UNIVERSIDADE FEDERAL DO PARÁ
NÚCLEO DE MEDICINA TROPICAL
PROGRAMA DE PÓS-GRADUAÇÃO EM DOENÇAS TROPICAIS
ÁREA DE CONCENTRAÇÃO EM PATOLOGIA DAS DOENÇAS TROPICAIS

ANÁLISE MOLECULAR DE ROTAVÍRUS TIPO G9 DE CRIANÇAS NA REGIÃO NORTE DO BRASIL

SYLVIA DE FÁTIMA DOS SANTOS GUERRA

Belém/PA
2016

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Tese de doutorado apresentado à banca examinadora ao programa de Pós-Graduação em Doenças Tropicais (área de concentração em Patologia das doenças Tropicais), Núcleo de Medicina Tropical, Universidade Federal do Pará, como requisito para a obtenção do grau de doutor em Doenças Tropicais.

Orientadora: Profª Dra. Joana D'Arc Pereira Mascarenhas

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2016

Dados Internacionais de Catalogação na Publicação (CIP)
Biblioteca do Instituto Evandro Chagas

Guerra, Sylvia de Fátima dos Santos.
Análise molecular de rotavírus tipo G9 de crianças na região norte do Brasil / Sylvia de Fátima dos Santos Guerra. – Belém, 2016.
142 f.: il.; 30 cm.

Orientadora: Profª Dra. Joana D'Arc Pereira Mascarenhas
Co-orientadora: Profª Dra. Luana da Silva Soares
Tese (Doutorado em Doenças Tropicais) – Universidade Federal do Pará, Núcleo de Medicina Tropical, 2016.

1. Rotavírus. 2. Genótipo G9. 3. Variantes genética. I. Mascarenhas, Joana D'Arc Pereira, *orient*. II. Soares, Luana da Silva, *co-orient*. III. Universidade Federal do Pará. IV. Título.

CDD: 618.923427

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Belém/PA

2016

A Deus e à minha família, pela constante presença na realização deste sonho

AGRADECIMENTO

A Deus, por dar-me forças para superar os obstáculos, guiando sempre pelos caminhos da vida e por mais esta vitória em minha vida

Ao Instituto Evandro Chagas por todo apoio técnico cientifico no desenvolvimento deste trabalho.

Ao Núcleo de Medicina Tropical/UFPa por proporcionar tanto a minha pós graduação em mestrado quanto no doutorado.

A CAPES e CNPq por todo aopio ao desenvolvimento do estudo

Minha eterna gratidão às minhas orientadoras Dra. Joana Mascarenhas e Dra. Luana Soares, por me conduzirem na vida científica, desde a iniciação científica até o doutorado, sempre acreditando em meu trabalho e me incentivando com suas amizades.

Ao Dr. Linhares, por toda por toda lição e incentivo científico, por confiar no trabalho desenvolvido.

À “família rotavírus” por todo apoio que uma equipe poderia proporcionar, em especial, Patricia Lobo, Alessilva Oliveira e Fabiolla Santos por toda amizade e apoio técnico.

A minha família, pais, irmãos, tios, primos, esposo e filhos, por todo incentivo, pela compreensão dos momentos de ausência e por toda força para superar os obstáculos.

A todos que de alguma forma contribuíram direta ou indiretamente para o desenvolvimento deste trabalho

“*O cientista não é o homem que fornece as verdadeiras respostas; é quem faz as verdadeiras perguntas*”.

(Claude Lévi-Strauss)

RESUMO

O rotavírus do grupo A (RVA) é o principal agente viral associado às gastrenterites agudas, ocasionando cerca de 200 mil óbitos entre crianças menores de cinco anos de idade anualmente. Pertencem à família *Reoviridae*, gênero *Rotavirus*, possui RNA de dupla fita (dsRNA) com 11 segmentos codificando 12 proteínas, sendo seis estruturais (VPs) e seis não estruturais (NSPs). Cada proteína designa um genótipo específico de RVA, sendo a proteína VP7 responsável pelo genótipo G, existindo, atualmente, 32 variantes genéticas. O genótipo G9 emergiu em escala global na década de 90, período este anterior a introdução da vacina de RVA no Brasil em 2006, sendo continuamente detectado até os dias atuais. Este estudo objetivou descrever a frequência e a constelação genética associada ao genótipo G9 circulantes na região Norte do Brasil. Foram selecionadas 50 amostras coletadas de 1999 a 2013, sendo 45 G9P[8], 2 G9P[4] e 3 G9P[6], nas quais se procedeu a suspensão e extração do dsRNA para posterior amplificação e sequenciamento de nucleotídeos. Foi observado que no período pré-introdução da vacina a frequência de G9 alcançou frequência de 43%, enquanto que após a introdução da vacina, a maior frequência obtida foi 12,5% (2008 a 2010). A análise filogenética do gene VP7 demonstrou que todas as amostras agruparam na linhagem III de G9, observando-se modificações aminoacídicas em sítios antigênicos quando comparadas às cepas vacinais. Tal fato foi observado também na análise do gene VP4-P[8], os quais agruparam na linhagem III de P[8], enquanto que VP4-P[4] agrupou na linhagem V e VP4-P[6] na linhagem I. Todas as amostras G9P[6] e G9P[4] foram associadas à constelação DS-1, genogrupo 2, enquanto que as amostras G9P[8] apresentaram a constelação Wa, genogrupo 1, com exceção de uma amostra que apresentou o gene NSP3 com perfil DS-1. As amostras G9 da região Norte analisadas associaram-se às constelações esperadas e descritas em outras partes do mundo, com exceção de uma amostra G9P[8] que apresentou uma reestruturação genética na proteína NSP3. O genótipo G9 pode ser considerado um tipo usual de RVA na região Norte e apesar de terem sido detectadas as mesmas linhagens circulantes no período antes e após a implantação da vacina, observou-se modificações em regiões antigênicas relevantes assim como reestruturação genetica, enfatizando a necessidade de contínua monitorização das variantes genéticas circulantes de RVA.

Palavras-chaves: Rotavirus, genótipo G9, variantes genética.

ABSTRACT

Group A rotavirus (RVA) is the most viral agent associated with acute gastroenteritis, responsible for about 200,000 deaths among children aged under five years annually. RVA belongs to *Reoviridae* family, *Rotavirus* genus, its genome is composed by double-stranded RNA (dsRNA) with 11 segments encoding 12 proteins, six structural (VPs) and six non-structural (NSPs). Each protein designating a specific RVA genotype, being VP7 protein responsible for G genotype and currently there are 32 genetic variants. G9 genotype emerged on a global scale in the 90s, a period before RVA vaccine introduction in Brazil that occurred in 2006, and is continuously detected until present day. This study aimed to describe the frequency and genetic constellation associated with the current G9 genotype in Northern Brazil. It was selected 50 samples collected between 1999 and 2013, being 45 G9P[8], 2 G9P[4] and 3G9P[6], for fecal suspension preparation and dsRNA extraction for further genome amplification and sequencing of nucleotides. It was observed that during pre-RVA vaccine introduction period G9 frequency rate was 43%, while after RVA vaccine introduction the most frequece obtained was 12.5% (2008 to 2010). Phylogenetic analysis of VP7 gene showed that all strains belong to lineage III of G9, observing aminoacidic substitutions in antigenic sites when compared with vaccine strains. It was demonstrated in VP4 gene that P[8] strains gathered in lineage III, whereas P[4] grouped into lineage V and P[6] strains into lineage I. All G9P[6] and G9P[4] samples were associated with DS-1 constellation, genogroup 2, while G9P[8] samples showed Wa constellation, genogroup 1, except for one sample showing NSP3 gene with DS-1 profile. G9 samples from Northern region analyzed were associated with the expected constellations described in other parts of the world, except for one G9P[8] sample that showed a genetic restructuration in NSP3 protein. In the present study the same G9 lineages have circulated during pre and post RVA vaccine introduction periods, and it was described aminoacidic substitutions in relevant antigenic regions, such as it was reported genetic restructuration phenomenon in one sample of this genotype, emphasizing the continuous monitoring of current genetic variants of RVA.

Keywords: Rotavirus, G9 genotype, genetic variants.

SUMÁRIO

1. INTRODUÇÃO

A mortalidade infantil tem significante impacto na saúde pública, acometendo principalmente crianças menores de cinco anos residentes em países em desenvolvimento. Em 2013, foram registrados cerca de 578 mil óbitos em crianças menores de cinco anos, sendo o rotavírus (RV) o agente responsável por 37% dos óbitos nesta idade (UNICEF; WHO 2013; LIU et al., 2015).

As doenças diarreicas são atribuídas a diversos agentes, principalmente os vírus que estão envolvidos nesta etiologia, com destaque para os RV da espécie A (RVA) que se configuram como um dos agentes de maior importância epidemiológica e ao qual é atribuído de 25% à 35% das hospitalizações por doenças diarreicas. Neste contexto, estima-se que mais de 95% das crianças menores de cinco anos de idade já foram infectadas ao menos uma vez por este agente (TAYEB et al, 2011; LIU et al., 2012; SANCHEZ-FAUQUIER et al., 2013; GREENWOOD, 2015).

A relevância clínico-epidemiológica atribuída aos RVA é relacionada à elevada frequência de infecções, as quais podem ser assintomáticas ou evoluir para um quadro clínico moderado ou grave que culminam em desidratação que, sem intervenção, pode ocasionar óbito infantil (PALUMBO et al., 2009; DESSELBERG, 2014).

A incidência de casos de gastroenterite ocasionados por RVA são similares em países desenvolvidos e em desenvolvimento, contudo, nestes últimos, observa-se maior mortalidade, possivelmente relacionada à maior prevalência de subnutrição e ao precário acesso aos serviços de saúde (UNICEF; OMS, 2009; TELMESANI, 2010, PATH, 2016).

Visando minimizar o impacto que os RVA acarretam à saúde pública, tornou-se primordial o desenvolvimento e implantação de imunizantes seguros e eficazes contra este agente, principalmente em países em desenvolvimento, nos quais o agravo causado por este agente viral tem maior impacto na saúde infantil (DESAI et al., 2011; GREENWOOD, 2015).

Desde o ano de 2006, imunizantes previamente testados e comprovadamente eficazes contra os tipos mais comuns de RVA foram incorporados ao calendário vacinal de diversos países, inclusive no Brasil. Tais imunizantes

compreendem a Vacina Oral de Rotavírus Humano (VORH), produzida pela Glaxo SmithKline, genótipo G1P[8] e a vacina Rotateq, produzida pela Merk, genótipo G1, G2, G3, G4 e P[8], disponibilizada na rede privada de imunizantes no Brasil, ambas apresentando resultados satisfatórios contra gastroenterites graves e hospitalizações (LINHARES et al., 2006a; LINHARES; VILLA, 2006; O'RYAN; LINHARES, 2009; CARVALHO-COSTA et al., 2009; YEN et al., 2011; BUCARDO et al., 2014).

Desde então, a OMS recomenda o desenvolvimento de estudos que visem à caracterização dos tipos de RVA circulantes no cenário pós-introdução da vacina, objetivando detectar e caracterizar o surgimento de novas variantes genéticas de RV, o que influenciaria em mudanças nas estratégias dos imunizantes ora vigentes (WHO, 2013).

Atualmente, 80 países introduziram a VORH em seu Programa de Vacinação, enfatizando a necessidade da realização de estudos de vigilância pós licenciamento (PATH, 2016).

Desta forma, visando compreender a dinâmica deste agente viral, principalmente no período pós-introdução da vacina, há a necessidade da contínua monitorização e caracterização dos tipos de RVA circulantes, tornando-se imprescindível para um melhor entendimento a respeito da genética viral, assim como a detecção de possíveis mudanças na epidemiologia molecular que possa servir a comprometer as estratégias dos imunizantes ora vigentes.

2. REFERENCIAL TEÓRICO

2.1- HISTÓRICO

Até meados da década de 1960, a maioria dos casos diarreicos mais graves possíam etiologia desconhecida, sendo incansável a busca pelos agentes etiológicos envolvidos nesse agravo (ESTES; GREENBERG, 2013).

A descoberta do vírus Norwalk no ano de 1972 e do RV em 1973 elucidaram a maioria das etiologias de casos diarreicos de origem não bacteriana, o que impulsionou bastante os estudos acerca da epidemiologia destes vírus (KAPIKIAN et al., 1972; BISHOP et al., 1973; ESTES; GREENBERG, 2013).

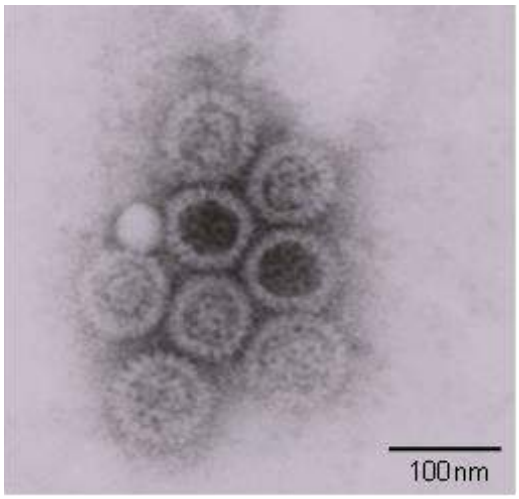
Em Melbourne, Austrália, Bishop et al. (1973) desenvolvendo estudo com crianças que apresentavam episódios diarreicos graves de origem não bacteriana, observaram pela primeira vez a partícula viral dos RV em finas seções do epitélio duodenal, utilizando a técnica de visualização direta por microscopia eletrônica.

Após a primeira caraterização do RV, estudos morfológicos e sorológicos de espécimes fecais diarreicos de animais demonstraram que este vírus não ocasionava gastroenterite apenas em seres humanos, circulando também em animais como suínos, equinos e roedores (WOODE et al. 1987).

Inicialmente, este vírus foi caracterizado como Duovírus, porém, considerando a sua similaridade morfológica ao de uma roda, foi posteriormente denominado de Rotavírus (RV) como demonstrado na figura 1 (FLEWETT; WOODE, 1978).

No ano de 1976, em um estudo desenvolvido no Hospital da Santa Casa de Misericórdia de Belém, Pará, conduzido em crianças com gastroenterite aguda sob atendimentos ambulatorial e hospitalar, Linhares et al. (1977) detectaram pela primeira vez o RV no Brasil, sendo desenvolvido, desde então, inúmeros estudos epidemiológicos, enfatizando a importância desses agentes virais em âmbito regional, nacional e mundial, demonstrando sua diversidade genética (WOODS et al*.,* 1992; LINHARES, 2000; LUZ et al*.*, 2005; SANTOS; HOSHINO, 2005; MATTHIJNSSENS et al., 2009; LUCHS et al, 2015).

Figura 1. Visualização da micrografia eletrônica de extrato fecal contendo partículas completas de RV.



Fonte: Cedida pelo setor de microscopia eletrônica do IEC (2004).

2.2 - ROTAVÍRUS

2.2.1 - Partícula Viral

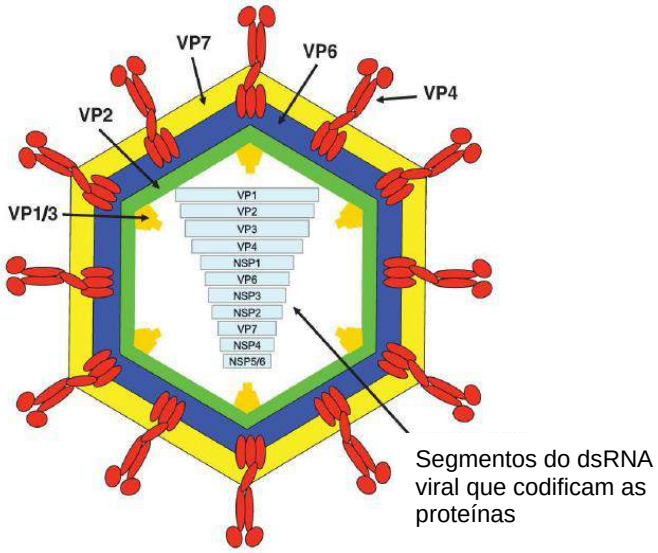
Os *Rotavirus* compreendem um gênero específico da família *Reoviridae*, sub-família *Sedoreovirinae,* sendo formados por ácido ribonucléico de dupla cadeia (dsRNA) de natureza segmentada, o qual codifica 6 proteínas estruturais e, geralmente, 6 proteínas não-estruturais (ESTES; GREENBERG, 2013).

A partícula viral íntegra é desprovida de envelope lipoproteico, exibindo simetria icosaédrica e diâmetro de aproximadamente 100 nanômetros (nm), sendo constituída por três camadas proteicas concêntricas, quais sejam capsídeo externo, intermediário e interno, estando este último intimamente associado ao genoma viral, como demonstrado na Figura 2 (ESTES; GREENBERG, 2013).

As proteínas VP1, VP2 e VP3 compõem o capsídeo interno, estando a proteína VP2 em maior quantidade e interagindo com a proteína VP6 e com o genoma viral (JAYARAM; ESTES; PRASAD , 2004; ESTES; GREENBERG, 2013).

O capsídeo intermediário é constituído pela proteína VP6, a qual compreende mais de 50% da partícula viral e designa os grupos e subgrupos de RV, sendo considerada uma das proteínas virais mais importantes (JAYARAM; ESTES; PRASAD , 2004; ESTES; GREENBERG, 2013).

Figura 2: Representação esquemática do RV, demonstrando a formação proteica dos capsídeos externo, intermediário, interno e o RNA viral.



Fonte: adaptada de ESTES; GREENBERG, 2013

A camada externa é composta pelas proteínas VP4 e VP7, as quais são independentes e estão envolvidas na imunidade do hospedeiro, sendo as proteínas mais estudadas do RV (SANTOS; HOSHINO, 2005; ESTES; GREENBERG, 2013; MOROZOVA et al., 2015).

A VP7 é uma glicoproteína que designa o genótipo G de RV, enquanto que a VP4 designa o genótipo P, por ser sensível à protease, e compõe as 60 projeções (espículas) com cerca de 10 a 12 nm de comprimento, compreendendo 1,5% da partícula viral (JAYARAM; ESTES; PRASAD , 2004; ESTES; GREENBERG, 2013).

As proteínas não estruturais codificadas pelo vírus não compõem a partícula viral morfologicamente, contudo exercem funções vitais para a infectividade e replicação do RV, estando presentes apenas na célula infectada (ESTES; GREENBERG, 2013).

2.2.2 – Classificação

Devido a reatividade sorológica e a variabilidade genética dos rotavírus (RV) podem ser classificados em 9 espécies distintas denominadas de A-I, com base na proteína VP6. As espécies A, B, C e H têm sido detectadas acometendo seres

humanos e animais, enquanto que as demais foram identificadas apenas em animais (MATTHIJNSSENS et al., 2012; MIHALOV-KOVÁCS et al., 2015).

O RVA apresenta maior relevância epidemiológica, estando envolvido na maioria dos estudos de caráter epidemiológico em seres humanos, contudo, na região amazônica além desta espécie, outros já foram descritos acometendo humanos, como a espécie C (RVC), e acometendo animais, como as espécies D, F e G (GABBAY et al., 1999; GABBAY et al., 2008; BEZERRA et al, 2012; ESTES; GREENBERG, 2013; SILVA et al., 2013; LOBO et al., 2016).

Para o RVA, são definidas as classificações em subgrupos (SG) e sorotipos/genótipos. Os SG compreendem em SG I, SG II, SG I+II e SG não-I/não-II, sendo, distinguidos em dois genogrupos baseados na caracterização molecular da proteína VP6: Genogrupo I correspondendo ao SG I e genogrupo II, o qual reúne os demais SG (DESSELBERG, 1996; ITURRIZA-GÓMARA et al., 2002).

Os sorotipos/genótipos consistem em uma classificação binária designada pelos genes que codificam as proteínas VP4 e VP7, correspondendo aos genótipos P e G, respectivamente. Os sorotipos são determinados por anticorpos monoclonais específicos e testes de neutralização, enquanto que os genótipos são definidos por métodos de biologia molecular, sendo descritos até o momento, 32 genótipos/sorotipos G e 46 diferentes genótipos P, com 14 sorotipos P (MATTHIJNSSENS et al., 2011; ESTES; GREENBERG, 2013; TROJNAR et al., 2013; LI et al., 2016).

Estudos epidemiológicos caracterizando as proteínas VP4 e VP7 foram os mais descritos, até que Matthijnssens et al. (2008), propuseram uma nova classificação para o RVA, baseada na caracterização molecular e análise filogenética dos demais genes, enfatizando a necessidade de estudos envolvendo a caracterização da constelação do RVA, tal como Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx que corresponde aos genótipos que codificam as proteínas VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6, respectivamente.

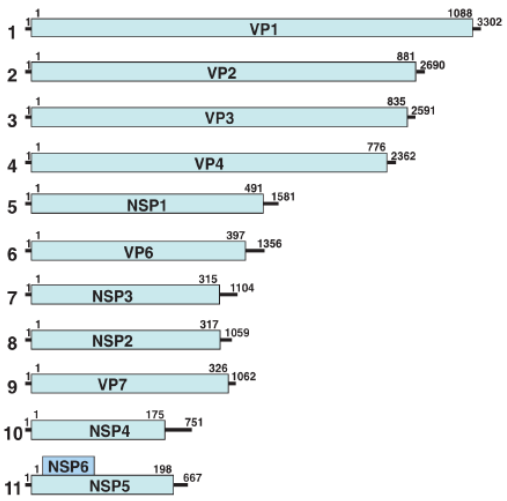
2.2.3 - Genoma Viral

O RVA possui genoma de RNA fita dupla reunindo 18.556 pares de bases (pb), os quais são divididos em 11 segmentos, que codificam 12 proteínas virais, sendo seis estruturais (VP1 – VP4; VP6 e VP7) e seis não estruturais (NSP1 –

NSP6). Os segmentos variam de 667 pb (segmento 11) a 3.302 pb (segmento 1), sendo que cada segmento é responsável pela síntese de uma única proteína, com exceção do 11º que pode codificar para duas proteínas (NSP5 e NSP6), conforme demonstrado na figura 3. O RVC não codifica para a proteína NSP6, sendo todos os segmentos monocistrônicos e tal proteína também não se expressa em algumas amostras adaptadas a cultura de células (ESTES; GREENBERG, 2013; DESSELBER, 2014; SANTOS; SOARES, 2015).

A característica segmentada do genoma do RV, aliada à diferença de massa molecular entre os segmentos, permite que o genoma viral seja caracterizado pelo seu padrão eletroforético de migração, que pode ser visualizado pela separação dos mesmos em eletroforese em gel de poliacrilamida (EGPA), sendo classificados como longo, curto e supercurto, além de caracterizar quanto a espécie de RV (ESTES; GREENBERG, 2013).

Figura 3: Representação do genoma segmentado do RV e as respectivas proteínas codificadas

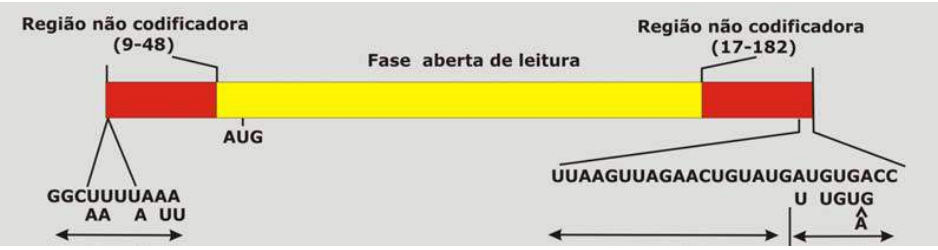


Fonte: Retirada de ESTES;GREENBERG, 2013

Geralmente, cada perfil do RVA corresponde a determinados genótipos G, como o perfil longo aos tipos G1, G3, G4 e G9, o perfil curto ao tipo G2 e o perfil super curto ao genótipo G8, Contudo, tal associação pode variar, tal como a detecção do perfil super curto associados ao G4, G5 e G9 em estudo desenvolvido por Nagai et al (2015) com suínos e o perfil curto associado aos genótipos G1 e G3 (MATSUI et al., 1990; TANIGUCHI; URASAWA, 1995; ESTES;GREENBERG, 2013; KOMOTO et al., 2015; COWLEY et al., 2016).

Os 11 segmentos genômicos do RV são semelhantes em sua estrutura e composição, sendo ricos em adenina e uracila e apresentando na fita positiva a extremidade 5'-guanidina seguida de uma sequência conservada contida na região 5' não codificadora. Após o códon de iniciação, encontra-se uma fase aberta de leitura (ORF), a qual codifica as proteínas virais, seguida do códon de finalização e outra região não codificadora, situada na extremidade 3', a qual finaliza com 3'-citidina, conforma observado na Figura 4 (ESTES; GREENBERG, 2013).

Figura 4: Estrutura do gene do rotavírus



Fonte: Retirado e adaptado de ESTES;GREENBERG, 2013

2.2.4 – Proteínas

2.2.4.1 - Estruturais

O capsídeo interno é constituído pelas proteínas VP1, VP2 e VP3. A VP1 é composta por 3302 nucleotídeos (nt) que codificam uma proteína de 1088 aminoácidos (aa), sendo considerada a RNA polimerase dependente de RNA que catalisa a síntese do RNA na partícula subviral e apresentando elevada afinidade com a porção 3' final do RNA de polaridade negativa, fato este imprescindível para a replicação do genoma viral (JAYARAM; ESTES; PRASAD , 2004; LU et al., 2008; ESTES; GREENBERG, 2013; ESTROZI et al., 2013).

A proteína VP2 é a mais abundante do capsídeo interno, constituída de 60 dímeros de proteína que envolvem o genoma, sendo, assim, considerada o suporte do core viral. Esta proteína é constituída por 2690 nt, 881 aa, e atua na replicação viral por estar envolvida na síntese do RNA de polaridade negativa para produzir o dsRNA, sendo necessária para a atividade de replicase da VP1. (JAYARAM; ESTES; PRASAD , 2004; GULHIELMI; MCDONALD; PATTON, 2010; ESTES; GREENBERG, 2013; SANTOS; SOARES, 2015).

Outra proteína que compõe o capsídeo interno é a VP3, a qual apresenta 2591 nt que codificam 835 aa e é considerada uma metil e guanililtransferase, estando envolvida nas funções enzimáticas requeridas na adição de um 5-CAP presente no RNA mensageiro viral, importante para o mecanismo de transcrição e revestimento do genoma viral (VALENZUELA et al., 1991; CHEN et al., 1999; JAYARAM; ESTES; PRASAD , 2004; MCCLAIN et al., 2010; ESTES; GREENBERG, 2013).

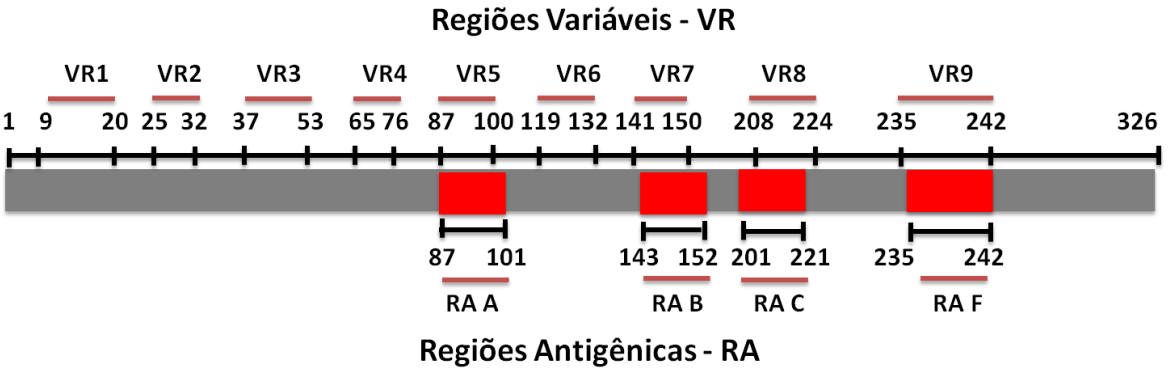
A camada intermediária é formada pela proteína VP6 que é codificada por um gene de 1356 nt. Devido sua organização na partícula viral, está envolvida nas duas principais funções do vírus, processo de entrada na célula hospedeira e a replicação viral, por meio de sua interação com a proteína VP2 e com as proteínas VP4 e VP7 (JAYARAM; ESTES; PRASAD , 2004; PATTON, 2012; ESTES; GREENBERG, 2013).

A VP7 é uma glicoproteína que compreende a maior parte do capsídeo externo e, junto com a VP4, induz a formação de anticorpos neutralizantes. A função precisa desta proteína nas primeiras interações do vírus com a célula hospedeira ainda não foi bem elucidada, contudo, acredita-se que ela possa estar envolvida na modulação da função da proteína VP4 no processo de aderência e penetração viral, podendo também interagir com as moléculas da superfície celular do hospedeiro, processo conduzido pela proteína VP4 (BEISNER et al., 1998; MENDEZ; ARIAS; LOPEZ , 1996; MENDEZ et al., 1999; PANTON, 2012; ESTES;GREENBERG, 2013).

O gene VP7 é composto por 1062 nt apresentando uma região de leitura de 326 aa, o qual apresenta elevada variabilidade genética. O gene que codifica esta proteína apresenta nove regiões variáveis (VR) e dentro destas, seis regiões antigênicas (RA) classificadas de A a F, as quais estão representadas na figura 5 (DYALL-SMITH et al., 1986; COULSON; KIRKWOOD, 1991; KOBAYASHI et al., 1991; DUNN et al., 1993; KIRKWOOD; MASENDYCZ; COULSON, 1993; CIARLET et al., 1994; LAZDINS et al., 1995; ZAO et al., 1999; MATTHIJNSSENS et al., 2011).

Estudo conduzido por Aoki et al (2009) descrevem epítopos neutralizantes no gene VP7 denominados 7-1a (resíduos 87, 91, 94, 96-100, 104, 123, 125, 129, 130, 291), 7-1b (resíduos 201, 211-213, 238, 242) e 7-2 (resíduos143, 145-148, 190, 217, 221, 264), sugerindo que mutações em tais regiãos podem modificar a resposta de anticorpos neutralizantes.

Figura 5: Demonstração das regiões variáveis VR1 (aa 9 - 20), VR2 (aa 25 - 32), VR3 (aa 37 - 53), VR4 (aa 65 - 76), VR5 (aa 87 - 100), VR6 (aa 119 - 132), VR7 (aa 141 - 150), VR8 (aa 208 - 224) e VR9 (aa 235 - 245) e das principais regiões antigênicas (A, B, C e F) presentes no gene VP7.



A proteína VP4, assim como a VP7, forma o capsídeo externo da partícula viral. A VP4 é clivada pela enzima proteolítica tripsina, dando origem a um fragmento N-terminal, VP8*, e um fragmento C-terminal, VP5*, processo relevante para a entrada do vírus na célula hospedeira. A VP4 tem importante papel na aderência e penetração do vírus, estando envolvida na hemaglutinação, neutralização e virulência. Estudos envolvendo a caracterização deste gene descrevem a existência de cinco epítopos de neutralização, dentro da subunidade VP8* da proteína VP4 denominados 8-1 (resíduos 100, 146, 148, 150, 188, 190, 192 – 196), 8-2 (resíduos 180 e 183), 8-3 (resíduos 113 – 116, 125, 131-133 e 135) e 8-4 (resíduos 87-89), enquanto que na subunidade VP5* observou-se os epítopos 5-1 (384, 386, 388, 393, 394, 398, 440, 441), 5-2 (resíduo 434), 5-3 (resíduo 459), 5-4 (resíduo 429) e 5-5 (resíduo 306) (JAYARAM; ESTES; PRASAD , 2004; DORMITZER et al., 2002, 2004; PATTON, 2012; ESTES; GREENBERG, 2013; TROJNAR et al., 2013).

2.2.4.2 - Não estruturais

A proteína não estrutural NSP1 é codificada pelo quinto segmento do dsRNA viral que compreende 1581 nt, sendo a proteína do RV que apresenta maior diversidade e a menos conservada. Esta proteína é associada ao citoesqueleto revelando-se determinante para uma replicação eficiente, além de demonstrar estar envolvida no escape imune viral, atuando como antagonista do interferon por diversos mecanismos, regulando assim a apoptose nas primeiras horas da infecção

e favorecendo a replicação viral (MERTENS, 2004; GRAFF et al., 2009; BAGCHI et al., 2010; FENG et al., 2013).

A NSP2 é codificada por 317 aa e se encontra no viroplasma, onde está envolvida na replicação e empacotamento do RNA viral juntamente com a proteína NSP5, também formando complexos com as proteínas VP1, VP2 e tubulina, sendo essencial na produção do viroplasma. É codificada pelo oitavo segmento do RNA viral e apresenta atividades de NTPase e de desestabilização de hélices de ácidos nucleicos (TARAPOREWALA; CHEN; PATTON, 1999; JAYARAM; ESTES; PRASAD , 2004; ESTES; GREENBERG, 2013; ARNOLDI et al., 2014).

A NSP3 é uma proteína codificada pelo sétimo segmento do genoma viral composta por 315 aa, a qual atua na regulação da tradução viral e previne a degradação do mRNA por nucleases celulares (MOSSEL; RAMIG, 2002; JAYARAM; ESTES; PRASAD , 2004; FUJITA et al., 2010; RUBIO et al., 2013).

O décimo segmento do dsRNA viral codifica para uma proteína composta por 175 aa e denominada de NSP4. É uma glicoproteína transmembrana e está localizada, especificamente, no retículo endoplasmático (RE) da célula infectada, com significativo papel na morfogênese, por interagir com a proteína VP6, e patogênese viral, por mobilizar íons Cálcio e, com isso, alterar a homeostase intracelular induzindo à apoptose celular, sendo denominada, devido à isto, como a primeira enterotoxina viral (JAGANNATH et al., 2006; KHAMRIN et al., 2008; HYSER et al., 2012; ESTES; GREENBERG, 2013).

O último segmento do genoma viral codifica para duas proteínas, a NSP5 e a NSP6, sendo a NSP5 a maior proteína codificada por este segmento. A NSP5 encontra-se no viroplasma e, assim como a NSP2, demonstrou ser essencial para a formação do mesmo, podendo ter atividade de autoquinase, contudo, sua função bioquímica ainda não está completamente elucidada (TARAPOREWALA; PATTON, 2004; CAMPAGNA et al., 2007; CONTIN et al., 2010; ESTES; GREENBERG, 2013; CRIGLAR et al., 2014).

A proteína NSP6 localiza-se no viroplasma e interage com a NSP5, propriedade esta que evidencia sua participação na replicação e encapsidação viral, contudo observou-se não ser essencial à todos os tipos de RV (TARAPOREWALA; PATTON, 2004; ESTES;GREENBERG, 2013; DESSELBERG, 2014).

2.2.5 - Mecanismos de evolução genética

Os vírus de RNA estão entre os organismos de maior adaptação evolutiva na biologia, sendo tal fato proporcionado por mecanismos de evolução. Por apresentar genoma segmentado, o RVA exibe elevada variabilidade genética, a qual pode ocorrer por distintos mecanismos, sendo: mutações pontuais, rearranjo genético (*rearrangements*), reestruturação genética (*reassortment*) e recombinação do genoma (ITURRIZA-GOMARA et al, 2003; DESSELBERG, 2014).

