[7], P[10], P[11], P[14], P[17], P[21], P[29] e P[33]) já foram detectados em bezerros (SUZUKI et al., 1993; RAO et al., 2000; FUKAI et al., 2002; OKADA; MATSUMOTO, 2002; ABE et al., 2009; MARTELLA et al., 2010; ABE et al., 2011).

Algumas combinações de genotipos G e P tendem a aparecer mais frequentemente na natureza (GORZIGLIA et al., 1988). Os genotipos G e P de RVA mais comumente encontrados em amostras fecais de bezerros com diarreia são G6, G8, G10, P[1], P[5] e P[11]. As combinações de genotipos G e P mais frequentes são: G6P[1] (NCDV - Lincoln), G6P[5] (UK); G6P[11] (KN-4), G10P[11] (B223), G8P[1] (A5) (SNODGRASS et al., 1990; ISHIZAKI et al., 1996; FUKAI et al., 1999, 2002; OKADA; MATSUMOTO, 2002; ALFIEIRI et al., 2004; BARREIROS et al., 2004; GARAICOECHEA et al., 2006; REIDY et

al., 2006; MONINI et al., 2008; BUZINARO et al., 2009; DHAMA et al., 2009; SWIATEK et al., 2009; CASHMAN et al., 2010; MARTELLA et al., 2010; BADARACCO et al., 2012; MIDGLEY et al., 2012; HASSINE-ZAAFRANE et al., 2014; MADADGAR et al., 2015).

Um estudo comparativo de genotipos de RVA em rebanhos bovinos de corte e leite realizado na Argentina (2004-2010) verificou que o genotipo G6P[5] (68.5%) foi a combinação de genotipos mais frequentemente encontrada em rebanhos de corte, enquanto os genotipos G6P[11] (21%), G10P[11] (17%), e G6P[5] (14%) foram os mais relatados em rebanhos leiteiros (BADARACCO et al., 2012). Genotipos semelhantes foram relatados por Garaicoechea et al. (2006), também na Argentina, em que G6P[5] foi o mais prevalente em rebanhos de corte e G6P[11] e G10P[11] em rebanhos leiteiros.

Os genotipos G6 e G10 de RVA são encontrados nas espécies bovina, caprina, suína e humana. Como estes genotipos são comumente encontrados em bovinos, existe grande possibilidade de que os genotipos G6 e G10 de origem humana sejam derivados de RVA bovino por transmissão interespécies (bovino e humano) (COONEY et al., 2001; PALOMBO, 2002).

Os genotipos G6 e G10 de RVA de origem bovina já foram identificados em humanos (YAMAMOTO et al., 2011). Estudos filogenéticos conduzidos com as cepas de RVA pertencentes aos genotipos G6P[14], G12P[11] e G10P[14], circulantes geralmente em bovinos, tem demonstrado relação com cepas descritas em crianças com diarreia em todo o mundo (MONDAL et al., 2012; TACHAROENMUANG et al., 2015). Sugere-se que as rotaviroses podem ocorrer independentemente em diferentes países por meio do ressortimento natural entre cepas locais (MUNFORD et al., 2007; YAMAMOTO et al., 2011). Variações na proteína VP7 dos genotipos G6, G8 e G10 podem ocorrer por transmissão interespécies e/ou ressortimento com diferentes genotipos seguido por seleção natural ou por pressão imune, podendo ocorrer constante evolução das proteínas VP7 e VP4 circulantes, mesmo em condições naturais (CHANG et al., 2000; MARTELLA et al., 2003).

Com base nas sequências de nucleotídeos (nt), sequências de aminoácidos (aa) e na análise filogenética é possível mostrar a heterogeneidade genética em cepas de genotipos G de RVA. Os genotipos G e P de RVA têm sido classificados em linhagens. Os genotipos G6 e G10 são classificados em cinco (I-V) e seis (I-VI) linhagens distintas, respectivamente (BADARACCO et al., 2013). No Brasil, até o presente momento, foram relatadas em rebanhos de corte as linhagens G6 (IV) e G6 (III) em associação com P[5] e P[11], respectivamente (MEDEIROS et al., 2014; DA SILVA MEDEIROS et al., 2015).

Os genotipos P[5] e P[11] são classificados atualmente em nove (I-IX) e três (I-III) linhagens, respectivamente (BADARACCO et al., 2013; DA SILVA MEDEIROS et al., 2015). No Brasil, foram relatadas em rebanhos de corte as linhagens P[5] (IX) e P11 (III) (MEDEIROS et al., 2014; DA SILVA MEDEIROS et al., 2015).

1.8 CONTROLE DAS DIARREIAS NEONATAIS

O bom manejo do rebanho e a imunização são fatores essenciais para o controle e profilaxia de infecções por RV e redução das perdas econômicas (CARUZO et al., 2010).

Em crianças, quando a vacina é administrada precocemente, atua na promoção da imunidade ativa, mimetizando a infecção primária, mas não ocasionando a doença (GLASS et al., 1997). Em bovinos, os anticorpos maternos transmitidos passivamente, mesmo em baixos títulos, inibem a síntese de imunoglobulinas em bezerros neonatos e impedem o sucesso da vacinação em animais jovens, tornando a vacinação precoce ineficiente (TIZARD, 2014a). Nesta espécie animal, a vacina é utilizada na vaca, ao final da gestação, com a intenção de aumentar o título de anticorpos específicos contra o RV no colostro ou leite para proteção do neonato (LU et al., 1994; ALFIERI et al., 2007).

As imunoglobulinas mais abundantes no colostro de bovinos são IgG1 e IgA. Os anticorpos séricos (IgG e IgM) não garantem boa imunidade no recém nascido, diferentemente da IgA, que tem a função de prevenir a aderência de bactérias e vírus na mucosa intestinal e não permitir que o vírus penetre na célula (ALFIERI et al., 2007). A IgA também pode ser transportada através das células intestinais, se ligar com proteínas virais recém sintetizadas, interrompendo desta forma a replicação viral (TIZARD, 2014b).

As vacinas comerciais contra a rotavirose bovina no Brasil são inativadas (Rotatec J5®/ Biogénesis Bagó e ScourGuard® 4KC/Zoetis) e contêm as cepas de RVA NCDV-Lincoln (G6P[1]) e B223 (G10P[11]); (Rotavec® Corona /MSD contém a cepa de RVA UK (G6P[5]). Além do RVA, este imunógeno contém agentes virais e bacterianos causadores de diarreia neonatal em bezerros, coronavírus e *Escherichia coli* (K99). A aplicação intramuscular ou subcutânea da vacina comercial na tábua do pescoço é recomendada em vacas ou novilhas em gestação entre a 3ª e 12ª semanas que antecedem o parto para obtenção de uma melhor qualidade de imunidade colostral aos bezerros (BIOGÉNESIS BAGÓ, 2016; ZOETIS, 2016; MSD, 2016).

Devido à diversidade da associação entre os diferentes genotipos G e P de várias cepas virais, o mecanismo de evasão antigênica é favorecido (PISANELLI et al., 2005; GURGEL et al., 2007), e vem sendo relatados casos de falhas vacinais homólogas e heterólogas tanto em humanos (GURGEL et al., 2007) e animais como suínos (LORENZETTI et al., 2011) e bovinos no Brasil (BARREIROS et al., 2004; DA SILVA MEDEIROS et al., 2015) e no mundo (CLARK et al., 1996; KIM et al., 2002; PHAN et al., 2007; RODRÍGUES-LIMA et al., 2009). Falhas vacinais podem ocorrer por várias razões, inclusive pelo manejo vacinal utilizado (má acondicionamento da vacina, subdose, aplicação em animais com hipertermia), no entanto ainda não é bem esclarecido como as divergências antigênicas que ocorrem entre os antígenos vacinais e de campo afetam a eficácia da imunização (ALKAN et al., 2010).

Desta forma, vigilância epidemiológicas constantes, avaliando diferentes tipos de exploração (corte e leite) com intuito de verificar as cepas de RVA circulantes em bovinos de determinada região ao decorrer do tempo são cruciais para a compreensão da evolução viral e epidemiologia da infecção do RVA.

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2 OBJETIVOS

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2.1 Objetivo geral

- Determinar a frequência de rotavírus A e os genotipos G (VP7) e P (VP4) em cepas identificadas em episódios de diarreia neonatal em bezerros de corte e leite, provenientes das três regiões geográficas brasileiras, no período de 2006 a 2015.

2.2 Objetivos específicos

- Determinar a frequência de diagnóstico de RVA por ss-PAGE em fezes diarreicas de bezerros;

- Avaliar as frequências de RVA em fezes diarreicas de bezerros de acordo com o tipo de produção (corte / leite); região geográfica e distribuição temporal em dois períodos de 5 anos (2006-2010 e 2011-2015);

- Realizar análises filogenéticas comparativas incluindo as cepas identificadas neste estudo e cepas de RVA de origem bovina, suína e humana disponíveis em bases públicas de dados (GenBank).

3 ARTIGOS PARA PUBLICAÇÃO

3.1 Neonatal diarrhea and rotavirus A infection in beef and dairy calves, Brazil, 2006-2015

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Neonatal diarrhea and rotavirus A infection in beef and dairy calves, Brazil, 2006-2015

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Abstract Calf diarrhea causes substantial economic losses in cattle industry worldwide. Bovine rotavirus A (RVA) is the main viral agent which leads to enteric infection and diarrhea outbreaks in calves throughout the world. The aim of this retrospective (2006-2015) study was to determine the frequency of RVA detection in diarrheic fecal samples of beef and dairy calves. It were evaluated by silver stained-polyacrylamide gel electrophoresis (ss-PAGE) technique 1,498 diarrheic fecal samples from 124 beef and 56 dairy cattle herds from the three main cattle producing regions of Brazil. The RVA double stranded-RNA was identified by ss-PAGE technique in 410 (27.4%) fecal samples. The frequency of positive samples found in beef calves (31.9%; 328/1,027) was significantly higher ($p \leq 0.05$) than the frequency identified in diarrheic fecal samples from dairy calves (17.4%; 82/471). RVA infection was identified in calves from three geographical regions of Brazil, however, the frequency of positive diarrheic calves in the Midwest region (39.4%), predominantly from beef cattle, was significantly higher ($p \leq 0.05$) than the South (19.4%) and Southeast (17.6%) regions. There was no significant difference when the temporal distribution of bovine rotavirus infected calves was evaluated in five years periods (first: 2006-2010; second: 2011-2015). Considering the wide regional and temporal scope of this study it can be concluded that RVA remains one of the major neonatal diarrhea etiologies in calves of Brazilian cattle herds.

Keywords Calf · Enteric infection · RVA · ss-PAGE · Epidemiology

1 Introduction

Neonatal diarrhea causes health problem in livestock production worldwide (Holland 1990). The diarrhea outbreaks in calves have severe direct and indirect economic consequences due to mortality and reduced growth rates, increase age at first calving, treatment costs, and time spent caring for the affected calves (Alfieri et al. 2006; Freitas et al. 2011; Windeyer et al. 2014).

Diarrhea in calves has a multifactorial etiology. Virus, bacteria, and protozoa infection; immunological conditions, and also management factors (housing, feeding, hygienic conditions) play an important role as determining and predisposing factors (Bendali et al. 1999; Alfieri et al. 2006; Windeyer et al. 2014). With regard to the enteropathogens, *Escherichia coli* K99, *Cryptosporidium parvum*, coronavirus, and rotavirus have been detected in most cases of intestinal infections in young calves (Nussbaum et al. 1999; Bartels et al. 2010).

Rotavirus infections have a worldwide distribution and is one of the most important etiological viral agents of severe gastroenteritis in young humans and many mammalian and avian species (Estes and Kapikian 2007; Attoui et al. 2012).

Rotaviruses belong to the *Reoviridae* family, genus *Rotavirus*. The virus has 70-100 nm in diameter and is characterized by a non-enveloped triple-layered protein capsid with a genome composed by 11 segmented double-stranded RNA (dsRNA) translated into six structural (VP1-VP4, VP6-VP7) and six non-structural (NSP1-NSP5/6) proteins (Estes and Kapikian 2007). Based on the antigenic properties of the VP6 protein that composes the middle layer of the viral capsid, rotaviruses are classified into eight species designated A through H (Attoui et al. 2012; Matthijnssens et al. 2012). Recently, a new rotavirus species was proposed and tentatively named I (Mihalov-Kovacs et al. 2015).

Infection by RV A (RVA), B, and C were already reported in cattle. However, RVA is the most common rotavirus specie causing neonatal diarrhea outbreaks in calves worldwide (Buzinaro et al. 2003; Ghosh et al. 2010; Medeiros et al. 2014; Otto et al. 2015).

In acute infections, high titers of RV is excreted in the feces (Murphy et al. 1999; Estes and Kapikian 2007). Therefore, the virus or viral proteins and genome identification can be performed by several laboratorial methods (Medeiros et al. 2014).

Silver stained-polyacrylamide gel electrophoresis (ss-PAGE) is a simple, fast, and low cost method for rotavirus dsRNA detection in diarrheic feces. The technique has high specificity and good sensitivity for the rotavirus identification in acute infections (Herring et al. 1982; Gregori et al. 2000; Campos et al. 2002).

This retrospective study (2006-2015) had the aim to describe the frequency of RVA diagnosis in diarrheic fecal samples of beef and dairy calves of the three main cattle producing regions of Brazil.

2. Materials and Methods

2.1 Study population

The diarrheic fecal samples included in this study were selected from the collection of fecal samples sent by convenience to the Animal Virology Laboratory that is a national reference for control and diagnosis of animal rotavirus. The samples were collected from January 2006 to December 2015 in beef and dairy herds from the three main cattle producing regions of Brazil (Midwest; South, and Southeast). A total of 1,498 fecal samples were included in the study, being 1,027 samples from diarrheic beef and 471 from dairy calves. It was included in the analysis only samples collected from diarrheic calves up to 60 days of age. Additional information about the origin of the fecal samples is presented in table 1. The fecal samples were stored at -80°C until analysis. All experimental procedures were approved and conducted following the Ethics Committee on Animal Experiments of the Universidade Estadual de Londrina under nº 072/2013.

Table 1 Fecal samples for rotavirus diagnosis in neonatal diarrhea distributed according to origin (region) and type (beef / dairy) of cattle production, Brazil, 2006-2015.

Region	State	County Number	Herds			Samples		
			Beef	Dairy	Total	Beef	Dairy	Total
South	RS / SC / PR	43	38	32	70	250	327	577
Southeast	SP / MG	33	20	20	40	174	122	296
Midwest	MS / GO / MT	44	66	4	70	603	22	625
Total		**120**	**124**	**56**	**180**	**1,027**	**471**	**1,498**

2.2 Nucleic acid extraction

Fecal suspensions 20% (w/v) in buffer Tris-Ca^{2+} pH 7.4 (50 mM Tris-HCl; 10 mM NaCl; 1.5 mM 2-mercaptoetanol; 3 mM $CaCl_2$) were homogenized and centrifuged at 2,000 x *g* for 5 min at 4ºC. Aliquots of 500 µL of supernatant were collected and treated with 50 µL of SDS (sodium dodecyl sulphate) at a final concentration of 1%. The nucleic acid was extracted using a combination of the methods phenol/chloroform/isoamyl alcohol (25:24:1) and silica/guanidinium isothiocyanate (Alfieri et al. 2006). The nucleic acid was eluted in 50 µL of ultrapure water treated with DEPC (Invitrogen Life Technologies, Carlsbad, CA, USA) and shortly stored at -20°C. The cell culture adapted NCDV-Lincoln strain of bovine RVA and aliquots of Tris-Ca^{2+} buffer were included as positive and negative controls, respectively, in all RNA extraction procedures.

2.3 ss-PAGE

The presence of RVA dsRNA in diarrheic fecal samples was evaluated by the ss-PAGE technique (Herring et al. 1982; Pereira et al. 1983).

2.4 Statistic analysis

The chi-square, performed through the software Minitab 16.1.1.0 adopting a *p* value of $p \leq 0.05$, was used to analyze the frequencies of RVA diagnosis in diarrheic calves according to the regional and temporal distribution.

3 Results

The dsRNA of bovine RVA was identified in 27.4% (410/1,498) of the diarrheic fecal samples included in this study. The rate of positive samples was significantly higher ($p \leq 0.05$) in calves from beef (31.9%; 328/1,027) than dairy (17.4%; 82/471) herds.

Rotavirus infection was identified in calves from three Brazilian geographical regions, however, the frequency of positive diarrheic calves in the Midwest region (39.4%) was significantly higher ($p \leq 0.05$) than the South (19.1%) and Southeast (17.6%) regions (Table 2).

Table 2 Rotavirus A identified by ss-PAGE in diarrheic fecal samples from calves distributed according the geographical origin of the cattle herds, Brazil, 2006-2015.

Geographical Region	**ss-PAGE**		
	Positive (%)	**Negative**	**Total**
South	112 (19.4)[a]	465	577
Southeast	52 (17.6)[a]	244	296
Midwest	246 (39.4)[b]	379	625
Total	**410 (27.4)**	**1,088**	**1,498**

Lowercase letters means significant difference (p <0.05) between Brazilian regions.

The frequencies of RVA diagnosis by ss-PAGE in diarrheic calves according to the temporal distribution is presented in Table 3.

Table 3 Temporal distribution of rotavirus A identified by ss-PAGE in diarrheic calves, Brazil, 2006-2015.

Years	ss-PAGE/ samples		Total
	Positive (%)	Negative	
2006 - 2010	123 (24.5)[a]	378	501
2011 - 2015	287 (28.6)[a]	710	997
Total	**410 (27.3)**	**1,088**	**1,498**

Lowercase letter means that was no significant difference ($p < 0.05$) between temporal distribution.

4 Discussion

Neonatal diarrhea is a major health problem in suckling animals in livestock production worldwide. The global burden of rotavirus disease in calf rearing is immense. Mortality, poor growth rate, detrimental effect on later performance, and the treatment costs are the major deleterious consequences of neonatal diarrhea in calves. In Brazil as well as around the world also diarrhea outbreaks are the main health problem in the beef and dairy calf rearing (Alfieri et al. 2004; Alfieri et al. 2006; Freitas et al. 2011; Medeiros et al. 2014).

The current study, with retrospective design, evaluated for a period of 10 years (2006-2015) the frequency of RVA diagnosis in diarrheic fecal samples of beef and dairy calves from three geographical regions representing about 65% of the Brazilian cattle industry (BRASIL 2016). The detection rate (27.4%) of RVA-positive fecal samples was similar to identified in other epidemiological studies performed in Brazil (Alfieri et al. 2006; Buzinaro et al. 2009), Argentina and Iran (Badaracco et al. 2012; Madadgar et al. 2015). Therefore, the present information highlight that similar frequency of RVA in different countries with the

same behavior of the disease, beside the different management system, temperature, humidity, and animals breed, between others risk factors.

The frequency of RVA-positive fecal samples in diarrheic calves from beef herds (31.9%) was significantly higher ($p \leq 0.05$) than in dairy herds (17.4%). Neonatal diarrhea in calves is a multifactorial syndrome. Some characteristics differentiated in the management of beef and dairy farms in Brazilian cattle breeding adopted in the last decade can partly explain the higher frequency of diagnosis of RVA infection in beef farms.

The use of fixed-time artificial insemination in breeding seasons of two or three months has been widespread in almost all the most technified extensive beef cattle herds and also those herds with the highest number of cows. As a result of this reproduction management there is a concentration of calves birth. Some farms also use farrowing picket and rotate pickets. Thus, the challenge is even greater, and consequently, also the risk of infection because the concentration of birth provide considerable increase in the number of animals susceptible to infection at any given time. Thus, in the field diarrhea outbreaks in beef calves has been frequent in the main Brazilian cattle producing regions (Buzinaro et al. 2003; Medeiros et al. 2014)

Cattle herds in three Brazilian geographical regions with different management system were evaluated in this study. Although in the Southern region was evaluated higher number of diarrheic fecal samples in relation to the Southeast region, there was no significant difference in the frequency of RVA-positive samples identified in these two regions.

The prevalence of diarrhea by RVA infection identified in the Midwest region was significantly higher ($p \leq 0.05$) than the other regions. Some herd and management characteristics present in most of the farms of this region may have contributed to the higher frequency of diarrhea. In this context we highlight some risk factors: 1) short breeding season with a concentration of calving; 2) herd size, since this region is characterized by the presence of large herds with extensive management; 3) greater frequency of crossbreeding producing calves (*B. indicus* x *B. taurus*) with lower rusticity than Nelore (*B. indicus*) calves; 4) farrowing picket; 5) population density.

The temporal distribution of bovine rotavirus infection in calves as a result of the number of diarrheic fecal samples evaluated each year was not uniform. In order to reduce this sampling bias we chose to analyze the results in two five-year periods represented by the years 2006-2010 (first) and 2011-2015 (second). Although in the second period were evaluated more fecal samples (n=995) compared to those analyzed in the first period (n=501)

there was no significant difference in the frequency of RVA-positive results found in both periods of time.

A study performed in 1998-2002, by our research group detected bovine RVA in 19.4% (369/1898) of the samples collected in calves with diarrhea, from beef and dairy cattle herds from four Brazilian geographical regions (South, Southeast, Midwest, and North). The proportion of positive samples collected was 22.8% (205/899) and 16.9% (169/999), from beef and dairy cattle herds respectively (Alfieri et al. 2006). Comparing the results, it can be observed that in nearly 20 years of these temporal studies the frequency of RVA diagnostic in Brazilian cattle herds stay practically the same.

Probably the highest rate of diagnosis of RVA in beef herds in the Midwest Brazilian region due to changes implemented in reproductive management, such as time-fixe artificial insemination, which culminated with an increased risk of enteric infections in neonates.

Protocols for the control of neonatal diarrhea in calves have been implemented in some countries and studies indicate that the commercial vaccines currently in use are appropriated to provide protection against RVA infection in bovines (Collins et al. 2014).

Considering the quantitative and qualitative increase in Brazilian cattle industry, measures of control and prevention of bovine neonatal diarrhea outbreaks is necessary and urgent. For this the mitigation of risk factors and the implementation of a vaccination program against bovine neonatal diarrhea are two important actions to be induced in the context of health programs in farm animals.

5 Conclusion

Based on the wide regional and temporal scope of this study, we can conclude that the RVA remains one of major neonatal diarrhea etiologies in calves of Brazilian beef cattle herds. There was no difference in the rate of RVA diagnosis when evaluating two five-year periods (2006-2010; 2011-2015). The RVA infection rate is higher in calves of beef cattle herds in relation to the dairy herds and that in the Midwest, where predominated diarrheic fecal

samples from beef herds, and the rate of RVA-positive diagnosis was significantly higher than the other regions (South and Southeast) included in the study.