As mutações pontuais são eventos em que há a substituição, inserção ou deleção de bases nucleotídicas podendo tanto ocorrer esporadicamente quanto se acumular nas sequências dos genes. Quando tais alterações não modificam sua sequência aminoacídica, são denominadas de sinonímias, no entanto quando ocorre mudança na sequência aminoacídica, são designadas então de não-sinonímias, o que pode contribuir para a modificação proteica e, com isso, comprometer ou não sua função. Neste contexto, as mutações não-sinonímias assumem relevância epidemiológica, pois podem originar novas linhagens ou sublinhagens genéticas podendo causar surtos e comprometer as estratégias vacinais implantadas em escala global (MAUNULLA; VON BONSDORFF, 1998; ITURRIZA-GÓMARA et al., 2001, 2003; MARTÍNEZ-LAZO et al., 2009; IANIRO et al., 2013; DE GRAZIA et al., 2014).

Outra forma de evolução dos RVA é o rearranjo genético, sendo uma forma especial de recombinação viral, no qual há deleções ou duplicações parciais das sequências de nucleotídeo dos segmentos, ocorrendo com mais frequência em genes que codificam para proteínas não estruturais, pois não comprometeriam a estrutura viral, permitindo a viabilidade da partícula (PEDLEY et al., 1984; DESSELBERGER, 1996; ITURRIZA-GÓMARA et al., 2001; CAO et al., 2008).

Quando ocorre a infecção de diferentes tipos de RVA em uma mesma célula e há permuta de material genético, caracteriza-se a reestruturação genética, a qual está frequentemente envolvida em transmissão zoonótica de RVA. Esse fenômeno ocorre principalmente em países em desenvolvimento, provavelmente por estar associado a fatores como maior convívio do homem com o animal, proporcionando maior probabilidade de ocorrer infecções mistas e provavelmente transmissão entre espécies (MASCARENHAS et al., 1989, 2002, 2007a, 2007b; ITURRIZA-GÓMARA et al., 2001; JAIN et al., 2001; TEODOROFF et al., 2005;

MARTELLA et al., 2006; MATTHIJNSSENS et al., 2008; McDONALD et al., 2009; MAESTRI et al., 2012).

A recombinação do genoma pode ocorrer entre segmentos de genes de diferentes tipos de RVA com grande homologia, contudo, como afirmado por Woods (2015), o conhecimento acerca deste mecanismo ainda é escasso, assim como sua contribuição para a diversidade do RVA, não havendo conhecimento se tal mecanismo sustentaria a transmissão e se todos os segmentos possuem taxas de recombinação similares. Apesar da necessidade de esclarecimentos acerca deste mecanismo de evolução, Phan et al. (2007) reportaram a recombinação intrasegmental ocorrendo entre epítopos de domínios antigênicos da proteína VP7.

2.2.6 – Epidemiologia molecular

Após a descoberta dos RV na etiologia das doenças diarreicas, diversos estudos conduzidos até o momento demonstraram a diversidade genética que o RVA (LEITE et al, 1996, 2008; GOUVEA; SANTOS, 1999; GUSMÃO et al., 1999; GONZÁLES-LOSA et al., 2005; SANTOS; HOSHINO, 2005; GURGEL et al., 2008; JUSTINO et al., 2011; SOARES et al, 2012; LUCHS et al., 2015).

Os estudos epidemiológicos visavam principalmente à caracterização dos genes VP7 e VP4 para a elucidação da combinação binária G e P demonstrando a variedade e circulação dos tipos de RVA, contudo, havia a necessidade do conhecimento da completa diversidade genética do RVA, a dinâmica de evolução apresentada por este vírus e o relacionamento entre os tipos circulantes (PATTON, 2012). Desse modo, Matthijnssens et al. (2008), propuseram a nova classificação a qual enfatiza a necessidade de estudos envolvendo a caracterização da constelação do RV, conservando a combinação binária de VP7 e VP4 e caracterizando as demais proteínas virais.

Atualmente já foram descritos G32-P46-I24-R18-C17-M17-A28-N17-T19-E24-H19 que corresponde aos genótipos que codificam as proteínas VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6, respectivamente. Até o momento, já foram caracterizados diversos tipos de constelação, com destaque para a Wa (I1-R1-C1-M1-A1-N1-T1-E1-H1) e DS-1 (I2-R2-C2-M2-A2-N2-T2-E2-H2) que são as mais detectadas em humanos como demonstrado na figura 6 (MATTHIJNSSENS et

al., 2008; PATTON, 2012; TROJNAR et al., 2013; DE GRAZIA et al., 2015; LI et al., 2016).

Figura 6: Constelações de RV em humanos, animais e vacinas anti-rotavirus

			Genótipos										
			VP7 (27)	VP4 (35)	VP6 (16)	VP1 (9)	VP2 (9)	VP3 (8)	NSP1 (16)	NSP2 (9)	NSP3 (12)	NSP4 (14)	NSP5 (11)
Cepas Comuns	Wa	human	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	DS-1	human	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
	P	human	G3	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	ST3	human	G4	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	WI61	human	G9	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
Cepas Vacinas	Rotarix RIX4414	human	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RotaTeq WI79-9	Hu x Bo reassort	G1	P[5]	I2	R2	C2	M1	A3	N2	T6	E2	H3
	RotaTeq SC2-9	Hu x Bo reassort	G2	P[5]	I2	R2	C2	M1	A3	N2	T6	E2	H3
	RotaTeq WI78-8	Hu x Bo reassort	G3	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
	RotaTeq BrB-9	Hu x Bo reassort	G4	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
	RotaTeq WI79-4	Hu x Bo reassort	G6	P[8]	I2	R2	C2	M2	A3	N2	T6	E2	H3
Cepas incomuns	Au-1	human	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
	MWI	human	G8	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
	GR 10924/99	human	G9	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2
	6717/2002/ARN	human	G10	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	Dhaka12-03	human	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	Dhaka25-02	human	G12	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
Cepas Animais	SA11-H96	simian	G3	P[2]	I3	R2	C5	M5	A5	N5	T5	E2	H5
	RRV	simian	G8	P[3]	I2	R2	C3	M3	A9	N2	T3	E3	H6
	OSU	porcine	G5	P[7]	I5	R1	C1	M1	A1	N1	T1	E1	H1
	WC3	bovine	G6	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3

Fonte: Retirado e adaptado de PATTON, 2012.

As combinações binárias de RVA consideradas usuais reúnem os genótipos G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] e, mais recentemente, o G12P[8], sendo tais combinações de maior relevância epidemiológica por serem frequentemente encontradas por isso denominadas usuais (SANTOS; HOSHINO, 2005; RAHMAN et al., 2007; URSU et al., 2009; MATTHIJNSSENS et al., 2009; TROJNAR et al., 2013).

A análise do genoma viral completo revelou que os RVA usuais do tipo G1P[8], G3P[8], G4P[8], G9P[8] e G12P[8] são geralmente caracterizados como genogrupo 1 (constelação Wa) enquanto que o G2P[4] é caracterizado como genogrupo 2 (constelação DS-1) (PATTON, 2012)

A combinação G1P[8] de RVA era a mais prevalente a nível mundial, responsável por 65% das infecções no período antes da introdução da vacina de RVA, obtendo um declínio de sua detecção para 3% logo após este evento, devido à predominante detecção do genótipo G2 (SANTOS; HOSHINO, 2005; LEITE et al., 2008). Apesar disso, atualmente, segundo a OMS (2015), o genótipo G1P[8] ainda é o mais detectado em todo o mundo, em menor escala, estando envolvido em 25,3%

dos casos de RVA, seguido do G2P[4] em 12,5% e G12P[8] em 10% das infecções (SOARES et al. 2010; OLIVEIRA et al., 2012; WHO, 2015).

A detecção do genótipo G2 no período pós-vacina re-emergiu na América Latina e em várias partes do mundo. No Brasil, observou-se a elevada detecção do genótipo G2 sobre os outros tipos de RV, provavelmente devido a uma seleção ocasionada após a implantação da vacina ou mesmo a um comportamento cíclico deste genótipo que circula a cada dez anos (LEITE et al., 2008; CARVALHO-COSTA et al., 2009; JUSTINO et al., 2010; OLIVEIRA et al., 2012; GÓMEZ et al., 2014; SOARES et al., 2014).

O estudo conduzido por Mascarenhas et al. (2010), no sul do Pará nos anos de 2006 e 2008, evidenciou o genótipo G2 em 90% das amostras analisadas, revelando uma flutuação do genótipo G2 e a identificação de duas novas possíveis sublinhagens na linhagem II (IIa e IIc). Neste estudo não foram observadas divergências significativas na composição aminoacídica, se comparados, crianças vacinadas e não vacinadas.

O genótipo G3 foi bastante detectado nos período pós-vacina em países que inseriram apenas a Rotateq em seu calendário vacinal, compreendendo 7,6% dos casos de diarreia por RVA em todo o mundo. Um novo tipo de G3P[8], VP7 equino e demais genes mais similares a RVA humanos, foi detectado na Ásia, Oceania e Europa, sendo considerado por Dóro et al (2016) uma possível cepa pandêmica (MATTHIJNSSENS et al., 2009; OLIVEIRA et al., 2012; SOARES et al., 2012; WHO, 2015; KOMOTO et al., 2016; COWLEY et al., 2016).

Outro genótipo que é considerado usual em escala global, contudo na região Norte do país não é detectado com frequência é o tipo G4 (JUSTINO et al., 2010; OLIVEIRA et al., 2012; DÓRO et al., 2014; SOARES et al., 2012, 2014). Tal fato também foi observado por Luchs et al (2015) ao analisar amostras coletadas de 2007 a 2012 de diferentes unidades federativas do Brasil.

As combinações consideradas não usuais são descritas em todo o mundo, com destaque para os países em desenvolvimento, como por exemplo, G5, G8, G10, P[6] e P[9], os quais podem representar um desafio aos imunizantes disponíveis em escala global (LEITE et al., 2008; SOARES et al., 2012, 2014; DÓRO et al., 2014; KAIANO, 2015).

A detecção de genótipos não usuais ou combinações binárias não usuais tais como G3P[4], G1P[4], G9P[4], podem surgir a partir de infecções mistas,

sugerindo uma constante troca de material genético entre as amostras de RVA humano ou entre amostras de RVA humano e animal (DUNN et al., 1993; GOUVEA et al, 1994; STEELE et al., 1995; MASCARENHAS et al, 1999, 2002; ITURRIZA-GÓMARA et al., 2001; MAUNULLA; VON BONSDORFF, 2002; SANTOS et al, 2003; SANTOS; HOSHINO, 2005; BARRIL et al, 2006; MASCARENHAS et al., 2007a; LUCHS et al., 2015).

O genótipo G12 era considerado não usual e foi caracterizado pela primeira vez nas Filipinas na década de 1980. A partir de 2000, foi reportado em diversas partes do mundo, sendo considerado, atualmente, o sexto genótipo usual, em combinação com o P[8], mas também já foi detectado em combinação com o P[6] e o P[9] (TANIGUCHI et al., 1990; GRIFFIN et al., 2002; PONGSUWANNA et al., 2002; DAS et al., 2003; SHINOZAKI et al., 2004; CASTELLO et al., 2006; PIETRUCHINSKI et al., 2006; SOARES et al., 2012).

Diante desse panorama, a importância da contínua vigilância dos genótipos circulantes RVA, torna-se imprescindível na atual cenário pós-implantação da vacina de RVA (RAMACHANDRAN et al., 1998; RAHMAN et al., 2005a; LEITE et al., 2008; KHAMRIN et al., 2009; MATTHIJNSSENS et al., 2009).

2.2.6.1 - Genótipo G9

Em meados da década de 1990, um novo tipo de RVA emergiu ou reemergiu no cenário mundial, o genótipo G9, apresentando relevância em vários aspectos incluindo a epidemiologia molecular, origem evolutiva e composição genética do vírus.

Este genótipo foi isolado pela primeira vez nos Estados Unidos (EUA) em 1983 proveniente de uma criança com 18 meses de idade hospitalizada por gastroenterite grave (CLARK et al., 1987). Posteriormente, o mesmo genótipo foi detectado no Japão, em 1985, não sendo mais observado em ambos os países por cerca de uma década (NAKAGOMI et al., 1988a). Foi também isolado na Índia em 1986 e na Tailândia em 1989 em casos esporádicos (DAS et al., 1993; URASAWA et al., 1992).

No início da década de 1990, o G9 foi novamente detectado em vários países, como na Índia e EUA (1993), Reino Unido e Bangladesh (1995), no Japão e Tailândia (1996), em Malawi (1997) e outras localidades (DAS et al., 1994;

UNICOMB et al., 1999; CUNLIFFE et al., 1999; CUBITT et al., 2000; GRIFFIN et al., 2000; MANEEKARN; USHIJIMA, 2000; OKA; NAKAGOMI; NAKAGOMI, 2000).

Desta forma, o G9 foi considerado um genótipo cosmopolita, sendo encontrado em diversos países dos cinco continentes com elevada incidência e com alguns estudos indicando um declínio nos últimos anos (KIRKWOOD et al., 2003; STEELE; IVANOFF, 2003; WILHELMI et al., 2003; PARRA et al., 2005; REIDY et al., 2005; STEYER et al., 2005; CARMONA et al., 2006; KHAMRIN et al., 2006; 2007; YANG et al., 2007; STUPKA et al., 2007, 2009; LEITE et al., 2008; NARANJO et al., 2008; SOLBERG et al., 2009; KHANANURAK et al., 2010; ZURIDAH et al., 2010; JUSTINO et al., 2011; OLIVEIRA et al., 2012; SOARES et al., 2012; 2014)

Na América Latina, estudo desenvolvido por Barril et al. (2006) na Argentina, comprovou a circulação do genótipo G9 na década de 80, tornando-se então o país latino com a descrição mais antiga de G9.

No Brasil, este genótipo foi caracterizado circulando em Belém, Rio de Janeiro, Goiânia, Salvador, São Paulo e Acre, disseminando-se assim por todo país (ARAÚJO et al., 2001; SANTOS et al., 2001, 2005; SOUZA et al., 2003; VOLOTÃO et al., 2005; CARMONA et al., 2006; MONTENEGRO et al., 2007; RIBEIRO et al., 2008; OLIVEIRA et al., 2012).

Este genótipo também é bastante detectado a partir de casos de RVA em animais, principalmente suínos, tal como descrito nos estudos desenvolvidos por Kim et al. (2010), Okitsu et al. (2011), Midgley et al. (2012) e Amimo, Vlasova e Saif (2013).

Este genótipo foi detectado em surtos tal como descrito por Siqueira et al. (2010) no Acre em 2005, onde ocorreu 12.145 casos diarreicos com sete óbitos por desidratação, estando o G9 envolvido em 71% das infecções por RV.

A variabilidade genética do G9 possibilita a distinção deste genótipo em linhagens e sublinhagens. Inicialmente, a filogenia do genótipo G9 apresentou três linhagens distintas: linhagem I, representada por amostras isoladas nos EUA (WI61) e no Japão (F45) em 1983; linhagem II, representada pela amostra 116E, isolada na Índia em 1986; linhagem III representada pelas amostras contemporâneas (CAO et al., 2008). Posteriormente, Rahman et al. (2005b) propuseram a classificação do G9 em quatro linhagens distintas.

Em 2007, estudo desenvolvido por Phan et al. (2007), propuseram nova classificação para a filogenia do G9 em 6 linhagens e 11 sublinhagens, contudo

Martinez-Laso et al. (2009), em análise de tal classificação, discordaram quanto a existência das sublinhagens propostas, confirmando apenas a distinção do G9 em 6 linhagens.

Os demais tipos usuais de RVA são geralmente detectados em combinação com um tipo P específico. O genótipo G9 apresenta a peculiaridade de ter sido detectado com diversos genótipos P, apresentando diferentes eletroferotipos e subgrupos específicos, além de ter sido detectado em várias localidades em todo o mundo, em seres humanos e animais, tornando, este genótipo o mais disseminado e sendo considerado “promíscuo” na comunidade científica (HOSHINO et al., 2004; GHOSH et al., 2012; PATTON, 2012; ESONA et al., 2013; LEWIS et al., 2014).

Apesar de sua variedade, este genótipo é detectado principalmente associado aos tipos P[6] e P[8], os quais representavam 4,1% das infecções detectadas em escala global, contudo, há estudo descrevendo também a caracterização de RVA G9P[4] (GENTSCH et al., 2005; SANTOS; HOSHINO, 2005; LEWIS et al., 2014; LUCHS et al., 2015).

2.3 – INFECÇÃO POR ROTAVÍRUS

2.3.1 - Transmissão

Na infecção por RVA o indivíduo apresenta muitas evacuações com elevada concentração da partícula viral, aproximadamente um trilhão de partículas virais por milímetro cúbico de espécime fecal, sendo o pico máximo de excreção entre o 3º ou 4º dias após o início dos sintomas. A transmissão então é fecal-oral, sendo necessários apenas dez vírions para desencadear uma infecção no homem (WARD et al., 1986; LEBARON et al., 1990; ESTES; GREENBERG, 2013).

Outras vias de infecção têm sido estudadas, tais como contaminação através de fômites ou pelo trato respiratório, apesar deste último haver estudos afirmando a inviabilidade de tal transmissão (VOLLET III; DUPON; PICKERING, 1981; COOK et al., 1990; ZHAORI et al., 1991; ZHENG et al., 1991; SATTAR et al., 1994; AZEVEDO et al., 2005; ESTES; GREENBERG, 2013; SANTOS; SOARES, 2015).

A estabilidade físico-química dos RV contribui para uma eficiente transmissão deste vírus, sendo por este motivo observada a persistência das

infecções em enfermarias neonatais e a elevada frequência de infecções nosocomiais (ESTES; GREENBERG, 2013). Teixeira (2010), em estudo desenvolvido no Rio de Janeiro em ambiente hospitalar, detectaram a contaminação de RVA em diversas superfícies, sugerindo a utilização deste vírus como marcador biológico de contaminação de superfícies hospitalares.

Em estudo conduzido em Belém, Linhares et al. (2002) observaram que a cada nove neonatos que desenvolveram diarreia por RVA, cinco casos foram de infecções nosocomiais.

A transmissão entre espécies tem sido registrada sendo mais provável de ocorrer nos países em desenvolvimento, onde há influência das condições insalubres de higiene e do maior convívio dos homens com animais, principalmente bovino, suíno e aves (JAIN et al., 2001; TEODOROFF et al., 2005; MARTELLA et al., 2006). Na Bélgica, Matthijnssens et al. (2006) detectaram amostra de RVA de uma criança com gastroenterite, sendo que todos os segmentos genômicos foram mais similares à amostra de RV lapino do que o humano. Em Belém, em um estudo com espécimes fecais de neonatos e crianças com diarreia foi observado similaridade com genes provenientes de amostras de origem suína (MASCARENHAS et al., 2007a; 2007b; MAESTRI et al., 2012).

2.3.2 – Replicação viral

A transmissão do RV ocorre geralmente pela via fecal-oral. Após a ingestão, as partículas virais alcançam o intestino delgado, onde penetram geralmente na membrana apical das células epiteliais maduras que recobrem as microvilosidades, replicando-se no citoplasma das células infectadas, comprometendo permanentemente as mesmas, o que impede a efetiva absorção de fluidos e nutrientes. O pico máximo apresentado na replicação ocorre de 10h a 12h após a infecção à 37°C (GRAY et al, 2008; SANTOS; SOARES, 2015).

O capsídeo externo está diretamente envolvido no processo infeccioso, atuando na adesão celular, penetração da membrana e entrada na célula, participando da interação inicial com a célula alvo (passo 1, figura 7). O processo de penetração do vírus inicia-se pela clivagem da proteína VP4 pela tripsina, processo este essencial para a penetração viral na célula, gerando os polipeptídeos VP5* e

VP8* os quais interagem com receptores contendo ácido siálico (VP8*) e posteriormente interação com os co-receptores, sendo sugerida principalmente a integrina α2β1 (VP5*). Contudo, existem RV que não se ligam a este receptor, os chamados ácido siálico independentes, parecendo ser um processo mediado apenas pela VP5*, sugerindo a existência de mais receptores. A proteína VP7 restringe a fragmentação da VP4 por reduzir o diâmetro de contato da protease à base VP5* (ZARATE et al., 2000; HEWISH et al., 2000; GUERRERO et al., 2000; LOPEZ; ARIAS, 2004; CIARLET; CRAWFORD; ESTES, 2001; CIARLET et al., 2002; ESTES; GREENBERG, 2013; SANTOS; SOARES, 2015).

Após a interação da partícula com seus respectivos receptores, a internalização da partícula viral ocorre de 60 a 90 minutos, para a qual foi proposta duas vias de internalização do RV: a penetração direta via membrana plasmática ou endocitose mediada por receptores, ocorrendo, em ambas as propostas, desnudamento da partícula, perda do capsídeo externo, devido às baixas concentrações de íons cálcio, dando origem a partículas de dupla camada (DLP), a qual será translocada através do plasma ou da membrana endossomal até o citoplasma celular (passo 2 e 3, figura 7) (RUIZ et al., 2009; SANTOS; SOARES, 2015)

Após o desnudamento da partícula do RV, a transcrição viral ocorre no citoplasma da célula, mediada pelo complexo de polimerase viral (VP1+VP3), com a proteína VP6 apresentando relevante papel na interação conformacional com a VP1, ocorrendo então a produção de RNA mensageiro (RNAm) tanto para a formação das proteínas virais como para constituir os moldes com vistas à formação das progênies (passo 4, 5 e 6, figura 7) (LIU et al., 1992; CHEN et al., 1999; ESTES; GREENBERG, 2013; SANTOS; SOARES, 2015).

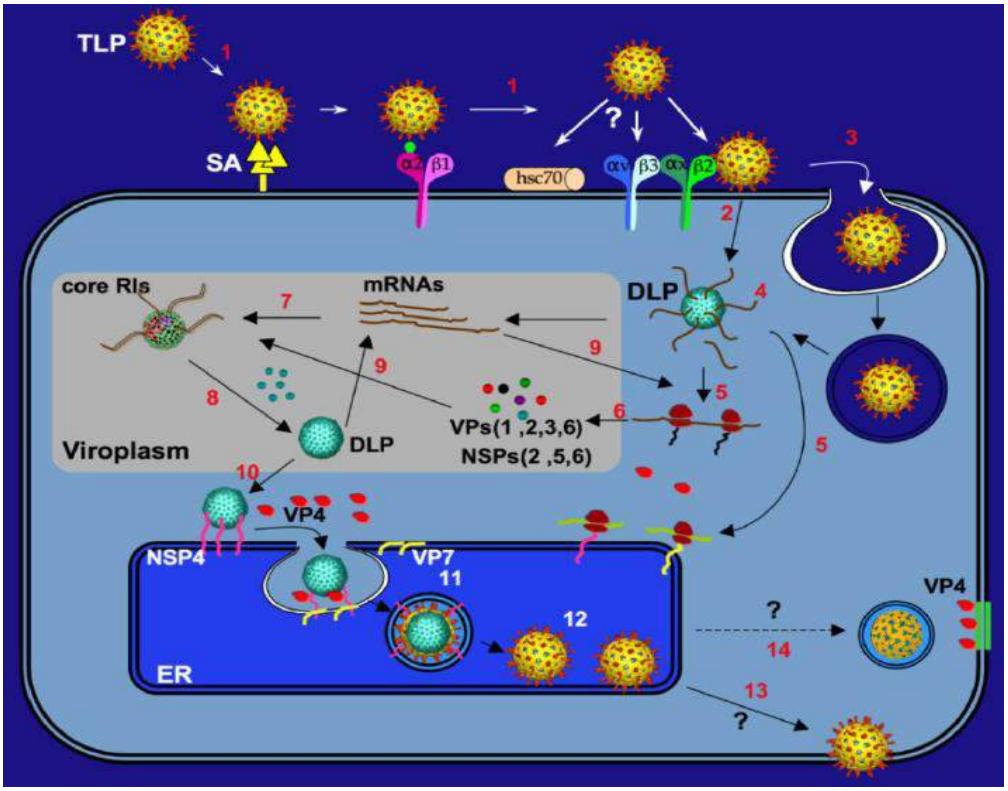
Como o RV é formado por dsRNA, a fita de RNA de polaridade negativa (-) servirá de molde para a fita de RNA polaridade positiva (+), sendo esta última utilizada como RNAm e molde para a produção de novas fitas de RNA (-), as quais se acumularão no citosol disponíveis aos ribossomos celulares para tradução das proteínas virais (passo 7, 8 e 9, figura 7) (ESTES; GREENBERG, 2013; SANTOS; SOARES, 2015).

As partículas subvirais são desenvolvidas e agrupadas, por intermédio das proteínas NSP2 e NSP5, em inclusões discretas e eletrodensas denominadas viroplasmas, localizadas próximas ao RE e formadas 2 a 3 horas após a infecção.

As proteínas NSP3 e NSP1 potencializam a tradução das proteínas e bloqueiam tal indução, respectivamente, para que ocorra o empacotamento e replicação do RNA viral (FABBRETTI et al., 1999; SILVESTRI et al., 2004; ESTES; GREENBERG, 2013, SANTOS; SOARES, 2015).

A partícula subviral se associa à proteína VP4 no citoplasma e atravessa para o interior do RE por ação da NSP4 (passo 10, figura 7), que atua como um receptor intracelular, momento este em que a VP7 é adicionada à partícula viral. Posteriormente, as mesmas adquirem um envelope transitório (passo 11, figura 7), o qual é perdido e substituído por uma fina camada de proteína que constitui o capsídeo externo (passo 12, figura 7). Por fim, as partículas virais maduras são liberadas por meio da lise celular (passo 13, figura 7). Outra hipótese é que a proteína VP4 esteja armazenada na membrana plasmática da célula infectada, sendo associada à partícula viral no momento de sua retirada da célula conforme figura 8 (passo 14, figura 7) (RAMIG, 1997; JAYARAM; ESTES; PRASAD, 2004; BALL et al., 2005; ESTES; GREENBERG, 2013; SANTOS; SOARES, 2015).

Figura 7: Replicação do RV



Fonte: RUIZ et al., 2009

2.3.3. – Patogenia da infecção pelo rotavírus:

A infecção pelo RVA pode variar de assintomática até desidratação grave e consequentemente óbito, contudo o conhecimento acerca da patogênese é baseado em modelos animais, ainda não sendo totalmente elucidado, sendo influenciada por fatores virais e fatores do hospedeiro, principalmente no que tange à idade do indivíduo acometido (GREENBERG; ESTES, 2009).

Sabe-se que a patogenia da infecção por RVA tem sido atribuída a diferentes mecanismos, incluindo a má absorção devido à destruição dos enterócitos e migração das células, o efeito enterotóxico da proteína não estrutural NSP4 e a ativação do sistema nervoso entérico (GRAY et al., 2008; ESTES; GREENBERG, 2013; SANTOS; SOARES, 2015).

A má absorção é desencadeada pela lesão dos enterócitos, o que determina principalmente a depressão transitória no nível das dissacaridases, em particular a lactase, gerando a diarreia de natureza osmótica. A descamação das células apicais, acelera a migração das células da cripta para as vilosidades, precursoras dos enterócitos que, contudo, não apresentam capacidade absortiva, secretando íons Cloro no lúmen intestinal (BALL et al., 1996; SANTOS, SOARES, 2015).

A NSP4 é uma proteína não estrutural que é considerada a primeira enterotoxina viral atuando no mecanismo patogênico, induzindo diarreia em camundongos jovens (BALL et al., 1996). Muitos estudos apontam a participação desta proteína no processo patogênico do RVA, promovendo a desestabilização da membrana do RE, com consequente lise do mesmo, ocorrendo um aumento do Ca^{++} intracelular e o acréscimo da secreção de íons Cl^{-}, causando diarreia de natureza secretória. Outra via a ser considerada é a possível interação exógena da NSP4 secretada de células infectadas ligando-se com células ainda não infectadas, sendo caracterizados receptores apicais e basolateriais (α1β1, α2β1) para esta proteína (TIAN et al., 1995; ESTES; MORRIS, 1999; HUANG et al., 2004; RAMIG, 2004; BALL et al., 2005; ESTES; GREENBERG, 2013; SANTOS; SOARES, 2015).

Outros estudos evidenciam que a NSP4 secretada de células infectadas, NSP4 exógena, possa interagir com receptores da serotonina, estimulando sua secreção das células enterocromafins, o que altera a secreção gástrica e de fluidos entéricos (HAGBOM et al., 2011, 2012; SANTOS; SOARES; 2015)

Observações subsequentes demonstraram que a secreção de fluidos e eletrólitos nas infecções por RV decorre por ativação direta do sistema nervoso entérico, o qual é induzido possivelmente por toxinas (LUNDGREN et al., 2000; LUNDGREN; SVENSSON, 2001; SANTOS; SOARES, 2015).

2.3.4 – Imunidade

Embora muitos estudos tenham sido norteados pela busca do conhecimento acerca do mecanismo imunológico envolvido em uma infecção causada por RV, o qual permanece ainda não totalmente esclarecido, empregando-se estudos em modelo animal, buscam elucidar o mecanismo no homem (LINHARES, 2000; LINHARES; BREESE, 2000; FRANCO et al., 2006; DESSELBERGER; HUPPERTZ, 2011; ESTES; GREENBERG, 2013; SANTOS;SOARES, 2015).

A necessidade da implantação de imunizantes contra RVA norteou diversos estudos para melhor entendimento sobre a proteção a este agente, observando-se que a primeira infecção pode proteger contra a forma mais grave ou reinfecções subsequentes (LINHARES, 2000; LINHARES; BREESE, 2000; LINHARES et al., 2002; DESSELBERG; HUPPERTZ, 2011; ESTES; GREENBERG, 2013).

Em estudo desenvolvido por Velázquez et al. (1996) no México monitorando 200 crianças com até dois anos de idade observaram que após a primoinfecção tais crianças apresentavam menor risco de desenvolver infecção por RVA quando comparadas à outras que não foram acometidas por este agente, ou seja, a segunda ou terceira infecção confere progressivamente maior proteção contra gastroenterite causada por RV. Desta forma, o RVA pode induzir a proteção imune homotípica e heterotípica. Na primeira infecção pelo RVA, primoinfecção, a proteção é homotípica sorotipoespecífica, enquanto que em uma reinfecção, ocorre a proteção heterotípica, a qual é associada a prevenção de infecções contra diferentes tipos de RVA (JIANG et al., 2002; GRAY et al., 2008; DESSELBERG; HUPPERTZ, 2011).

As proteínas VP4 e VP7 demonstraram ser relevantes na resposta imune do hospedeiro, por estimularem a produção de anticorpos neutralizantes, conferindo resposta homotípica e heterotípica, conceitos estes que norteiam as vacinas ora

vigentes, já que a Rotarix® é um imunizante monovalente objetivando induzir proteção heterotípica, enquanto que a Rotateq® é uma vacina pentavalente, baseando-se no conceito de proteção homotípica (OFFIT, 1996; DESSELBERG; HUPPERTZ, 2011).

Os anticorpos específicos IgG e IgA para as proteínas VP4, VP7, NSP4 e VP6 estão envolvidos, sendo esta última detectada em níveis mais elevados, contudo, ainda não houve comprovação se, individualmente ou combinadas, estas proteínas possam proporcionar completa proteção clínica (GRAY et al., 2008).

A IgA foi detectada em níveis elevados em espécimes fecais estando envolvida na resposta heterotípica e são dirigidas para a proteína VP6, sendo detectada após 7 a 28 dias da infecção por RVA (LINHARES, 2000; LINHARES et al., 2002; GLASS et al., 2005; SANTOS; SOARES, 2015)

A transferência de anticorpos maternos podem conferir proteção passiva ao lactante, sendo esta a hipótese utilizada para justificar as infecções assintomáticas nos primeiros 6 meses de vida, contudo, o mecanismo para proteção ainda é controverso (LINHARES et al., 1989; RAY et al., 2007; SANTOS; SOARES, 2015).

A imunidade celular é importante na infecção por RVA, contudo encontra-se sob investigação. Estudos abordando a contribuição de citocinas, tais como IFN-α, IFN-γ, IL-10 e TNF-α demonstraram que as mesmas podem estar envolvidas no processo de proteção contra a infecção por RVA por meio de mecanismos como a inibição da entrada do vírus nas células do hospedeiro e o aumento da atividade dos linfócitos T (CD8+ e CD4+) (BASS, 1997; ROLLO et al., 1999; JIANG et al., 2002; GRAFF et al., 2009).

Há a hipótese de que em princípio, os linfócitos B assumiriam importância frente à infecção viral ao se associar à produção de anticorpos específicos das classes IgA e IgG, enquanto os linfócitos T atuariam posteriormente na resolução do processo infeccioso propriamente dito (BERNSTEIN, 1994; WARD, 1996; WEITKAMP et al., 2003; AIYEGBO et al., 2013)

2.3.5. – Características clínicas

As infecções ocasionadas pelo RVA podem ser assintomáticas ou sintomáticas, expressando-se estas como gastroenterite moderada ou grave. O

período de incubação viral é de um a três dias, evoluindo geralmente para a tríade clássica: febre moderada/alta, vômito e diarreia aquosa que podem perdurar por 5 a 8 dias, podendo culminar em desidratação e óbito nos casos de ausência de intervenção terapêutica (ESTES; GREENBERG, 2013).

A forma assintomática geralmente ocorre em adultos e em crianças com até 6 meses de idade possivelmente devido a proteção conferida pelos anticorpos de origem materna (LINHARES et al., 1989; BISHOP et al., 1996, LINHARES, 2000; GLASS et al., 2006; RAY et al., 2007).

A infecção sintomática ocorre principalmente em crianças de 6 a 24 meses, tal como observado em estudo desenvolvido por Justino et al. (2011) em Belém, Pará, no qual 53,2% das casos eram provenientes de crianças diarreicas com 12 a 23 meses de idade.

A gravidade da infecção possivelmente pode ser explicada pela variação da virulência entre os genótipos de RVA, imunidade deficiente do indivíduo ou a um genótipo considerado não usual inserido na população, tal como observado na emergência do genótipo G9, culminando em internações e óbitos por desidratação (DESSELBERG, 1996; CUBITT et al., 2000; LINHARES et al., 2006b; PATEL et al., 2009; SIQUEIRA et al., 2010).

As infecções por RVA com manifestações clínicas graves em crianças e adultos também têm sido associados ao genótipo G2, contudo, há estudos que discutem a pouca correlação entre genótipos e gravidade clínica (BARNES et al., 1992; POLANCO-MARIN et al., 2003; CLARK et al., 2004a; MIKAMI et al., 2004; ARISTA et al., 2005a; MARTELLA et al., 2005; AUPIAIS et al., 2009).

Outras manifestações clínicas consideradas atípicas já foram relatadas, tais como síndrome de Reye, encefalites, doença de Kawasaki, enterocolite necrotizante, gastroenterite hemorrágica em neonatos e diarreia prolongada em pacientes imunocomprometidos, ressaltando os casos de intussuscepção intestinal observados nos EUA posteriormente à administração da vacina quadrivalente RRV-TV contra RVA (MASCARENHAS; LINHARES, 2005; ESTES; GREENBERG, 2013).

Embora proteínas e RNA do RVA tenham sido detectados em sangue e fluido cérebro espinhal, o significado clínico de tal fato ainda deve ser explorado (MORRISON; GILSON; NUEVO, 2001; ITURRIZA-GÓMARA et al., 2002; LYNCH et al., 2003; ZAHN; MARSHALL, 2006; BLUTT et al., 2007; DICKEY et al., 2009).