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3.2 G and P genotypes profile of field strains of rotavirus A bovine circulating in beef and dairy cattle herds in Brazil, 2006 - 2015

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G and P genotypes profile of field strains of rotavirus A bovine circulating in beef and dairy cattle herds in Brazil, 2006 - 2015

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Abstract Group A rotavirus (RVA) is one of the main causes of neonatal calf diarrhea worldwide. The VP7 and VP4 genes of RVA determine the binary RVA classification in G and P genotypes, respectively. Bovine RVA strains mainly possess combinations of genotypes G6P[1], G6P[5], G6P[11], G8P[1], and G10P[11]. The aim of this study was to describe the G and P genotypes of Brazilian bovine RVA wild-type strains detected on diarrheic calves. Samples were selected from a collection that consisted of 1,589 fecal samples, collected from 2006 to 2015, and previously evaluated for the presence of RVA by silver-stained polyacrylamide gel electrophoresis (ss-PAGE) technique. Of these, 417 (26.2%) samples were RVA-positive. To increase the genetic diversity of RVA strains as inclusion criteria for the selection of samples to be genotyped in this study it was chosen one or two RVA-positive fecal samples per cattle herd. With this, for the genotyping study, 155 RVA strains from 70 beef and 30 dairy cattle herds from all Brazilian geographical regions were selected. The G and P genes of RVA strains were amplified by RT-PCR and sequenced. The Brazilian bovine RVA strains included in this study belonged to the G6, G10, P[5], and P[11] genotypes. A different distribution of G and P genotypes combination was found according to the type of farm being the G6P[5] genotype more prevalent (65.5%) in beef herds and the G10P[11] (38.4%) and G6P[11] (30.8%) genotypes prevalent in dairy herds (Fisher's exact test $p<0.05$). The distribution of G and P genotype of bovine RVA showed different aspects depending of the Brazilian geographical region analyzed. Midwest was the region with the highest number of genotyped RVA strains, but only the genotype G6 was identified. South and Southeast were the regions with the greatest genetic diversity of G and P genotypes. In the nucleotide phylogenetic tree some Brazilian bovine RVA strains could be divided into lineages. The genotype combination between P[5] and G6(IV), prevalent in beef herd, and between P[11] and G6(III) or G10(V), prevalent in dairy herds, were found grouped together with human origin RVA strains. In addition, for the first time in Brazil was detected one RVA strain that the sequence of genotype P[5] grouped together with lineage II. Worldwide, 14 and 11 different G and P genotypes, respectively, have been described in bovine RVA strains. Considering that a great number of RVA strains from the main Brazilian geographical regions of cattle industry was evaluated, the diversity of G and P genotypes found in this study was limited. The importance of genotyping studies particularly with retrospective (2006-2015) design carried out in several different geographical regions or in different production types (beef and dairy) of cattle herds contribute to comprehension of viral evolution and understanding of the epidemiology of the RVA infection.

Keywords: Cattle · diarrhea · RVA · genotypes · lineages.

1 Introduction

Rotaviruses are the main viral etiology of diarrhea in children and young animals of a wide variety of species of mammals and birds worldwide [3, 4, 8, 15].

Rotaviruses belong to the *Reoviridae* family, genus *Rotavirus*. The virus has 70-100 nm in diameter characterized by a non-enveloped triple-layered protein capsid with a genome composed by 11 segments of double-stranded RNA (dsRNA) translated into six structural and six non-structural proteins [16]. Based on antigenic characteristics of the VP6 protein that compose the middle layer of the viral capsid, rotaviruses can be classified in eight (A-H) distinct serological groups [5, 33]. Recently a new group of rotavirus, tentatively named I was proposed, found in dogs [36].

The group A rotavirus (RVA) is the most common cause of acute gastroenteritis in young children and neonatal diarrhea in production animals such as piglets and calves [3, 49]. According to the antigenic and molecular characteristics of the two proteins (VP7 and VP4) present in the outer layer of viral capsid, the RVA can be classified into serotypes / genotypes G and P, respectively [16]. So far, 32 G genotypes and 47 P genotypes have been described in RVA strains identified in human and animal hosts [18, 32].

Several rotavirus genotypes have been detected in calves, at least 14 different G genotypes (G1–G8, G10, G11, G15, G18, G21, and G24) and 11 P genotypes (P[1], P[3], P[5], P[7], P[10], P[11], P[14], P[17], P[21], P[29], and P[33]) [1, 2, 19, 20, 31, 40, 44, 46]. The G6P[1] (NCDV strain), G6P[5] (UK strain), G6P[11] (KN-4 strain), G8P[1] (A5 strain), and G10P[11] (B223 strain) genotype combinations are considered epidemiologically important in beef and dairy cattle herds worldwide [4, 6, 9, 12, 14, 31, 38].

Based on nucleotide (nt), deduced amino acid (aa) sequences and in phylogenetic analysis it is possible to show genetic heterogeneity in G and P genotypes of wild-type RVA strains. The G and P genotypes of RVA has still been phylogenetically classified into lineages. The G6 and G10 genotypes are classified in five (I-V) and six (I-VI) distinct lineages, respectively, while in P[5] and P[11] genotypes eight (I-VIII) and three (I-III) lineages, respectively, has been described so far [7].

The aim of this study was to describe the G (VP7 gene) and P (VP4 gene) genotypes of wild-type Brazilian RVA strains identified in diarrheic calves from 2006 to 2015.

2 Materials and Methods

2.1 Inclusion criteria

The diarrheic fecal samples included in this study were selected from fecal samples sent to the Animal Virology Laboratory that is a national reference for control and diagnosis of animal rotavirus. The collection consisted of fecal specimens of dairy and beef calves aging until 60 days old from all Brazilian geographical regions from 2006 to 2015, which were maintained stored at -80ºC. The sampling comprising 1,589 diarrheic fecal specimens was previously evaluated by silver-stained polyacrylamide gel electrophoresis (ss-PAGE) technique for RVA dsRNA detection, 417 (26.2%) were RVA-positive. In order to obtain major diversity in relation to the geographic regions, states, counties, and herds included in the analysis one or two fecal samples were selected per farm. Even as inclusion criteria in those herds with more than two RVA-positive samples it selected the specimens with best results in the ss-PAGE technique including staining intensity of the bands. Based on these inclusion criteria, 155 RVA-positive fecal samples from 116 beef and 39 dairy calves were selected to determine the G and P genotypes. The calves were from 70 beef and 30 dairy farms located in five Brazilian geographical regions. All experimental procedures were approved and conducted following the Ethics Committee on Animal Experiments of the Universidade Estadual de Londrina under nº 072/2013.

2.2 Nucleic acid extraction

The viral dsRNA extraction was performed using fecal suspensions 20% (w/v) in buffer Tris-Ca^{2+} pH 7.4 (50 mM Tris-HCl; 10 mM NaCl; 1.5 mM 2-mercaptoetanol; 3 mM $CaCl_2$), centrifuged at 2,000 x *g* for 5 min at 4ºC. Aliquots of 500 µL of supernatant and 50 µL of SDS (sodium dodecyl sulphate) were homogenized and incubated at 56°C for 20 min. The nucleic acid was extracted using a combination of the methods phenol/chloroform/isoamyl alcohol (25:24:1) and silica/guanidinium isothiocyanate [3]. The RNA was eluted in 50 µL of ultrapure water treated with DEPC (Invitrogen Life Technologies, Carlsbad, CA, USA) and shortly stored at -20°C until use. Aliquots of Tris-Ca^{++} buffer were included as negative controls in all viral RNA extraction procedures.

2.3 RT-PCR assay

The nucleic acid was submitted to RT-PCR assay using rotavirus VP7 and VP4 consensus primers that amplifies 1,010 [26] or 1,062 bp [23] and 863 [26] or 876 bp [22, 30], to determine G and P genotypes, respectively.

The RT-PCR products were analyzed by electrophoresis on 2% agarose gel in TBE buffer pH 8.4 (89 mM Tris; 89 mM boric acid; EDTA 2 mM) containing 0.5 μg/mL ethidium bromide. After electrophoresis at constant voltage (100V) for 40 min, the gel was visualized under UV light.

2.4 Sequencing analysis

RT-PCR products were purified using a GFX PCR DNA and Gel Band purification commercial kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and quantified with a Qubit® Fluorometer (Invitrogen Life Technologies, Eugene, OR, USA). The samples were sequenced with an sequencer ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the forward and reverse primers used in the RT-PCR assay. The nucleotide sequence quality analysis and contig assembly of the RVA gene sequences were performed with Phred and CAP3 software, respectively, and the sequences were accepted if the base quality score was ≥20. Similarity searches were performed against sequences deposited in database using the BLASTn software. Multiple and pairwise alignments with RVA strains available in GenBank were performed with MEGA software v5 and the sequence identity matrix was constructed using BioEdit software version 7.0.8.0. Phylogenetic trees based on the nucleotide sequences were obtained using the neighbor-joining method with the kimura two-parameter model using MEGA v5 software. The bootstrapping probabilities were calculated using 1,000 replicates. The VP7 and VP4 genes of the Brazilian bovine RVA strains described in this study are available in GenBank.

2.5 Statistic analysis

The frequencies of G and P genotypes identified in diarrheic beef and dairy calves were analyzed by Fisher's exact test through the statistical software Minitab 16.1.1.0 adopting a value of $p \leq 0.05$.

3 Results

The most frequent G (VP7) and P (VP4) genotypes detected in the Brazilian wild-type bovine RVA strains evaluated in this study were G6 and P[5]. The G6 genotype was identified in combination with P[5], P[11], and P not-determined (P[x]). With the exception of two RVA strains in which the G genotype could not be identified the P[5] genotype was found only in combination with G6 genotype (Table 1).

Table 1. G (VP7) and P (VP4) genotypes of bovine RVA strains found in diarrheic calves of Brazilian cattle herds, 2006-2015

P (VP4 gene)	G (VP7 gene)			Total
	G6	G10	Gx	
P[5]	85	–	2	87
P[11]	34	19	–	53
P[x]	14	1	–	15
Total	**133**	**20**	**2**	**155**

A different distribution of G and P genotypes combination was found according to the herd type, being the G6P[5] (65.5%) genotype combination that occurred more frequently in beef, while the genotypes G10P[11] and G6P[11] were more frequent, 38.4 and 30.8% respectively, in dairy cattle herds (Fisher's exact test $p<0.05$) (Table 2).

Table 2. G and P genotypes of group A rotavirus (RVA) strains identified in diarrheic beef and dairy calves in Brazil, 2006-2015.

RVA Genotype	Calves		Total (%)
	Beef (%)	Dairy (%)	
G6P[5]	76 (65.5)[a]	9 (23.1)[ab]	85 (54.8)
G6P[11]	22 (19.0)[b]	12 (30.8)[a]	34 (21.9)
G10P[11]	4 (3.4)[cd]	15 (38.4)[a]	19 (12.3)
G6P[X]	11 (9.5)[c]	3 (7.7)[b]	14 (9.0)
G10P[X]	1 (0.9)[d]	–	1 (0.7)
GXP[5]	2 (1.7)[d]	–	2 (1.3)
Total	**116**	**39**	**155**

Lowercase letters indicate significant difference ($p < 0.05$) between genotypes (beef-dairy).

The bovine RVA G and P genotypes distribution showed different aspects according to the geographical regions evaluated. The Midwest which is the major in the Brazilian cattle industry, was the region with the highest number of RVA strains genotyped, but only the G6 genotype was identified in this region. South and Southeast were the regions with the greatest genetic diversity of RVA G and P genotypes. The G6 genotype was demonstrated in RVA strains of all the five Brazilian regions included in the study. The P[5] and P[11] genotypes of RVA strains were found in the principal bovine producing regions of Brazil (Table 3).

Table 3. Most common genotypes (G – P) of RVA strains identified in diarrheic calves, according to the herds of the Brazilian geographical regions, 2006-2015.

RVA Genotype	Geographical regions of Brazil					Total
	South	Southeast	Midwest	North	Northeast	
G6P[5]	17	9	56	–	3	85
G6P[11]	5	10	18	1	–	34
G10P[11]	15	4	–	–	–	19
G6P[X]	4	2	8	–	–	14
G10P[X]	1	–	–	–	–	1
GXP[5]	–	1	1	–	–	2
Total	**42**	**26**	**83**	**1**	**3**	**155**

- none sample showed this genotype combination

G and P genotypes combination of RVA strains distributed according the year of fecal sample collection identified G6P[5] genotype in rotavirus strains from each year evaluated. G6P[11] genotype was not found only in two years of study (2006 and 2008). The G10P[11] genotype only was identified since 2011 in the present study (Table 4).

Table 4. G and P genotype combination in Brazilian wild-type group A rotavirus (RVA) strains identified in diarrheic calves in cattle herds distributed according the year of fecal sample collection.

Year	Bovine RVA G and P genotypes						
	G6P[5]	G6P[11]	G10P[11]	G6P[X]	G10P[X]	GXP[5]	Total
2006	3	–	–	–	–	–	3
2007	4	4	–	–	–	–	8
2008	2	–	–	2	–	–	4
2009	20	2	–	1	–	–	23
2010	1	3	–	–	–	–	4
2011	5	7	1	1	–	–	14
2012	7	3	1	–	–	–	11
2013	11	6	8	5	1	–	31
2014	28	8	9	2	–	–	47
2015	4	1	–	3	–	2	10
Total	**85**	**34**	**19**	**14**	**1**	**2**	**155**

The sequences of the VP7 gene of the Brazilian RVA strains identified were compared with G6 and G10 genotype sequences deposited in the GenBank. The G6 genotype fragment with 673 nt (145-877 nt) presented elevated (93.3 to 95.6%) nt identity with the prototypes of the lineage G6(III) Hun3 strain (G6P[9] genotype) from human and a buffalo strain 10733 (G6P[3] genotype). When compared with G6(IV) lineage the Brazilian RVA sequences presented high (90.3 to 96.2%) nt identity with the equine prototype Erv99 strain (G6P[X] genotype) and UK strain (G6P[5] genotype), respectively. The phylogenetic tree constructed using nt sequences displayed all Brazilian G6 genotype sequences from this study grouped with G6(III)P[11] or G6(IV)P[5] lineages, respectively (data not shown), as demonstrated in Figure 1.

The G10 lineages were aligned with the Brazilian RVA sequences, showing a high (87.8 to 95.1%) nt identity to G10(V) lineage prototypes of human I321 and with cow B223, respectively, both G10P[11] genotype (Figure 2).

The VP4 sequences showed products with two different fragments, depending to the pair of the primer used in the RT-PCR for P genotyping. The Brazilian bovine RVA strains were identified and compared with P[5] and P[11] genotypes sequences from GenBank. The RVA strains with a fragment with 612 nt (105-718 nt) and 648 nt (1,166-1,815 nt) showed a high identity to P[5](IX) lineage prototype, clustering in a new branch. However, using the primers described by Isegawa et al. (1993) (Figure 3) one RVA strain showed a high (95.5%) nt identity with a P[5]-II lineage from a cow, 791_BA prototype, (G10P[5] genotype), described in Argentina.

The nucleotide sequences of Brazilian bovine RVA P[11] strains with 697 nt (125-821 nt), clustered with the strains belonging to the genetic lineage III showing a higher similarity (91.5 to 97.5 %) with bovine K33 and giraffe GirRV-2-Gir strains, respectively, both G10P[11] genotypes (Figure 4).

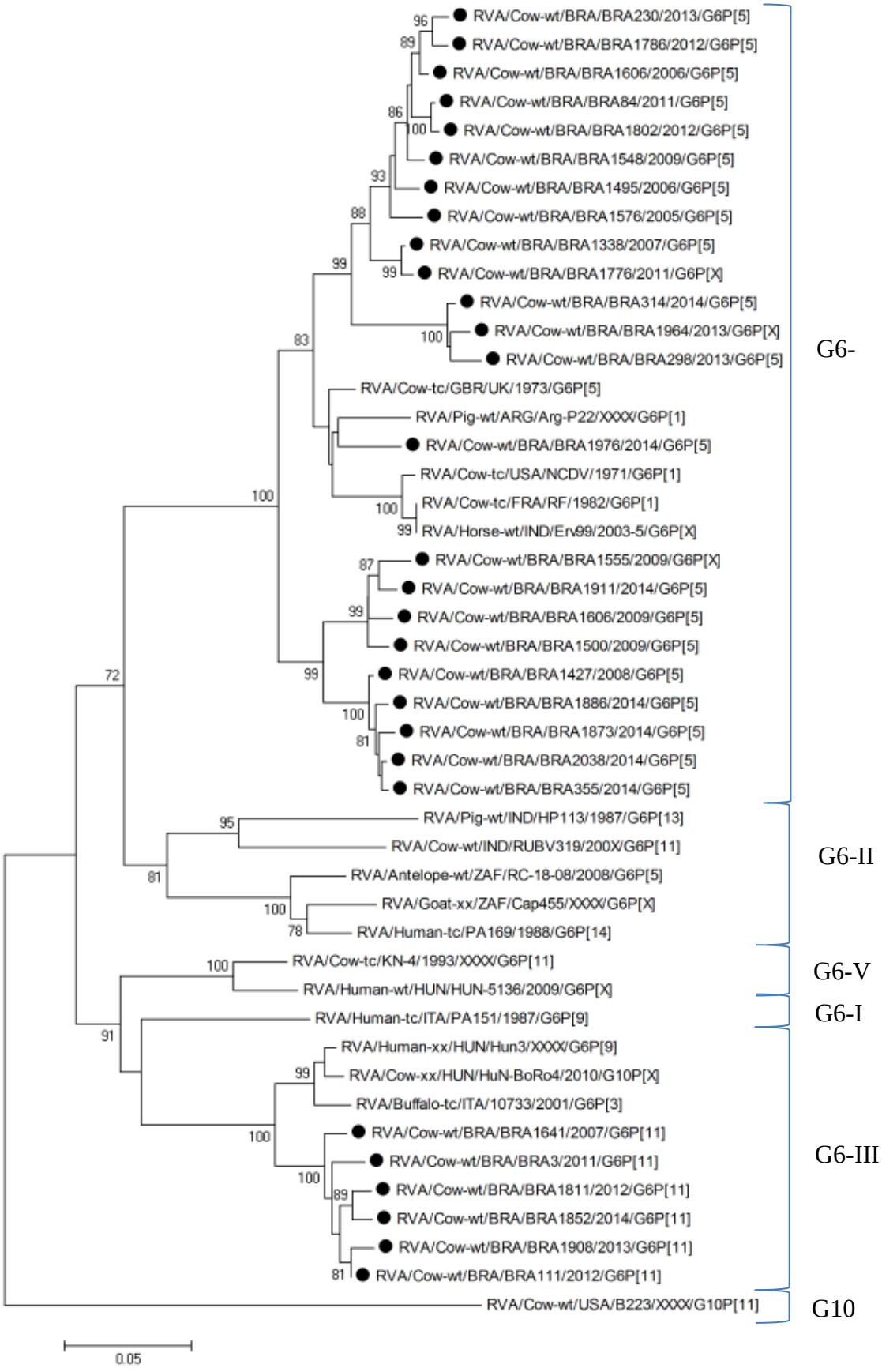
RVA/Cow-wt/BRA/BRA230/2013/G6P[5]
RVA/Cow-wt/BRA/BRA1786/2012/G6P[5]
RVA/Cow-wt/BRA/BRA1606/2006/G6P[5]
RVA/Cow-wt/BRA/BRA84/2011/G6P[5]
RVA/Cow-wt/BRA/BRA1802/2012/G6P[5]
RVA/Cow-wt/BRA/BRA1548/2009/G6P[5]
RVA/Cow-wt/BRA/BRA1495/2006/G6P[5]
RVA/Cow-wt/BRA/BRA1576/2005/G6P[5]
RVA/Cow-wt/BRA/BRA1338/2007/G6P[5]
RVA/Cow-wt/BRA/BRA1776/2011/G6P[X]
RVA/Cow-wt/BRA/BRA314/2014/G6P[5]
RVA/Cow-wt/BRA/BRA1964/2013/G6P[X]
RVA/Cow-wt/BRA/BRA298/2013/G6P[5]
RVA/Cow-tc/GBR/UK/1973/G6P[5]
RVA/Pig-wt/ARG/Arg-P22/XXXX/G6P[1]
RVA/Cow-wt/BRA/BRA1976/2014/G6P[5]
RVA/Cow-tc/USA/NCDV/1971/G6P[1]
RVA/Cow-tc/FRA/RF/1982/G6P[1]
RVA/Horse-wt/IND/Erv99/2003-5/G6P[X]
RVA/Cow-wt/BRA/BRA1555/2009/G6P[X]
RVA/Cow-wt/BRA/BRA1911/2014/G6P[5]
RVA/Cow-wt/BRA/BRA1606/2009/G6P[5]
RVA/Cow-wt/BRA/BRA1500/2009/G6P[5]
RVA/Cow-wt/BRA/BRA1427/2008/G6P[5]
RVA/Cow-wt/BRA/BRA1886/2014/G6P[5]
RVA/Cow-wt/BRA/BRA1873/2014/G6P[5]
RVA/Cow-wt/BRA/BRA2038/2014/G6P[5]
RVA/Cow-wt/BRA/BRA355/2014/G6P[5]
RVA/Pig-wt/IND/HP113/1987/G6P[13]
RVA/Cow-wt/IND/RUBV319/200X/G6P[11]
RVA/Antelope-wt/ZAF/RC-18-08/2008/G6P[5]
RVA/Goat-xx/ZAF/Cap455/XXXX/G6P[X]
RVA/Human-tc/PA169/1988/G6P[14]
RVA/Cow-tc/KN-4/1993/XXXX/G6P[11]
RVA/Human-wt/HUN/HUN-5136/2009/G6P[X]
RVA/Human-tc/ITA/PA151/1987/G6P[9]
RVA/Human-xx/HUN/Hun3/XXXX/G6P[9]
RVA/Cow-xx/HUN/HuN-BoRo4/2010/G10P[X]
RVA/Buffalo-tc/ITA/10733/2001/G6P[3]
RVA/Cow-wt/BRA/BRA1641/2007/G6P[11]
RVA/Cow-wt/BRA/BRA3/2011/G6P[11]
RVA/Cow-wt/BRA/BRA1811/2012/G6P[11]
RVA/Cow-wt/BRA/BRA1852/2014/G6P[11]
RVA/Cow-wt/BRA/BRA1908/2013/G6P[11]
RVA/Cow-wt/BRA/BRA111/2012/G6P[11]
RVA/Cow-wt/USA/B223/XXXX/G10P[11]
0.05
G6-
G6-II
G6-V
G6-I
G6-III
G10

Figure 1 Phylogenetic tree with 673 bp (145-877 nt) of the G6 lineage of wild-type Brazilian bovine RVA strains. The following accession numbers are available on GenBank: UK (X00896), Arg-P22 (EF474079), NCDV-Lincoln (M12394), RF (X65940); HP113 (DQ003292), RUBV319 (EF199501), RC-18-08 (FJ495133), Cap455 (AY128708), PA169 (EF554131); KN-4 (D12710), Hun-5136 (HQ315855); PA151 (L20881); Hun3 (AJ487831), HUN-BoRo4 (HQ315856), 10733 (AY281360); B223 (X57852).