2.3.6 – Tratamento

O tratamento para as infecções ocasionadas por RVA é essencialmente a reposição de fluidos e eletrólitos por via oral ou endovenosa, dependendo da gravidade, devido a depleção ocasionada por vômito e diarreia (ESTES; GREENBERG, 2013; ESONA; GAUTAM, 2015).

Geralmente, os episódios diarreicos possuem uma evolução para cura espontânea, devendo o indivíduo ser reidratado com soro e dieta adequada, pois preservará o estado nutricional impedindo o agravamento do quadro com a instalação da desnutrição. A administração de antimicrobianos, medicamentos adsorbantes ou antiespasmódicos com a finalidade de interromper o curso da diarreia não são recomendados, sendo indicados apenas em infecções com coinfecção bacteriana (BRASIL, 2005; OMS, 2005; OLIVEIRA et al., 2007; ESONA; GAUTAM, 2015).

A OMS (2006) recomenda a suplementação de zinco, devido possivelmente estar envolvido na recuperação imune e intestinal durante o episódio diarreico, sendo empregado principalmente em países em desenvolvimento, no qual a deficiência de zinco é bastante acentuada.

Outros estudos destacam possíveis estratégias de tratamento, tal como a utilização de nitazoxanida, racecadotril, probióticos (*Lactobacillus* GG) e drogas que inibem funções neuronais (LUNDGREN et al., 2000; SALAZAR-LINDO, et al., 2004; CEZSARD; SALAZAR-LINDO, 2005; OLIVEIRA et al., 2007; PANT et al., 2007; TERAN; TERAN-ESCALERA; VILLARROEL, 2009).

2.3.7 – Diagnóstico laboratorial

A sintomatologia manifestada nas infecções por RVA é semelhante aos demais agentes virais, impossibilitando um diagnóstico clínico, sendo então necessária a elucidação do agente por análise laboratorial para manejo adequado do paciente e controle pela Vigilância epidemiológica (ESONA; GAUTAM, 2015).

O método laboratorial utilizado primeiramente para detecção direta do RVA foi a microscopia eletrônica, técnica esta que permitiu as primeiras descrições deste agente viral (BISHOP et al., 1973; LINHARES et al., 1977).

Atualmente, o método mais difundido nos laboratórios como método de triagem é o ensaio imunoenzimático (EIA), devido sua sensibilidade, especificidade e praticidade, comercializado em kits que detecta os antígenos virais (HERRMANN et al., 1985; ESTES; GREENBERG, 2013; SANTOS; SOARES, 2015).

Outros kits comerciais são utilizados rotineiramente em laboratório para detecção de antígenos virais com as técnicas de aglutinação em látex; a qual apresenta considerável sensibilidade e a imunocromatografía, a qual também apresenta sensibilidades e especificidades satisfatórias (FLEWETT et al*,* 1989; GABBAY et al., 2005; DESSELBERG, 2014; ESONA; GAUTAM, 2015; SANTOS; SOARES, 2015).

Tais testes empregando kits comerciais detectam geralmente o RVA, enquanto que o emprego da EGPA permite a caracterização dos demais tipos de RV, devido o genoma viral ser segmentado e os segmentos possuírem distintas massas moleculares (ESTES; GREENBERG, 2013).

A técnica de reação em cadeia pela polimerase, precedida de transcrição reversa (RT-PCR) é considerada a “padrão ouro” para RVA, revelando-se altamente sensível e específica, permitindo a genotipagem do vírus presente na amostra, devendo os iniciadores serem submetidos à avaliações periódicas devido à mutações virais (ITURRIZA-GÓMARA; KANG; GRAY, 2004; SIMMONDS et al., 2008; DESSELBERG, 2014).

Outras técnicas têm sido empregadas para a detecção do genoma viral envolvendo a biologia molecular, tais como a hibridização (*dot-blot*), PCR quantitativa, sequenciamento de nucleotídeos e microarranjo (*microarray).* Contudo, o emprego de tais métodos ainda se restringe a pesquisa, devido o custo gerado para sua aplicação (GOUVEA et al., 1990; HONMA et al., 2007; PANG et al., 2010).

2.3.8. – Controle e prevenção

As habituais práticas de controle, como o acesso amplo à água potável e a implementação de medidas visando o saneamento básico, parecem não exercer impacto significativo nas infecções por RVA, o que é evidenciada pelo fato das taxas de incidência da doença nos países desenvolvidos e naqueles em desenvolvimento serem comparáveis. O mais efetivo controle das infecções por RVA se condiciona ao

uso de uma vacina segura e eficaz contra este agente (LINHARES, 2000; LINHARES; BREESE, 2000; FISCHER et al., 2004; GLASS et al., 2004).

2.3.8.1 - Vacinas contra rotavírus

O impacto que o RVA causa a saúde pública revelou a necessidade de combater ou minimizar a gravidade desta doença, sendo então relevante o desenvolvimento e implantação de imunizantes seguros e eficazes contra esse vírus. Várias candidatas a vacinas foram desenvolvidas objetivando oferecer proteção contra as gastroenterites de maior gravidade, contudo apenas duas foram disseminadas em escala global, a Rotarix® e a Rotateq® (PARASHAR et al., 2003; MASCARENHAS; LINHARES, 2005; RUIZ-PALADIOS et al., 2006; VESIKARI et al., 2006).

Uma das primeiras vacinas a ser testada, propondo um imunizante polivalente de RVA de origem animal e humano, foi a RRV-TV (RotashieldTM), derivada de coinfecção em culturas celulares das amostras MMU18006 (macaco Rhesus) sorotipo 3 e de RVA humano sorotipos 1, 2 e 4. Este imunizante foi testado em vários países, inclusive no Brasil, obtendo resultados satisfatórios, proteção variando de 66% a 57% no primeiro ano de vida, principalmente contra o genótipo G1, o que possibilitou seu licenciamento em 1998. Contudo, casos de intussuscepção intestinal associados a sua administração culminou na suspensão deste imunizante em 1999 nos EUA (BERNSTEIN et al., 1995; LANATA et al., 1996; LINHARES et al., 1996; CDC/MMWR, 1999; HOSHINO et al., 2003).

Após tal fato, estudos foram conduzidos a fim de desenvolver imunizantes seguros e eficazes e, em 2006, as vacinas Rotarix® e Rotateq® foram licenciadas e adotadas por diversos países, sendo em 2009, recomendado pela OMS a inclusão destas vacinas em programas de imunizações em escala global devido ter significante impacto na saúde pública (OMS, 2009a).

A vacina Rotarix®® foi produzida pela Glaxo Smith-Kline (Biologicals, Rixsensart, GSK), é de origem humana e caracterizada como genótipo G1P[8], sendo testada na Finlândia e em onze países da América Latina, obtendo eficácia de até 85% contra os episódios mais graves de diarreia por RVA e reduzindo em 42% as admissões hospitalares devido às diversas causas de gastroenterite (RUIZ-PALACIOS et al., 2006). Estudo conduzido por Linhares et al., (2008) na América

Latina demonstrou que duas doses deste imunizante revelaram ser eficazes contra a diarreia grave por RVA durante os dois primeiros anos de vida, enfatizando que a inclusão desta vacina nos programas de imunizações infantis podem reduzir os casos de gastroenterite por RVA em todo o mundo. Tal imunizante teve seus testes desenvolvidos também em Belém, Pará, onde apresentou eficácia de 86% e 93% contra gastroenterites graves e hospitalizações por RVA, respectivamente (LINHARES et al., 2006a).

Esta vacina monovalente demonstrou induzir tanto proteção homóloga em 92%, quanto heteróloga em 87%, aos genótipos circulantes na época do estudo (G3, G4, G9 e P[8]), apresentando menor proteção ao genótipo G2P[4] (41%), dado este que não é estatisticamente significativo devido à baixa circulação do G2 nos anos de desenvolvimento dos estudos (LINHARES et al., 2006a; O'RYAN; LINHARES, 2009).

Em estudo desenvolvido em Belém, Pará por Araújo et al., (2007a) com crianças que receberam duas doses deste imunizante, observaram índices satisfatórios (81,5%) contra gastroenterite ocasionada por RVA, principalmente nos casos que envolveram o genótipo G9 (79,8%).

No Brasil, a partir de 6 de março de 2006, a Rotarix® foi implantada no calendário proposto pelo Programa Nacional de Imunizações, estando disponível nos postos de saúde para ser administrada em duas doses via oral, aos segundo e quarto meses de idade, demonstrando resultados satisfatórios na redução de gastroenterite grave (BRASIL, 2006; JUSTINO et al., 2010; LANZIERI et al., 2010; LINHARES; JUSTINO, 2014).

Outra vacina que demonstrou ser bastante eficaz em testes clínicos foi a Rotateq®, produzida pela Merck e licenciada nos EUA desde 2006, a qual foi construída a partir do protótipo viral WC3, de origem bovina e reestruturada com cepas humanas, formando assim, o imunizante pentavalente de origem humano-bovino, que confere imunidade contra os genótipos G1, G2, G3, G4 e P[8] e proteção cruzada contra o genótipo G9, demonstrando eficácia de até 100% nos episódios diarréicos mais graves e de 68,8% a 76,6% contra gastroenterites por RV (CLARK et al., 2004b; HEATON et al., 2005; VESIKARI et al., 2006; 2007).

Esta vacina pentavalente foi responsável pela redução de 86% na incidência de consultas médicas, de 94% nas emergências, de 96% de redução nas

hospitalizações ocasionadas por RVA e de 59% na gastroenterite causada por outros agentes etiológicos (DENNEHY, 2008).

Os países em desenvolvimento, como Ásia e África, representam os grandes desafios para tais imunizantes devido ao elevado número de coinfecções de RVA, maior possibilidade de diversidade genética do vírus, subnutrição da população e infecções com outros patógenos, sendo então recomendado pela OMS, estudos de eficácia da vacina de RVA nestes locais (LINHARES; BRESEE, 2000; GLASS et al., 2006; BRESEE et al., 2005; O'RYAN; LINHARES, 2009; OMS, 2009b; ARMAH et al., 2010).

Apesar da eficácia das vacinas ser elevada em países desenvolvidos, acima de 85%, tal realidade não se reflete nos países em desenvolvimento, em especial na África a Ásia (ARMAH et al., 2010; LOPMAN et al. 2012; GLASS et al., 2014).

Em janeiro de 2016, 80 países tinham introduzido ao menos uma desta vacina em seu Programa de Imunização, portanto, aumentou-se a necessidade de realização de estudos de vigilância pós licenciamento (PATH, 2016). Embora tais estudos tenham fornecido dados satisfatórios para o impacto da vacina em casos graves de RVA, a pressão seletiva induzida pelos imunizantes pode levar a um impacto nas cepas circulantes, questão esta ainda debatida (GENTSCH; PARASHAR; GLASS, 2009; TATE et al., 2010; PATEL et al., 2011; MATTHIJNSSENS et al., 2012).

3 - RELEVÂNCIA DO ESTUDO

O genótipo G9 é um tipo de RVA bastante disseminado em todo o mundo, apresentando destaque na epidemiologia molecular por ser detectado em diferentes combinações binárias. Em face à classificação dos RVA baseada nos 11 genes virais, torna-se relevante o desenvolvimento de estudos que visem a elucidação da constelação deste tipo de RVA circulante na região amazônica, a fim de observar a variabilidade genética, possível transmissão entrespécies envolvendo RVA de origens humana e animal, bem como o impacto que tais alterações possam ocasionar sobre as estratégias de vacinação.

A implantação de imunizantes no cenário mundial demonstrou resultados satisfatórios sobre os casos de gastroenterite aguda em crianças, em todo mundo, contudo, a introdução poderá influenciar na epidemiologia molecular do RVA. Desta forma, é de extrema importância a realização de estudos sobre a variabilidade genética deste vírus, principalmente do tipo G9, que possui elevada adaptação à outros tipos de RVA circulantes e que não está presente na formulação de nenhum dos imunizantes licenciados no Brasil.

Desta forma, a presente investigação proporcionará melhor compreensão a respeito da complexidade genética do RVA G9 e sua evolução na região norte, já que esse genótipo se apresenta amplamente difundido em escala global.

4 – OBJETIVOS

4.1 – OBJETIVO GERAL

Realizar a caracterização molecular dos genes que codificam para as proteínas estruturais e não estruturais do genótipo de RVA G9 provenientes de crianças com gastroenterite aguda na região norte do Brasil.

4.2 – OBJETIVOS ESPECÍFICOS

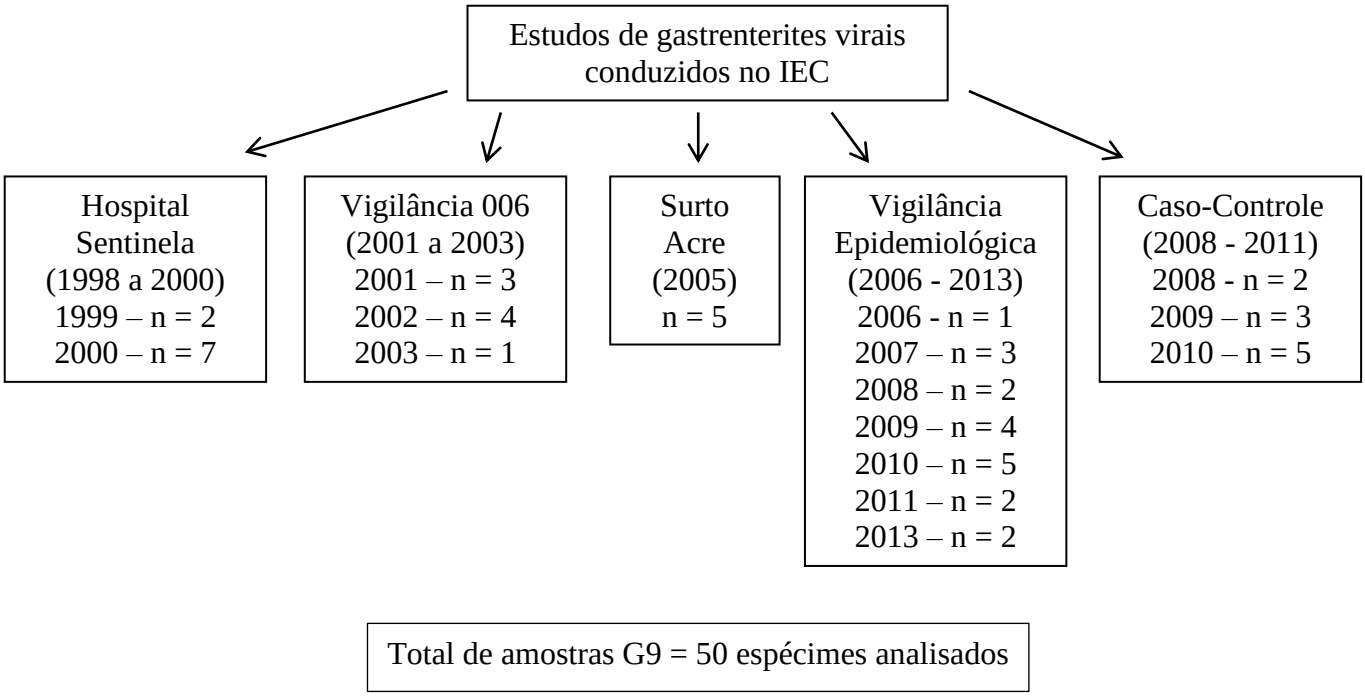
a) Determinar a frequência do genótipo G9 no região Norte;
b) Determinar a constelação do genótipo G9 circulante na região Norte;
c) Determinar a relação filogenética dos 11 genes do RVA G9.

5 – MATERIAL E MÉTODOS

5.1 – MATERIAL

Os espécimes clínicos analisados foram provenientes de quatro estudos desenvolvidos no Instituto Evandro Chagas (IEC) conduzidos no período de 1998 a 2000 (Projeto Hospital Sentinela), 2001 a 2003 (Projeto Vigilância 006), 2006 a 2013 (Projeto Rede de Vigilância) e 2008 a 2011 (Projeto Rotavírus Efetividade).

Figura 9 – Espécimes clínicos analisados no presente estudo



Todas as amostras selecionadas para o presente estudo foram previamente caracterizadas como RVA genótipo G9, por meio das técnicas de reação em cadeia mediada pela polimerase precedida de transcrição reversa e posteriormente por nested-PCR.

O critério de inclusão dos genótipos G9 no estudo foi a quantidade suficiente de espécimes fecais disponíveis para as análises e a distribuição deste genótipo no período do estudo:

1. Estudo de vigilância da doença diarréica por RV em Belém, Pará, antes da implementação de vacinas (Estudo Hospital-Sentinela).

Realizado em Belém, Pará, no período de maio de 1998 a maio de 2000 e objetivou analisar espécimes fecais de crianças com diarreia aguda, não sanguinolenta, de até três anos de idade, internadas no Hospital Santa Terezinha ou fazendo uso de terapia de reidratação oral (TRO), no Posto de Saúde do Marco. Foram coletadas 281 positivas para RV, destas, 24 (8,5%) pertenceram ao genótipo G9.

2. Estudo da imunogenicidade, segurança e eficácia de duas doses da vacina atenuada de origem humana contra RV (RIX4414) em crianças saudáveis de Belém, Brasil – Vigilância 006.

Estudo conduzido de maio de 2001 a abril de 2003 e objetivou avaliar a imunogenicidade, segurança e eficácia de duas doses da vacina atenuada de origem humana (RIX 4414), em crianças saudáveis em Belém, Brasil. Das amostras fecais colhidas, 136 foram amostras positivas para RV, sendo o genótipo G9 detectado em 58 espécimes (42,7%).

3. Estudo de vigilância epidemiológica das gastroenterites por RV no Brasil: determinação do impacto da doença

Este estudo iniciou em fevereiro de 2006 incluindo municípios de cada região do país e tem como objetivo estabelecer uma rede nacional de vigilância epidemiológica das gastroenterites por RV, a fim de definir-se o impacto da doença causada por esses patógenos no país e monitorar a diversidade antigênica das amostras virais circulantes. Foram colhidas amostras fecais de crianças menores de cinco anos internadas com diarreia aguda ou sob terapia de reidratação oral, nas unidades de saúde de referência para o projeto.

4. Projeto Rotavirus Caso-Controle

Este estudo teve seu início em maio de 2008, coletando amostras em 4 clínicas pediátricas: PIO XII, Pediátrica Serzedelo Côrrea, Policlínica do Pará e Santa Terezinha. O critério de inclusão seria ter nascido após a data da implantação da vacina (6 de março de 2006), ter 15 dias de vida e estar hospitalizado menos que 48 horas. A partir de 2009, as clínicas contempladas no estudo foram a PIO XII e Serzedelo Côrrea, abordando o mesmo critério de inclusão.

As amostras analisadas coletadas após a introdução da vacina foram classificadas em vacinadas, crianças que administraram ao menos uma dose da vacina contra RVA, e não vacinadas nos casos de ausência de vacinação ou de

informação acerca do esquema vacinal. No presente estudo obteve-se 9 amostras de crianças consideradas vacinadas.

5.2 – ASPECTO ÉTICO

O estudo não apresentou qualquer risco ou prejuízo para as crianças ou seus responsáveis. A presente investigação foi avaliada pelo Comitê de Ética em Pesquisa do Instituto Evandro Chagas a aprovada sob parecer de n° 0047/2011, em respeito às normas nacionais e internacionais que regulamentam tal atividade (Anexo A).

5.3 – MÉTODOS

5.3.1 Preparo da suspensão fecal

A partir dos espécimes fecais selecionados, foram preparadas suspensões fecais a 10% em tampão Tris-Ca^{++} 0,01M pH 7,2. Tais suspensões foram homogeneizadas e clarificadas por centrifugação a 5.000 xg durante 10 minutos a 4º C. O sobrenadante obtido foi coletado e estocado a -20º C para a realização dos testes posteriores.

5.3.2 Extração do genoma viral

A extração do genoma viral foi desenvolvida segundo protocolo descrito por Boom et al. (1990) a partir da suspensão supracitada.

Foi coletado em um tubo de 1,5 mL 300 µL da suspensão fecal. Adicionou-se 20 µL de Proteinase K (20 mg/mL) e 800 µL de Tampão L6, agitando-se posteriormente em vótex. O material foi incubado em banho maria a 56°C por 10 minutos e logo após adicionado ao tubo 200 µL de Etanol absoluto (4ºC) e 20 µL de sílica. Homogeneizou-se em agitador tipo kline por 20 minutos à temperatura ambiente e posteriormente centrifugado a 16.000 x g por 40 segundos.

Descartou-se o sobrenadante em frasco contendo NaOH 10 N, adicionando-se logo após 500 µL de Tampão L2 e homogeneizando em vortex visando centrifugação a 16.000 x g por 30 segundos. Descartou-se o sobrenadante

em frasco contendo NaOH 10N e adicionou-se 500 µL de Etanol 70%. Homogeneizou-se em vortex e centrifugou-se a 16.000 x g por 40 segundos para posteriormente se descartar o sobrenadante em frasco contendo hipoclorito de sódio.

Secou-se o sedimento em banho maria a 56° C por 15 minutos e, em seguida, adicionou-se 60 µL de água ultra pura (livre de DNAse e RNAse) e homogeneizou-se em vortex.

Posteriormente, o material foi incubado em banho maria a 56° C por 15 minutos e homogeneizado em vórtex. O tubo foi centrifugado à 16.000 x g por 4 minutos, a fim de realizar a coleta do sobrenadante (30 a 40 µL) o qual foi transferido para um tubo previamente identificado e armazenado à -20° C para a realização dos testes.

Durante o processo de extração todas as medidas de controle de contaminação foram realizadas, inclusive a utilização de controles positivo (amostra positiva para RV) e negativo (água ultra pura).

5.3.3 – Reação em cadeia mediada pela polimerase, precedida de transcrição Reversa (RT-PCR)

Após a extração viral, procedeu-se à RT-PCR, a qual compreendeu duas etapas e foi realizada segundo protocolo descrito por Das et al. (1994), Gentsch et al. (1992) e Gouvea et al (1990) com modificações de Leite et al. (1996).

A primeira etapa inicia-se com a formação do DNA complementar (cDNA), utilizando-se iniciadores específicos para os 11 genes do RV, por, desnaturação a 97º C em termociclador por 7 minutos, seguido de imersão em banho de gelo (0º C) por 5 minutos. Esses iniciadores amplificaram fragmentos específicos conforme o gene amplificado, como demonstrando no quadro 1.

Após desnaturação, foi adicionado uma mistura, visando à obtenção do cDNA por meio de transcrição reversa, durante 1 hora a 42° C (quadro 2).

Quadro 1. Sequência dos iniciadores utilizados na RT-PCR para os 11 genes de RV

Iniciador	Sequência	Gene	Amplicon (pb)	Referência
VP1F	5' GGC TAT TAA AGC TGT ACA ATG GG 3'	VP1	686	Varghese et al., 2006
VP1R	5' TAA TCC TCA TGA GAA AAC ACT GAC 3'			
VP2F	5' GGC TAT TAA AGG GCT CAA TGG CG 3'	VP2	686	
VP2R	5' CTT CAT CTT GAA ATA TAG CAA TCA C 3'			
VP3F	5' GGC TAT TAA AGC AGT CCA GTA G 3'	VP3	702	
VP3R	5'GTA AAC ATA GAT TCA TTA CGC GGA CC 3'			
4con3F	5' TGG CTT CGC CAT TTT ATA GAC A 3'	VP4	875	Gentsch et al., 1992
4con2R	5' ATT TCG GAC CAT TTA TAA CC 3'			
VP6F	5’ GGC TTTT AAA CGA AGT CTT 3’	VP6	1356	Both et al., 1984
VP6R	5’ GGT CAC ATC CTC TCA CTA CA 3’			
Beg9F	5' GGC TTT AAA AGA GAG AAT TTC CGT CTG G 3'	VP7	1062	Gouvea et al., 1990
END9R	5' GGT CAC ATC ATA CAA TTC TAA TCT AAG 3'			
NSP1F	5' GGG CTT TTT GAA AAG TC 3'	NSP1	1590	Matthijnssens et al., 2006
NSP1R	5' GGT CAC ATT TTA TGC TGC CTA 3'			
NSP2f	5' GGC TTT TAA AGC GTC TCA G 3'	NSP2	1059	
NSP2R	5' GGT CAC ATA AGC GCT TTC 3'			
NSP3F	5' GGC TTT TAA TGC TTT TCA GTG 3'	NSP3	1078	
NSP3R	5' ACA TAA CGC CCC CTA TAG C 3'			
JRG30F	5' GGC TTT TAA AAG TTC TGT T 3'	NSP4	738	Cunliffe et al., 1997
JRG31R	5' ACC ATT CCT TCCATT AAC 3'			
NSP5F	5' GGC TTT TAA AGC GCT ACA G 3'	NSP5	667	Matthijnssens et al., 2006
NSP5R	5' GGT CAC AAA ACG GGA GT 3'			

Quadro 2. Reagentes usados na RT-PCR para a obtenção do cDNA

RT	**1X/µL*****
H_2O	16,25
dNTP* 25Mm	1
Tampão 10X	2,5
$MgCl_2$ 50mM	1
RT** 20U	0,25
Total	21

* Mistura dos quatro desoxirribonucleotídeos trifosfato: dATP, dCTP, dGTP e dTTP.
**II RNAse Reverse Transcriptase 10000U, 200 U/µL
*** O correspondente a uma reação

Após a obtenção do cDNA, iniciou-se a segunda etapa, a qual consistiu na amplificação dos genes pela PCR. Adicionou-se uma segunda mistura (quadro 3) com as seguintes condições: 1 ciclo de 94º C por 2 minutos (desnaturação prévia), seguido de 35 ciclos de 94º C por 30 segundos (desnaturação), 42º C por 30 segundos (hibridização) e 72º C por 1 minuto (extensão), finalizando com um ciclo

de 72º C por 10 minutos de extensão final.A seguir, os produtos da RT-PCR foram armazenados a -20º C.

Quadro 3. Reagentes usados na PCR para a amplificação dos 11 genes do RV

PCR	1X/µL
H_2O	20,25
dNTP 25Mm	1
Tampão 10X	2,5
$MgCl_2$ 50Mm	1
Taq DNA Polimerase	0,25
Total	25

5.3.4 – Eletroforese em gel de agarose

Os produtos obtidos na RT-PCR foram submetidos à eletroforese horizontal em gel de agarose a 1,5% com *Sybr safe DNA gel stain*® (Invitrogen) em tampão tris-borato-EDTA (TBE) 1x. Os produtos da RT-PCR foram aplicados com azul de bromofenol juntamente com o marcador de peso molecular de 123 pb diluído em TBE 1x e submetidos a condições de 120 V e 400 mA por 30 minutos.

Os produtos da RT-PCR em que foi visualizado o amplicon foram submetidas à purificação e posterior sequenciamento; e nas que não amplificaram, a RT-PCR foi repetida.

5.3.5 – Purificação do produto da RT-PCR

Após a RT-PCR, o DNA foi purificado, visando o sequenciamento de nucleotídeos. Utilizou-se o kit comercial de purificação do produto de PCR da EasyGen, baseando-se na inexistência de amplificações secundárias observadas no gel de agarose, segundo protocolo descrito pelo fabricante, como descrito abaixo:

Adicionou-se 5 vezes o volume do tampão de ligação para cada volume do produto de PCR usado, seguido da homogeneização. Transferiu-se a mistura obtida para a coluna acoplada com o tubo coletor de 2 mL, centrifugando-se por 60 segundos a 4.000 x g. Descartou-se o líquido do tubo coletor o qual foi recolocado na coluna. Adicionou-se 0,75 mL do tampão de diluição já na coluna, a qual foi centrifugado por 60 segundos para lavagem da coluna a 4.000 x g.

Descartou-se o líquido do tubo coletor e, para remover resíduos de reagentes, centrifugou-se a coluna novamente por 60 segundos sem adicionar nenhum reagente. Posteriormente, transferiu-se a coluna do tubo coletor para um tubo limpo de 1,5 mL sem tampa. Em seguida, para eluir o DNA, adicionou-se 50 µL do tampão EB (10mM Tris-Cl, pH 8,5) com muito cuidado no centro da membrana, incubando-se em temperatura ambiente por 1 minuto. Logo após, centrifugou-se por 1 minuto, para então recolher e armazenar o produto final a -20ºC.

5.3.6 – Quantificação do DNA

A quantificação do DNA para a determinação da concentração do mesmo foi realizada segundo o protocolo descrito pelo fabricante com o uso do marcador de peso molecular Low Mass Ladder (Invitrogen). O DNA purificado foi aplicado, juntamente com azul de bromofenol, em gel de agarose a 2% com sybr safe DNA gel stain (Invitrogen) diluído em TBE a 1X, sendo aplicado também em um outro orifício o peso molecular.

Em seguida, os amplicons de interesse no gel foram comparados com o peso molecular, segundo a intensidade da banda, e a concentração do DNA foi expressa em nanogramas (ng), o qual serviu de parâmetro para a reação de sequenciamento.

5.3.7 – Reação de sequenciamento de nucleotídeos

A reação de sequenciamento foi conduzida segundo o protocolo descrito pelo fabricante do kit Big Dye Terminator® (Applied Biosystems), utilizando-se a mistura da reação conforme o quadro 4. A mistura foi adicionada a placa, juntamente com o DNA purificado e colocada no temociclador nas seguintes condições: 25 ciclos de 96° C por 30 segundos (desnaturação), 50° C por 15 segundos (hibridização) e 60° C por 3 minutos (extensão).

Quadro 4. Reagentes usados na reação de sequenciamento

Reagentes	1X/µL
Iniciador*	2
Tampão	2
Big Dye Terminator	2
DNA	Depende da concentração (máximo 4µL)
H_2O	Depende da quantidade de DNA (máximo 3µL)
Total	10

* Iniciadores descritos no quadro 1

5.3.8 – Purificação do produto da reação de sequenciamento

O produto dessa reação foi purificado por precipitação com isopropanol e etanol, com a finalidade de retirar o excesso de terminadores não incorporados na reação de sequenciamento.

Primeiramente, adicionou-se 40 µL do isopropanol 65% ao produto da reação da placa, agitando posteriormente em vortex por 3 a 5 segundos. Em seguida, incubou-se à temperatura ambiente (TA) por 15 minutos, deixando a placa dentro da centrífuga e em seguida centrifugando por 45 minutos a 4000 xg a TA.

Após a centrifugação, retirou-se o sobrenadante por inversão da placa. Em seguida, adicionou-se 200 µL em cada orifício de etanol a 70% e centrifugou-se a placa por 10 minutos a 4000 Xg a TA, retirando o sobrenadante novamente por inversão. Todavia, desta vez, manteve-se a placa invertida para, então, dar-se uma rápida centrifugação na mesma sobre papel absorvente, repetido a ação.

Secou-se a placa em termobloco a 50 ºC por 5 a 10 minutos para, posteriormente, ressuspender o sedimento.

5.3.9 – Eletroforese em sequenciador automático

As amostras foram ressuspensas em 10 µL de formamida, desnaturadas a 96ºC por 5 minutos em termociclador e colocadas em banho de gelo a 0ºC por 3 minutos. Em seguida, a placa foi adicionada ao sequenciador automático ABI PRISM 3130 xl (Applied Biosystems), mediante a utilização de marcadores com partículas fluorescentes que ao contato com raios laser, emitem fluorescência em distintos

comprimentos de onda, correspondendo a cada nucleotídeo na extremidade 3’ (SAGER; NICKLEN; COULSON, 1977).

5.3.10 – Alinhamento e edição das sequências para construção da árvore filogenética

Após o término da eletroforese foram obtidas as sequências para os 11 genes de RVA, as quais foram montadas utilizando o software CAP3, alinhadas com MAFFT v.7.221 e editadas com a suíte de Bioinformática Geneious v.8.1.7 e comparadas com sequências de outros vírus isolados e disponíveis no banco de genes “GenBank”, a partir do programa BLAST.

As árvores filogenéticas foram construídas a partir das sequencias alinhadas no programa MEGA 7 no qual foi calculada uma matriz de distancia usando a fórmula de dois parâmetros de Kimura adotando-se assim, o método de Neighbour-joining. A análise de *bootstrap* foi realizada usando 2.000 réplicas objetivando a obtenção de resultados reprodutíveis além de proporcionar maior confiabilidade aos grupamentos (KIMURA, 1980; FELSENSTEIN, 1985).

6 - RESULTADOS:

Os resultados relacionados ao objetivo específico 1 foram apresentados nos manuscritos 6.1, 6.2, 6.3, 6.4 e 6.5, enquanto que os objetivos específicos 2 e 3 foram apresentados no manuscrito 6.6

6.1 – ROTAVIRUS SEROTYPE DISTRIBUTION IN NORTHERN BRAZIL TRENDS OVER A 27 YEAR PERIOD PRE AND POST NATIONAL VACCINE INTRODUCTION

Trials in Vaccinology 1 (2012) 4–9

Contents lists available at SciVerse ScienceDirect

Trials in Vaccinology

journal homepage: www.elsevier.com/locate/trivac

Review Article

Rotavirus serotype distribution in northern Brazil trends over a 27 year period pre and post national vaccine introduction

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Este manuscrito avaliou a epidemiologia molecular do RVA na região Norte com amostras coletadas no período antes da implantação da vacina (1981 a 2005) e logo após a introdução da vacina no calendário de imunização nacional (2006 a 2008).

Observou-se a emergência do genótipo G9 na região na década de 90, sendo detectado pela primeira vez na região em um estudo conduzido de 1990 – 1992. Posteriormente, reemergiu nas infecções por RVA em estudo conduzido de 1998 a 2000 e, no ano de 2005, foi associado a maioria das infecções ocorridas no Acre (43%).

Desta forma, descreve-se a circulação do genótipo G9 em 14% no período analisado.

Trials in Vaccinology 1 (2012) 4–9

Contents lists available at SciVerse ScienceDirect

Trials in Vaccinology

journal homepage: www.elsevier.com/locate/trivac

Review Article

Rotavirus serotype distribution in northern Brazil trends over a 27 year period pre and post national vaccine introduction

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ARTICLE INFO

Article history:
Received 28 October 2011
Received in revised form 16 December 2011
Accepted 21 December 2011

Keywords:
Rotavirus strains
Genotypes
Gastroenteritis
National vaccine
North Brazil
Serotype

ABSTRACT

In Brazil, a rotavirus vaccine was included in the public sector in March 2006. In order to identify a possible effect of vaccination on rotavirus strains we evaluated the distribution of serotypes/genotypes in northern Brazil during pre (1981–2005) and early post (2006–2008) national rotavirus vaccine introduction periods. Of 1286 rotavirus strains, 993 (77.2%) were successfully G typed. G1 strains were detected throughout the years, accounting for the majority of typed isolates ($n = 426$; 42.9%). G2 rotaviruses displayed a cyclic pattern of occurrence over time, re-emerging recently in early 2006, with detection rates as high as 91%, and remained the predominant circulating strain through 2008. G9 rotaviruses appeared during 1990–1992, re-emerged from 1998 to 2000 and rose to 43% in a gastroenteritis outbreak in northwestern Brazil in 2005. The most common combinations overall were G2P[4] (55.1%; 136/247), and G1P[8] (24.7%; 61/247). Although our data show the predominance of G2P[4] early after vaccine introduction, there is a need for continuous, long-term surveillance of circulating strains to better assess a possible effect of rotavirus vaccination on the strain ecology.