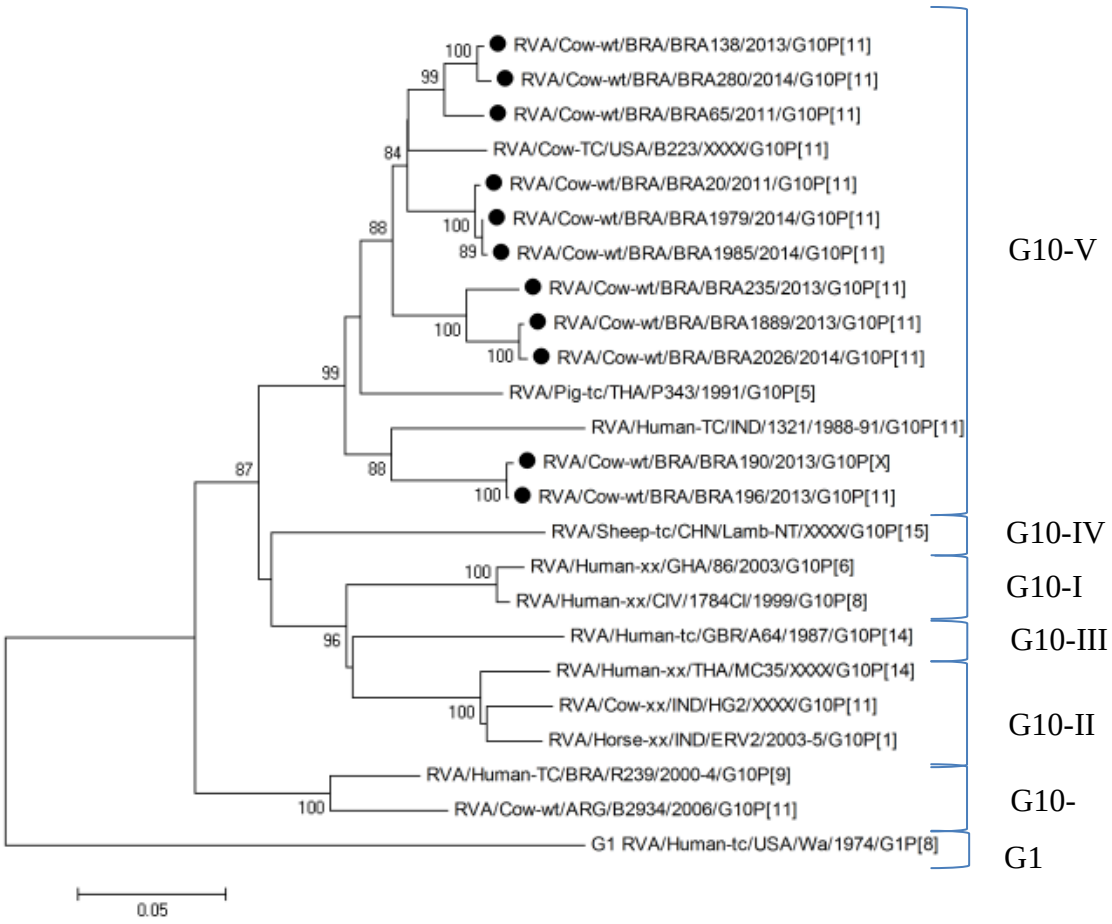

Figure 2 Phylogenetic tree with 760 bp (151-910 nt) of the G10 lineage of wild-type Brazilian bovine RVA strains. The following accession numbers are available on GenBank: P343 (AB972861), I321 (L07658); Lamb-NT (FJ031029); 86 (GU984762), 1784CI (AY816181); A64 (X63156); MC35 (D14033), Hg2 (AF386916), Erv2 (DQ981476); R239 (AY855063), B2934 (KC895802); Wa (JX406755).

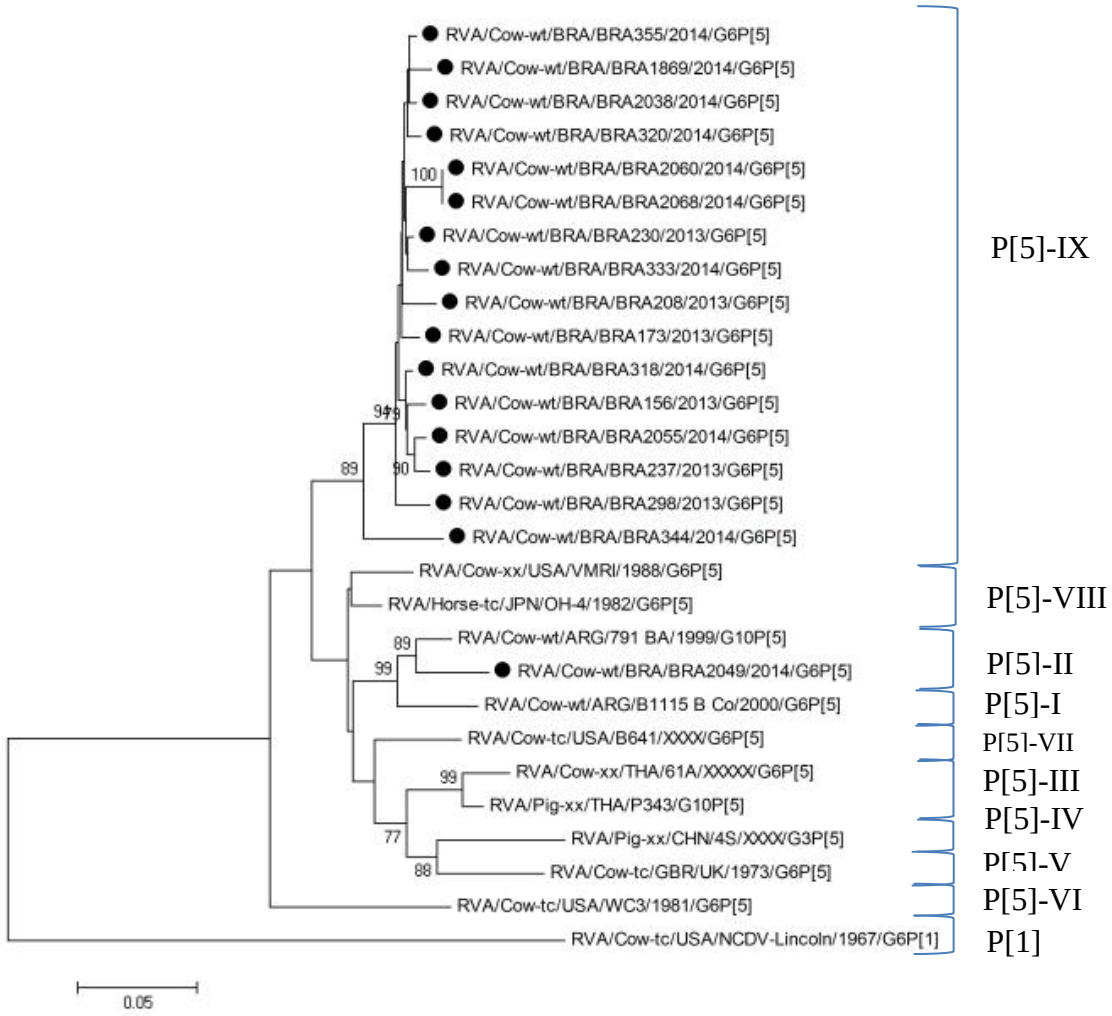

Figure 3 Phylogenetic tree with 648 bp (1,166-1,815 nt) of the P[5] lineage of wild-type Brazilian bovine RVA strains. The following accession numbers are available on GenBank: VMRI (U53923), OH-4 (KC815661); 791_BA (KC895826); B1115_B_Co (KC895815); B641 (M63267); 61A(D13396), P343 (AB972859); 4S (L10358); UK (M22306); WC3 (AY050271); NCDV (AB119636).

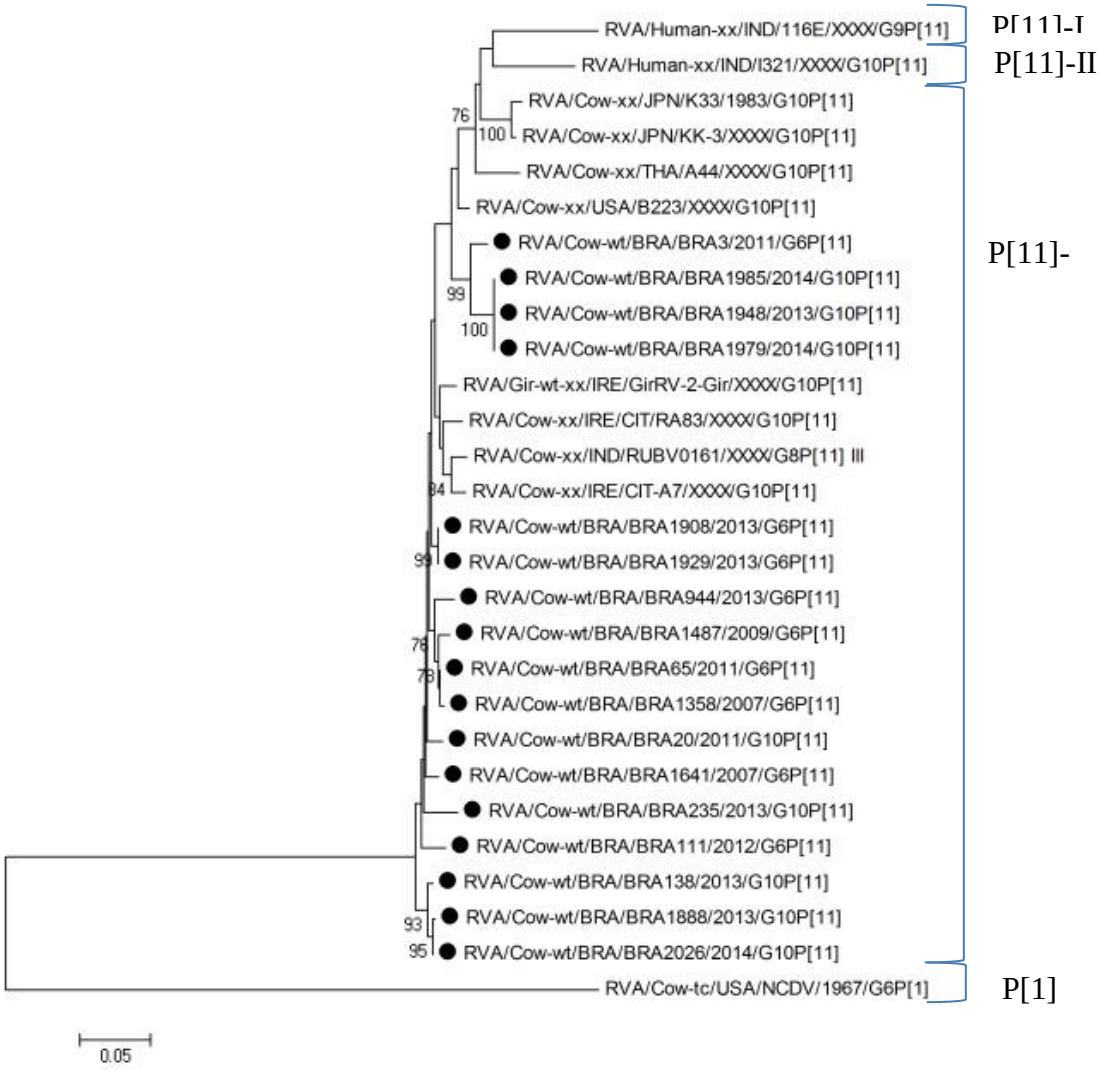

Figure 4 Phylogenetic tree with 708 bp (125-832 nt) of the P[11] lineage of wild-type Brazilian bovine RVA strains. The following accession numbers are available on GenBank: 116E (L07934); I321 (L07657); K33 (D13393), KK-3 (D14367), A44 (D13392), B223 (D13394), GirRV-2-Gir (D13394), CIT/RA83 (EU164421), RUBV0161 (EF200558), CIT-A7 (GQ414747), NCDV-Lincoln (AB119636).

4 Discussion

Even considering the great number of diarrheic fecal samples from beef and dairy herds from the most important Brazilian geographical regions of cattle breeding the neonatal diarrhea by rotavirus infection in calves evaluated in this study was predominantly due RVA strains genotype G6P[5]. This bovine RVA genotype was also reported as the most common genotype combination in other studies carried in Brazil and around the world [4, 6, 10, 21, 35, 45, 47].

A small range of genotypes was found in the RVA strains included in this study being only two G genotypes (G6 and G10) and two P genotypes (P[5] and P[11]). In contrast, genotyping studies in RVA strains of porcine and human origin demonstrate the occurrence of a wide variety of genotypes circulating simultaneously or causing diarrhea outbreaks with reemergent genotypes or the emergence of new genotypes [32, 35, 39, 41].

A different distribution of G and P genotype combinations was found according to the herd type whereas the G6P[5] genotype combination was more frequent (65.5%) in beef herds and the G10P[11] (38.4%) and G6P[11] (30.8%) in dairy herds. In the Argentina (2004-2010) the G6P[5] was also the most frequent genotype combination in RVA strains found in beef cattle herds. Despite genotype G10P[11] found mainly in dairy calves diarrheic fecal samples in the Brazilian study, a different distribution of G6P[11] (21%), G10P[11] (17%), and G6P[5] (14%) genotypes was found in Argentinean dairy herds [6].

Even the diversity of RVA genotypes found in dairy herds being considered low, it was higher than in beef herds. Although the number of RVA strains from the Brazilian Midwest exceed the analyzed number of other regions the genotype G10P[11] was identified only in strains from the South and Southeast regions.

G6P[11] genotype was not found in two years of study (2006 and 2008), and the G10P[11] genotype only was identified from 2011, in addition other studies previously conducted in Brazil, identified this genotype combination before (1996-1999) [4] (2003) [17]. The though small number of samples analyzed in these first years of analysis may have given this bias, due to decreasing of age heifer included in the reproductive management, the use of fixed-time artificial insemination, immune pressure induced by the commercial vaccine.

Probably the genotype profile of bovine RVA strains has been changing over time. The G6P[1] was not detected in any RVA strains included in this study, beside the period (10 years) and number of strains (*n*=155) evaluated. During 1996-1999, our research group genotyped 50 RVA strains from eight dairy and/or beef cattle herds located in the South, Southeast, and Midwest regions of Brazil. The G6P[1] (NCDV-Lincoln like) genotype was found in 12% (6/50) of the RVA strains analyzed while the G6P[5] genotype was described in 40% (20/50). Additionally, unusual associations of G and P genotypes, G8P[11] and G5P[1], were identified in two RVA strains [4]. Thus, it is observed that there are already over 20 years that the G6P[5] has been the most frequent genotype combination found in the Brazilian wild-type bovine RVA strains identified in diarrheic calves.

G8 is a frequent genotype in bovine RVA strains detected worldwide [6, 29, 42]. However this genotype was not found in any sample analyzed in this study and in accordance with other surveys performed in Brazil [10, 11, 13, 17], and other countries as Argentina, Tunisia, and Iran [7, 24, 28].

Considering the importance of the Brazilian cattle industry the rotavirus vaccines are still little used in both beef and dairy herds for the prophylaxis of RVA infection. However, compared with almost a decade ago there is no doubt that there was a considerable increase in the use of rotavirus vaccines in Brazil, mainly containing genotype G6P[1]. However vaccine failures may occur, as recently, have been described diarrhea outbreaks in calves of regularly vaccinated herds in Brazil, with rotavirus vaccine containing RVA genotype G6P[1] [13].

The G6 genotype of Brazilian bovine RVA sequences were distributed in G6(III) and G6(IV) lineages, according to association with the P[11] and P[5] genotype, respectively, that were not necessarily occurred in Argentina [6]. Beside only two G6 lineages were demonstrated still there are great diversity in the G6(IV) lineage, in which sequences clustered with several different prototypes. The G10 genotype clustered only with the G10(V) lineage, even though were described VI different lineages in this genotype [7].

We highlight that the P[5](II) lineage was described for the first time in the RVA strains identified in Brazilian cattle herds. The P[5] genotype of bovine RVA sequences also clustered with P[5](IX) lineage, beside the extremely diversity with IX lineages described for this genotype [7, 13]. The P[11] genotype clustered in P[11](III) lineage, previously detected circulating in Brazilian Midwest region by our group [34].

In this study in just two RVA strains it has not been possible to characterize the G genotype and this result can be considered expected. However, with respect to P genotype it was not possible to obtain amplicon of VP4 gene in 15 RVA strains, even performing several attempts and using three different pairs of primers [22, 26, 30].

The mistyping of VP7 (G) or VP4 (P) gene has been documented in RVA strains of human and animal origin worldwide [4, 11, 21]. This can be caused by the presence of inhibitors of RT-PCR reaction in the stool; fecal samples conservation problems or viral infection with lower titers. However this should not be the reason for the failure in P genotype determination since in all 15 bovine RVA strains were P genotype was not determined was possible to determine the G genotype. Probably aspects related to accumulation of point mutations or homologous recombination and hybridization of primers to the target sequences may have been responsible for not determining the P genotype in 15 bovine RVA strains included in this study [17, 21, 25, 27, 43].

Molecular characterization of RVA strains circulating in animals is of great importance either in relation to aspects concerning animal health as the public health. Phylogenetic studies of RVA strains G6P[14], G12P[11], G10P[14] circulating in cattle has been related in children with diarrhea throughout the world showing evidence for bovine to human interspecies transmission and reassortment events [37, 48].

The importance of genotyping studies, mainly with retrospective design, carried out in several geographical regions, and in different types of cattle breeding (beef and dairy farms) contribute to viral comprehension and epidemiology surveillance of the genotypes of RVA more frequent.

5 Conclusion

Compared to studies conducted in Brazil with RVA strains of human and porcine origin the G and P genotypes diversity in bovine RVA strains identified in this study can be considered low, but the diversity of G and P genotype increased after year 2011.

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4 CONCLUSÕES

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- RVA continua sendo um dos principais agentes etiológicos de diarreia em bezerros de rebanhos bovinos leiteiros e, principalmente, de corte no Brasil;

- A frequência de RVA em bezerros diarreicos foi maior nos rebanhos de corte em relação aos rebanhos leiteiros;

- Na região Centro-Oeste, a frequência de diagnóstico de RVA foi significativamente superior às outras regiões (Sul e Sudeste) avaliadas;

- Não houve diferença significativa quando a frequência de diagnóstico de RVA em fezes diarreiras de bezerros foi distribuída em dois períodos (2006-2010; 2011-2015) de cinco anos, mas aumentou a diversidade de combinações de genotipos G e P no segundo período avaliado.

- A diversidade de genotipos G e P de cepas de RVA bovino no Brasil pode ser considerada pequena em relação à identificada em estudos realizados também no Brasil com cepas de RVA de origem humana e suína;

- A heterogeneidade de genotipos de RVA encontrada em bezerros diarreicos de rebanhos leiteiros foi superior à identificada em rebanhos de corte;

- As cepas classificadas como G6P[5] continuam a representar a combinação de genotipos mais frequentes em cepas de RVA bovino identificadas no Brasil.

- A linhagem P[5](II) foi descrita pela primeira vez no Brasil, nas cepas de RVA bovinas identificadas nos rebanhos bovinos brasileiros.

5 ANEXOS

ANEXO A: Lista de reagentes

1. 100 mM dNTP *Set*, 4 x 250 µL; 25 µmol cada (100 mM dATP *Solution*, 100 mM dCTP *Solution*, 100 mM dGTP *Solution*, 100 mM dTTP *Solution*) (*Invitrogen™ Life Technologies, EUA*)
2. 10 x PCR-*Buffer* (200 mM Tris-HCl, pH 8,4, 500 mM KCl) (*Invitrogen™ Life Technologies, EUA*)
3. 123 bp DNA *Ladder* (*Invitrogen™ Life Technologies, EUA*)
4. 2-Mercaptoetanol ($C_2H_6O_5$) P.M. 78,13 (Fluka®)
5. Acetona P.A. (CH_3COCH_3) P.M. 58,08 (Dinâmica®)
6. Ácido acético glacial P.A. (CH_3COOH) P.M. 60,05 (Nuclear®)
7. Ácido bórico (H_3BO_3) P.M. 61,83 (Sicalab®)
8. Ácido clorídrico (HCl) P.M. 36,46 (Reagen®)
9. Ácido etilenodiaminotetraácido sal di-sódico - EDTA P.A. ($C_{10}H_{14}N_2O_8Na_2.2H_2O$) P.M. 372,24 (Reagen®)
10. Acrilamida P.M. 71,08 (Gibco BRL®)
11. Agar Noble (Difco®)
12. Agarose (*Invitrogen™ Life Technologies, EUA*)
13. Água DEPEC (Dietil pirocarbonato) (*Invitrogen™ Life Technologies*®, EUA)
14. Álcool etílico absoluto (C_2H_2OH) P.M. 46,07 (Nuclear®)
15. Álcool isoamílico ($(CH_3)2CHCH_2CH_2OH$) P.M. 88,15 (Synth®)
16. Azul de bromofenol (Sigma®, EUA)
17. Bicarbonato de sódio P.A. ($NaHCO_3$) P.M. 84,01 (Biotec®)
18. *BigDye Terminator v3.1 Cycle Sequencing kit* (*Applied Biosystems*®, EUA)
19. Bis-acrilamida P.M. 154,2 (Sigma®, EUA)
20. Borohidreto de sódio P.M. 37,83 (Sigma®, EUA)
21. Brometo de etídeo ($C_{21}H_{20}N_3Br$) P.M. 394,3 (Sigma®, EUA)
22. Cloreto de Cálcio Puro ($CaCl_2$) P.M. 110,94 (*Invitrogen™ Life Technologies, EUA*)
23. Cloreto de Potássio P.A. (KCl) P.M. 74,56 (Reagen®)
24. Cloreto de Sódio P.A. (NaCl) P.M. 58,45 (Reagen®)
25. Clorofórmio P.A. ($CHCl_3$) P.M. 119,38 (Dinâmica®)