Contents

1. Introduction

Rotavirus remains the most common cause of severe acute gastroenteritis in infants and young children worldwide, being responsible for an estimated 527,000 deaths annually. Parashar et al. [1] have estimated that 82% of these deaths occur in children in the less developed regions of the world, where a rotavirus vaccine is most needed.

Currently, there are two licensed oral live attenuated rotavirus vaccines, *Rotarix*™ (GlaxoSmithKline, Rixensart, Belgium) and *Rotateq*™ (Merck Research Laboratories, USA). In large, phase III

Abbreviations: UMV, Universal Mass Vaccination; MoH, Ministry of Health; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; ST, G serotyping.

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doi:10.1016/j.trivac.2011.12.002

trials in Latin America, Europe and the United States these two vaccines proved to be safe and have demonstrated excellent protection (>85%) against severe rotavirus disease [2,3].

Rotarix™ and *Rotateq*™ have been pre-qualified by WHO and are increasingly being introduced in national immunization programs of many countries worldwide, mainly in those regions where successful phase III efficacy trials had been conducted [4]. In this context, Brazil was one of the first countries to introduce universal immunization in March 2006 with *Rotarix*™. Brazil is also the largest country to introduce *Rotarix*™ into Universal Mass Vaccination (UMV), covering a birth cohort of 3.3 million. According to the Brazilian Ministry of Health [5], since the introduction of nation-wide rotavirus vaccination it has been observed an increase in the uptake of vaccine over time, with 81% of children having had two doses of the vaccine by 2008. However, the coverage rates of the second dose remain suboptimal in the Northern region, ranging from 27% to 65% in 2006 and 2008, respectively.

Of importance, trials recently completed in Africa and Asia have provided evidence that both vaccines are quite beneficial in poor settings and this warranted a further WHO global recommendation to include rotavirus vaccines in every nation's immunization program [6–9].

While *Rotarix*™ (monovalent; G1P[8]) and *Rotateq*™ (pentavalent; G1, G2, G3, G4 and P[8]) differ in strain composition, both vaccines appear to provide significant protection against a variety of rotavirus strains [10,11]. Nevertheless, a continuous monitoring of circulating rotavirus strains is needed to detect the possible emergence of uncommon or novel types in the community that may pose a challenge to the efficacy of the available vaccines [4].

Although recent recommendations have been proposed for the classification of group A rotaviruses using all 11 genomic RNA segments, currently a system exists for the dual classification of serotype specificities are based mainly on the segregation of VP4 (protease-sensitive; P types) and VP7 (glycoproteins; G types) genes [12]. Based on the diversity of VP4 and VP7 proteins present on the outer shell, rotaviruses are classified into 23 G and 31P types [13]. Currently, the most common strains in human disease belong to G1, G2, G3, G4, and G9 types in combination with either P[4], P[6] or P[8] types [14]. Several studies have reported that approximately 90% of the human rotavirus strains include G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] combinations.

Several serotyping/genotyping studies have been conducted across Brazil during the past two decades, underscoring the broad diversity of circulating strains over time, including the common human rotavirus strains as well as uncommon strains (G5) or globally emerging (G9) types [15]. A number of surveys conducted beginning in early 2006, coinciding with the year that the vaccine was introduced, indicate an apparent predominance of G2P[4] strains in northern, northeastern and southeastern regions of Brazil [16–19] and led to speculation that the vaccine was causing strain replacement [20].

This review assesses the diversity of rotavirus strains in northern region of Brazil between 1981 and 2008, highlighting the distribution profiles of circulating serotypes/genotypes before and early after introduction of *Rotarix*™ into the national immunization program. The patterns of genotype distribution over time provided us with the opportunity of an early insight on the issue of whether changes following introduction of universal rotavirus vaccination in Brazil might be due to natural fluctuation or possible vaccine pressure.

2. Material and methods

We reviewed data from 11 studies assessing diarrhoea burden in children and adults, conducted between 1981 and 2006 in northern Brazil [21–28]. In addition, a review was made using specifically data from the Brazilian Ministry of Health's (MoH) surveillance network, established in February 2006. This national, hospital-based surveillance was part of a program implemented by Pan American Health Organization in Latin American and Caribbean countries to assess the burden of rotavirus disease and monitoring circulating strains in the pre-vaccine era. While samples from the 11 studies were obtained from the states of Pará ($n = 10$) and Maranhão ($n = 1$), the official MoH's network included seven states located in the Amazonian region of Brazil-Acre, Amazonas, Roraima, Pará, Amapá, Maranhão and Tocantins (Table 1). Overall, there were nine hospital-based studies, one community-based study and two vaccine trials comprising variable age groups: 0–3 years (3 studies), 0–5 years (2), 0–2 years (1), 0–28 days (1), 1 month–2 years (1), 2 months–1 year (2), 1 month–29 years (1), and 0–55 years (1). The surveillance network implemented by the MoH included diarrhoeic children of ⩽5 years.

Stool specimens were obtained as soon as possible after an episode of diarrhoea was detected. An aliquot of each sample was stored at 2–8 °C for a maximum of 24 h until being transported on ice to Instituto Evandro Chagas, a MoH's Rotavirus National Reference Centre. All samples were screened for the presence of group A rotaviruses by commercially available enzyme-linked immunosorbent assays (Dakopatts, Denmark or Rotaclone, USA). Serotyping/genotyping was performed using monoclonal antibodies, solid phase immuno-electron microscopy or reverse-transcription polymerase chain reaction (RT-PCR), depending on the techniques available during the conduct of each study over time. In order to assess the RNA electrophoretic profiles, polyacrylamide gel electrophoresis (PAGE) was carried out on selected faecal suspensions using a standard method which includes extraction of nucleic acid by using glass powder. G serotyping (ST) using monoclonal antibodies was performed in five studies; ST and solid-phase immune electron microscope in one, ST and RT-PCR genotyping (GT) in 2, and GT only in 4. Samples from the MoH's surveillance network were genotyped only by PCR.

3. Results

Of the 1286 stool specimens that yielded group A rotavirus antigen positive result by ELISA, a G serotype could be assigned to 993 (77.2%). These isolates (either single or mixed) comprised the five globally relevant G serotypes, that is, G1, G2, G3, G4 and G9, which make up the majority of strains associated with gastroenteritis in humans. The predominant G serotypes detected from 1981 to 2008 were G1 (426; 43.0%), followed by G2 (337; 33.9%), G9 (142; 14.3%), G4 (45; 4.5%) and G3 (15; 1.5%). In addition, 28 (2.8%) rotavirus strains had mixed serotype-specificities (Fig. 1).

G1 strains were identified throughout the years, being the predominant serotype detected in 6 out of the 11 periods of observation of each study, at prevalence rates that ranged from 24.6% to 66.7%. Rotavirus strains bearing G2 type-specificity occurred at rates that varied from 14.8% to 26.3% during studies conducted during 1981–1990, 1982–1986, and 1990–1992 and became predominant in 1992–1994 (76.7%). In the 1998–2000 period G2 types were recognized at a rate comparable to that of G1 (34.1% and 30.3%, respectively) and this was followed by an abrupt decrease in prevalence rates (0–2.3%) in studies conducted during the following 5 years. G2 then re-emerged as the leading serotype beginning early in 2006 (Study K; just before and a few months after introduction of rotavirus vaccine in Brazil), as well as during the official national surveillance (2006–2008), at rates that ranged from 60.6% to 91.0%.

With the exception of one isolate during a 1990–1992 vaccine trial in Belém, Brazil, G9 could not be recognized in six studies

Table 1
Characteristics of rotavirus studies conducted in northern Brazil during 1981–2008.

Study, short title	Characteristics	Study period	Setting	Age-group	Laboratory method	Reference
(A) National surveillance	Hospital-based	1981–1990	Belém, Brazil	0–5 years	Serotyping	Pereira [21] (1993)
(B) Longitudinal study	Community-based	1982–1986	Belém, Brazil	0–3 years	Serotyping	Linhares [22] (1989)
(C) Hospital-based study	Hospital-based	1988[a]	Belém, Brazil	1 month–2 years	Serotyping	Linhares [23] (1993)
(D) RRV-TV trial	Double-blind placebo-controlled trial	1990–1992	Belém, Brazil	1 month–2 years	Serotyping, SPIEM	Linhares [24] (1996)
(E) Nosocomial/community infection	Hospital-based	1992–1994	Belém, Brazil	0–5 years	Serotyping	Gusmão [25] (1999)
(F) Hospital-based study in São Luís	Hospital-based	1997–1999	São Luís, Maranhão	0–2 years	Serotyping	Luz [26] (2005)
(G) Hospital-based surveillance	Hospital-based	1998–2000	Belém, Brazil	0–3 years	Serotyping/ genotyping	Gabbay (personal communication)
(H) Vaccine trial, phase II	Double-blind placebo-controlled trial	2001–2003	Belém, Brazil	2 months–1 year	Genotyping	Salinas [27] (2005)
(I) Pre-trial surveillance	Hospital-based	2003[b]	Belém, Brazil	0–3 years	Genotyping	Abate [28] (2004)
(J) Outbreak in Rio Branco	Hospital-based	2005[c]	Rio Branco, Acre	1 month–29 years	Genotyping	Siqueira [41] (2010)
(K) Salobo study	Hospital-based	2006[d]	Parauapebas, Pa, Brazil	0–55 years	Genotyping	Mascarenhas (personal communication)
(L) Official national surveillance	Hospital-based, outbreaks	2006–2008	Northern, north-eastern	0–5 years	Genotyping	CGLAB*, MoH's database

SPIEM: Solid-Phase Immune Electron Microscopy; CGLAB: Coordenação de Laboratórios de Saúde Pública, Ministério da Saúde.
[a] March–September, 1988.
[b] March–September, 2003.
[c] August–September, 2005.
[d] January–September, 2006.

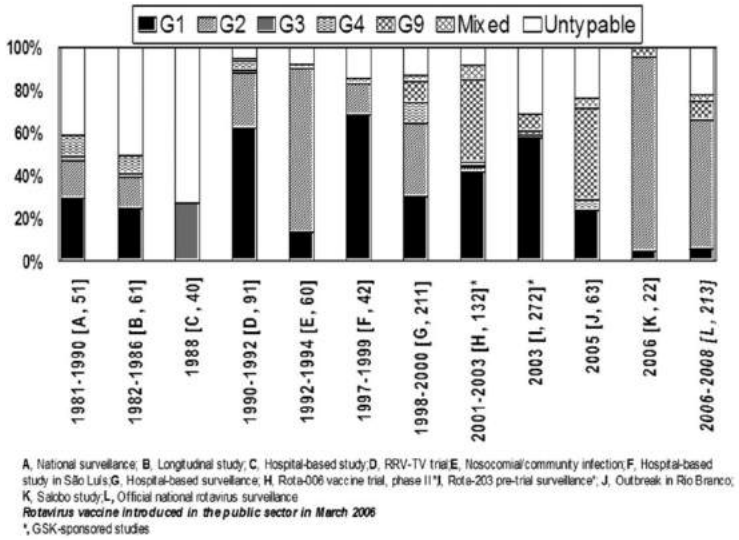

Fig. 1. Occurrence of rotavirus G types in 12 studies and an official Ministry of Health's survey conducted between 1981 and 2008 in northern Brazil. This figure shows the prevalence rates of circulating rotavirus types over time (1981–2008) in 12 studies and the official Ministry of Health's survey carried out in Northern Brazil. Overall, G1 rotavirus serotype was found to be predominant, accounting for 43.0% of isolates.

conducted from 1981 to 1999; it re-emerged during a study carried out from 1998 to 2000 and rose to 42.9% as the predominant serotype during an outbreak of gastroenteritis in north-western Brazil. G3 was the most prevalent type during a hospital-based surveillance for gastroenteritis in 1988. G4 occurred at low prevalence rates (1.5–9.8%) across seven studies conducted between 1981 and 2005.

Table 2 shows the results of 247 samples from five surveys (B, D, F, K, L), as shown in Fig. 1) conducted between 1982 and 2008, which could be both G- and P-typed. The most common combinations were G2P[4] (55.1%; 136 out of 247 samples) and G1P[8] (24.7%; 61/247). While G or P mixed serotypes were identified in 17 (6.7%) rotavirus strains, a G or P type-specificity could not be assigned to 28 (11.3%) samples. The electrophoretic RNA

Table 2
Combination of G and P rotavirus genotypes of 247 samples from 1982 to 2008 in northern Brazil.

G genotype	P genotype					
	P[8]	P[4]	P[6]	P mixed	P NT	Total
G1	55	5	4	6	9	79
G2	1	130	7	2	1	141
G3	0	0	0	0	1	1
G4	0	0	6	0	0	6
G9	5	1	0	0	0	6
G mixed	0	0	1	4	0	5
G NT	0	0	1	0	8	9
Total	61	136	19	12	19	247

profile could be identified in 253 samples from 5 studies (B, D, E, F and L, as shown in Fig. 1). Of these, 155 (61.5%) and 82 (32.5%) specimens displayed "long" and "short" electropherotypes, respectively, with the remainder of samples showing unclear profiles (data not shown).

4. Discussion

The conduct of several surveillance studies and (more recently) vaccine trials in northern Brazil, during almost 3 decades, allowed an assessment of the diversity of rotavirus strains that have circulated over time. One of these trials was conducted during 1990–1992, when a first generation rhesus–human reassortant rotavirus vaccine (RRV-TV, *Rotashield*™) was evaluated for safety, immunogenicity and efficacy in Belém. Altogether these studies have generated a large amount of data on the temporal distribution of rotavirus serotypes/genotypes for the northern region of Brazil, highlighting their circulation profiles before and early after countrywide introduction of rotavirus vaccine.

Overall our findings have shown that the degree of diversity of co-circulating strains was remarkable in northern Brazil during the past 27 years, particularly when considering data from studies conducted between 1981 and 2005 are analyzed. Data showing that rotaviruses bearing VP7 G1 serotype-specificity were the most common strain are consistent with numerous studies reporting worldwide predominance of G1P[8] strains [14]. Furthermore, the overall highest frequency (42.9%) of G1 in our study is similar to the prevalence rates of G1 type for all over Brazil (~43%) reported in two recent reviews by Gurgel et al. [18] and Leite et al. [15]. Both above mentioned reviews covered only a small proportion of the studies from northern Brazil, included in this current review paper.

It should be pointed out that unlike G1 strains, which were detected throughout the review period, rotaviruses belonging to G2 serotype displayed a cyclic pattern of occurrence, as it has been previously documented in Brazil and elsewhere [25,29,30]. A major finding in our study was the remarkable re-emergence (detection rates as high as 91%) of G2 strains during 2006–2008, which likely reflects a continental phenomenon [15]. This remarkable G2P[4] predominance appears indeed to have occurred all over Latin America, even in those countries that had not introduced rotavirus vaccination into their national immunization programs. In support of this, a recent epidemic of G2P[4] related rotavirus gastroenteritis in Honduras (Ferrera et al. [31]), as well as the high incidence rates (68–81%) of G2 strains during surveillance studies in El Salvador, Guatemala, Honduras, Argentina and Paraguay [32–34]. Interestingly, outside Latin America there are also recent reports of increasing detection rates of G2P[4] strains in Bangladesh [15,35].

A recent, post-vaccine licensure surveillance conducted in northeastern region of Brazil led some investigators to claim that predominance of G2 serotype may be a result of possible vaccine-induced selective pressure [17]. However, based on previous observations, the current high prevalence of G2 most likely reflects its cyclic pattern of occurrence in Brazil and elsewhere [36]. In a recent study in Recife, Brazil, Nakagomi et al. [20] showed that G2 strains were on increasing trend, since rates were of 7% in 2004–2005 and yielded 47% even at the onset of rotavirus vaccination program, To reinforce this hypothesis, it is known that some south-American countries bordering Brazil (e.g. Argentina and Paraguay), where G2P[4] is currently predominant, have not yet introduced rotavirus vaccine into the public sector. The current issue concerning predominance of G2 strains during early post-vaccine introduction may only be fully elucidated through the development of well-designed post-marketing studies already under way in Belém, Brazil, Panama and El Salvador, to assess both vaccine impact and strain surveillance, in compliance with recent World Health Organization recommendations [4].

The other important finding that has emerged from this analysis is the detection of G9 strains in large numbers, accounting for 14% of typed isolates. In our 27-year analysis of circulating serotypes in northern Brazil, a single G9 isolate was reported in a vaccine trial during 1990–1992, it re-emerged during a hospital-based surveillance from 1998 to 2000 and rose to >40% in late 2005 as the predominant serotype in a gastroenteritis outbreak in north-western Brazil (unpublished data). Of importance, G9 rotaviruses are currently considered emerging pathogens and its emergence as an important serotype in the northern Brazil parallels reported global increases of this ST [14,15,37,38]. Also relevant in regard to this serotype is its reported association with an increased severity of illness, with outbreaks occurring in multiple settings [39,40]. Conditions similar to these were recognized in 2005 (Fig. 1) during an extensive epidemic of severe G9-related gastroenteritis involving children living in Rio Branco, Acre State, Brazil's Western Amazon [41].

In general rotaviruses other than G1, G2 and G9 were detected at low prevalence rates during the 27-year review period, including G3, G4 and strains bearing mixed (dual) serotype-specificities. Furthermore, we found significant numbers (22.7%; 293 out of 1286 specimens) of non-typeable rotavirus strains, which may in part have been due to a lack of sensitivity of methods used in the initial studies. However, it is likely that a proportion of samples were rotaviruses of G types other than G1–G4 and G9.

Among the 247 rotavirus strains that could be analyzed for G- and P-genotype specificities, 190 (77%) samples comprised the globally common G1P[8], G2P[4] and G9P[8] combinations, although less common types such as G1P[4], G1P[6], G2P[8], G2P[6], G4P[6], and G9P[4] were also identified at lower frequencies. Interestingly, some of these unusual viruses possess P[6] VP4 type-specificity and are found commonly infecting neonates [40]. Following a pattern similar to that generally reported worldwide, G1, G4 and G9 types had "long" electropherotypes, whereas G2 viruses displayed a "short" RNA pattern. It is notable that G2P[4] strains isolates from children and adults from January to September 2006 in Parauapebas, Southern Pará state, Brazil (Study K), showed identical electrophoretic profiles, suggesting a possible common source of infection.

A major limitation of our analysis was the fact that the numerous reported studies conducted during 1981–2008 are often difficult to compare due to differences in methodologies such as age groups, time of surveillance, and severity of disease definitions. Nonetheless, the large amount of data gathered throughout 27 years in northern Brazil provides useful information on the dynamics of rotavirus strains circulation profiles in a pre-vaccination period and early after introduction of rotavirus vaccine in Brazil.

The availability of data on the rotavirus strain characterization during 27 years in the northern region highlights a variable (and fluctuating) type composition of co-circulating serotypes. These conditions can trigger quick changes over time, resulting in rotavirus

evolution via natural gene reassortment events and other mechanisms, and therefore posing a challenge to currently adopted vaccines. It is therefore strongly recommended to pursue continuous longitudinal surveillance and epidemiological studies in the region and elsewhere in Brazil, in order to determine the true incidence of rotavirus serotypes during the post-vaccine era. This would allow assessing the possible effect of vaccination on rotavirus strain circulation, including the potential for serotype replacement.

Conflict of interest

All investigators were funded through their institutions by GlaxoSmithKline Biologicals.

Nervo Sánchez and Romulo E. Colindres were employed by GlaxoSmithKline Biologicals at the time of this study.

Alessilva Oliveira, Joana D'Arc P. Mascarenhas, Luana S. Soares, Sylvia F.S. Guerra, Yvone B. Gabbay, Maria Cleonice A. Justino and Alexandre C. Linhares declare no conflict of interests.

Role of funding source

This study was sponsored by GlaxoSmithKline Biologicals. Study sponsors were involved in the study design, in the analysis and interpretation of data, in the writing of the manuscript, and in the decision to submit the manuscript for publication.

Alessilva Oliveira: Contribution to the laboratory methods, analysis of the data and reviewing of the manuscript.

Joana D'Arc P. Mascarenhas: Contribution to the laboratory methods, analysis of the data and reviewing of the manuscript.

Luana S. Soares: Contribution to the laboratory methods.

Sylvia F.S. Guerra: Contribution to the laboratory methods.

Yvone B. Gabbay: Contribution to the laboratory methods, analysis of the data and reviewing the manuscript.

Nervo Sánchez: Analysis of data and reviewing of the manuscript.

Romulo E. Colindres: Analysis of data and reviewing of the manuscript.

Maria Cleonice A. Justino: Contribution to clinical evaluation of children, analysis of the data and reviewing of the manuscript.

Alexandre C. Linhares: Contribution to the laboratory methods, analysis of the data and writing of the first draft.

All authors were involved in the critical revision of drafts and have approved the final manuscript version for submission.

Trademarks

Rotarix™ is a trademark of the GlaxoSmithKline group of companies.

Rotateq™ is a trademark of Merck & Co, Inc.

Acknowledgements

The authors would like to acknowledge the technical assistance given by the laboratory personnel at the Virology Section of the *Instituto Evandro Chagas*, Health Surveillance Secretariat, Brazilian Ministry of Health.

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6.2 - EFFECTIVENESS OF THE MONOVALENT G1P[8] HUMAN ROTAVIRUS VACCINE AGAINST HOSPITALIZATION FOR SEVERE G2P[4] ROTAVIRUS GASTROENTERITIS IN BELÉM, BRAZIL.

ORIGINAL STUDIES

Effectiveness of the Monovalent G1P[8] Human Rotavirus Vaccine Against Hospitalization for Severe G2P[4] Rotavirus Gastroenteritis in Belém, Brazil

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Neste manuscrito, analisou-se amostras coletadas de maio de 2008 a maio de 2009 em Belém, Pará, observando-se o declínio da detecção de G9 na região, o qual foi associado a 1% das infecções por RVA que teve como genótipo predominante o G2P[4] (87% das infecções por RVA).

Effectiveness of the Monovalent G1P[8] Human Rotavirus Vaccine Against Hospitalization for Severe G2P[4] Rotavirus Gastroenteritis in Belém, Brazil

Maria Cleonice A. Justino, MD, MSc, Alexandre C. Linhares, MD, PhD,* Tatiana M. Lanzieri, MD, MSc,† Yllen Miranda, MD,* Joana D'Arc P. Mascarenhas, BPharm, PhD,* Erika Abreu, MD,* Sylvia F. S. Guerra, BPharm, MSc,* Alessilva S. L. Oliveira, BPharm, MSc,* Veronilce B. da Silva, RN,* Nervo Sanchez, MD,† Nadia Meyer, MD,‡ Fakrudeen Shafi, MSc,§ Eduardo Ortega-Barria, MD,† Montse Soriano-Gabarró, MD, MSc,‡ and Romulo E. Colindres, MD, MPH†*

Background: Brazil initiated universal immunization of infants with the G1P[8] human rotavirus (RV) vaccine in March 2006. This study evaluated vaccine effectiveness (VE) against severe rotavirus gastroenteritis (RVGE) hospitalizations.

Methods: Matched case-control study conducted at 4 hospitals in Belém from May 2008 to May 2009. Cases were children hospitalized with RVGE age-eligible to have received 2 doses of the human RV vaccine (≥12 weeks of age and born after March 6, 2006). For each case, 1 neighborhood and 1 hospital control without gastroenteritis was selected, matching by birth date (±8 and ±6 weeks, respectively). Matched odds ratio of 2-dose RV vaccination in cases versus controls was used to estimate VE (1 − odds ratio × 100%).

Results: Of 538 RVGE cases, 507 hospital controls and 346 neighborhood controls included, 54%, 61%, and 74% had received both RV vaccine doses. VE against RVGE hospitalization was 75.8% (95% confidence interval [CI]: 58.1–86.0) using neighborhood controls and 40.0% (95% CI: 14.2–58.1) using hospital controls. VE in children 3 to 11 months and ≥12 months of age was 95.7% (95% CI: 67.8–99.4) and 65.1% (95% CI: 37.2–80.6) using neighborhood controls, and 55.6% (95% CI: 12.3–77.5) and 32.1% (95% CI: −3.7–55.5) using hospital controls. G2P[4] accounted for 82.0% of RVGE hospitalizations. G2P[4]-specific VE was 75.4% (95% CI: 56.7–86.0) using neighborhood controls and 38.9% (95% CI: 11.1–58.0) using hospital controls.

Conclusions: Although fully heterotypic G2P[4] was the predominant RV strain, good VE was demonstrated. VE was highest in children aged 3 to 11 months. However, protection in children ≥12 months of age, important for optimal public health impact, was significantly sustained based on estimates obtained using neighborhood controls.

Key Words: gastroenteritis, rotavirus, hospitalizations, human rotavirus vaccine, Brazil

(*Pediatr Infect Dis J* 2011;30: 000–000)

Rotavirus (RV) is the most common cause of acute gastroenteritis (GE) requiring medical attention or hospitalization in young children worldwide, accounting for approximately 2.4 million hospitalizations and more than half a million deaths annually among children less than 5 years of age.[1–4] The availability of safe and effective vaccines against RV offers the potential to reduce the global burden of rotavirus gastroenteritis (RVGE).[2,5,6] The World Health Organization (WHO) recommends inclusion of RV vaccination of infants into all national immunization programs.[5] Two oral RV vaccines are now available in many countries—a 2-dose human G1P[8] RV vaccine (Rotarix, GlaxoSmithKline Biologicals, Rixensart, Belgium) and a 3-dose live bovine-human reassortant pentavalent vaccine (Rotateq, Merck Vaccines, Whitehouse Station, NJ).[7] Both vaccines have been shown to be safe and highly effective for the prevention of RVGE in large-scale clinical trials[8–14] and postlicensure studies.[7,15–17]

The human RV vaccine is currently licensed in 13 Latin American countries. Brazil was the first country in the region to incorporate this vaccine into the national Expanded Program on Immunization, with RV vaccination available free of charge at public primary healthcare centers throughout the country since March 2006. Prior to RV vaccine introduction, RV accounted for an annual 3.5 million episodes of GE, 650,000 visits to outpatient healthcare facilities, 92,000 hospitalizations and 850 deaths among Brazilian children less than 5 years of age.[18] RV accounted for approximately 43% of all GE hospitalizations in this age group[19] and 46% of GE hospitalizations in children younger than 3 years.[20]

With RV vaccines increasingly being introduced into childhood immunization programs, monitoring vaccine effectiveness (VE) under normal operational conditions is a high priority.[21,22] In parallel, continuous surveillance of circulating RV strains is warranted during the postintroduction period to evaluate any potential impact of RV vaccination on genotype diversity.[23] We assessed the effectiveness of the human RV vaccine for the prevention of severe RVGE hospitalizations in children age-eligible to have received both vaccine doses in Belém, Brazil. To mimic the real-life scenario, the effectiveness of partial vaccination was also assessed.

Accepted for publication November 2, 2010.

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Supported by the GlaxoSmithKline Biologicals. The study sponsor designed the study in collaboration with investigators, and coordinated data gathering, analysis and interpretation, and writing of the report. All investigators at study clinical sites were funded though their institutions to do the study protocol. Supported by GlaxoSmithKline Biologicals (to M.C.A.J. and A.C.L.).

Presented at the 28th Annual Meeting of the European Society for Pediatric Infectious Diseases, Nice, France, May 4–8, 2010. Abstract 463.

Authors had full access to all study data and final responsibility for the decision to submit for publication. Authors T.M.L., N.S., N.M., F.S., E.O.B., R.E.C., and M.S.G. are employees of GlaxoSmithKline Biologicals at the time of this study.

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Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.pidj.com).

ISSN: 0891-3668/11/3005-0001
DOI: 10.1097/INF.0b013e3182055cc2

METHODS

Study Setting and Design

This was a hospital-based, age-matched case-control study in Belém, a large city in Northeastern Brazil with a population of 1,437,604 and an annual birth cohort of 24,054.[24,25] Active surveillance for RVGE was conducted at 4 large urban pediatric clinics/hospitals with a total of 294 pediatric beds, accounting for approximately 80% of all pediatric admissions for severe GE in Belém. RV strain surveillance was initiated in parallel with the case-control study and is currently ongoing.

Study design was based on the WHO generic protocol for monitoring the impact of RV vaccination on GE disease burden.[21] The protocol was reviewed and approved by the independent ethics committee of the investigational center at Instituto Evandro Chagas, Secretaria de Vigilância em Saúde, Brazilian Ministry of Health. Written informed consent was obtained from the parents/guardians of all participating children prior to study entry.

Case Definition and Enrollment

Cases were children at least 12 weeks of age born after March 6, 2006 hospitalized with laboratory-confirmed severe RVGE, defined as diarrhea (3 or more looser than normal stools within 24 hours), with or without vomiting, of less than 14 days duration requiring at least an overnight stay and intravenous rehydration therapy in one of the participating centers during the study period. As part of routine clinical practice, stool samples were collected within 48 hours of admission and tested for the presence of RV by enzyme-linked immunosorbent assay (ELISA) at the Ministry of Health's National Rotavirus Reference Laboratory, Instituto Evandro Chagas. Only children with ELISA-confirmed RVGE were eligible for inclusion as a case. Children with onset of severe RVGE more than 48 hours after hospital admission (nosocomial infections) were excluded. All ELISA-positive stool samples were tested by polymerase chain reaction at Instituto Evandro Chagas for determination of RV G and P type.

Control Definition and Enrollment

For each case, we planned to enroll 1 hospital and 1 neighborhood control. Hospital controls were children hospitalized for any reason except GE or another vaccine-preventable disease identified through review of the hospital admission log book and matched progressively to cases by date of birth (from ±2 weeks, up to a maximum of ±6 weeks). Neighborhood controls were children without any signs or symptoms of GE who had resided in the same neighborhood as the case for at least 3 months. Neighborhood controls were selected by interviewing neighbors to the right and left of the case home in a sequential manner until a child born within ±8 weeks of the case was enrolled. This wider age range for neighborhood controls was used because of logistical difficulties to facilitate the enrollment of subjects.

Data Collection

After informed consent had been obtained, parents/guardians of all cases/controls were interviewed by a pediatrician or nurse to obtain information on demographics, medical history, GE symptoms and treatment prior to hospitalization (cases only), and diagnosis at hospital admission and discharge (cases and hospital controls). Study staff also reviewed medical records and recorded appropriate information for cases and hospital controls. For both cases and controls, vaccination history was confirmed by vaccination card review during the interview with the parent/guardian.

Statistical Analysis

All statistical analyses were performed using SAS statistical software (version 9.1; SAS Institute Inc., Cary, NC). Considering RV vaccine coverage of 69.1% for the first dose and 52.1% for the full 2-dose course among controls (Pará State Secretary of Public Health, September 2007), through simulation (2000 runs) and conditional logistic regression we estimated that a total of 230 cases and 230 controls would provide a power of 97% to demonstrate that VE is higher than 50%, with an alpha level of 5%, when the true VE is 80%.

For calculation of VE of the full 2-dose course of human RV vaccine, the analysis included only pairs for which the case and the controls had received either 0 or 2 vaccine doses and who met all protocol-defined criteria. Cases were required to have received the first dose of human RV vaccine at least 14 days before the onset of severe GE to be included in this analysis. VE (%) was estimated as 1 minus the matched odds ratio of vaccination multiplied by 100 for each control group. Conditional logistic regression was used to estimate the matched odds ratio (hazard ratio using SAS code of PROC PHREG), with 95% Wald confidence limits,[26] and was repeated to include potential confounders, for which a backward elimination strategy was used to retain variables with $P \leq 0.20$.[16,27] VE of the full 2-dose course of human RV vaccine was estimated according to age (3–11 months and ≥12 months), severity of RVGE hospitalizations determined using the Vesikari scale,[28] and RV genotype. VE was also estimated in children who had received at least 1 vaccine dose. Finally, to include some of the subjects with missing/unknown vaccination history, in addition to the actual VE, VE was also calculated using a sensitivity analysis assuming cases and controls with missing/unknown vaccination history were vaccinated and unvaccinated, respectively, for the worst case scenario (sensitivity −), or the opposite for the best case scenario (sensitivity +).

The proportion of hospital admissions for severe GE and the proportion of severe GE hospitalizations attributable to RV were calculated with exact 95% confidence interval [CI]. Demographic characteristics, age distribution, disease seasonality, severity and distribution of RV G and P types were also tabulated. Due to non-normality, sparse or unbalanced data, demographic characteristics were compared between cases and each set of controls using the Fisher exact test for categorical variables and the Mann-Whitney Wilcoxon 2 sample test for continuous variables. Two-sided P values of <0.05 were considered statistically significant.

RESULTS

Study Population

Between May 14, 2008 and May 28, 2009, 10,828 age-eligible children were hospitalized at the participating centers, 4692 (43.3%) of whom were hospitalized due to severe GE. Of these, 80.2% (3763/4692) provided stool samples for testing, 24.1% of which were positive for RV by ELISA (906/3763). The proportion of ELISA-confirmed severe RVGE hospitalizations varied by age and was highest in children aged 12 to 23 months, with 53.2% (482/906) of RVGE hospitalizations occurring in this age group. The proportion of severe RVGE hospitalizations varied over time, peaking during July and August 2008 and April 2009 (Fig. 1).

A total of 538 RV-positive children were enrolled as cases (59.4% of those testing positive for RV in the screened population), 522 of whom had a matched hospital and/or neighborhood control (97.0%). Differences between the screened and enrolled RV-positive subjects, with respect to age distribution or area of residence that could impact the results, were not identified. Overall, 368 RV-positive children were not included as a case in the final according-to-protocol analysis. Because of logistic reasons (eg, child was discharged from the hospital and moved away from the study area, address could not be found, etc.), it

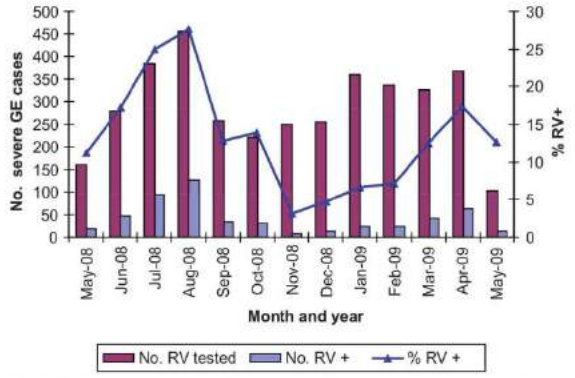

FIGURE 1. Seasonal distribution of severe GE cases and the proportion of cases attributable to RVGE by month of year in Belém, Brazil (May 14, 2008–May 28, 2009).

was not possible to obtain written informed consent from parents/guardians for 317 subjects. In the additional 51 subjects for whom informed consent had been obtained, reasons for exclusion from the according-to-protocol analysis were stool sample collected more than 48 hours after admission (15 subjects), nosocomial diarrhea (15 subjects), diarrhea did not meet the protocol definition (13 subjects), previous enrollment (3 subjects), persistent diarrhea (2 subjects), doubtful history of diarrhea (1 subject), outside the age group (1 subject), and birth date unknown (1 subject). It was not possible to enroll both a matched hospital and a neighborhood control for all cases. Among children screened as hospital controls, 516 of 885 (58.3%) were enrolled and 507 met the criteria for inclusion in the case-control analysis. There was an uneven temporal distribution of cases and hospital controls (>90% of the hospital controls were acute respiratory infections), leading to a significant difference in the time period of their recruitment into the study (month, year). While most of the cases occurred during July to August 2008 (150/538; 28%), respiratory illnesses (hospital controls) peaked in October to November 2008 (145/507; 28.6%). In the former period, for instance, there were too few hospitalizations for acute respiratory infections to match a large number of RVGE hospitalizations.