26. Dióxido de sílica (SiO_2) P.M. 60,08 (Sigma®, EUA)
27. Dodecil Sulfato de Sódio - Lauril Sulfato de Sódio - SDS ($C_{12}H_{25}NaO_4S$) P.M. 288,38 (BDH)
28. Fenol (C_6H_5OH) P.M. 94,11 (*Invitrogen™ Life Technologies®*, EUA)
29. Fosfato de sódio dibásico anidro (Na_2HPO_4) P.M. 141,96 (Synth®)
30. Fosfato de sódio monobásico ($NaH_2PO_4 . 2H_2O$) P.M. 155,99 (Reagen®)
31. Fosfato de sódio dihidratado ($Na_2HPO4 . 2H_2O$) P.M 177,99 (Merck®)
32. GFX PCR DNA and *Gel Band Purification Kit* (*GE Healthcare*, UK)
33. Glicina P.A. P.M. 75,07 (Nuclear®)
34. Glicose $C_6H_{12}O_6$ P.M. 180,16 (Reagen®)
35. Hidróxido de Sódio P.A. (NaOH) P.M. 40,00 (*Mallinckrodt Chemicals®*)
36. Hidroximetil amino metano - TRIS 99% P.M. 121,14 (Merck)
37. Isotiocianato de guanidina P.M. 118,16 (*Invitrogen™ Life Technologies, EUA*)
38. Metanol, P.A. (CH_3OH) P.M. 32,04 (Allkimia®)
39. Oligonucleotídeo iniciador (*primer*) Con3 (VP4) (forward; 5´-TGGCTTCGCCATTTTATAGACA -3´; nucleotídeo 11-32) GENTSCH et al. (1992) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
40. Oligonucleotídeo iniciador (*primer*) Con2 (VP4) (reverse; 5´-ATTTCGGACCATTTATAACC -3´; nucleotídeo 868-887) GENTSCH et al. (1992) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
41. Oligonucleotídeo iniciador (*primer*) con3M (P) (forward; 5´-GGCTATAAAATGGCTTCGCTCATTTATAGACA -3´; nucleotideo 11-32) MARTELLA et al. (2006) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
42. Oligonucleotídeo iniciador (*primer*) Beg9 (VP7) (forward; 5´-GGCTTTAAAAGAGAGAATTTCCGTCTGG -3´; nucleotídeo 1-28) GOUVEA et al. (1990) - 200 pmol (*Invitrogen™ Life Technologies*, EUA®)
43. Oligonucleotídeo iniciador (*primer*) End9 (VP7) (reverse; 5´-GGTCACATCATACAATTCTAATCTAAG -3´; nucleotídeo 1062-1036) GOUVEA et al. (1990) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)

44. Oligonucleotídeo iniciador (*primer*) End9 (UK) (G) (reverse; 5´- GGTCACATCATACAACTCTAATCT -3´; nucleotideo 1062-1039) GOUVEA et al. (1993) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
45. Oligonucleotídeo iniciador (*primer*) End9 (CRW8) (G) (reverse; 5´- GGTCACATCTTACAGCTTTAACCT -3´; nucleotideo 1059-1036) GOUVEA et al. (1993) - 200 pmol (*Invitrogen™ Life Technologies®*, EUA)
46. Oligonucleotídeo iniciador (*primer*) Bov9com5 (RVA) (VP7) (forward; 5´- TGT ATG GTA TTG AAT ATA CCA C -3´; nucleotídeo 50-71) ISEGAWA et al. (1993) - 200 pmol (*Invitrogen ™ Life Technologies®*, EUA)
47. Oligonucleotídeo iniciador (*primer*) Bov9com3 (RVA) (VP7) (reverse; 5´- TCA CAT CAT ACA ACT CTA ATC T -3´; nucleotídeo 1038-1060) ISEGAWA et al. (1993) - 200 pmol (*Invitrogen ™ Life Technologies®*, EUA)
48. Oligonucleotídeo iniciador (*primer*) Bov4com5 (RVA) (VP4) (forward; 5´- TTC ATT ATT GGG ACG ATT CAC A -3´; nucleotídeo 1067-1088) ISEGAWA et al. (1993) - 200 pmol (*Invitrogen ™ Life Technologies®*, EUA)
49. Oligonucleotídeo iniciador (*primer*) Bov4com3 (RVA) (VP4) (reverse; 5´- CAA CCG CAG CTG ATA TAT CAT C -3´; nucleotídeo 1909-1930) ISEGAWA et al. (1993) - 200 pmol (*Invitrogen ™ Life Technologies®*, EUA)
50. *Platinum Taq DNA Polymerase* 500 unidades (Invitrogen™ Life Technologies, BRA)
51. *Quant-iT™ Working Solution* (*Invitrogen™ Life Technologies*, EUA)
52. Sacarose, P.A. - sucrose ($C_{12}H_{22}O_{11}$) P.M. 342,31 (Reagen®)
53. *Superscript TM II RNase H - Reverse Transcriptase* – 200 unidades/µL (*Invitrogen™ Life Technologies, EUA*)
54. Triton x-100 (J.T.Baker®)

ANEXO B: Soluções e tampões

- **Hidratação da sílica**
  - 6 g de sílica (O_2Si)
  - Adicionar 50 mL de água bidestilada
  - Agitar lentamente e manter em repouso durante 24 h
  - Por sucção, desprezar 44 mL do sobrenadante
  - Ressuspender a sílica em 50 mL de água bidestilada
  - Manter em repouso durante 5 h para sedimentar
  - Desprezar 44 mL do sobrenadante
  - Adicionar 100 μL de HCl fumegante para ajustar o pH=2,0.
  - Aliquotar

- **Solução L6**
  - 120 g de isotiocianato de guanidina (GUSCN)
  - 100 mL de TRIS-HCl 0,1 M pH 6,4
  - 22 mL de EDTA 0,2 M pH 8,0
  - 2,6 mL de Triton x-100

- **Solução L2**
  - 120 g de isotiocianato de guanidina (GUSCN)
  - 100 mL de TRIS-HCl 0,1 M pH 6,4

- **Tampão de amostra para eletroforese em gel de agarose**
  - 0,25 g de azul de bromofenol (0,25%)
  - 45 g de sacarose - sucrose ($C_{12}H_{22}O_{11}$) (45%)
  - Água bidestilada q.s.p. 100 mL

- **Tampão de amostra para eletroforese em gel de poliacrilamida (PAGE)**

  - 0,2 mL de azul de bromofenol 1%
  - 6,0 mL de SDS 10%
  - 1 mL de 2-mercaptoetanol
  - 2,5 mL de TRIS-HCl 0,5 M
  - 6 g de ureia
  - Água bidestilada q.s.p. 20 mL

- **Tampão de corrida: TBE (TRIS - Ácido bórico - EDTA) 10 x [ ]**

  - 107,78 g de TRIS (0,89 M)
  - 55,03 g de ácido bórico (0,89 M)
  - 7,45 g de EDTA (0,02 M)
  - Água bidestilada q.s.p. 1 litro

- **Tampão de corrida para PAGE**

  - 3 g de TRIS (0,24 M)
  - 14,4 g de ácido aminoacético (glicina) (NH_2CH_2COOH) (0,19 M)
  - Água bidestilada q.s.p. 1 litro

- **Tampão de estabilização para o rotavírus (TRIS/Ca^{++}) 10 x - pH 7,2**

  - 12,12 g de TRIS (0,89 mM)
  - 2,2 g de cloreto de cálcio (1,5 mM)
  - Água bidestilada autoclavada q.s.p. 1 litro

- **Fenol/clorofórmio-álcool isoamílico**

  - 25 mL fenol saturado
  - 24 mL clorofórmio
  - 1 mL álcool isoamílico

- **SDS 10%**

  - 5 g de dodecil sulfato de sódio - Lauril sulfato de sódio - SDS ($C_{12}H_{25}NaO_4S$)
  - Água bidestilada q.s.p. 50 mL

- **Lower TRIS pH 8,8 para PAGE**
  - 36,34 g de TRIS (1,5 M)
  - Água bidestilada q.s.p. 200 mL

- **Upper TRIS pH 6,8 para PAGE**
  - 12,12 g de TRIS (0,5 M)
  - Água bidestilada q.s.p 200 mL

- **Solução Acrilamida / Bisacrilamida**
  - 0,8 g de bisacrilamida
  - 30 g de acrilamida
  - Água bidestilada q.s.p 100 mL

- **Solução fixadora para PAGE**
  - 30 mL de álcool etílico absoluto
  - 1,5 mL de ácido acético
  - Água bidestilada q.s.p. 300 mL

- **Solução de prata para PAGE**
  - 0,55 g de nitrato de prata
  - Água bidestilada q.s.p. 300 mL

- **Solução reveladora para PAGE**
  - 9 g de hidróxido de sódio
  - 2,5 mL de formaldeído
  - 0,06 g de borohidreto de sódio
  - Água bidestilada q.s.p. 300 mL

- **Solução stop da coloração para PAGE**
  - 15 mL de ácido acético P.A.
  - Água bidestilada q.s.p. 300 mL

- **Solução conservadora para PAGE**

  - 15 mL de álcool etílico P.A.
  - Água bidestilada q.s.p. 300 mL

- **Gel inferior (7,5%) da PAGE**

  - 5 mL de Lower TRIS
  - 3 mL de acrilamida/bisacrilamida
  - 50 µL de TEMED
  - 0,56 de mL persulfato de amônio 2%
  - 11,44 mL de água bidestilada

- **Gel superior (3,5%) da PAGE**

  - 2,5 mL de Upper TRIS
  - 1 mL de acrilamida/bisacrilamida
  - 100 µL de TEMED
  - 0,60 mL de persulfato de amônio 2%
  - 6,20 mL de água bidestilada

- **Gel de agarose 2%**

  - 1 g de agarose
  - 50 mL de tampão TBE 1x
  - 30 µL de brometo de etídeo

- **Diluição de dNTP**

  - solução estoque - concentração 100 mM
  - solução uso - concentração 10 mM - 10 µl da solução estoque + 90 µl de água ultrapura

- **Diluição dos *primers***

RT-PCR P (VP4) consensual

- *Primer* Con 3 *forward*

Concentração: 20,46 nmol

Data de fabricação: Jul.2012

20,46 x 1000 = 20460 pmol

20460 / 200 = 102,3 µL de água ultrapura

- *Primer* Con 2 *reverse*

Concentração: 27,42 nmol

Data de fabricação: Dez.2008

27,42 x 1000 = 27420 pmol

27420 / 200 = 137,10 µL de água ultrapura

- *Primer* Con 3M *forward*

Concentração: 20,46 nmol

Data de fabricação: Jul.2012

20,46 x 1000 = 20460 pmol

20460 / 200 = 102,3 µL água ultra-pura

- *Primer* Bov4com5 VP4 *forward*

Concentração: 20,84 nmol

Data de fabricação: Out.2014

20,84 x 1000 = 20840 pmol

20840 / 200 = 104,2 µL de água ultrapura

- *Primer* Bov4com3 *reverse*

Concentração: 22,31 nmol

Data de fabricação: Out.2014

22,31 x 1000 = 22310 pmol

22310 / 200 = 111,55 µL de água ultrapura

RT-PCR G (VP7) consensual

- *Primer* Beg9 *forward*

Concentração: 19,28 nmol

Data de fabricação: Set.2012

19,28 x 100 = 19280 pmol

19280 / 200 = 96,4 µL de água ultrapura

- *Primer* End 9 *reverse*

Concentração: 22,52 nmol

Data de fabricação: Set.2011

22,52 x 1000 = 22520 pmol

22520 / 200 = 112,6 µL de água ultrapura

- *Primer* End 9 (UK) *reverse*

Concentração: 23,38 nmol

Data de fabricação: Set.2010

23,38 x 1000 = 23380 pmol

23380 / 200 = 116,9 água ultra-pura

- *Primer* End 9 (CRW8) *reverse*

Concentração: 22,87 nmol

Data de fabricação: Set.2010

22,87 x 1000 = 22870 pmol

22870 / 200 = 114,35 água ultra-pura

- *Primer* Bov9com5 *forward*

Concentração: 18,9 nmol

Data de fabricação: Out.2014

18,9 x 1000 = 18900 pmol

18900 / 200 = 94,5 µL de água ultrapura

- *Primer* Bov9com3 *reverse*

Concentração: 19,75 nmol

Data de fabricação: Out.2014

19,75 x 1000 = 19750 pmol

19750 / 200 = 98,75 µL de água ultrapura

Solução estoque: ressuspender em água ultrapura para obtenção de solução 10x [ ]