The absolute median (range) difference between date of birth of cases and hospital controls was 1 (0–6) week. The median duration of hospitalization for both cases and hospital controls was 5 days. Among children screened as neighborhood controls, 348 of 387 (89.9%) were enrolled and 346 met the criteria for inclusion in the case-control analysis. The absolute median (range) difference between date of birth of cases and neighborhood was 3 (0–8) weeks.

Demographic characteristics are shown in Table 1. Median age of study participants was 17 months, 52.6% were male, all were American Hispanic or Latino and 90.4% lived in Belém. Of note, the proportion of hospital controls that lived in Belém was lower than for neighborhood controls (87.6% and 95.7%, respectively).

RV Vaccination History

In all, 68.0% of cases, 76.3% of hospital controls, and 85.3% of neighborhood controls had received at least 1 dose of the human RV vaccine, with 53.7% of cases, 60.7% of hospital controls, and 74.0% of neighborhood controls having completed the full 2-dose course (Table, Supplemental Digital Content 1, http://links.lww.com/INF/A695). The percentage of vaccinated subjects that received the first vaccine dose between 8 and 12 weeks of age was 87.2%, 80.4%, and 86.8%, in the 3 groups, respectively. The respective proportions of vaccinated subjects who had completed the full 2-dose course by 24 weeks of age were 77.3%, 76.2%, and 85.4%.

Most subjects had received other recommended vaccinations. However, for most vaccines, coverage rates were 5% to 6% lower in hospital controls than in neighborhood controls.

Vaccine Effectiveness

VE of 2 doses of the human RV vaccine for the prevention of severe RVGE hospitalization was 75.8% (95% CI: 58.1–86.0) using neighborhood controls and 40.0% (95% CI: 14.2–58.1) using hospital controls (Table 2). VE in cases and hospital controls that lived in the same district was 52.9% (95% CI: −9.1 to 79.7). VE was higher in children aged 3 to 11 months than in those aged ≥12 months (95.7% [95% CI: 67.8–99.4] versus 65.1% [95% CI: 37.2–80.6] using neighborhood controls and 55.6% [95% CI:

TABLE 1. Demographic Characteristics of Study Participants in Belém, Brazil (May 14, 2008–May 28, 2009)

Characteristic	Cases N = 538	Hospital Controls N = 507	*P*	Neighborhood Controls N = 346	*P*	Total N = 1391
Gender, %						
Male	50.7	52.9	0.500	54.9	0.240	52.6
Female	49.3	47.1		45.1		47.4
Age, mo						
Median (range)	16 (3–36)	18 (3–36)	0.008	17 (3–36)	0.240	17 (3–36)
Age group, %						
3–5 mo	2.6	2.4		1.4		2.2
6–11 mo	22.9	19.3		19.1		20.6
12–23 mo	53.9	53.6		57.5		54.7
≥24 mo	20.6	24.7		22.0		22.4
Living in Belém at the time of the study, %						
Yes	89.8	87.6	0.280	95.7	0.001	90.4
No	10.2	12.4		4.3		9.6

P values were calculated using exact Fisher exact test for categorical variables and Mann-Whitney-Wilcoxon 2 sample test for continuous variable.

TABLE 2. Effectiveness of the Human RV Vaccine for the Prevention of Hospital Admissions for Severe RVGE in Belém, Brazil (May 14, 2008–May 28, 2009)

	Neighborhood Controls					Hospital Controls				
	Vaccine Effectiveness			Sensitivity		Vaccine Effectiveness			Sensitivity	
	N	%	95% CI	−	+	N	%	95% CI	−	+
Full 2-dose series										
Overall	249	75.8	58.1–86.0	71.2	77.8	312	40.0	14.2–58.1	21.3	52.0
3–11 mo	64	95.7	67.8–99.4	95.7	95.8	77	55.6	12.3–77.5	48.1	58.6
≥12 mo	185	65.1	37.2–80.6	58.1	68.8	235	32.1	−3.7–55.5	7.5	49.3
Full 2-dose series by RVGE severity (Vesikari score)										
Mild/moderate (1–10)	149	72.7	43.0–87.0	66.7	75.7	187	25.6	−20.2–54.0	2.6	42.0
Severe (≥11)	100	78.8	52.1–90.6	75.8	80.0	125	53.7	20.2–73.1	39.0	62.0
Very severe (≥15)	23	90.0	21.9–98.7	90.0	90.0	25	28.6	−125.1–77.3	0.0	37.5
Full 2-dose series against fully heterotypic G2P[4]	222	75.4	56.7–86.0	70.5	77.6	286	38.9	11.1–58.0	22.2	51.6
Full 2–dose series against pooled non-G2P[4] types	42	70.0	−9.0–91.7	70.0	70.0	46	50.0	−33.2–81.2	8.3	60.0
Full or partial series vaccination										
Overall	331	62.3	42.3–75.4	58.4	65.5	444	44.2	23.1–59.6	26.0	54.3
3–11 mo	91	88.9	63.4–96.6	88.9	89.3	120	60.5	28.2–78.3	52.6	62.5
≥12 mo	240	48.0	16.5–67.6	42.0	53.6	324	34.9	4.3–55.6	10.6	50.6

Sensitivity −, cases and controls with other or unknown RV vaccination status are assumed respectively vaccinated and unvaccinated.
Sensitivity +, cases and controls with other or unknown RV vaccination status are assumed respectively unvaccinated and vaccinated.
N indicates number of matched pairs; RV, rotavirus; RVGE, rotavirus gastroenteritis.

12.3–77.5] versus 32.1% [95% CI: −3.7 to 55.5] using hospital controls in the 2 age groups, respectively).

During hospitalization, 58.4% of RVGE cases were rated as mild/moderate (Vesikari score, 1–10), 41.6% as severe (Vesikari score, ≥11), and 9.7% as very severe (Vesikari score, ≥15). Using neighborhood controls, VE of 2 doses of the human RV vaccine was 72.7% (95% CI: 43.0–87.0), 78.8% (95% CI: 52.1–90.6), and 90.0% (95% CI: 21.9–98.7) for the prevention of mild/moderate (Vesikari score, 1–10), severe (Vesikari score, ≥11), and very severe (Vesikari score, ≥15) RVGE, respectively. Respective VE estimates using hospital controls were 25.6% (95% CI: −20.2 to 54.0), 53.7% (95% CI: 20.2–73.1), and 28.6% (95% CI: −125.1 to 77.3).

Strain characterization was conducted on all enrolled 538 RV-positive cases. G2P[4] was the most common RV type, accounting for 82.0% (441/538) of cases. Of the 97 (18%) non-G2P[4] strains identified, 1 was G1P[6] (0.2%), 11 were G1P[8] (2.0%), 16 were G2P[6] (3.0%), 2 were G9P[4] (0.4%), 1 was G9P[6] (0.2%), 2 were G9P[8] (0.4%), 11 were G12P[6] (2.0%), 48 were mixed types (8.9%), and 5 were untypeable (0.9%).

VE of 2 doses of the human RV vaccine for the prevention of severe G2P[4] RVGE was 75.4% (95% CI: 56.7–86.0) using neighborhood controls and 38.9% (95% CI: 11.1–58.0) using hospital controls. For RVGE caused by pooled non-G2P[4] types, VE of 2 doses of the human RV vaccine was 70.0% (95% CI: −9.0 to 91.7) using neighborhood controls and reached 50% (95% CI: −33.2 to 81.2) using hospital controls.

In children who had received at least 1 vaccine dose, VE using neighborhood controls was 62.3% (95% CI: 42.3–75.4) overall, 88.9% (95% CI: 63.4–96.6) in children aged 3–11 months, and 48.0% (95% CI: 16.5–67.6) in those aged ≥12 months. Corresponding VE using hospital controls was 44.2% (95% CI: 23.1–59.6) overall and 60.5% (95% CI: 28.2–78.3) and 34.9% (95% CI: 4.3–55.6) in the 2 age groups, respectively.

After controlling for potential confounders (or risk factors) in the conditional logistic regression model (Table, Supplemental Digital Content 2, http://links.lww.com/INF/A696 and Table, Supplemental Digital Content 3, http://links.lww.com/INF/A697), VE was 73.6% (95% CI: 53.9–84.9) and 43.3% (95% CI: 8.4–64.8) using neighborhood and hospital controls, respectively. Age ≥1 year was significantly associated with RVGE using neighborhood controls. For hospital controls, time period of recruitment into the study, the presence of underlying medical conditions and diet including breast-feeding also had a significant impact on VE. Results of the sensitivity analysis for the primary objective ranged from 71.2% to 77.8% using neighborhood controls and from 21.3% to 52.0% using hospital controls (Table 2).

DISCUSSION

This study demonstrated the effectiveness of 2 doses of the human RV vaccine for the prevention of severe RVGE hospitalizations, predominantly due to the G2P[4] strain, in Belém, Brazil, one of the settings where the pivotal Latin American Phase III trial of this vaccine was conducted.[8,11] The effectiveness of 2 doses and at least 1 dose of human RV vaccine was comparable to the findings of previous clinical trials in the region.[11,29]

The effectiveness of RV vaccines against the fully heterotypic G2[P4] strain is currently of particular interest, since this strain seems to be showing natural re-emergence in Latin America and many other parts of the world.[19,30–35] The human RV vaccine has been shown to provide broad protection against circulating G1 and non-G1 strains in randomized controlled clinical trials, with G2P[4]-specific efficacy ranging from 45% to 86%.[8,10–12] A meta-analysis of results from 6 randomized controlled clinical trials indicated a VE of 81% against G2P[4] RVGE of any severity and of 71% against severe G2P[4] RVGE.[36] We found the human RV vaccine to provide a high level of protection against hospitalization for G2P[4] RVGE, in line with the results of another recent study in Recife, Brazil, which had an unmatched case-control design and demonstrated VE of 77% against severe G2P[4] RVGE requiring hospital admission or emergency department treatment in children 6 to 11 months of age.[17]

Duration of protection is another important factor influencing the potential public health impact of RV vaccines. VE of 83% to 85% against hospitalization for severe GE caused by the fully

heterotypic G2P[4] type was seen in children aged 6 to 11 months and nonsignificant results in children ≥12 months in the Recife study.[17] We also observed highest VE in children 3 to 11 months of age (96%), with lower but still significant protection in children ≥12 months of age (65%) using neighborhood controls. Vaccine efficacy was also found to be slightly lower during the second year of follow-up in the Phase III study in Latin America (79% vs. 83% during the first year).

The proportion of severe GE hospitalizations attributable to RV was low in this study compared with that reported in Belém and other regions in Brazil prior to RV vaccine introduction (43%–46%).[19,20] Similarly, the proportion of RV-positive cases among children with diarrhea accessing emergency services decreased from 24% in 2006 to 7% in 2008 in Aracaju, Brazil, with greatest reductions seen in the youngest age groups.[37] A marked decline in hospitalizations for all-cause gastroenteritis among children younger than 1 year following the introduction of RV vaccination in Brazil has also recently been reported.[38] While earlier studies in Brazil and Latin America found approximately 50% of RV cases to occur in children aged <12 months and 80% in those aged <24 months,[20] most RVGE occurred in children 12 to 23 months of age in this study, followed by infants aged 3 to 11 months and children aged >24 months. It is possible that the age distribution and severity of RVGE may change after vaccine introduction, with less severe cases occurring among older children. However, protection against RVGE during the first 2 years of life is particularly important, as this is the time when RV infections are most severe.

Although we used an age-matched case-control design and obtained a large sample size, the marked differences in VE using the 2 different control groups are striking. Case-control studies are an effective method of monitoring VE in real-life conditions, particularly during the early phases of vaccine introduction.[21] However, choice of control group can have a significant impact on VE estimates.[21,39–41] For RV infection, neighborhood controls provide the advantage of controlling for key potential confounding factors which could impact on risk of developing severe RVGE, particularly sociodemographic status and general access to vaccination and medical care. In contrast, greater variability in such factors may occur among hospitalized controls. Hospitalization and emergency department visits are recognized to be potential markers of under-vaccination, even in children with access to healthcare.[42] In Belém, vaccination is performed at primary healthcare centers with well-defined catchment areas and not in hospitals, and vaccination coverage varies greatly geographically.[43] VE increased when we attempted to augment comparability of hospital controls to neighborhood controls by restricting analysis to cases and hospitals controls that lived in the same districts in Belém. In this study, a higher proportion of hospital controls than cases and neighborhood controls resided outside of the Belém area. Furthermore, coverage rates for most routine childhood vaccines were lower in hospital controls than neighborhood controls. In addition, a higher proportion of hospital controls had missing or unknown RV vaccination status which may explain the greater variability in the sensitivity analysis using this group. One possible limitation of our study in this context was that hospital controls were not screened for RV infection. According to routine clinical practice in each participating hospital and the generic WHO protocol followed,[21] stool samples were only tested for the presence of RV by ELISA if GE was present at admission.

In summary, results of this study show a considerable reduction in the proportion of severe GE hospitalizations attributed to RV in children younger than 3 years in Belém, Brazil following introduction of the human RV vaccine. Good VE was demonstrated versus fully heterotypic G2P[4], which was the predominant RV strain throughout the study period. VE was highest in children 3 to 11 months of age. However, our results also suggest that the vaccine affords protection in older children ≥12 months of age, based on estimates obtained using neighborhood controls. Ongoing surveillance studies should further demonstrate the public health benefits afforded by this RV vaccine in community settings.

ACKNOWLEDGMENTS

The authors thank Dr. Helena Progênio, all the staff at the Clínica Santa Terezinha, Clínica Pio XII, Hospital Serzedelo Correa (formerly, Clínica Pediátrica do Pará) and Policlínica Infantil de Nazaré in Belém who were involved in the conduct of this study, and all the participating children and their parents/guardians. The authors also thank the clinical operations team at GlaxoSmithKline Biologicals, Brazil lead by Amanda Novaes; Kusuma Gopala, Pryia Rajagopal, and other members of the CDOC-B team at GlaxoSmithKline Biologicals who provided statistical input to the protocol, scientific writing support, and assisted with data management; Thomas Verstraeten (GlaxoSmithKline Biologicals) for critical review of the manuscript; Jennifer Coward (Independent Medical Writer, Bollington, United Kingdom) for medical writing assistance and Jessica Mattos for publication coordination on behalf of GlaxoSmithKline Biologicals. Rotarix is a registered trademark of the GlaxoSmithKline group of companies. Rotateq is a registered trademark of Merck and Co, Inc.

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6.3 – IDENTIFICATION OF LINEAGE III OF G12 ROTAVIRUS STRAINS IN DIARRHEIC CHILDREN IN THE NORTHERN REGION OF BRAZIL BETWEEN 2008 AND 2010.

Arch Virol
DOI 10.1007/s00705-011-1111-z

BRIEF REPORT

Identification of lineage III of G12 rotavirus strains in diarrheic children in the Northern Region of Brazil between 2008 and 2010

Luana da Silva Soares · Patrícia dos Santos Lobo · Joana D'Arc Pereira Mascarenhas · Darivaldo Luz Neri · Sylvia de Fátima dos Santos Guerra · Alessilva do Socorro Lima de Oliveira · Régis Piloni Maestri · Darleise de Souza Oliveira · Euzeni Maria de Fátima Costa de Menezes · Alexandre da Costa Linhares

O manuscrito aborda a detecção do genótipo G9 em 12,5% das infecções por RVA caracterizadas na região Norte, de amostras coletadas de 2008 a 2010, reportando a identificação do genótipo G12 na epidemiologia molecular da região, assim como o primeiro relato da linhagem III de G12 na América Latina.

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BRIEF REPORT

Identification of lineage III of G12 rotavirus strains in diarrheic children in the Northern Region of Brazil between 2008 and 2010

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Received: 23 May 2011 / Accepted: 9 September 2011

Abstract This study reports on the surveillance for rotavirus genotypes and the identification of G12 human rotavirus in the Northern Region of Brazil. Rotavirus-positive samples were collected from children <5 years of age with acute diarrhea from January 2008 to October 2010. G2P[4] was the most prevalent genotype, accounting for 45.6% (126/303) of cases. Five rotavirus strains bearing G12P[6] genotype specificity were detected. Phylogenetic analysis of the VP7 gene showed that G12 strains clustered into lineage III. This is the first detection of G12 strains from lineage III in Latin America, broadening the current evidence for the worldwide emergence of this genotype.

Keywords Acute gastroenteritis · G12 rotavirus · Genotypes

Worldwide, rotaviruses are recognised as a major cause of acute gastroenteritis in infants and young children, and in the young of many mammalian and avian species. It is estimated that rotavirus disease is responsible for approximately 36% of hospitalizations for diarrhea among children less than five years of age, resulting in 527,000 deaths annually, mostly (85%) in developing countries [1–3]. Efforts toward the development of effective rotavirus vaccines have been made during the past two decades to reduce the global burden of rotavirus diarrhea. In 2009, the World Health Organization (WHO) recommended inclusion of rotavirus vaccination in all national immunization programs [4]. Currently two live, attenuated, oral-administrable rotavirus vaccines, Rotarix™ (Glaxo SmithKline) and RotaTeq™ (Merck & Co., Inc.), are licensed in >100 countries and have been incorporated into the childhood immunization programs of several countries [5–8].

Rotavirus is a member of the family *Reoviridae*, and its genome consists of 11 double-stranded (ds) RNA segments, which encode 12 proteins: 6 structural and 6 non-structural proteins. Rotavirus particles are composed of three concentric protein layers: outer capsid, inner capsid and core [9]. Two viral surface proteins, VP7 and VP4, which make up the outer capsid shell, allow classification of rotavirus in G and P genotypes, respectively. To date, 27 G and 35 P genotypes have been reported by sequence analysis. Recently, a novel classification system has been proposed based on the nucleotide sequences of all rotavirus genes to provide a complete characterization of strains and possibly identify reassortment events [10, 11].

The most common G/P combinations of human rotaviruses are G1P[8], G3P[8], G4P[8], G9P[8] and G2P[4]. Despite the high prevalence of such strains, several studies have described the circulation of unusual and/or novel G and P genotypes, such us G5, G8 and P[9], mainly in African, Asian and American countries [12–17]. The occurrence of mixed infections involving G and P genotypes appears to be more common in developing countries where, in general, a broad genotype diversity can be seen.

G12 is currently recognised as a globally emerging rotavirus genotype that appears to be spreading more rapidly in recent years. G12 rotavirus was first identified in the Philippines in 1987, causing diarrhea in children [18, 19]. Subsequently, G12 rotavirus was detected in the United

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Published online: 27 September 2011

States, in several Asian countries, in Europe and in South America [20–23]. Four lineages of the VP7 gene of G12 strains have been described. Lineage I comprises the prototype L26 with P[4] specificity detected in the Philippines; lineage II clusters G12P[9] strains isolated in Asia and South America; lineage IV comprises prototype RU172 with P[7] genotype-specificity, the only porcine G12 strain; and lineage III consists of G12P[8] and G12P[6] human rotaviruses that are currently circulating and have been able to spread across the world [21, 24–27].

Current rotavirus vaccination programs require a continuous monitoring of circulating rotavirus strains in order to detect the emergence of possible uncommon and novel types, as well as to assess their potential impact on the effectiveness of vaccines. It has been argued by some authors that the increasing use of rotavirus vaccines might have an impact on viral ecology through a potential replacement of circulating genotypes [28]. The oral, live attenuated, monovalent (G1P[8]) rotavirus vaccine Rotarix™ was adopted by the public-health sector in Brazil in March 2006 to target an annual 3,000,000-birth cohort. Since then the Brazilian Ministry of Health (MoH) has implemented a nationwide surveillance network to assess the burden of rotavirus disease, as well as to monitor circulating strains. Taking advantage of this country's surveillance system, we were able to gather data on the occurrence of rotavirus genotypes among diarrhoeic children and, in particular, could characterize the emergence of G12 human rotavirus isolates in the Northern Region of Brazil.

Between January 2008 and October 2010, a total of 787 stool samples were collected from children with acute gastroenteritis through a surveillance program carried out by Instituto Evandro Chagas, a Brazilian MoH's Rotavirus National Reference Center located in Belém, Pará state. These specimens were obtained from five states in the Northern Region of Brazil (Acre [110 samples], Amazonas [325], Amapá [33], Pará [86] and Roraima [233]) and screened routinely for the presence of rotavirus using a commercial ELISA kit (Premier Rotaclone, Meridian Bioscience, Inc). Rotavirus-positive samples were further subjected to dsRNA extraction using guanidinium isothiocyanate-silica, as described previously [29]. Polyacrylamide gel electrophoresis (PAGE) was carried out in Tris-glycine buffer, and the rotavirus genome profile was determined following electrophoresis of extracted dsRNA through vertical 5% acrylamide bisacrylamide gels [30].

In order to determine the rotavirus genotype specificity, stool samples that were positive by both ELISA and PAGE were subjected to RT-PCR using Super-Script TM (Invitrogen, Carlsbad, CA), and the resulting cDNAs were amplified to generate fragments of 1062 and 876 bp, corresponding to portions of the genes encoding the VP7 and VP4 protein, respectively. The primers used in the first amplification were Beg9/End9 and 4con3/4con2 for the G and P type, respectively [31, 32]. G-typing was done using primer RVG9 in combination with primers BT1 (G1), CT2 (G2), ET3 (G3), DT4 (G4) and FT9 (G9) [31]. Characterization of P genotypes was performed using primer 4con3 in combination with type-specific primers 1T-1 (P[8]), 2T-1 (P[4]), 3T-1 (P[6]), and 4T-1 (P[9]) as described elsewhere [32].

The VP7 and VP4 (VP8* portion) genes of G12 strains were partially sequenced using the primers Beg9/End9 and 4con3/4con2 and a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The products were analyzed using an automatic ABI Prism 3130*xl* DNA sequencer (Applied Biosystems). The nucleotide sequences of these genes were aligned and edited using the BioEdit Sequence Alignment Editor (version 7.0.5.2) program and compared with the corresponding gene fragments of rotavirus strains available in GenBank. Phylogenetic analysis was performed with MEGA software version 4.0.1 by the neighbour-joining (NJ) method. For NJ, a distance matrix calculated from the aligned sequences using the Kimura two-parameter formula was used [33]. To determine the reliability of the tree topology, a bootstrap test of 2,000 replicates was performed [34]. The partial nucleotide sequences of the VP7 gene determined in this study have been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) and assigned the accession numbers GU250828 (RV98670), GU250829 (RV98660), JF980340 (RV102320), JF980341 (RV105659), and JF980342 (RV108214).

Of the 787 stool specimens screened for rotavirus antigen by ELISA, 318 (40.4%) were positive for rotavirus. PAGE was performed with 687 samples, of which 214 (31.1%) displayed a typical RNA electrophoretic migration pattern. Of these, 119 (55.6%) specimens showed a short profile and 95 (44.4%) showed a long profile. All G2 rotavirus strains exhibited identical short electropherotypes, and G12 strains yielded a long pattern. Among the rotavirus-positive children, 93 (29.3%) received rotavirus vaccine (Rotarix®), 92 (28.9%) were not vaccinated, and for 133 (41.8%), information on rotavirus vaccination was not available. There was no significant difference ($P = 0.1280$) when rates of occurrence of genotypes were compared between vaccinated and unvaccinated infants.

The G genotype could be determined for 303 (95.3%) samples. G2 was the most prevalent type (47.5%, n = 144), followed by G1 (29.4%, n = 89) and G9 (12.5%, n = 38). With regard to the VP4 gene, 290 strains were P-typed (91.2%); the most frequent genotypes were P[4] (47.2%, n = 137), P[8] (38.2%, n = 111) and P[6] (6.2%, n = 18). Using the binomial (G and P genotype) nomenclature system, it could be seen that the G2P[4] was the most

prevalent genotype, responsible for 45.6% (n = 126) of cases, followed by G1P[8] (22.1%, n = 61) and G9P[8] (12.7%, n = 35). Mixed infections, as defined by the identification of more than one G- or P-type specificity in the same sample, were detected in 11.6% (n = 32) of the strains (Fig. 1).

Of a total of fifteen G non-typeable samples, seven showed the full-length VP7 gene amplified in the first round of PCR. They were selected for VP7 gene sequencing, and all of them were recovered from unvaccinated children. Two samples not could be characterized due to their low cDNA concentration, five G12 rotaviruses displayed long RNA patterns, and the VP7 gene partial sequences (924 bp) demonstrated levels of nucleotide and amino acid homologies of 99–100% and 98.3–100%, respectively. G12 isolates grouped into VP7 lineage III, showing a high degree of nucleotide homology (>87.9%) and amino acid homology (>91.4%) when compared with other human G12 rotavirus strains reported worldwide (Fig. 2). Alignment of VP7 amino acid sequences from G12 strains did not show any major amino acid changes in protein coding (data not shown). The nucleotide sequences of the VP4 genes of G12 strains were also determined. The VP4 gene of the five G12 strains belonged to lineage P[6]-Ia, as proposed by Martella et al. [35] (data not shown).

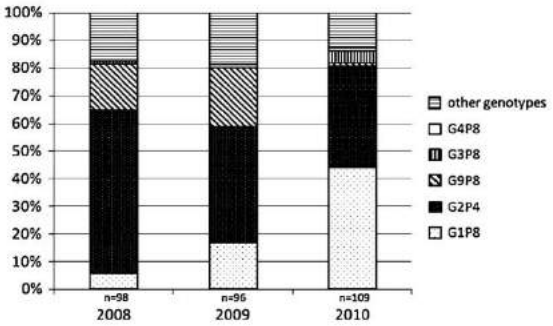

Fig. 1 Distribution of rotavirus G and P genotypes in Northern Region, Brazil, during 2008-2010

In the present study, G2P[4] was found to be the predominant genotype, accounting for 45.6% of isolates, mostly in 2008, when 59% of strains belonged to this genotype. Recently, a systematic review and meta-analysis study reported that the G2P[4] genotype accounted for 85% of circulating serotypes in the post-rotavirus-vaccine era in Latin America [36]. Although some studies in Latin America have suggested that the predominance of G2P[4] genotype in recent years may be associated with vaccine (Rotarix®)-induced selective pressure, such a continental phenomenon could merely reflect a natural fluctuation in co-circulating serotypes over time. The latter hypothesis is supported by the fact that G2 has also been a dominant strain in some Latin American countries where universal rotavirus vaccination has not yet been introduced into the public sector [15, 36–40].

Our study shows a significant increase in the prevalence of G1P[8] strains in 2010 compared to 2009 and 2008. Several studies have demonstrated that continuous circulation of G1 rotavirus strains may be due to their broad genetic diversity, as demonstrated by the occurrence of

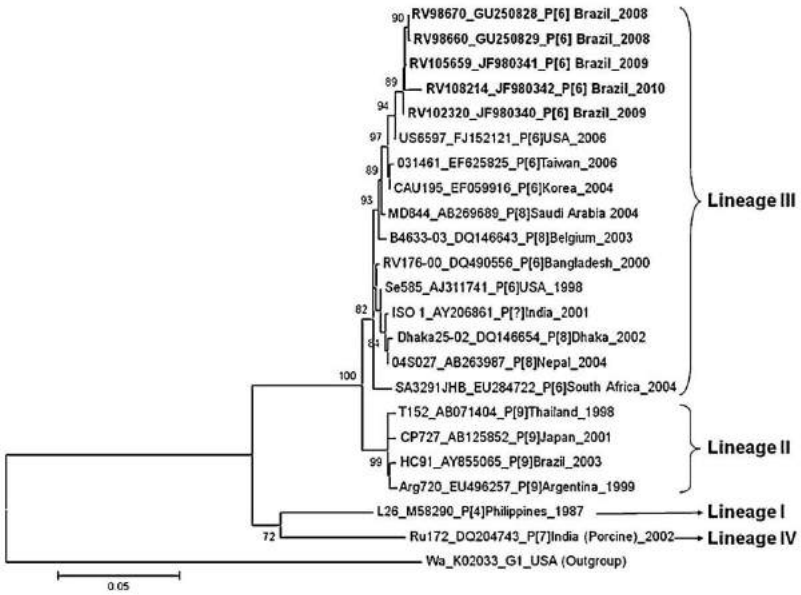

Fig. 2 Dendrogram based on the partial nucleotide sequences of the VP7 gene (924 bp; nt 1-924; aa 1-308). The numbers adjacent to the nodes represent the percent bootstrap support for that cluster. Bootstrap values lower than 70% are not shown. The calibration bar indicates substitutions per nucleotide. G12 strains analyzed in this study are in bold

strains with VP7 genes of different lineages and sublineages in the population [41, 42].

Five strains were typed as G12P[6] by sequence analysis. These samples were recovered from diarrhoeic children aged 16 months to 3 years and with no history of rotavirus vaccination. These G12-positive specimens were collected in Amazonas (3 samples) and Pará (2 samples) states between 2008 and 2010. Initially these samples were found to be G-untypeable by conventional RT-PCR methods, and they were genotyped by nucleotide sequencing of the VP7 fragment. Nucleotide homology and phylogenetic analysis of these samples showed 98.8% homology with US6597 (FJ152121), a strain from the USA that was recently isolated from a child with diarrhea [43].

Here, we describe the spread of G12P[6] rotavirus strains (clustering in lineage III of the VP7 gene) into Latin America. G12 rotavirus with P[4] specificity was first isolated in the Philippines in 1987 from children < 2 years, [18, 19]. In Brazil, the first G12 strain, bearing the P[9] genotype and displaying a long electropherotype, was reported in 2003 as having been recovered from an 11-month-old boy with diarrhea in Paraná State, Southern Brazil [23]. Unlike these findings, the recent isolates were combined with the P[6] genotype and demonstrated a high degree of homology with G12 strains currently circulating elsewhere. These findings are suggestive of a recent introduction of G12P[6] rotaviruses in the Northern Region of Brazil. In addition, the combination of the G12 and P[6] genotypes in the new isolates strongly suggests the possibility of a zoonotic transmission, as described elsewhere [44]. Some studies suggest that G12 strains are emergent worldwide [12, 27, 45]. In a pattern of occurrence similar to that noted for G9, the G12 genotype was found rarely for several years after its first detection, with subsequent increasing prevalence rates during the past few years. It should be considered whether G12 rotaviruses potentially challenge current rotavirus vaccination strategies, since the G12P[6] genotype rotavirus is not included in the composition of the two currently licensed vaccines [7].

The fact that G12 has been found to have been circulating in most parts of the world during the past decade and that it will probably soon become the sixth major human rotavirus genotype warrant the inclusion of G12 primers in routine genotyping procedures. Furthermore, complete genome characterization of G12 rotavirus strains should be encouraged in order to broaden our understanding on their evolution and possible impact on vaccine development. Surveillance studies to monitor prevalent genotypes of rotavirus after vaccine introduction are crucial, mainly to detect the spread of unusual genotypes, such as G9 and G12, as well as to detect the emergence of strains of possible novel genotype composition.

Acknowledgments This work was supported by a grant from Instituto Evandro Chagas and Brazilian National Council for the Development of Science and Technology (CNPq).

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6.4 - DIVERSITY OF ROTAVIRUS STRAINS CIRCULATING IN NORTHERN BRAZIL AFTER INTRODUCTION OF A ROTAVIRUS VACCINE: HIGH PREVALENCE OF G3P[6] GENOTYPE.

Journal of Medical Virology 86:1065–1072 (2014)

Diversity of Rotavirus Strains Circulating in Northern Brazil After Introduction of a Rotavirus Vaccine: High Prevalence of G3P[6] Genotype

Luana da Silva Soares,* Sylvia de Fátima dos Santos Guerra, Alessilva do Socorro Lima de Oliveira, Fabiola da Silva dos Santos, Euzeni Maria de Fátima Costa de Menezes, Joana d'Arc Pereira Mascarenhas, and Alexandre C. Linhares

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Neste manuscrito, observou-se a detecção do G9 em 3% das infecções por RVA em amostras coletadas de 2011 e 2012 na região Norte. Destacou-se a elevada prevalência do genótipo G3P[6], combinação não usual circulando na região.

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Diversity of Rotavirus Strains Circulating in Northern Brazil After Introduction of a Rotavirus Vaccine: High Prevalence of G3P[6] Genotype

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Rotavirus A (RVA) is the most common cause of severe acute gastroenteritis in infants and young children worldwide, causing 453,000 deaths annually. In Brazil, the most frequent genotype identified was G1 during almost three decades in the pre-vaccination period; however, after anti-rotavirus vaccine introduction, there was a predominance of G2 genotype. The aim of this study was to determine the G and P genotypes of rotaviruses isolated from children under 5 years of age with acute gastroenteritis in the Northern region of Brazil, and discuss the emergence of G3P[6] genotype. A total of 783 stool specimens were obtained between January 2011 and March 2012. RVA antigen was detected in 33% (272/783) of samples using a commercial enzyme-linked immunosorbent assay and type-specificity was determined by reverse-transcription polymerase chain reaction. The most common binary combination was G2P[4], representing 41% of cases, followed by G3P[6] (15%), G1P[8] (8%), G3P[8] (4%), G9P[8] (3%), and G12P[6] (2%). G3P[6] strains were analyzed further and phylogenetic analysis of VP7 gene showed that G3 strains clustered into lineage I and showed a high degree of amino acid identity with vaccine strain RV3 (95.1–95.6%). For VP4 sequences, G3P[6] clustered into lineage Ia. It was demonstrated by the first time the emergence of unusual genotype G3P[6] in the Amazon region of Brazil. This genotype shares neither VP7 nor VP4 specificity with the used vaccine and may represent a challenge to vaccination strategies. A continuous monitoring of circulating strains is therefore needed during the post-vaccine era in Brazil. ***J. Med. Virol. 86: 1065–1072, 2014.***

KEY WORDS: diarrhea; Rotarix™; G3P[6] rotavirus A

INTRODUCTION

Rotavirus A (RVA) is the most common cause of severe acute gastroenteritis in infants and young children worldwide being responsible for 453,000 deaths annually [Tate et al., 2012]. In Brazil, there were approximately 3.5 million cases, 650,000 clinic visits, 100,000 hospitalizations, and 850 deaths caused by rotavirus gastroenteritis each year during the pre-vaccine era [Sartori et al., 2008; Dennehy, 2012].

Currently, two oral RVA vaccines are licensed and widely available: Rotarix™ (GlaxoSmithKline, Rixensart, Belgium) and Rotateq™ (Merck Research, Whitehouse Station, NJ). These vaccines were found to be efficacious against severe rotavirus disease and demonstrated substantial reductions in childhood morbidity and mortality in middle and low-income countries [Munos et al., 2010; Lanzieri et al., 2011; O'Ryan et al., 2011; Dennehy, 2012]. In 2009, the World Health Organization (WHO) recommended the inclusion of rotavirus vaccination into national immunization programs and recently reinforced its implementation as a priority [WHO, 2009, 2013].

Authors' Contributions: L.S.S. was responsible for molecular genetic studies, sequence analysis, and drafted the manuscript. S.F.S.G., A.S.L.O., F.S.S. carried out molecular genetic studies. E.M.F.C.M. contribution to the laboratory methods. J.D.P.M. supervized laboratory work and training. Involved in analysis and provided critical review of manuscript. A.C.L. supervized laboratory work. Contributed to study design and review final versions manuscript. All authors read and approved the final manuscript.

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Accepted 29 August 2013

DOI 10.1002/jmv.23797
Published online 17 October 2013 in Wiley Online Library (wileyonlinelibrary.com).

Rotaviruses on classified into 8 major species (A–H) but most human strains belong to group A, although groups B and C have occasionally been associated with human illness [Estes and Kapikian, 2007; Matthijnssens et al., 2012]. Based on the two outer capsid proteins, VP7 and VP4, RVA are classified into 27 G and 37 P types, respectively [Matthijnssens et al., 2011; Trojnar et al., 2013]. Several surveillance and epidemiologic studies have been conducted around the world and the most common strains are G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8], representing approximately 90% of the human RVA strains. Recently, G12 emerged associated with either P[8] or P[6], which is considered the sixth most common global genotype [Santos and Hoshino, 2005; Rahman et al., 2007; Matthijnssens et al., 2010].

In Brazil, the most frequent genotype identified was G1 during almost three decades in the pre-vaccination period, however, after anti-rotavirus vaccine introduction there was a predominance of G2 genotype with a frequency in the average of 74%; however in recent years it was observed a trend for continuous decline of this genotype [Leite et al., 2008; Carvalho-Costa et al., 2011; Oliveira et al., 2012; Soares et al., 2012]. Although rotavirus G3 is an usual genotype in the Northern region of Brazil it has rarely been detected in sporadic cases of gastroenteritis in humans, mostly in combination with P[8] [Oliveira et al., 2012; Soares et al., 2012].

Rotavirus G3 has been detected in several animal hosts, such as cats, dogs, pigs, and birds, mostly combined with P[3] and P[9] VP4 genes and recently a study showed that G3 rotavirus may be associated with severe diarrhea [Martínez-Laso et al., 2009; Martella et al., 2010; Grant et al., 2011; González and Rivero, 2013]. P[6] is in general associated with neonatal rotavirus infections with a wide variety of G-types [Martella et al., 2006; Mascarenhas et al., 2007; Stupka et al., 2009; Lorenzetti et al., 2011; Nordgren et al., 2012].

Recently it has been proposed a new rotavirus classification system based on the molecular characteristics of the 11 genes to achieve a better understanding of the function of each protein and of the evolutionary relationship among species [Matthijnssens et al., 2011]. Three genotype constellations of the non-G and non-P genes have been shown to circulate worldwide among humans: I1-R1-C1-M1-A1-N1-T1-E1-H1 (Wa-like); I2-R2-C2-M2-A2-N2-T2-E2-H2 (DS-1-like); and I3-R3-C3-M3-A3-N3-T3-E3-H3 (AU-1) [Matthijnssens and Van Ranst, 2012]. G3P[6] genotype has been described as a rotavirus with Wa-like genotype constellation and very closely related to attenuated RV3 vaccine strain [Rippinger et al., 2010].

The aim of this study was to characterize the G and P genotypes of RVA isolated from children under 5 years of age with acute gastroenteritis in Northern Brazil, between January 2011 and March 2012, arising from a Brazilian Ministry of Health's nationwide surveillance network to monitor circulating strains. In addition, it was focused on the emergence of the G3P[6] strains, which may suggest a reassortment among common human strains. This may theoretically pose a challenge to current rotavirus vaccination strategies.

MATERIALS AND METHODS

Clinical Specimens

The samples from this study were collected from hospitalized children who presented with symptoms of acute gastroenteritis and were selected from six states in the Northern region of Brazil (Table I). During January 2011 to March 2012 a total of 783 samples were collected and an aliquot of each sample was stored at 2–8°C and transported to Instituto Evandro Chagas, a Brazilian Ministry of Health's National Rotavirus Reference Laboratory.

Ethical Considerations

This study was part of an official Brazilian Ministry of Health's surveillance, therefore there was no need for ethical clearance.

Rotavirus Screening and RNA Extraction

All fecal samples were screened for the presence of RVA by a commercially available enzyme-linked immunosorbent (ELISA) assay according to the manufacturer's instructions (Premier Rotaclone, Meridian Bioscience, Cincinatti, OH). The results were determined by absorbance readings. Viral RNA was extracted using guanidinium isothiocyanate-silica method [Boom et al., 1990]. Polyacrylamide gel

TABLE I. RVA-Positivity Associated With Age Group, Brazilian State, Clinical Characteristics, and Vaccination Status

	RV-A positive/tested (%)
Age group (year)	
0–1	134/407 (33.0)
1–2	63/190 (33.2)
2–5	47/106 (44.3)
>5	19/61 (31.1)
Brazilian state	
Acre	87/189 (46.0)
Amazonas	134/470 (28.5)
Amapá	8/21 (38.0)
Pará	15/57 (26.3)
Rondônia	15/21 (71.4)
Roraima	13/25 (52.0)
Clinical characteristics	
Fever	73/239 (30.5)
Vomiting	132/375 (35.2)
Rotavirus vaccination history (Rotarix™)	
Ineligible	22/61 (36.0)
Unknown	163/398 (41.0)
Unvaccinated	20/66 (30.0)
Vaccinated (received at least one dose)	67/258 (26.0)

electrophoresis (PAGE) was carried out in Tris–glycine buffer and the rotavirus genome profile was determined following electrophoresis of extracted dsRNA through vertical 5% acrylamide bisacrylamide gels [Pereira et al., 1983].

RT-PCR and Genotyping

All RVA-positive samples were subjected to reverse transcription-polymerase chain reaction (RT-PCR). First round was performed with consensus primers Beg9/End9 and 4con3/4con2 to amplify VP7 and VP4 genes, respectively. G and P genotyping was performed using seminested type-specific multiplex PCR using specific primers for G (G1, G2, G3, G4, G9, and G12) and P-types (P[4], P[6], P[8], and P[9]), as described previously [Gouvea et al., 1990; Gentsch et al., 1992; Banerjee et al., 2007]. The G and P-types were determined by the specific sizes of the amplicons on agarose gels.

Nucleotide Sequencing and Phylogenetic Analysis

Sequencing of the PCR amplicons for VP7 and VP4 genes of G3 strains were performed using the same primers as those used in the PCR and carried out with a Big Dye Terminator cycle sequencing kit v 3.1 (Applied Biosystems, Foster City, CA). The sequences were collected from an automated ABI Prism 3130*xl* DNA sequencer (Applied Biosystems). Phylogenetic analyses were carried out using MEGA software program version 4.0.1 by the neighbor-joining (NJ) method [Kimura, 1980]. The statistical significance of the genetic relationships was estimated by bootstrap resampling analysis (2,000 replications). The sequences of G3 strains were submitted to GenBank under the accession numbers JX987024–JX987034, JX996189–JX996193, and KC164357– KC164370.

Data Analyses

The frequencies of RVA infection and genotype combinations were calculated using Microsoft Excel software. Comparisons of RVA infection rates in distinct groups were performed using χ^2 test through BioEstat 5.0 [Ayres et al., 2007]. Statistical significance was established at P values <0.05.

RESULTS

Overall samples were screened for RVA antigen by ELISA yielding a positivity of 33% (272/783, range 23–56%). Figure 1 shows the monthly frequencies of RVA detection, with two peaks where RVA rates were over 50%, February and June 2011.

Table I summarizes the RVA positivity associated with major clinical and epidemiologic characteristics of patients. The mean age of patients with rotavirus gastroenteritis was 25 months, with mean age of non-rotavirus acute gastroenteritis of 31 months. A higher RVA positivity (44%) was observed among children aged 2–5 years. Among children who were age-eligible for rotavirus vaccine, 67 (26%, 67/258) were RVA-positive and received Rotarix™, with mean age of 18 months, most of them infected by G2P[4] genotype (46%, 31/67). PAGE was performed in samples, of which 198 (25%) displayed a typical RNA electrophoretic migration pattern. Of these, 41 (21%) and 157 (79%) specimens showed long and short profile, respectively. All G3P[6] rotavirus strains exhibited identical short electropherotypes (RNA pattern not shown).

The most common binary combination was G2P[4], responsible for 41% (106/258) of cases, followed by

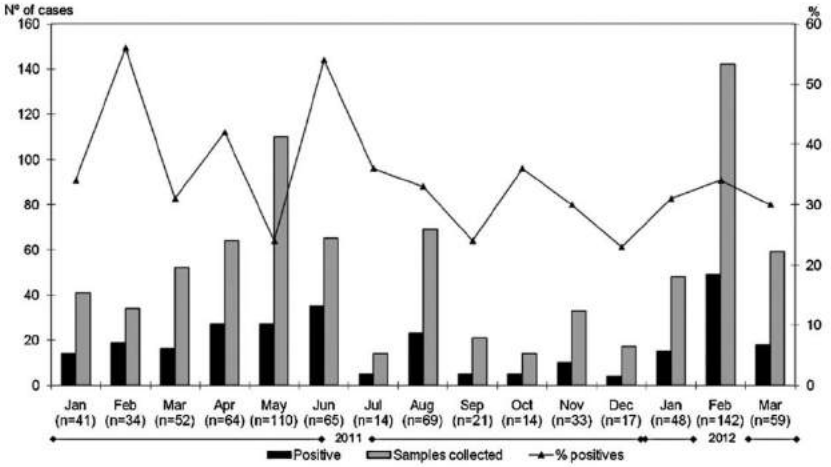

Fig. 1. Temporal distribution of RVA positivity in cases of gastroenteritis in Northern region of Brazil, between January 2011 and March 2012.

G3P[6] (15%, 39/258), G1P[8] (8%, 21/258), G3P[8] (4%, 11/258), G9P[8] (3%, 7/258), and G12P[6] (2%, 5/258). Mixed infections were detected in 31 samples (12%). Forty-one RVA-positive specimens could not be assigned to a specific G or P type. The distribution of G and P RV-A genotypes is shown in Figure 2. It was observed that G2P[4] genotype was identified throughout the study period and G3P[6] genotype was detected initially in November 2011. There was no significant difference ($P = 0.569$) when the rates of G2 genotype were compared among vaccinated and unvaccinated infants.

Sixteen G3 samples, all of which collected during 2012, were subjected to partial sequencing analyses of VP7 and VP4 genes. With regards to VP7 gene, G3 strains formed two distinct groups and clustered into lineage I according with Martínez-Laso et al. [2009]. One group was composed by 14 G3P[6] samples and were highly similar to each other (nt: 99.0–100%) as well as to African samples, ETH44 and BFA, collected in 2009 and 2010, showing with these a nucleotide similarity higher than 99%. Two G3P[8] strains gathered in another cluster with samples from USA, Thailand, Russia, and Spain (nt and aa: 98.5–99.3%). Likewise, a comparison of VP7 sequences of own Brazilian G3 samples clustered with vaccine strain RV3 showing a high degree of amino acid identity (95.1–95.6%; Fig. 3). Brazilian G3 strains showed amino acid substitutions at 96 and 213 positions, both of which from aspartic acid to asparagine, in antigenic regions A and C (data not shown).

With regards to VP4 sequences, G3P[6] clustered into lineage Ia. The degree of nucleotide identity among Brazilian strains was higher than 99% and when compared to the RV3 strain the median of nucleotide identity was 94% (Fig. 4). G3P[8] samples grouped into lineage III (data not shown).

DISCUSSION

In the present study, RVA gastroenteritis was associated with 33% of pediatric inpatients less than 5 years of age, a rate similar to those studies conducted in Latin America countries such as Guatemala, Venezuela, and Chile, where RVA frequency ranged from 20% to 40% [González et al., 2011; Linhares et al., 2011; Cortes et al., 2012; Lucero et al., 2012]. G1P[8] genotype was the most frequent binary combination found before rotavirus vaccine implementation [Santos and Hoshino, 2005; Patton, 2012]. In post-vaccine era, several studies reported the striking increase of G2P[4] circulating strains, mainly in Latin America countries, leading to the hypothesis of vaccine-induced selective pressure. Nevertheless, it cannot be ruled out that such phenomenon just reflect temporal fluctuation of G2P[4] [Gurgel et al., 2007; Linhares et al., 2011; O'Ryan et al., 2011; Assis et al., 2013]. Further long-term surveillance studies are needed to clarify this yet controversial issue.

It was observed a 26% rate of RVA detection among children vaccinated with Rotarix™. Similar results were found in two recent studies performed from 2005 to 2010 in Brazil, where prevalence rates ranged from 23% to 29% [Carvalho-Costa et al., 2011; Soares et al., 2012]. In the present study, G2P[4] genotype occurred in 46% of children who received

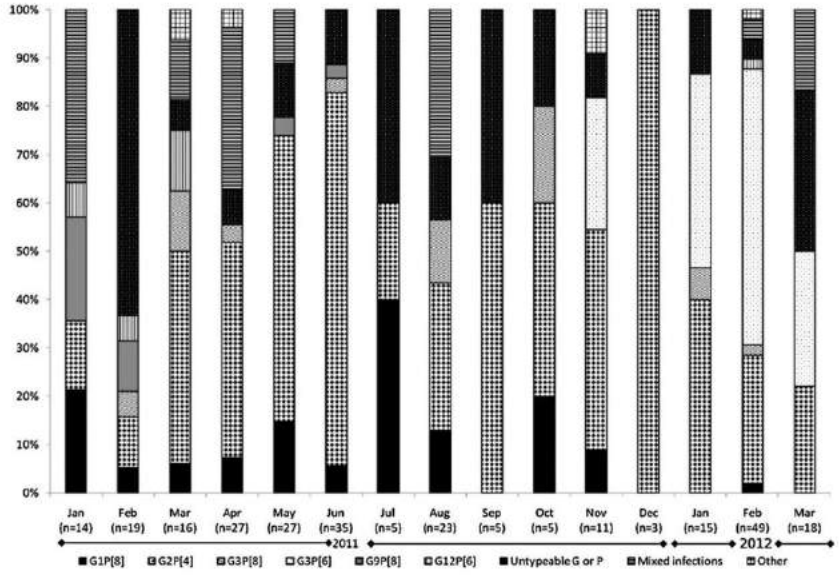

Fig. 2. G and P RVA genotype distribution in Northern region of Brazil, between January 2011 and March 2012.

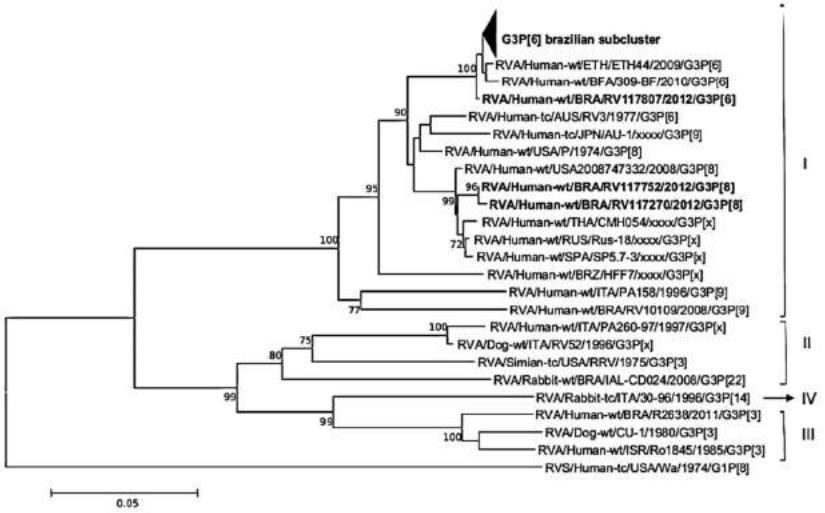

Fig. 3. Phylogenetic analysis of the VP7 protein of circulating G3 Brazilian RVA strains. Neighbor-joining tree was constructed using on the partial nucleotide sequences of VP7 gene (764 bp; nt 112–875; aa 38–291). Bootstrap values above 70% are given at branch nodes. G3 strains analyzed in this study are in bold and condensed into a black triangle.

Rotarix™, a rate slightly lower than that of an investigation conducted in Rio de Janeiro, where this type was associated in 57% of vaccinated patients [Carvalho-Costa et al., 2011]. Even though this genotype possesses distinct antigens compared to Rotarix™, recent studies have shown significant vaccine efficacy against G2P[4] rotavirus [Correia et al., 2010; Justino et al., 2011; Patel et al., 2013]. Furthermore, there was no significant difference when compared the rate of G2P[4] genotype between

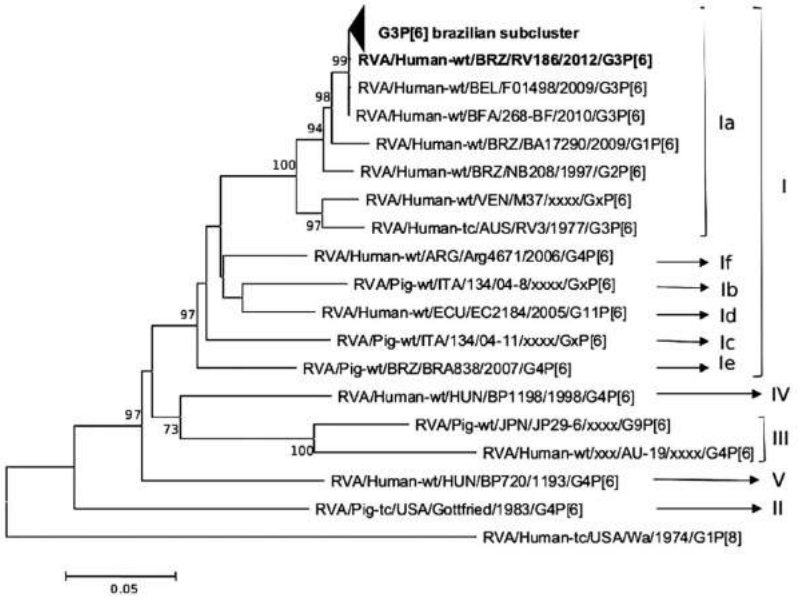

Fig. 4. Phylogenetic analysis of the VP4 protein of circulating G3 Brazilian RVA strains. Neighbor-joining tree was constructed using on the partial nucleotide sequences of VP4 gene (778 bp; nt 51–828; aa 18–276). Bootstrap values above 70% are given at branch nodes. G3 strains analyzed in this study are in bold and condensed into a black triangle.

vaccinated and unvaccinated infants, a finding similar to that reported in another study in Brazil [Soares et al., 2012]. It was detected a trend for a higher frequency of RVA diarrhea among children aged 2–5 years. This is likely to be due to the age for rotavirus vaccination that is for children under 6 months, so unvaccinated children older than 2 years may be more prone to acquire rotavirus infection; however this increase was not statistically significant ($P = 0.32$).

G2P[4] was the predominant genotype (40%) followed by G3P[6] (15%) and G1P[8] (8%). Soares et al. [2012] have reported similar results in a previous study from Northern region of Brazil, between 2008 and 2010, where G2P[4] rotavirus was detected in 46% of patients with acute diarrhea, followed by G1P[8] (22%). Interestingly, it was observed the increase in the rate of G3P[6] genotype, which was the second most frequent genotype circulating in the study population. G3P[6] genotype was detected as from November 2011 and circulated in Amazonas and Acre states only.

G3 rotavirus is a genotype associated with a broad range of hosts. Some studies have shown the increase of G3 frequency associated with P[8] specificity, mostly in Asian countries [Yang et al., 2008; Bányai et al., 2012; Thongprachum et al., 2013]. Recently, G3P[8] was the most frequently detected genotype in Argentina, responsible for about 40% of strains [Stupka et al., 2012]. Human G3 with P[3] and P[9] types have been detected showing higher similarities to feline or canine strains [Grant et al., 2011; Mitui et al., 2011; Maestri et al., 2012]. G3P[6] is a genotype found rarely. In a study conducted in Malawi, G3P[6] strains were responsible for 1% of RVA cases circulating in the nineties [Cunliffe et al., 2010]. In Latin America, G3P[6] strains were detected in 0.2% of RVA genotypes [Linhares et al., 2011].

Partial sequences were obtained for VP7 genes of G3 strains showing nucleotide similarity higher than 99% to each other over the study period, all of them clustering into lineage I. Trinh et al. [2007] have suggested that two amino acid substitutions (Asp96Asn and Asp213Asn) could be responsible for emergence of G3 rotavirus in China. Similarly these amino acid changes in the VP7 genes were noted in all Brazilian G3 strains identified in the present study. Moreover, Yang et al. [2008] have detected these changes in rotavirus G3 isolated for 1996–2005. Further studies are therefore warranted to explain why there was such a marked emergence of G3 strains in the region.

Brazilian G3 strains showed a high homology with RV3, a human neonatal G3P[6] candidate vaccine, and grouped into lineage I. Previous results during phase II study demonstrated that RV3 protected partially infants against severe diarrhea during successive winter months, even though further studies are needed to better assess the efficacy of this candidate vaccine [Barnes et al., 2002].

Since there was recent introduction of G12P[6] rotavirus in Brazil, the emergence of G3P[6] genotype may be associated possibly with reassortment between G12P[6] and G3P[8] strains. Recently, Heylen et al. [2013] described a full characterization for G3P[6] strains with DS-1-like genotype constellation and proposed a reassortment between different G-genotypes strains. The fact that G3P[6] Brazilian strains exhibited short electropherotypes and some G12P[6] strains possess the I2-R2-C2-M2-A2-N2-T2-E2-H2 genotype constellation supports the hypothesis of genetic exchanging between G3P[8] and DS-1-like strains. Nonetheless, further studies on the molecular characterization of rotavirus genes are needed to better assess a possible origin for these strains. Recently, Maestri et al. [2012] described interspecies transmission of rotavirus detected in Amazon region, including G3P[9] strains that supports a close relationship between human and animal rotavirus genes. Although G3 and P[6] genotypes may be associated with zoonotic transmission this seems unlikely for Brazilian strains, since a high similarity was seen with human strains.

In conclusion, these findings demonstrate the emergence of unusual genotype G3P[6] in Amazon region of Brazil and reinforces the need for continuous long-term monitoring of circulating strains through the national surveillance network, in order to better understand the complex dynamics of RVA molecular epidemiology. Furthermore, the monitoring of unusual genotype emergence that might represent possible challenges to current licensed rotavirus vaccines that do not contain strains with DS-1 genotype constellation.

ACKNOWLEDGMENTS

The authors would like to acknowledge the staff of State Central Laboratories (LACENs) and the technical assistance given by the laboratory personnel at the Virology Section of the Instituto Evandro Chagas, Health Surveillance Secretariat, Brazilian Ministry of Health.

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6.5 - ROTAVIRUS STRAIN SURVEILLANCE FOR THREE YEARS FOLLOWING THE INTRODUCTION OF ROTAVIRUS VACCINE INTO BELÉM, BRAZIL.

Journal of Medical Virology

Rotavirus Strain Surveillance for Three Years Following the Introduction of Rotavirus Vaccine into Belém, Brazil

Sylvia F.S. Guerra,[1] Alexandre C. Linhares,[1]* Joana D'Arc P. Mascarenhas,[1] Alessilva Oliveira,[1] Maria Cleonice A. Justino,[1] Luana S. Soares,[1] Elza Caroline Müller,[1] Patrícia Brasil,[2] Suely Tuboi,[3] Eduardo Ortega-Barria,[4] and Romulo Colindres[3]

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Neste manuscrito, determinou-se a epidemiologia molecular das amostras circulantes em Belém, Pará, no período de 2008 a 2011 de crianças hospitalizadas. Observou-se inicialmente a predominância do genótipo G2 e, a partir de 2010, do genótipo G1. O genótipo G9 esteve envolvido em 4,1% dos casos neste período na região, sendo detectado, além da combinação binária G9P[8], a G9P[6] e G9P[4].

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The monovalent human rotavirus (RV) vaccine, RIX4414 (Rotarix™, GlaxoSmithKline Biologicals) was introduced into Brazil's Expanded Program on Immunization in March 2006. One year after vaccine introduction, the G2P[4] strain was found to be predominant, with an apparent extinction of many non-G2 strains. This study investigated the diversity of circulating strains in the three years following RIX4414 introduction. Between May 2008 and May 2011, stool samples were collected from children aged ≥12 weeks who were hospitalized for severe lab confirmed RV-gastroenteritis (≥3 liquid or semi-liquid motions over a 24-h period for <14 days, requiring ≥1 overnight hospital stay and intravenous rehydration therapy) in Belém, Brazil. RV-gastroenteritis was detected by ELISA and the G- and P-types were determined by RT-PCR assays. During the first year of surveillance nucleotide sequencing was used for typing those samples not previously typed by RT-PCR. A total of 1,726 of 10,030 severe gastroentertis hospitalizations (17.2%) were due to severe RVGE. G2P[4] was detected in 57.2% of circulating strains over the whole study period, however it predominated during the first 20 months from May 2008 to January 2009. G1P[8] increased in the last part of the study period from May 2010 to May 2011 and represented 36.6% (112/306) of the circulating strains. G2P[4] was the predominant RV strain circulating during the first 20 months of the study, followed by G1P[8]. These findings probably reflect a natural fluctuation in RV strains over time, rather than a vaccine-induced selective pressure. ***J. Med. Virol.*** © 2015 Wiley Periodicals, Inc.

KEY WORDS: rotavirus; Belém, Brazil; genotypes; post-vaccination; gastroenteritis

INTRODUCTION

Rotavirus (RV) is the leading cause of acute gastroenteritis among children younger than 5 years of age worldwide [Parashar et al., 2006]; it accounts for approximately 40% of all cases of severe infant diarrhea [CDC, 2011]. The World Health Organization (WHO) estimates that in 2008 around 453,000 annual child deaths were due to RV [WHO, 2013a,b].

Abbreviations: CI, confidence interval; ELISA, enzyme linked immunosorbent assay; IV, intravenous; RV, rotavirus; RVGE, rotavirus gastroenteritis; SD, standard deviation; EPI, Expanded Program on Immunization; RT-PCR, reverse transcriptase polymerase chain reaction.

Grant sponsor: GlaxoSmithKline (to S.F.S.G., A.C.L., J.D.P.M., A.O., M.C.A.J., L.S.S., E.C.M.)

Conflict of interest: EOB and RC are employees of the GlaxoSmithKline group of companies and own restricted shares in the GlaxoSmithKline group of companies. ST was an employee of GlaxoSmithKline group of companies and owned restricted shares in the GlaxoSmithKline group of companies at the time of the study. PB reports no competing interests.

Institution where study took place: Instituto Evandro Chagas

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Accepted 2 February 2015

DOI 10.1002/jmv.24183
Published online in Wiley Online Library (wileyonlinelibrary.com).

Recent estimates from Latin America and the Caribbean revealed that, in the absence of vaccination, RV causes up to 229,656 hospitalizations and 6,302 deaths each year among children younger than 5 years of age [Desai et al., 2011]. In Brazil during the pre-vaccine period, RV infections have been estimated to cause 850 annual deaths and 92,453 hospitalizations in children less than five years of age [Sartori et al., 2008].

Two live oral RV vaccines are currently available: a pentavalent, human-bovine reassortant vaccine with RV types G1–G4 and P[8] (RotaTeq®, Merck, NJ, USA) and a monovalent vaccine with an attenuated human G1P[8] RV strain, (RIX4414, [Rotarix™, GSK Biologicals, Rixensart, Belgium]) [Grimwood and Lambert, 2009]. RV strains carrying either G1–G4, or G9, combined with P[4] or P[8] have been found to be the most prevalent causes of RV disease in humans [WHO, 2013a,b; Trojnar et al., 2013]. However, substantial temporal and geographical changes in strain prevalence can lead to the emergence of G- and P-types such as G12 carrying either P[8] or P[6] [Santos and Hoshino, 2005; O'Ryan, 2009] which theoretically could evade immunity provided by the RV vaccines, although P[8], specifically, is included in the composition of both currently available rotavirus vaccines [Matthijnssens et al., 2011].

In early rotavirus vaccine adopter countries the effectiveness of either RotaTeq® or Rotarix® has been demonstrated, as well as the substantial impact on childhood morbidity and mortality due to gastroenteritis [Tate and Parashar, 2014].

In March 2006, Brazil was one of the first countries to introduce the monovalent human rotavirus vaccine into their Expanded Program on Immunization (EPI), which covers a birth cohort of around 2.9 million [DATASUS, 2013]. By December 2014, 73 countries had introduced RV vaccines into their EPI's, therefore increasing the need for conducting post-licensure surveillance studies [PATH, 2014]. Although such studies have provided reassuring evidence for the monovalent human rotavirus vaccine impact and effectiveness, whether vaccine-induced selective pressure might impact circulating RV strains is still debated [Gentsch et al., 2009; Tate et al., 2010; Patel et al., 2011; Matthijnssens et al., 2012]. The implementation of the monovalent human rotavirus vaccine into the Brazilian EPI in 2006 coincided with a dramatic increase in circulating G2P[4], leading some investigators to suggest that a serotype replacement had occurred as a result of vaccine-induced selective pressure mechanisms [Gurgel et al., 2008; Leite et al., 2008; Nakagomi et al., 2008; van Doorn et al., 2009; Carvalho-Costa et al., 2011; Linhares et al., 2011; Dulgheroff et al., 2012; Oliveira et al., 2012]. However, as most of these studies covered just a short surveillance period following vaccine introduction, the data obtained could reflect a natural fluctuation of G2P[4] over time, rather than a consequence of vaccination.

In this study results from a long-term (2008–2011) hospital-based surveillance study of RV strains among children with severe RV gastroenteritis in Belém, Northern Brazil is reported.

MATERIALS AND METHODS

Study Setting and Design

This hospital-based study was conducted in Belém, Brazil between May 2008 and May 2011. Belém has a population of 2.08 million and an annual birth cohort of 24,054 [Justino et al., 2011]. Strain surveillance was performed in two stages: May 2008–May 2009 in parallel with a case-control study to estimate the effectiveness of RIX4414 at four large urban hospitals [Justino et al., 2011]; and for an additional two years (May 2009–May 2011) at two of these hospitals, which received 50% of all gastroentertis-related pediatric hospitalizations in this area, the covered population was still considered to be representative of Belém as a whole.

The protocol was approved by the Independent Ethics Committee of the Brazilian Ministry of Health's National Rotavirus Reference Laboratory, Instituto Evandro Chagas (IEC) and the Brazilian Ministry of Health. The study was carried out in accordance with the Declaration of Helsinki and Good Clinical Practice. Written informed consent was obtained from the parents/legal guardians of children before enrolment.

Case Definition

Cases were defined as children at least 12 weeks of age, who had been born after March 6, 2006, and were hospitalized for laboratory-confirmed severe RVGE ($\geq$3 liquid or semi-liquid motions over a 24-h period for $<$14 days, requiring $\geq$1 overnight hospital stay and intravenous rehydration therapy) [Justino et al., 2011]. This ensured that children were eligible to have received at least one vaccine dose at enrolment; each child was included only once in the study.

Assessments

Parents/guardians were interviewed to collect demographic data and relevant medical history. Individual vaccination history was not collected since evaluating vaccine effectiveness was not the purpose of the study.

As part of routine practice, stool samples were collected within 48 hr of admission and transported to the IEC, for RV testing using enzyme-linked immunosorbent assay (ELISA) (RIDASCREEN® Rotavirus; R-Biopharm, Darmstadt, Germany). The tests were performed according to the manufacturer's instructions and included positive and negative controls.

Exclusion criteria included logistical reasons, late screening or collection, insufficient sample. However, genotyping was completed for 1,076 samples as two samples had insufficient quantities. Genotyping was

done using reverse transcriptase-polymerase chain reaction (RT-PCR), to determine G- and P-types. RT-PCR was performed using a two-step amplification process as previously described [Boom et al., 1990; Gouvea et al., 1990; Gentsch et al., 1992; Das et al., 1994; Leite et al., 1996]. During the case-control study only (first year of monitoring), nucleotide sequencing was performed with strains not typed previously by RT-PCR. Briefly, amplified first round products of the VP7 and VP4 genes were sequenced using the Big Dye Terminator Cycle Sequencing kit (Applied Byosystems, Foster City, CA), following the manufacturer's instructions. As per protocol nucleotide sequencing was not performed during the second and third years of surveillance.

The second step was a nested PCR using G or P specific oligonucleotide primers targeted at G (G1–G4 and G9) and P (P[4], P[6], P[8], and P[9]) RV types. Genotyped RV strains were categorized according to their possible origin, as reported before by Iturriza-Gómara et al. [Iturriza-Gómara et al., 2011].

Statistical Analyses

Data analyses were performed using SAS version 9.1 (SAS Institute, Inc., Cary, NC).

The distribution of RV G- and P-types was tabulated and compared with respect to age (3–5 months, 12–23 months and >24 months), origin (common human strains, reassortants among common human strains, possible zoonotic strains and possible animal human hybrids) and time of the year using chi-square and Fischer exact tests. All tests were two-tailed and differences between variables were considered statistically significant at P-values ≤ 0.05.

RESULTS

Of 10,030 severe gastroenteritis hospitalizations that were screened for RV (between May 2008 and May 2011), RV was identified in 1,726 (17.2%) cases by ELISA-525 (out of 538 samples collected from May 2008 to April 2009, during the case-control study), 260 from May 2009 to April 2010, and 293 from May 2010 to May 2011. Among 1,726 ELISA-positive stool samples, a subset consisting of 1,078 samples was further analyzed (62%).

The mean age of the subjects was 18.5 (±9.4) months and 52.8% were male (Table I). The highest percentage of hospitalizations for severe RV gastroenteritis was seen in children ≥12 months of age (76.1%; 820/1,078).

RV genotyping by RT-PCR was done on 1,076 samples and enabled G- and P-types to be successfully determined in 88.7% (954/1,076) of cases. Strains that could not be fully G- and/or P-typed represented 11.3% (122/1,076) of the total samples. Single G and P strains were present in 88.6% (845/954) of samples and 11.4% (109/954) had mixed RV strains. G2P[4] was the most commonly observed RV strain (57.2% [615/1,076]) followed by G1P[8] (14.9% [160/1,076]). The most common mixed RV strains were G2P[4]-+P[6] (2.9%; 31/1,076) and G1+G2P[4] (2.2%; 24/1,076) (Fig. 1).

RV strains were classified according to their possible origins as: common human strains (74.1%; 797/1,076); reassortant among common human strains (0.6%; 6/1,076); potential zoonotic strains (0.2%; 2/1,076) and possible human-animal hybrids (2.7%; 29/1,076) (Table II).

G2P[4] was the most common strain in all age groups: 3–5 months (46.9% [95% CI: 29.1-65.3]; 6–11 months (56.0% [95% CI: 49.3–62.6]); 12–23 months (60.9% [95% CI: 56.6–65.0]); ≥24 months (52.0% [95% CI: 46.0–58.0]). G1P[8] was the second most prevalent RV strain across the four age groups ranging between 6.3% and 18.6% (data not shown).

Between May 2008 and April 2009, the most commonly found multiple combinations were G2P[-Mixed] (79.5%; 31/39). GMixedP[4] (47.2%; 17/36) was frequently seen between May 2009 and April 2010. The majority (70.5%; 86/122) of either partially typed or fully untypeable RV strains were detected from May 2010 to May 2011 (Table II).

G2P[4] strains were identified throughout the study period, but predominated from May 2008 until December 2009. Frequency rates ranged from 100% (May and June 2008) to 29.4% (June 2009). An increase in G1P[8] strains was observed from

TABLE I. Baseline Characteristics of Children <5 Years of Age (N = 1,078)

Characteristics	Categories	n	Value	%
Age (months)	Mean	1,078	18.5	–
	SD		9.4	
Gender	Female	509	–	47.2
	Male	569	–	52.8
Race	African heritage	35	–	3.3
	Asian heritage	1	–	0.1
	White Caucasian	11	–	1.0
	Other*	1,030	–	95.6
	Missing	1	–	0.1
Currently live in Belem	Yes	971	–	90.1
	No	107	–	9.9

N, number of severe RVGE hospitalizations; n, number of subjects in a given category; value, value of the considered parameter; % = n/N × 100; Other*, mixed race; SD, standard deviation.