Solução de uso de todos os *primers*: diluir 1:10 a solução estoque em água ultrapura. Concentração final: 20 pmol / µL

ANEXO C: Protocolo de técnicas

- **Suspensão fecal - Extração bruta**

  - Pesar 1 g do material fecal em balança de precisão
  - Adicionar 9 mL de tampão TRIS/Ca^{++} 1x (para amostra líquidas estabelecer a proporção 1:2)
  - Homogeneizar
  - Calibrar os tubos
  - Centrifugar 2.000 x *g* / 5 min
  - Recolher sobrenadante
  - Identificar e estocar em frascos a 4ºC

- **Extração do RNA: Associação das técnicas fenol/clorofórmio-álcool isoamílico e sílica/isotiocianato de guanidina**

  - Aliquotar 500 µL da suspensão fecal
  - Adicionar 50 µL de SDS 10 %
  - Homogeneizar em vórtex
  - Banho-maria 56 °C / 20 min
  - Centrifugar 10.000 x *g* / 30 s
  - Adicionar 500 µL de fenol/clorofórmio-álcool isoamílico (25:24:1)
  - Homogeneizar em vórtex
  - Banho-maria 56 °C / 15 min
  - Homogeneizar em vórtex
  - Centrifugar 10.000 x *g* / 10 min
  - Recolher o sobrenadante em outro microtubo
  - Adicionar 500 µL da solução L6
  - Adicionar 25 µL de sílica hidratada
  - Homogeneizar em vórtex
  - Agitar em temperatura ambiente / 30 min
  - Centrifugar 10.000 x *g* / 30 s
  - Desprezar o sobrenadante em solução contendo NaOH 10 M
  - Adicionar 500 µL de solução L2
  - Homogeneizar em vórtex
  - Centrifugar 10.000 x *g* / 30 s
  - Desprezar o sobrenadante em solução contendo NaOH 10 M
  - Adicionar 500 µL de solução L2
  - Homogeneizar em vórtex
  - Centrifugar 10.000 x *g* / 30 s
  - Desprezar o sobrenadante em solução contendo NaOH 10 M
  - Adicionar 500 µL de etanol 70% gelado
  - Homogeneizar em vórtex
  - Centrifugar 10.000 x *g* / 30 s
  - Desprezar sobrenadante em descarte comum
  - Adicionar 500 µL de etanol 70% gelado
  - Homogeneizar em vórtex
  - Centrifugar 10.000 x *g* / 30 s

- Desprezar sobrenadante em descarte comum
- Adicionar 1000 μL de acetona P.A. gelada
- Homogeneizar em vórtex
- Centrifugar 10.000 x *g* / 30 s
- Desprezar sobrenadante em descarte comum
- Secar o sedimento em termo bloco à 60ºC (aproximadamente 2 min) ou banho-maria à 56ºC (15 min)
- Adicionar 50 μL de água DEPC
- Homogeneizar em vórtex
- Banho-maria 56ºC / 15 min
- Homogeneizar em vórtex
- Centrifugar 13.000 x *g* / 4 min
- Recolher o sobrenadante em microtubo de 500 μL
- Estocar à 4ºC ou - 20ºC até a utilização

Quadro 1. *Primers* consensuais - gene VP4 (P tipo) e VP7 (G tipo)

Genotipo	*Primer* consensual	Sequência	Posição / Polaridade	Produto pb	Referência
G	Beg9	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1-28 (+)	1062	(1)
	End9/UK	GGTCACATCATACAACTCTAATCT	1062-1039 (-)	1062	(2)
	End9/CRW8	GGTCACATCTTACAGCTTTAACCT	1059-1036 (-)	1062	(2)
	End 9	GGTCACATCATACAATTCTAATCTAAG	1062-1036 (-)	1062	(1)
	Bov9com5	TGT ATG GTA TTG AAT ATA CCA C	50-71(+)	1010	(3)
	Bov9com3	TCA CAT CAT ACA ACT CTA ATC T	1038-1060 (-)	1010	
P	Con3M	GGCTATAAAATGGCTTCGCTCATTTATA GACA	11-32 (+)	877	(4)
	Con2	ATTTCGGACCATTTATAACC	868-887 (-)	876	(5)
	con3	TGGCTTCGCTCATTTATAGACA	11-32 (+)	876	(5)
	Bov4com5	TTC ATT ATT GGG ACG ATT CAC A	1067-1088 (+)	863	(3)
	Bov4com3	CAA CCG CAG CTG ATA TAT CAT C	1909-1930 (-)	863	(3)

(1) GOUVEA et al. (1990). (2) GOUVEA et al. (1993). (3) ISEGAWA et al. (1993). (4) MARTELLA et al. (2006). (5) GENTSCH et al. (1992).

- **RT-PCR P consensual para o rotavírus grupo A**

- Mix desnaturação

Mix Desnaturação P (VP4) e G (VP7)
Primer foward (20 pmol) - 1 μL *Primer reverse* (20 pmol) - 1 μL Água ultrapura - 3 μL
Volume final - 5 μL

Mix Desnaturação G tipo (VP7) GOUVEA et al. (1990;1993)
Beg 9 (20 pmol) – 1 μL End 9 (20 pmol) – 1 μL End 9 UK (20 pmol) – 1 μL End 9 CRW8 (20 pmol) – 1 μL Água 1 μL
Volume final – 5 μL

- Mix transcrição reversa (RT-MIX)

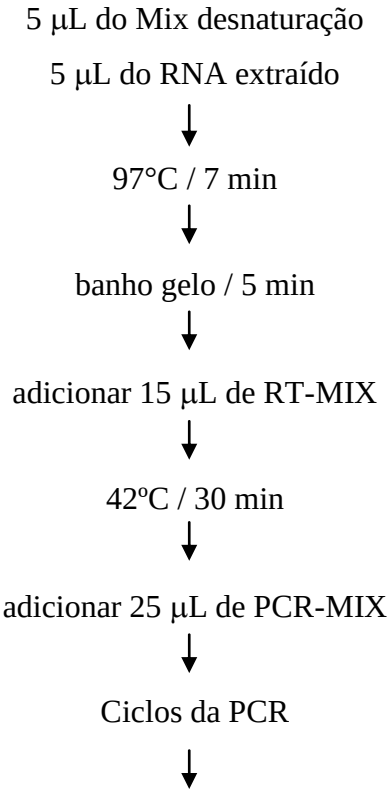
Mix RT-PCR
Tampão 10 x pH 8,4 - 2,5 μL $MgCl_2$ 50 mM - 2,5 μL dNTP 2,5 mM - 4 μL *SuperScript* II® 200 U / μL - 0,15 μL Água ultrapura - 5,85 μL
Volume final - 15 μL

- Mix da reação em cadeia da polimerase (PCR-MIX)

Mix PCR G (VP7) e P (VP4)
Tampão 10 x pH 8,4 - 2,5 μL dNTP 2,5 mM - 4 μL *Platinum®Taq DNA Polymerase* 5 U / μL - 0,25 μL *Primer foward* (20 pmol) - 1 μL *Primer reverse* (20 pmol) - 1 μL Água ultrapura - 16,25 μL
Volume final - 25 μL

Mix PCR G tipo (VP7) GOUVEA et al. (1990;1993)
Tampão 10 x pH 8,4 - 2,5 μL dNTP 2,5 mM - 4μL *Platinum®Taq DNA Polymerase* 5U/μL - 0,25 μL Água - 18,25 μL
Volume final - 25 μL

Esquema da RT-PCR



Etapas da PCR	
Ciclos	VP4 e VP7
Desnaturação inicial	94ºC / 3 min
Desnaturação	94ºC / 30 s
Anelamento	42ºC / 30 s
Extensão	72ºC / 45 s
Nº de ciclos térmicos	40
Extensão final	72ºC / 7 min

Eletroforese em gel de agarose 2%

- 1 g de agarose
- 50 mL TEB *buffer* (Tris 89 mM; ácido bórico 89 mM; EDTA 2 mM) pH 8,4
- 30 μL de brometo de etídeo (0,5 μg / mL)

São utilizados 8 μL do *amplicon* e 2 μL do tampão de amostra, onde a eletroforese sob voltagem (100 V) e amperagem (80 A) constantes, perdura por aproximadamente 60 min

- **Purificação de produto de PCR excisado do gel**

1. Pesar o fragmento excisado do gel em microtubo de 1,5 mL
2. Adicionar 10 μL do *Capture buffer type* 2 para cada 10 mg de gel
3. Incubar o tubo a 60°C / 15 min, homogeneizando a cada 3 min
4. Centrifugar a 16.000 x *g* / 30s
5. Transferir 600 μL da amostra com o *Capture buffer type* 2 em um tubo coletor com coluna
6. Incubar em temperatura ambiente por 1 min
7. Centrifugar a 16.000 x *g* / 30s
8. Descartar o filtrado e recolocar a coluna no mesmo tubo
9. Adicionar 500 μL do *Wash buffer type 1* na coluna com tubo coletor
10. Centrifugar a 16.000 x *g* / 30s
11. Descartar o filtrado e transferir a coluna para um microtubo de 1,5 mL
12. Adicionar 30 μL do *Elution buffer type 6*
13. Incubar a temperatura ambiente por 1 min
14. Centrifugar a 16.000 x *g* / 1 min
15. Estocar o DNA purificado em - 20°C

- **Quantificação de produto de PCR**

1. Preparar a solução *Quant-iT™ Working Solution* diluindo o reagente *Quant-iT™* no *Buffer Quant-iT™*, 1:200. São necessários 200 μL desta solução por amostra e para os padrões 0 e 100
2. Homogeneizar em vórtex
3. No microtubo das amostras adicionar 198 μL da solução *Quant-iT™ Working Solution* a 2 μL do DNA purificado
4. No microtubo do padrão 0 adicionar 190 μL da solução *Quant-iT™ Working Solution* a 10 μL do padrão 0
5. No microtubo do padrão 100 adicionar 190 μL da solução *Quant-iT™ Working Solution* a 10 μL do padrão 100
6. Homogeneizar os microtubos em vórtex por 2-3 s
7. Incubar os microtubos em temperatura ambiente por 2 min
8. Realizar a leitura usando *Qubit™ fluorometer* (*Invitrogen™ Life Technologies, EUA*)
9. Multiplicar pelo fator de diluição para determinar a concentração correta da amostra

ANEXO D: Lista de *softwares*

- *Electropherogram quality analysis - Phred e CAP3*

(http://asparagin.cenargen.embrapa.br/phph/)

- *BLAST The Basic Local Alignment Search Tool*

(http://blast.ncbi.nlm.nih.gov/Blast.cgi)

- *MEGA package software version 5.05*

(http://www.megasoftware.net/mega5/mega5.html)

- *BioEdit software version 7.1.3.0*

(http://www.mbio.ncsu.edu/bioedit/bioedit.html)