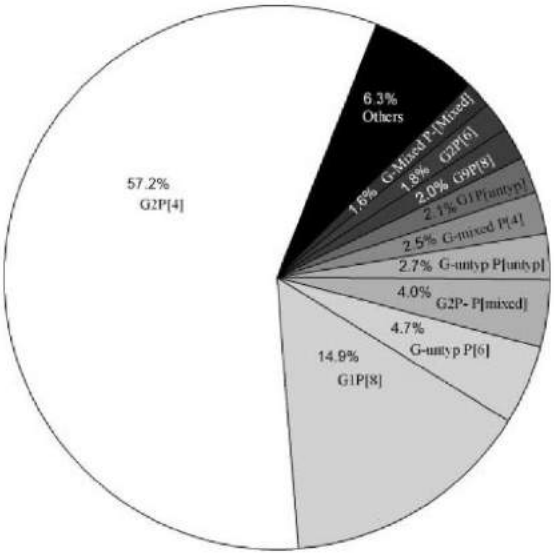

Fig. 1. Strain distribution (N = 1,076). Others = G12P[6], G2UNTYP, G1P-Mixed, G1P[6], G-Mixed P[8], G-Mixed, UNTYP P, G1P[4], G9P[4], G-Mixed P[6], G12P-Mixed, G9P[6], G9UNTYP, GUNTYPP-Mixed, G3P[8] and G4P-Mixed.

May 2010 to May 2011, representing 38.2% (112/293) of the circulating strains (Fig. 2). G1P[8] RV strains were not detected during the first 9 months of the study but were found at monthly low frequencies ranging from 2.5% (1/40) to 21.6% (6/37) from February 2009 until January 2010.

DISCUSSION

The present analysis is an extension of a previously published 12-month case-control study, which assessed the effectiveness of a full 2-dose series of RIX4414 vaccine in preventing severe RV gastroenteritis hospitalization in Belém [Justino et al., 2011]. The total duration of our RV strain surveillance was 36 months during which time it was essentially assessed if any significant changes in the temporal distribution of RV strains were evident following the introduction of the monovalent human rotavirus vaccine in the Brazilian EPI.

Overall, RV was identified in 17.2% of children who received treatment for severe gastroenteritis between May 2008 and May 2011. Although this follow up study was not designed to assess effectiveness or impact of the vaccine over the 3 years of study, this proportion of RV-positive cases among all GE cases was lower than that previously reported (46%) for Belém in 2002–2003 [Linhares et al., 2012], as well as other regions around Brazil (>30%) before the introduction of the vaccine [Carvalho-Costa et al., 2011; O'Ryan et al., 2011; Munford et al., 2009]. This is consistent with recent findings demonstrating a marked decline (59%) in hospitalizations of RV gastroenteritis among infants in the immediate post-vaccine era as compared with the pre-vaccine era [Sáfadi et al., 2010].

Although not individual rotavirus vaccination history was collected from participants, the majority of hospitalizations for severe RV gastroenteritis were seen in children age at least 12 months. These observations may warrant further investigation to assess the extension of long-term protection after 12 months of age, as demonstrated in pre-licensure efficacy studies. Indeed, phase III trials in Latin America and Europe have reported an efficacy for the first 2 years of life of 83% (73.1–89.7) and 96% (83.8–99.5), respectively, against hospital admission for rotavirus gastroenteritis [Vesikari et al., 2007; Linhares et al., 2008;]. Furthermore, in developed countries within Asia, vaccine efficacy against severe RV gastroenteritis was 96.9% (95% CI: 88.3–99.6%) during the first three years of life [Phua et al., 2012].

A marked increase in the relative prevalence of G2P[4] was observed during 2008 and 2009. This is consistent with findings from Brazil and Latin America, where a sharp increase in the fully heterotypic G2P[4] RV strain was seen during this time period. This trend was seen in countries with nationwide introduction of the monovalent human rotavirus vaccine [Munford et al., 2009; Carvalho-Costa et al., 2011; Dulgheroff et al., 2012; Oliveira et al., 2012] and more notably, also in countries where RV vaccination had not been implemented. Some South-American countries, such as Argentina and Paraguay had predominant G2P[4] strains even before introduction of rotavirus vaccine [Patel et al., 2011; Oliveira et al., 2012]. Furthermore, in Nicaragua, where a pentavalent rotavirus vaccine was introduced in 2006, one year later, G2P[4] was also identified in 88% of the rotavirus cases that required hospitalization [Patel et al., 2009]. During the first year of surveillance in a case-control study in Belém, G2P[4] accounted for 82.0% of RV gastroenteritis hospitalizations [Justino et al., 2011]. In contrast to the results of this study which showed an increase in the prevalence of G1P[8] starting in 2010, a recent 4-year follow-up study in Triângulo Mineiro, Brazil, showed that G2P[4] largely predominated over the other circulating strains in 2010, possibly reflecting a continuation of an "epidemic cycle" in this particular region [Dulgheroff et al., 2012]. These contrasting findings highlight the well-known temporal and geographical patterns in RV strains circulation [Santos and Hoshino, 2005; O'Ryan et al., 2011].

While it has been hypothesized that the 'emergence' of the G2P[4] strain may reflect a true shift in the RV strain distribution due to vaccine-induced selective pressure [Gurgel et al., 2008; Leite et al., 2008; Nakagomi et al., 2008; van Doorn et al., 2009; Linhares et al., 2011], it could possibly be due to natural strain fluctuation [Munford et al., 2009; van Doorn et al., 2009; Esteban et al., 2010; Kirkwood

TABLE II. Distribution of Rotavirus Strains Between 2008 and 2011 in Belém, Brazil (N = 1076)[a]

	May 2008–April 2009[b]		May 2009–April 2010		May 2010–May 2011		Total	
Genotype	N	%	n	%	n	%	n	%
Common human strains								
G1P[8]	11	2.1	37	14.5	112	38.0	160	14.9
G2P[4]	434	82.7	123	48.0	57	19.3	614	57.1
G3P[8]	0	0.0	0	0.0	1	0.3	1	0.1
G9P[8]	2	0.4	18	7.0	1	0.3	21	2.0
Reassortants among common human strains								
G1P[4]	0	0.0	3	1.2	0	0.0	3	0.3
G9P[4]	2	0.4	1	0.4	0	0.0	3	0.3
Potential zoonotic strains								
G9P[6]	1	0.2	1	0.4	0	0.0	2	0.2
Possible human-animal hybrid strains								
G1P[6]	1	0.2	3	1.2	6	2.0	10	0.9
G2P[6]	14	2.7	4	1.6	1	0.3	19	1.8
G12P[6]	11	2.1	0	0.0	0	0.0	11	1.0
Mixed infections (single G-genotype with multiple P-genotypes								
G1P[4] + P[6]	0	0.0	1	0.4	0	0.0	1	0.1
G1P[4] + P[8]	2	0.4	1	0.4	3	1.0	6	0.6
G1P[6] + P[8]	1	0.2	1	0.4	1	0.3	3	0.3
G2P[4] + P[6]	25	4.8	4	1.6	2	0.7	31	2.9
G2P[4] + P[8]	1	0.2	2	0.8	5	1.7	8	0.7
G4P[4] + P[6] + P[8]	1	0.2	0	0.0	0	0.0	1	0.1
G12P[4] + P[6]	2	0.4	0	0.0	0	0.0	2	0.2
G2P[4] + P[6] + P[8]	5	1.0	0	0.0	0	0.0	5	0.5
Mixed infections (multiple G-genotypes with a single P-genotype)								
G1 + G2P[4]	2	0.4	14	5.5	8	2.7	24	2.2
G1 + G2P[6]	0	0.0	0	0.0	2	0.7	2	0.2
G1 + G2P[8]	1	0.2	1	0.4	0	0.0	2	0.2
G1 + G9P[4]	0	0.0	1	0.4	0	0.0	1	0.1
G1 + G9P[8]	0	0.0	2	0.8	3	1.0	5	0.5
G1 + G2 + G9P[4]	0	0.0	2	0.8	0	0.0	2	0.2
Mixed infections (multiple G- and P-genotypes)								
G1 + G2P[4] + P[6]	1	0.2	3	1.2	0	0.0	4	0.4
G1 + G2P[4] + P[8]	0	0.0	1	0.4	6	2,0	7	0.7
G1 + G2P[6] + P[8]	1	0.2	0	0.0	0	0.0	1	0.1
G2 + G9P[4] + P[8]	0	0.0	2	0.8	0	0.0	2	0.2
G1 + G2P[4] + P[6] + P[8]	2	0.4	1	0.4	0	0.0	3	0.3
Partially genotyped (G-genotyped and P-untypeable)								
G1P[NT]	0	0.0	8	3.1	15	5.1	23	2.1
G2P[NT]	1	0.2	4	1.6	5	1.7	10	0.9
G9P[NT]	0	0.0	1	0.4	0	0.0	1	0.1
G1 + G2P[NT]	0	0.0	1	0.4	1	0.3	2	0.2
G1 + G9P[NT]	0	0.0	4	1.6	1	0.3	5	0.5
Partially genotyped (G-untypeable and P-genotyped)								
GNTP[6]	2	0.4	8	3.1	41	13.9	51	4.7
GNTP[4] + P[6]	1	0.2	0	0.0	0	0.0	1	0.1
G and P-untypeable								
GNTP[NT]	1	0.2	4	1.6	24	8.1	29	2.7
Total	525	100	256	100	295	100	1076	100

n, number of subjects in a given category; N, number of severe RVGE hospitalizations.
[a]Two samples were excluded from RV-testing by PCR.
[b]Nucleotide sequencing was performed with strains untyped by RT-PCR during this period only.

et al., 2011; Matthijnssens and Van Ranst, 2012]. This 3-year RV strain distribution surveillance study in Belém provides additional evidence to support the latter hypothesis, as the sharp decline in the relative prevalence rates of G2P[4] was followed by an increase in the detection of G1P[8] strains. Another recent study from Northern Brazil found similar patterns: G2 strains displayed a typical cyclical pattern of occurrence and re-emergence during the 2006–2008 period [Oliveira et al., 2012].

However, these findings remain potentially inconclusive for two reasons: firstly, the monovalent human rotavirus vaccine is composed of a G1P[8] species A, an RV strain related to the Wa-like genotype constellation, that fully differs from G2P[4], which possesses the DS-1-like genotype constellation [Matthijnssens et al., 2012]. Secondly, the decline in prevalence rates of G2P[4] in this study might also be influenced by an increasing proportion of children aged below 5 years who might had previously been

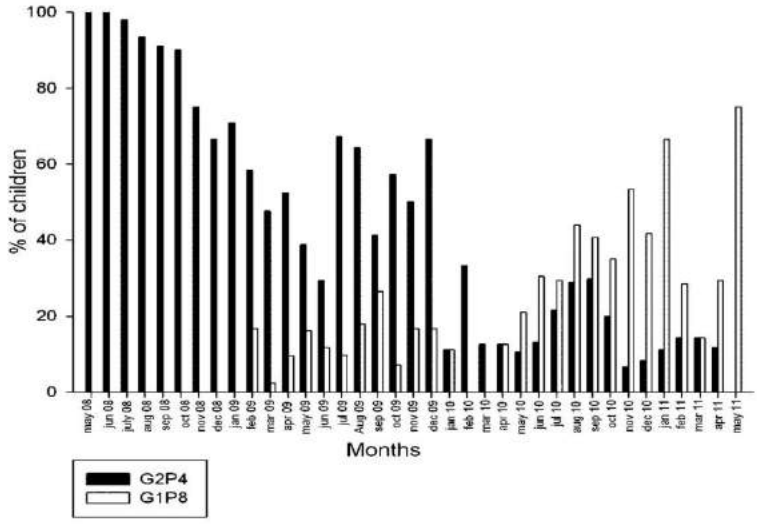

Fig. 2. Annual distribution of G2P[4] and G1P[8]. % = n/N × 100 N, number of severe RVGE hospitalizations; n, number of subjects in a given category.

infected with circulating G2P[4] strains and had developed homotypic immunity.

The trend for higher prevalence rates of G1P[8] could be interpreted as resulting from an apparent decline in protection after 1 year of age, as suggested by post-licensure studies conducted in Brazil and Latin America, even though further investigation on this particular issue is required [Gentsch et al., 2005; Correia et al., 2010; de Palma et al., 2010; Justino et al., 2011; O'Ryan et al., 2011; Patel et al., 2012].

A remarkable variability was observed in co-circulating strains from January 2010 onwards; the majority was either partially typed or fully untypeable strains, or mixed infections. Mixed infections, which may have occurred due to exposure of children to a heavily contaminated environment, were predominantly represented by G2P[4] + P[6] and G1 + G2P[4] strains which were common throughout the study period and could possibly challenge the RV vaccine effectiveness [Gentsch et al., 1996; Fischer et al., 2005; Santos and Hoshino, 2005].

A finding of particular interest was the detection of a high proportion of untypeable RV strains during 2010–2011, in comparison with the previous follow-up period. This occurrence may reflect the circulation of common RV strains that underwent genetic variation, and is supported by studies showing that standard RT-PCR methods may fail to determine genotype-specificities, due to possible silent mutations in the primer-binding site [Iturriza-Gómara et al., 2000; Soares et al., 2012]. We were unable to detect RVs bearing G12 type-specificity during the second and third years of follow-up, which, according to a study in Northern Brazil, is a recently emerging strain [Matthijnssens et al., 2010; Soares et al., 2012].

Another plausible explanation for the emergence of new RV strains is that in this study, potential zoonotic strains (G9P[6]) and strains, which are likely to originate from reassortment between human and animal RV strains (G1P[6] and G2P[6]) were detected at very low frequencies, suggesting that they do not spread efficiently among humans. Nonetheless, one cannot rule out the possibility that such unusual strains were generated through reassortant events involving common circulating human strains and the emerging G12P[6] strain.

The main limitation in this study was that vaccine protection was assessed only during the first year of surveillance where effectiveness against G2P[4] was75%. This study was not designed to evaluate vaccine protection during the remaining two years of follow-up. A possible limitation of this study is the difference in sample size over the four year study period: in the first two years we covered 80% of severe gastroenteritis cases in Belem compared with only 50% of cases in the remaining two years. In addition, the set of primers that were used did not target either the G12 or G5 type-specificities at least for the second and third years of surveillance, where nucleotide sequencing was not performed, we may have missed detecting G12 RV strains bearing either P[6] or P[8] types. These are known to have emerged worldwide and may possibly have arrived in the Northern region of Brazil [Matthijnssens et al., 2010; Soares et al., 2012]. An additional limitation of the current study was the lack of complete analysis of the entire RV genotype constellations for a long-term

assessment of vaccine effect on strain type, as based on the currently adopted classification of rotaviruses [Matthijnssens et al., 2012]. In this regard, molecular analyses to identify lineages from G1P[8] and G2P[4] genotypes are worth to be done for a better understanding of strain fluctuation over time.

Finally, although the monitoring of RV strains in our study was conducted over a relatively extended period, continued surveillance would be useful in detecting trends in the occurrence of the prevailing and potentially emerging new strains that may pose a challenge to the currently licensed RV vaccines. In conclusion, G2P [4] was predominantly observed during the first 20 months of our study, followed thereafter by G1P[8], which is suggestive of natural RV strain fluctuation over time, rather than vaccine-induced selective pressure on circulating RV strains. Future strain surveillance activities will be beneficial to further clarify the overall impact of RV vaccines.

TRADEMARK

Rotarix is a registered trademark of the Glaxo SmithKline group of companies.

Rotateq is a registered trademark of Merck & Co. Inc.

RIDASCREEN is a registered trademark of R-Biopharm, Darmstadt, Germany.

ACKNOWLEDGMENTS

We gratefully acknowledge all the staff at Clínica Santa Terezinha, Clínica Pio XII, Hospital Serzedelo Correa and Policlínica Infantil de Nazaré, in Belém, Pará, Brazil, who were involved in the conduct of the study. We also acknowledge all participating children and their parents/guardians. The authors thank Julia Donnelly for language editing on behalf of Glaxo SmithKline group of companies, Preethi Govindarajan for publication writing, and Ingrid Leal and Vinicius Costa for publication coordination (all employed by GlaxoSmithKline group of companies). We would also like to thank Dr. Tatiana Lanzieri for her involvement in this study and Dr. D. Fermin Arguello who contributed to the analysis of the results and the preparation and review of the first draft. All the authors had full access to the data and the corresponding author took the final responsibility for submitting the manuscript.

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A análise da frequência do genótipo G9 no ano de 2013 não foi descrita nos manuscritos acima. Em 2013, observou-se uma frequência de 20,6% das infecções por RVA nos casos diarreicos, estando o genótipo G9 envolvido em 7,5% dos casos de RVA caracterizados.

6.6 - MOLECULAR ANALYSIS OF G9 ROTAVIRUS DURING 15 YEAR PERIOD IN NORTHERN BRAZIL.

O presente manuscrito teve como objetivo descrever a análise dos 11 genes de RVA genótipo G9 associados aos tipos P[6], P[6] e P[8].

A análise do gene que codifica a proteína VP7 demonstrou que todas as amostras agruparam na linhagem III de G9 (linhagem contemporânea). Quanto ao gene que codifica a proteína VP4, as amostras P[8] agruparam na linhagem III, enquanto que as P[6] na linhagem I e as amostras P[4] foram identificadas na linhagem V.

Quanto aos demais genes analisados (VP1, VP2, VP3, VP6, NSP1 – NSP5), observou-se que as amostras G9P[8] agruparam no genogrupo 1 dos respectivos genes (R1, C1, M1, I1, A1, N1, T1, E1, H1), com exceção da amostra RVA/Human-wt/BRA/PA1A2053/2010/G9P[8] que apresentou genogrupo 2 no gene que codifica a proteína NSP3. As amostras G9P[4] e G9P[6] agruparam no genogrupo 2 dos respectivos genes (R2, C2, M2, I2, A2, N2, T2, E2, H2).

Desta forma, as constelações observadas nas amostras G9 da região Norte foram Wa-like (genogrupo 1) e DS1-like (genogrupo 2).

Molecular analysis of G9 rotavirus during 15 year period in Northern Brazil

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Rotavirus (RV) is a very common cause of severe diarrhoea in children aged <5 worldwide, estimating that is responsible for 215 000 deaths in children of this age group in 2013 (Tate et al., 2016).

This viral agent belongs to *Reoviridae* Family, *Rotavirus* genus and is classified into nine groups/species (A – I), being Rotavirus A (RVA) account for the majority of infections in humans (Matthijnssens et al., 2012; Mihalov-Kovács et al., 2015). Its genome comprising 11 double-stranded RNA segments that encodes six structural proteins (VP1-VP4, VP6 and VP7) and five or six non-structural proteins (NSP1-NSP5, eventually NSP6), with each protein present specific function in morphology and viral pathogenesis, assigns a specific genotype, being recognized currently 32G-46P-24I-18R-17C-17M-28A-17N-19T-24E-19H (Matthijnssens et al., 2008; Estes; Greenberg, 2013; Li et al., 2016).

On the basis of whole genome analyses of most human RVA , two genogroups constellations are known to circulate including Wa-like (I1-R1-M1-A1-N1-E1-H1), usually associated to G1P[8], G3P[8], G4P[8], G9P[8], G12P[8] strains, or DS-1-like (I2-R2-M2-A2-N2-E2-H2) related to G2P[4] and G9P[6] strains (Matthijnssens et al., 2008; Patton, 2012)

Due to epidemiologic impact of RVA, two oral vaccines, a monovalent human vaccine, *Rotarix*^{TM} (GlaxoSmithKline Biologicals,Rixensart, Belgium) and a pentavalent bovine–human reassortant vaccine, RotaTeq^{TM} (Merck Vaccines, Whitehouse Station, NJ, USA), are recommended for worldwide by WHO, with both immunizants providing protection against a broad variety of RVA strains, mostly to common genotypes, and have been reduced significantly gastroenteritis-related hospitalizations and deaths frequency worldwide (Costa et al., 2016; Greenwood, 2014; Linhares & Justino, 2014).

The common genotypes frequently detected in RVA infections are: G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8], being G9 and G12 emerged

and spread worldwide at 1990s and 2000s years , respectively, and now are considered genotypes more detected among the human infections (Guerra et al., 2015; Soares et al., 2014; Matthijnssens *et al.*, 2009).

G9 genotype was first reported in United States in 1983 and soon in Japan, India and Thailand (Clark et al., 1987; Nakagomi et al., 1990; Das et al., 1993; Urasawa et al., 1992). This genotype became undetectable for about one decade and then reemerged worldwide demonstrating highly incidence (Das et al., 1994; Unicomb et al., 1999; Cunliffe et al., 1999; Cubitt et al., 2000; Griffin et al., 2000; Maneekarn; Ushijima, 2000; Oka; Nakagomi; Nakagomi, 2000).

In Latin America, a study conducted by Barril et al. (2006) showed G9 circulation in the 1980s, becoming the Latin country with the oldest description of G9. In Brazil, this genotype was described circulating in Rio de Janeiro, Goiânia, Salvador, São Paulo, Rio Branco, Belém and anothers settings, demonstrating the high frequency of G9 (Linhares et al., 1996; Oliveira et al., 2012; Araújo et al., 2001; Santos et al., 2001, 2005; Souza et al., 2003; Volotão et al., 2005; Carmona et al., 2006; Montenegro et al., 2007; Ribeiro et al., 2008).

In Brazil was observed G9 genotype circulation in pre vaccine era, from 1996-2005, with 27% of frequency, and, in pos vaccine period, was detected in 11% of cases, showing constant circulation of this genotype (Leite; Carvalho-Costa; Linhares, 2008).

Epidemiological studies on RVA G9 infections G9 have demonstrated significant genetic diversity of this genotype, being associated with different P types and possessing Wa-like or DS-1-like genomic constellation or combination of both (Hoshino et al., 2004; Page et al., 2010; Matthijnssens & Van Ranst, 2012; Ghosh et al., 2012; Patton, 2012; Esona et al., 2013; Lewis et al., 2014).

This study aimed to describe genetic constellation of G9 genotype during fifteen years to explore genetic variability of this genotype during pre and post-vaccine era.

MATERIALS AND METHODS

Clinical Specimens

Clinical specimens selected for this study were recovered from children with RV gastroenteritis, who participated of viral gastroenteritis studies conducted in

Northen region of Brazil, between 1998 and 2013. Children were classified into vaccinated (collected samples from children after implementation of RVA vaccine and received at least one dose) and non vaccinated (collected samples before RVA vaccine implementation and after implementation, however, unvaccinated or status not informed)

Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR)

Viral RNA of samples previously genotyped as G9 was extracted using guanidinium isothiocyanate-silica, as described by Boom et al (1990). Structural and nonstructural genes were partially amplified by RT-PCR using primers described by Varguese et al. [2006], Gentsch et al. [1992], Both et al. [1984], Gouvea et al. [1990], Matthijnssens et al. [2006] and Cunliffe et al. [1997], for amplification of VP1 (686 bp), VP2 (686 bp), VP3 (702 bp), VP4 (876 bp), VP6 (1356 bp), VP7 (1062 bp), NSP1 (1565 bp), NSP2 (1038 bp), NSP3 (1062 bp), NSP4 (738 bp), and NSP5 (664 bp) genes. PCR amplicons were purified using the QIAquick PCR purification kit (QIAGEN®) posteriorly.

Nucleotide Sequencing and Phylogenetic Analysis

Nucleotide sequencing of PCR amplicons for all genes were performed using the same primers as those used in RT-PCR and were sequenced using Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) according to manufacturer instructions. The sequences were collected from an automated ABI Prism 3130xl DNA sequencer (Applied Biosystems). Phylogenetic analysis was conducted using MEGA software program version 7 by neighbor-joining (NJ) method [Kimura, 1980]. The statistical significance of genetic relationships was estimated by bootstrap resampling analysis (2,000 replications). The partial nucleotide sequences determined in this study were deposited in GenBank under the accession numbers XXX

Ethics

This study was approved by Evandro Chagas Institute's Human Research Ethics Committee, protocol number 0047/2011, in accordance with National Health Council's Resolution 466/2012.

Results

A total of 50 RVA G9 were obtained and analyzed for all RVA genes , being 50 VP7, 50 VP4, 50 VP1, 50 VP2, 49 VP3, 47 VP6, 40 NSP1, 48 NSP2, 50 NSP3, 50 NSP4 and 48 NSP5 with 45 G9P[8], 3 G9P[6] and 2 G9P[4], including vaccinated and non vaccinated children.

VP7 and VP8* sequence and phylogenetic analysis

Phylogenetic analyses based on VP7 gene showed that G9 strains clustered into lineage III, with similarity rates between 95.9-100% and 97-100% for nucleotide (nt) and amino acids (aa), respectively (Fig 1a). It was observed amino acid (aa) substitutions between G9 Brazilian strains and Rotateq^{TM} in neutralizing epitopes (7-1a, 7-1b) in residues 87, 94 and 242, whereas with Rotarix^{TM} showed substitutions in 7-1 epitope (residues 87, 94, 96, 123, 125, 129, 212, 213, 238 and 242) and 7-2 epitope (146, 147, 217 and 221)(Figure 2).

With regards VP4 gene, VP8* portion, phylogenetic analyses demonstrated that all P[8] strains belonged to lineage III, obtained nucleotide and amino acid similarities ranging from 95.5- 100% and 95.9-100%, respectively, that grouped in several clusters: (a) strains detected between 2008 and 2010, including vaccinated and non vaccinated children; (b) strains detected in 2006 and 2008 to 2010, including vaccinated and non vaccinated children; (c) strains detected from 1999 to 2001 and 2013; (d) strains detected between 2002 and 2008 and (e) strains detected in 2002 (Fig 1b). The aa sequence of neutralizing epitopes of VP4 was compared with vaccines strains (Rotarix^{TM} and Rotateq^{TM}), observing substitutions in residues 150, 195 and 196 from 8-1 epitope and 113 from 8-3 epitope. In residues 125, 131 and 135 from 8-3 epitope Brazilian G9P[8] strains showed aa substitution only with Rotarix^{TM} (Figure 3).

Phylogenetic analyses of P[4] and P[6] genotypes showed that strains belonged to lineage P[4]-V and P[6]-I, respectively (data not show).

Sequence analysis of genes encoding structural proteins (VP) The VP1, VP2, VP3 and VP6 gene analysis demonstrated that each gene of Brazilian strains grouped in two genotypes (1 and 2) (Figure 4)

Phylogenetic analysis based on VP1 gene showed that G9P[8] Brazilian strains grouped into R1 genotype with nt and aa similarities ranged from 91.5-100% and 95.9 – 100%, respectively, whereas for G9P[4] and G9P[6] strains grouped into R2 genotype with 95- 99.8% and 99-100% of nt and aa similarities, respectively.

It was observed similar characteristic in VP2 gene, where G9P[8] Brazilian strains with C1 genotype demonstrated similarity of 88.8 - 100% for nt and 93.1-100% for aa. For C2 genotype, G9P[4] and G9P[6] strains nt and aa similarity ranged from 97.6 - 99.3% and 97.1-100%, respectively.

Phylogenetic analysis of VP3 gene showed that G9 Brazilian strains grouped into two genotypes, M1 and M2, being that M1 genotype G9P[8] strains presented 88.5-100% and 92.4-100% of nt and aa similarities, respectively. In M2 genotype was observed G9P[4] and G9P[6] strains with nt similarity ranged drom 97.1-100% and aa similarity was 98.4-100%.

VP6 gene was analyzed and demonstrated that G9 strains clustered into I1 and I2 genotypes. G9P[8] Brazilian strains that grouped into I1 showed of nt identity values of 88.2-99.9% and aa identity was 94.2-100%. In I2 genotypegrouped G9P[4] and G9P[6] strains, that showed nt and aa similarity ranged from 96.2-99.6% and 97.2-99.5%, respectively.

Sequence analysis of genes encoding nonstructural proteins (NSP)

Phylogenetic analysis based on NSP1-NSP5 nucleotide sequences demonstrated that Brazilian strains grouped into two diferentes genotypes in all genes.

NSP1 gene was observed grouping in A1 and A2 genotype. Our G9P[8] samples grouped in A1 genotype with nt and aa similarity among then of 79.7-99.5% and 75.7-99.1%, while in A2 was 95% and 94.33% with nt and aa identity, respectively

NSP2 gene showed two groups in phylogenetic analysis, N1 and N2, grouped in N1 G9P[8] strains with nt and aa similarity among then of 82-99.2% and 84.1-99.7%, respectively. In N2 group, observed 96.3-99.9% of nt similarity and 96.3-100% of aa identity.

Analysis of NSP3 gene showed that G9P[8] Brazilian strains grouped into T1 genotype with nt and aa similarity among then of 90.9-100% and 91.8-100%, respectively, while in T2 genotype clustered G9P[4] and G9P[6] Brazilian strains, as

well as the strain RVA/Human-wt/PA2053/2010/G9P[8], with nt similarity of 96.9-99.8% and aa similarity of 96.2-100%.

Phylogenetic analysis based on NSP4 gene demonstrated that G9 Brazilian strains were E1 and E2 genotypes, being G9P[8] strains E1 genotype presented nt and aa identities ranged from 91.1-100% and 93.9-100%, respectively, whereas E2 genotype grouped G9P[4] and G9P[6] strains with nt identity among samples ranged from 87-100% and aa identity values of 90.8-100%.

With regards NSP5 gene, nucleotide sequence analysis showed that our samples grouped into H1 and H2 genotypes, with G9P[8] strains bearing H1 genotype with nt and aa similarities ranged from 95.7-100% and 97.8-100% respectively. In H2 genotype grouped G9P[4] and G9P[6] strains with nt and aa identity values among 95.7-100% and 98.5-100%, respectively.

Genome Constellation G9 RVA strains

The constellation observed in G9 samples isolated in Northern region of Brazil was G9-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1, G9-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2, G9-P[6]-I2-R2-C2-M2-A2-N2-T2-E2-H2 and one sample G9-P[8]-I1-R1-C1-M1-A1-N2-T1-E1-H1, revealing Wa-like and DS1-like genoma constellations.

DISCUSSION AND CONCLUSION

G9 RVA was considering an emergent genotype, revealing significant relevance in epidemiology of RVA in worldwide. In Brazil, this genotype was the protagonist of an outbreak has obtained many deaths, in pre vaccine period (Siqueira et al., 2010).

G9 genotype was historically associated with differs P-types (Hoshino et al., 2004; Bányai et al., 2004; Santos; Hoshino, 2005; Amino et al., 2013; Okitsu et al., 2013; Midgley et al, 2014). In this study, were characterized G9 strains associated with P[4], P[6] and P[8], being G9P[8] RVA the most detected strains in another studies during pre and pos vaccine introduction (Khamrin et al., 2006; donato et al., 2012; Esona et al., 2013; Dóro et al., 2014; Gómez et al., 2014)

In the present study it was observed that VP7 gene of G9 strains grouped into lineage III , as well as described in several studies conducted in worldwide since

mid-1990s, being this lineage cited as cosmopolitan(Kirkwood et al. (2003), Hoshino et al. (2005), Parra et al., 2005; Lin et al., (2006); Esona et al (2013) is therefore named lineage cosmopolitan, although had been related the existence of six lineages of G9 RVA (Martinez-Laso et al., 2009). Anothers studies with G9 RVA Brazilian was developed by Araújo et al., (2007); Tort et al. (2010) and Gómez et al., (2014), which observed predominance lineage III G9, contemplating samples pre and pos vaccine introduction, such as in the present study.

Analysis of VP7 neutralizing epitopes of G9 strains demonstrated aa substitutions when compared compared to vaccine strains. These modifications presents in pre and pos vaccine G9 strains were describe by Page et al (2010), Zeller et al (2012), Esona et al (2013), Than et al (2013) and Gómez et al (2014) in studies conducted in Africa, Belgium, Cameroon, South Korea and Brazil, respectively, reveling aa changes presented in G9 strains before and after RVA vaccine introduction in worldwide.

Analysis of VP4 gene showed that all P[8] samples grouped into lineage III and, despite having formed groups, showed high nt and aa similarities. The lineage P[8]-IIIwas associated to G9 in studies developed by Espínola et al. (2008), Paul et al. (2008) Rahman et al (2008), Donato et al. (2012) and Ianiro et al. (2013). Araújo et al (2007), Tort et al. (2010) and Gómez et al (2014) observed the same association in Brazilian G9P[8] strains. Gómez et al. (2014) also described the formation of clusters, showing the existence of different alleles circulating among G9 RVA Brazilian population. Although P[8] vaccines grouped into different lineages (P[8]-I and P[8]-II), tests prior to vaccines introduction demonstrated satisfactory results against G9 RVA strains (Araújo et al., 2007; O'ryan; Linhares, 2009; Justino et al., 2010; Lanzieri et al., 2010)

Analysis of aa neutralizing epitopes of VP4 geneshowed more aa substitutions when compared with Rotarix^{TM}, vaccine adopted in the public health system. The changes observed in present study was reported by Zeller et al (2012) in VP4 gene of G9P[8] samplesisolated in Belgium and by Gómez et (2014) with G9P[8] Brazilian strains.

P[4] and P[6] were associated to G9 and grouped into lineageP[4]-V and P[6]-I, respectively. One of the first reports in Latin America of G9P[4] was in Brazilian samples by Santos et al (2001) during pre-vaccine era. Posteriorly, Yen et al. (2011) detected G9P[4] strains in Mexico in era pos vaccineand this was the

second predominant genotype in Honduras, as reported by Quaye et al., 2013. Lewis et al (2014) described detection of G9P[4] genotype in USA and observed more similaries of P[4] associated with G2 and not to G9, such related in this study This fact may be associated with high prevalence of G2P[4] RVA in pos-vaccine era, mainly lineage P[4]-V as stated by Gómez et al (2014) with Brazilian samples. P[6] genotype is frequently associated to neonatal and animal infections (mainly pigs) (Mascarenhas et al, 2007). In this study, was more similar to human G9P[6] RVA strains described by Kirkwwod et al (1999) in United States and Page et al (2010) in Africa.

Phylogenetic analysis of others structural and non-structural genes of G9 samples Brazilian showed that G9P[8] was associated to genogroup 1 (R1 [VP1], C1 [VP2], M1 [VP3], I1 [VP6], A1 [NSP1], N1 [NSP2], T1 [NSP3], E1 [NSP4] and H1 [NSP5]), whereas G9P[4] and G9P[6] grouped in genogroup 2 (R2 [VP1], C2 [VP2], M2 [VP3], I2 [VP6], A2 [NSP1], N2 [NSP2], T2 [NSP3], E2 [NSP4] and H2 [NSP5]) presenting varied similarity because reported, some samples, highest homology with genes of different G genotypes, such as observed in study by Esona et al (2013) with G9P[8] Cameroon strains, indicating the high genetic variability obtained by genome reassortment.

The RVA/Human-wt/BRA/PA1A2053/2010/G9P[8] was an exception, exhibiting NSP3 gene with genogroup 2 (T2), another genome reassortment that can occur in one or more genes of RV, which was described by Nyaga et al (2013) with G9P[6] and G9P[8] strains in African, Dóro et al. (2014) with G9P[8] Hungary strains, Lewis et al. (2014) with G9P[4] USA strains, Kuzuya et al (2014) with G1P[8] Japan strains, Komoto et al (2015) with G1P[8] Thailand strains, Silva et al (2015) with G1P[8] Brazilian samples, Arana et al (2016) with G3P[8] Spain samples and Cowley et al (2016) with G3P[8] Australian samples, various genotypes in different locations, allowing greater viral genetic variability.

In this study, was observed the association of G9P[8] samples to Wa-like constellation, while G9P[6] showed DS1-like constellation. This association were previously described by Jere et al. (2011) with G9P[6] African strains, Ianiro et al (2013) with G9P[8] Italian samples, Esona et al (2013) with G9P[8] strains from Cameroon, Nyaga et al (2013) in African with characterization of G9P[6] and G9P[8] strains. Gómez et al (2014) related the circulation in Brazil in pre and pos introduction

vaccine of G9P[8] strains associated with Wa-like constellation, both vaccinated and unvaccinated children, like this study.

Not many studies involving the characterization of all genes from G9P[4] samples were developed, due to be considered a unusual combination, however, this samples showed high similarity with DS1-like constellation of G2P[4], being such fact observed in pos vaccine introdution era, where the circulation of G2P[4] was related in worldwide, including Brazil (Leite et al., 2008; Patel et al., 2008; Carvalho-Costa et al., 2009; Kirkwood et al., 2009; Mascarenhas et al., 2010; Esteban et al, 2010; Oliveira et al., 2012; Justino et al., 2012).

Thus, observed in present study that G9 samples in Brazilian, pre and pos introduction of vaccine period and vaccinated/unvaccinated children, not showed high divergence during the period analyzed, and although there is genetic diversity, was detected in almost samples regardless of the period of collection and vaccination situation of children.

However, genome reassortment were detected, showing the potential genetic variability of this genotype and need for continued surveillance of virus types circulating which can pose a challenge vaccines used worldwide over time.

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Figure 1: Phylogenetic analysis of the VP7 (A) and VP4 (B) proteins of RVA strains circulating in northen Brazil with known human RVA strains from GenBank database. Neighbor-joining trees were constructed using nucleotide sequences of VP7 and VP4. Our strains are indicated with a black triangle in pré vaccine strains, black circle in pos vaccine era strains from non vaccinated children and black quadrate in pos vaccine era strains from vaccinated children.

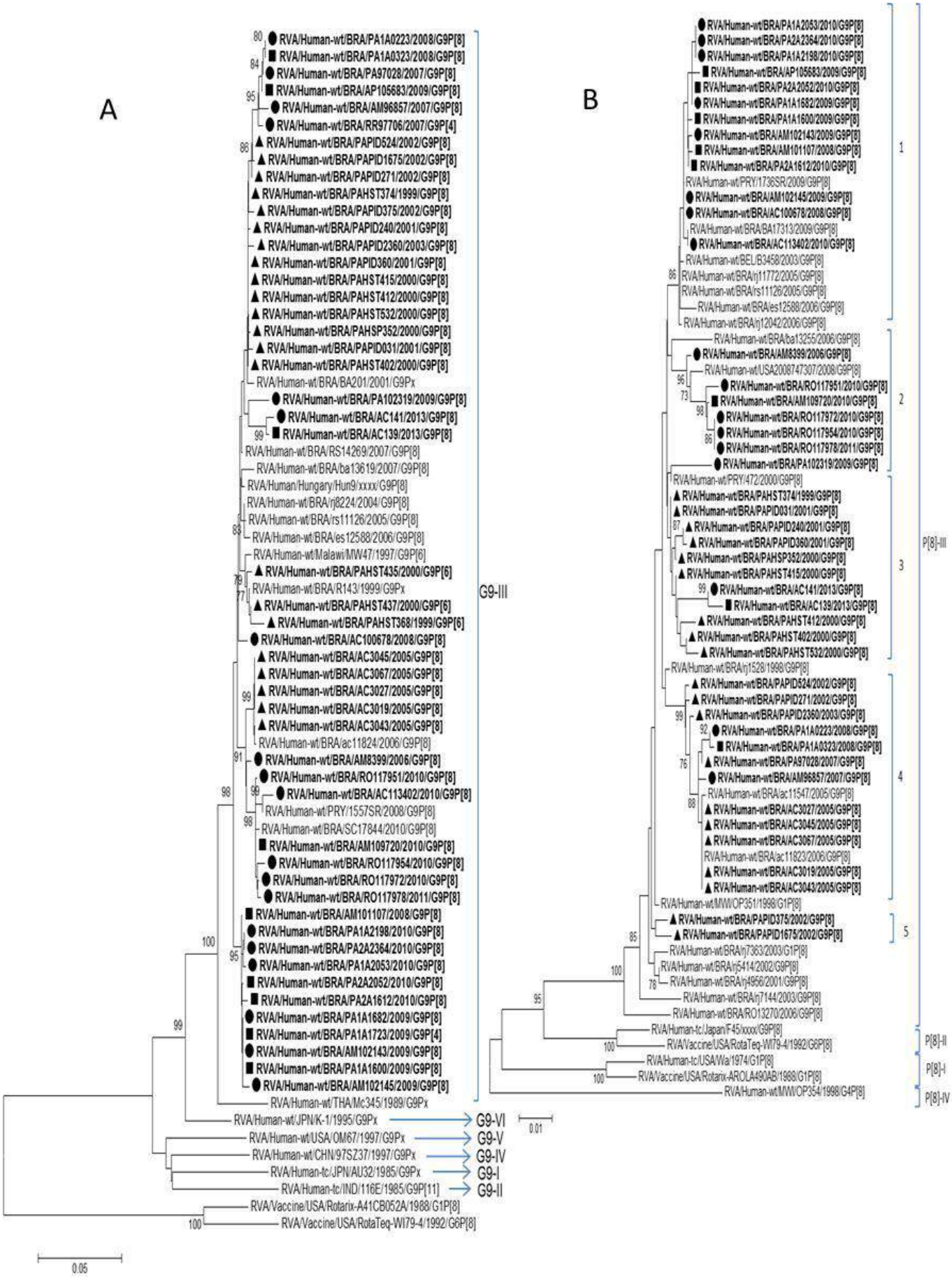
Figure 2: Alignment of antigenic residues in VP7 of the strains contained in Rotarix and RotaTeq and G9 strains circulating in northen Brazil from 1999 to 2013

Figure 3: Alignment of antigenic residues in P[8] VP4 of the strains contained in Rotarix and RotaTeq and G9 strains circulating in northen Brazil from 1999 to 2013

Figure 4: Phylogenetic analysis of the VP1 (A), VP2 (B), VP3 (C) and VP6 (D) proteins of RVA strains circulating in northen Brazil with known human RVA strains from GenBank database. Neighbor-joining trees were constructed using nucleotide sequences of each gene. Our strains are indicated with a black triangle in pré vaccine strains, black circle in pos vaccine era strains from non vaccinated children and black quadrate in pos vaccine era strains from vaccinated children.

Figure 5: Phylogenetic analysis of the NSP1 (A), NSP2 (B), NSP3 (C), NSP4 (D) and NSP5 (E) proteins of RVA strains circulating in northen Brazil with known human RVA strains from GenBank database. Neighbor-joining trees were constructed using nucleotide sequences of each gene. Our strains are indicated with a black triangle in pré vaccine strains, black circle in pos vaccine era strains from non vaccinated children and black quadrate in pos vaccine era strains from vaccinated children.

Figure 1


A
B
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G9-VI
G9-V
G9-IV
G9-I
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P[8]-III
P[8]-II
P[8]-I
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PAH5T374/99 G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K		Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
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AC3019/2005/G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K		Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
PA97028/2007G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K		Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RR97706/2007G9P[4]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K		Q	N	A	A	D	N	K	D	S	T	L	S	E	S	G
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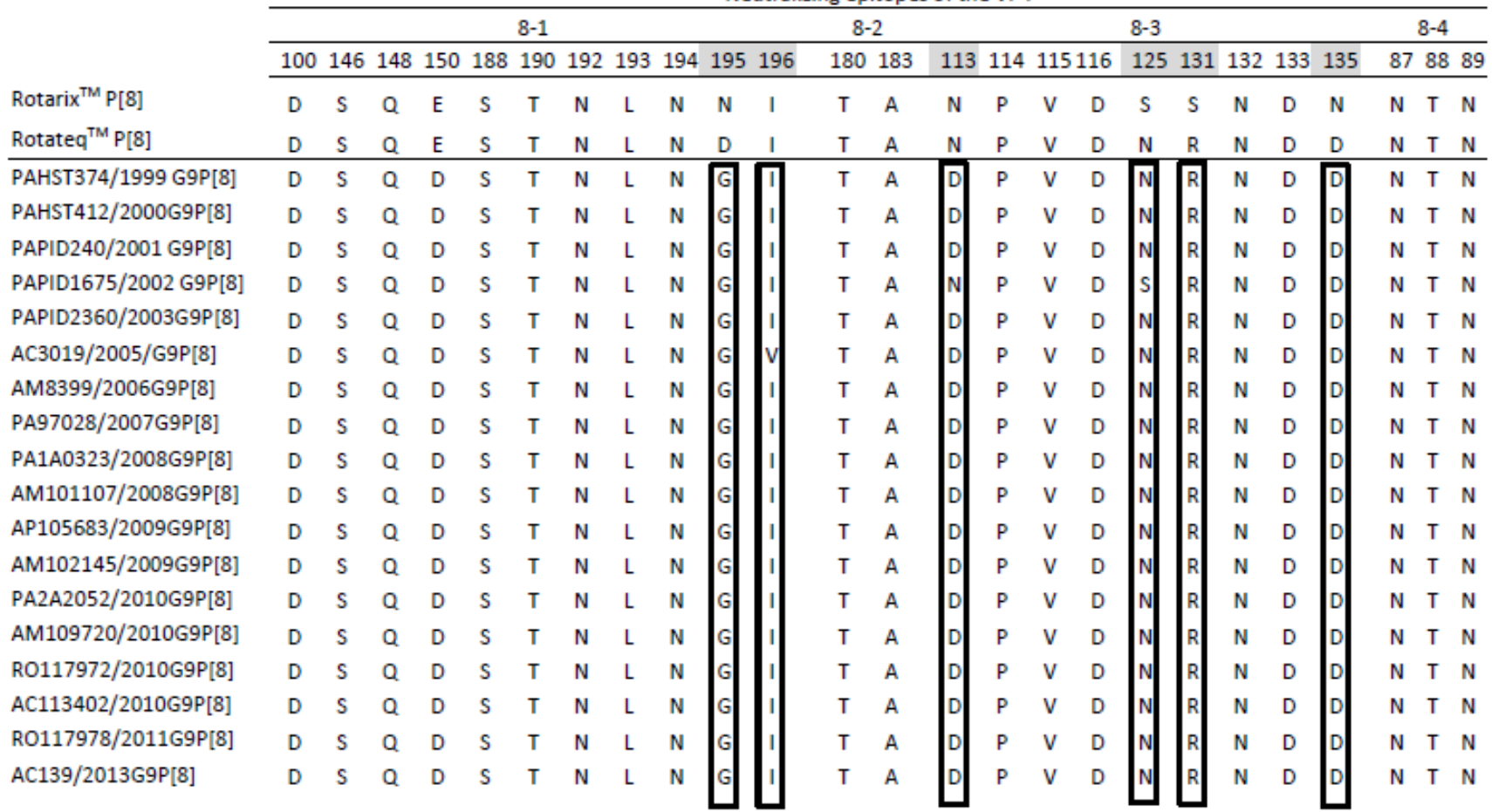
RO117951/2010G9P[8]	T	T	G	T	E	W	K	N	Q	D	A	I	D	K		Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RO117972/2010G9P[8]	T	T	G	T	E	W	K	N	Q	D	A	I	D	K		Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RO117954/2010G9P[8]	T	T	G	T	E	W	K	N	Q	D	A	I	D	K		Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
AC113402/2010G9P[8]	T	T	G	T	E	W	K	N	Q	D	A	I	D	K		Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
RO117978/2011G9P[8]	T	T	G	T	E	W	K	N	Q	D	A	I	D	K		Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G
AC139/2013G9P[8]	T	T	G	T	E	W	K	D	Q	D	A	I	D	K		Q	N	T	A	D	N	K	D	S	T	L	S	E	S	G



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Rotateq™ P[8]	D	S	Q	E	S	T	N	L	N	D	I	T	A	N	P	V	D	N	R	N	D	D	N	T	N
PAHST374/1999 G9P[8]	D	S	Q	D	S	T	N	L	N	G	I	T	A	D	P	V	D	N	R	N	D	D	N	T	N
PAHST412/2000G9P[8]	D	S	Q	D	S	T	N	L	N	G	I	T	A	D	P	V	D	N	R	N	D	D	N	T	N
PAPID240/2001 G9P[8]	D	S	Q	D	S	T	N	L	N	G	I	T	A	D	P	V	D	N	R	N	D	D	N	T	N
PAPID1675/2002 G9P[8]	D	S	Q	D	S	T	N	L	N	G	I	T	A	N	P	V	D	S	R	N	D	D	N	T	N
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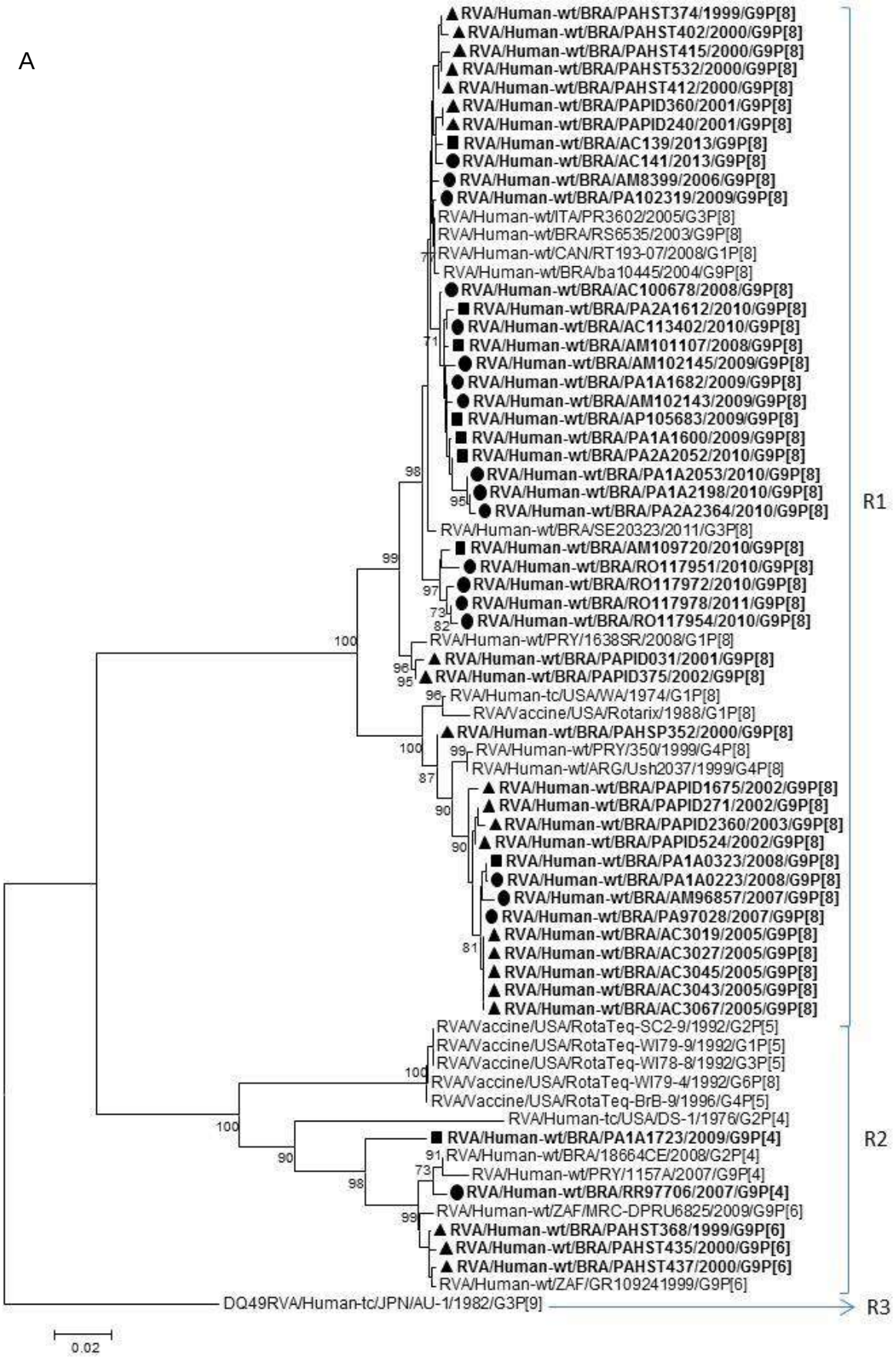
Figure 4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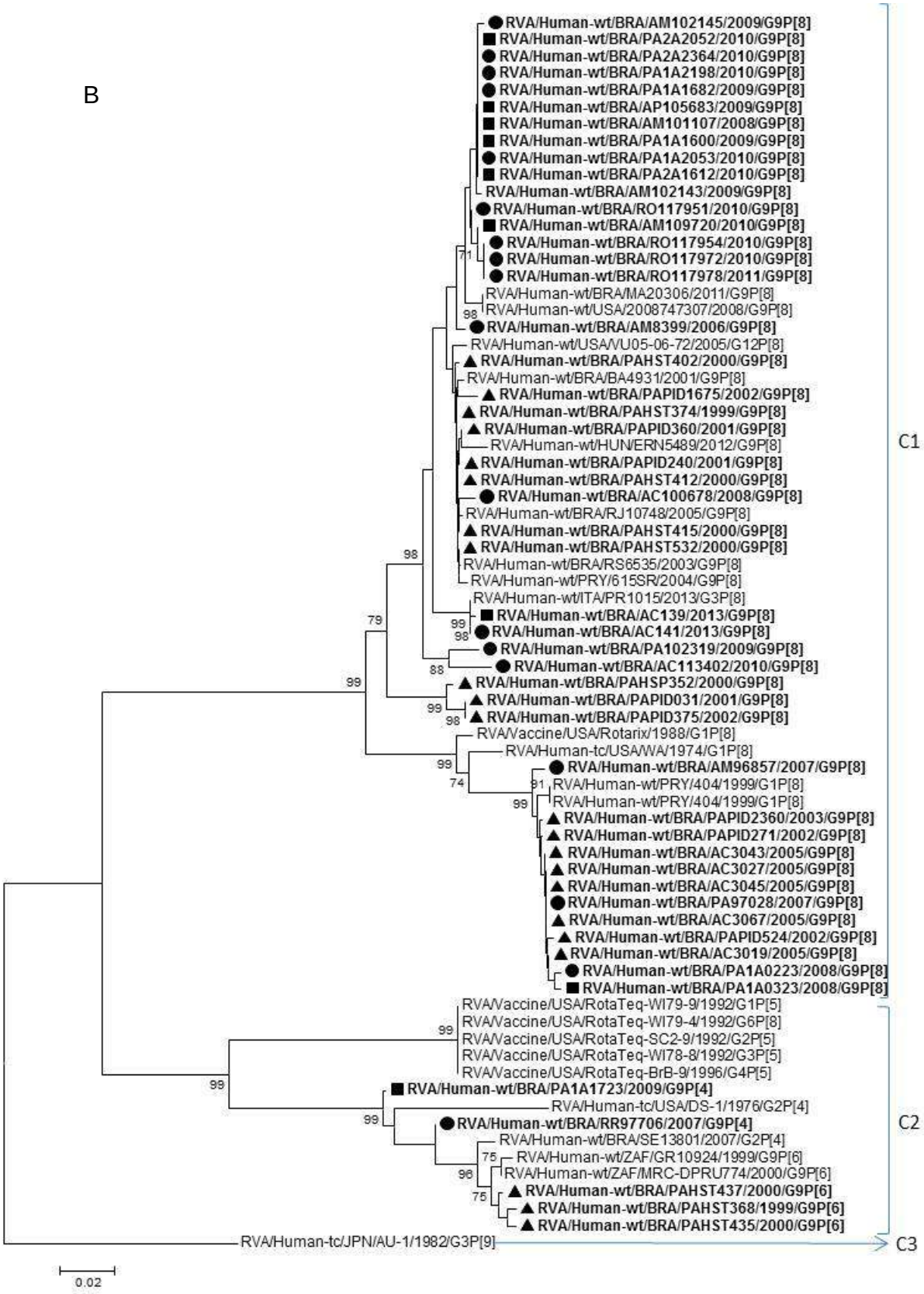
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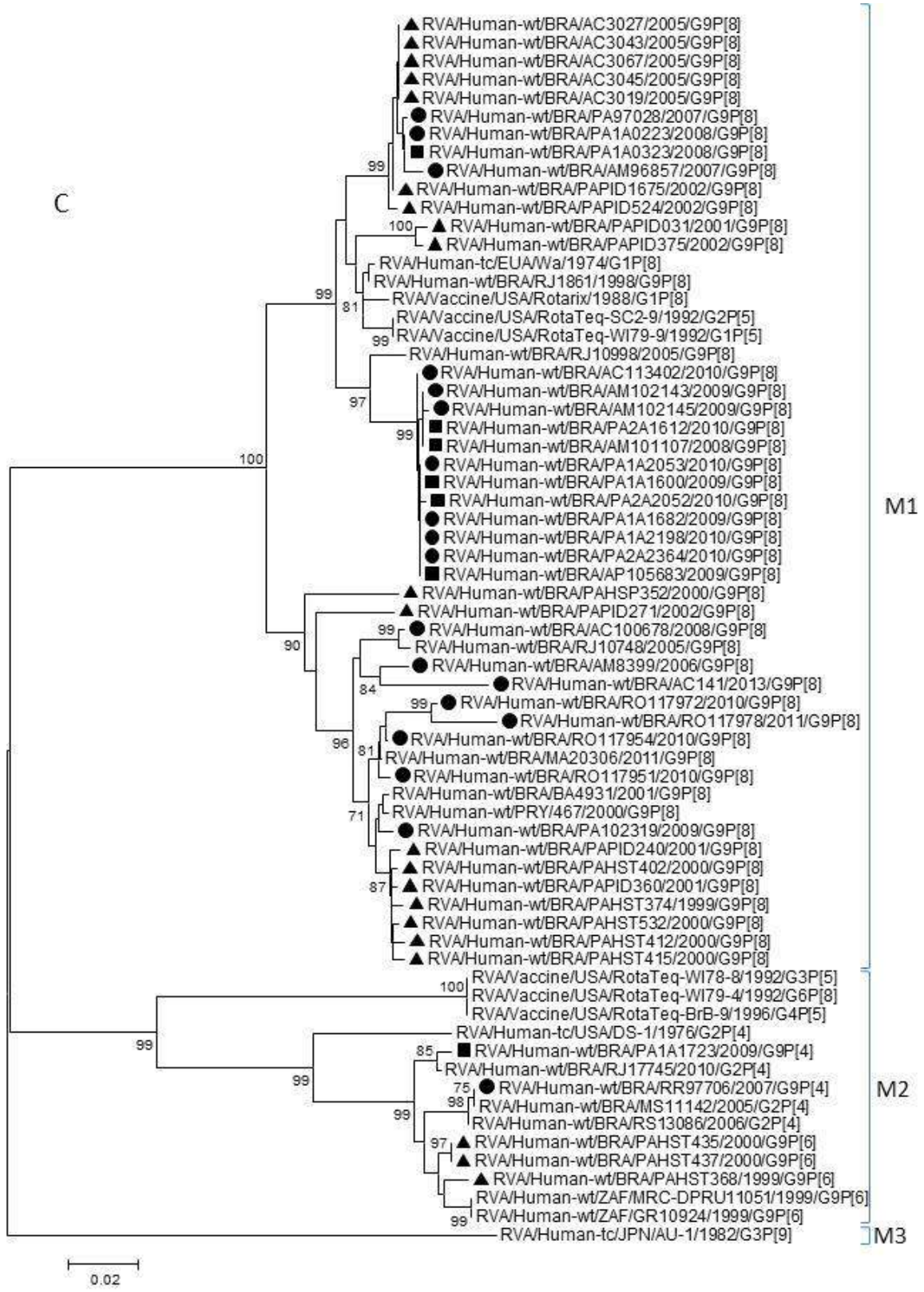
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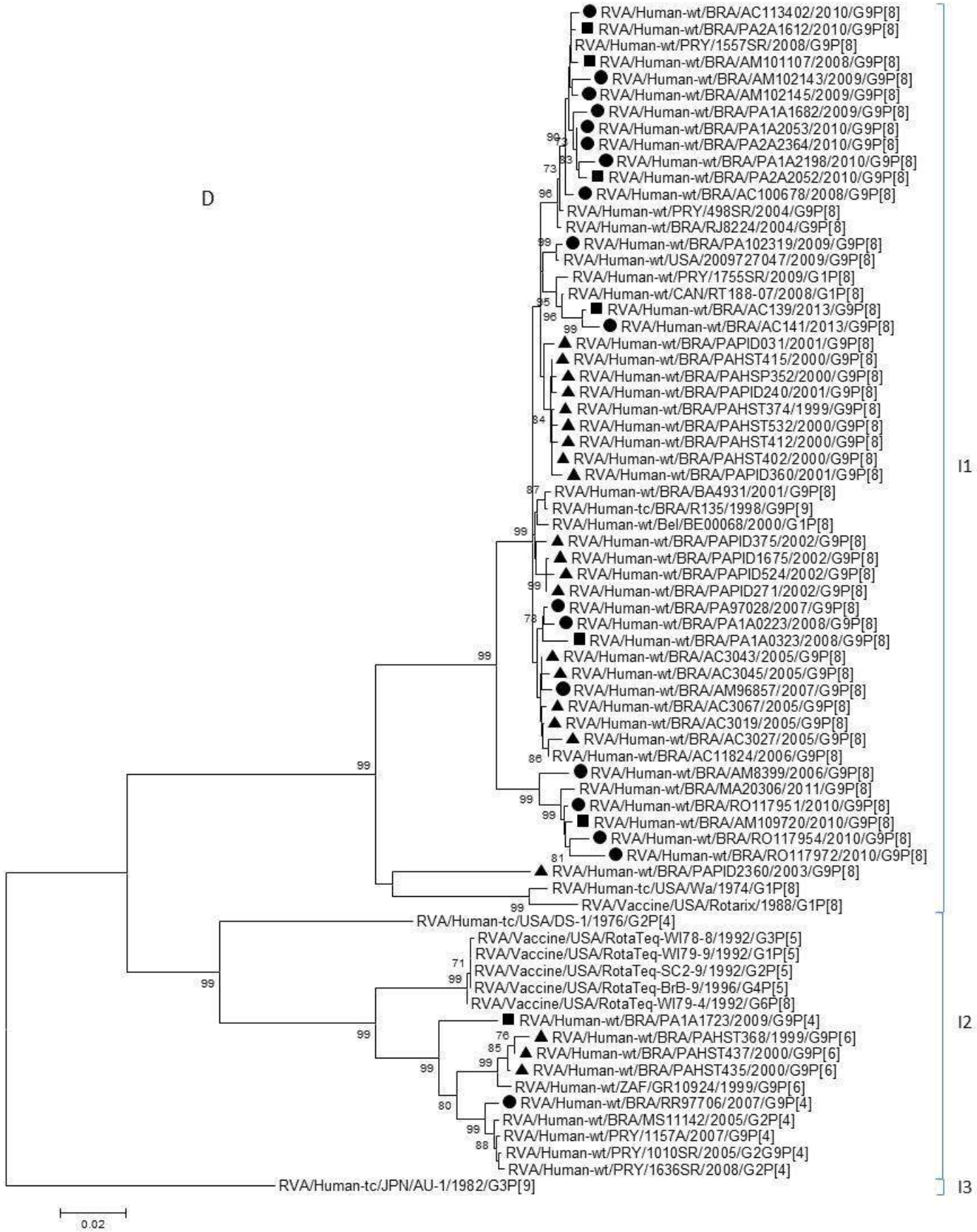
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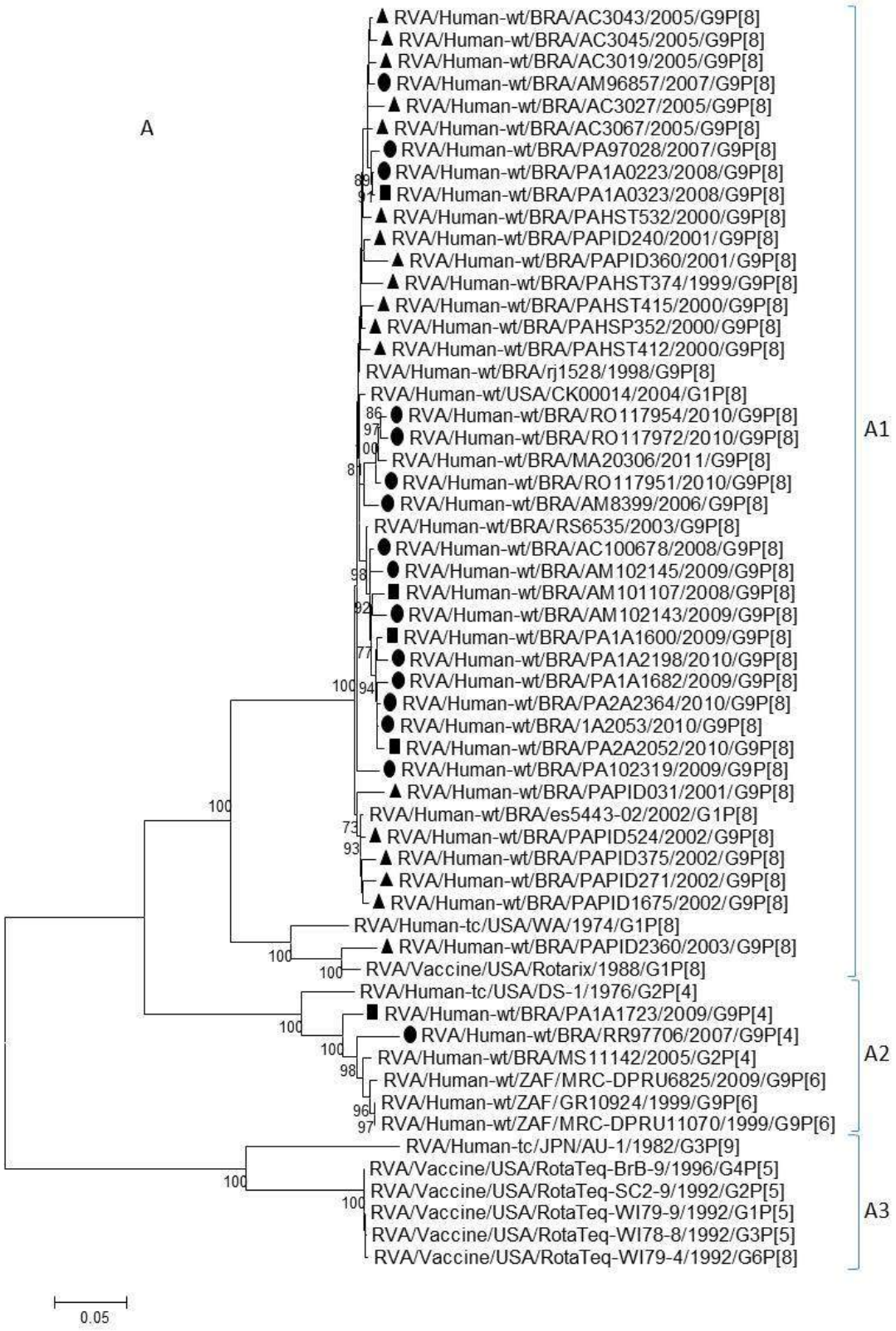
Figure 5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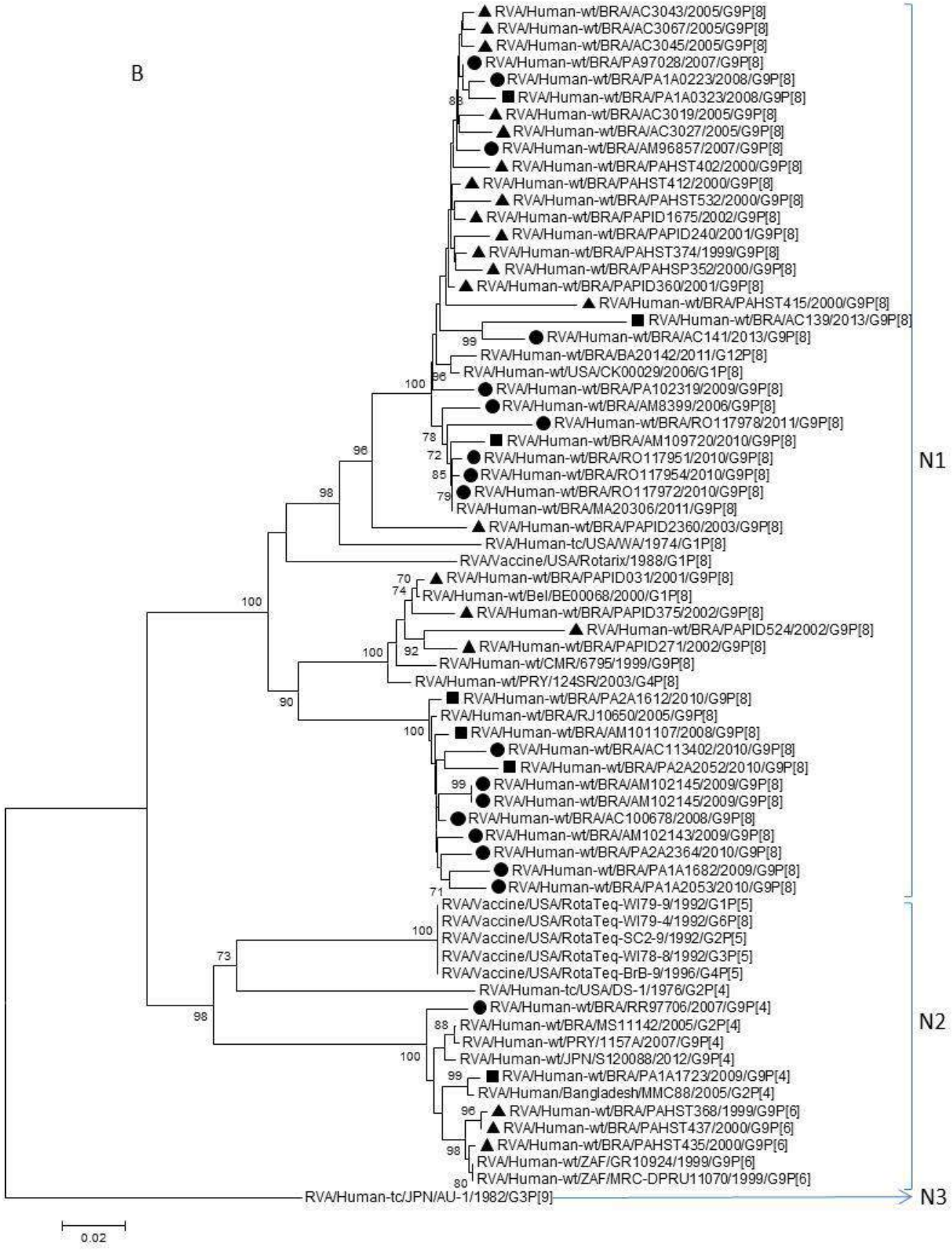
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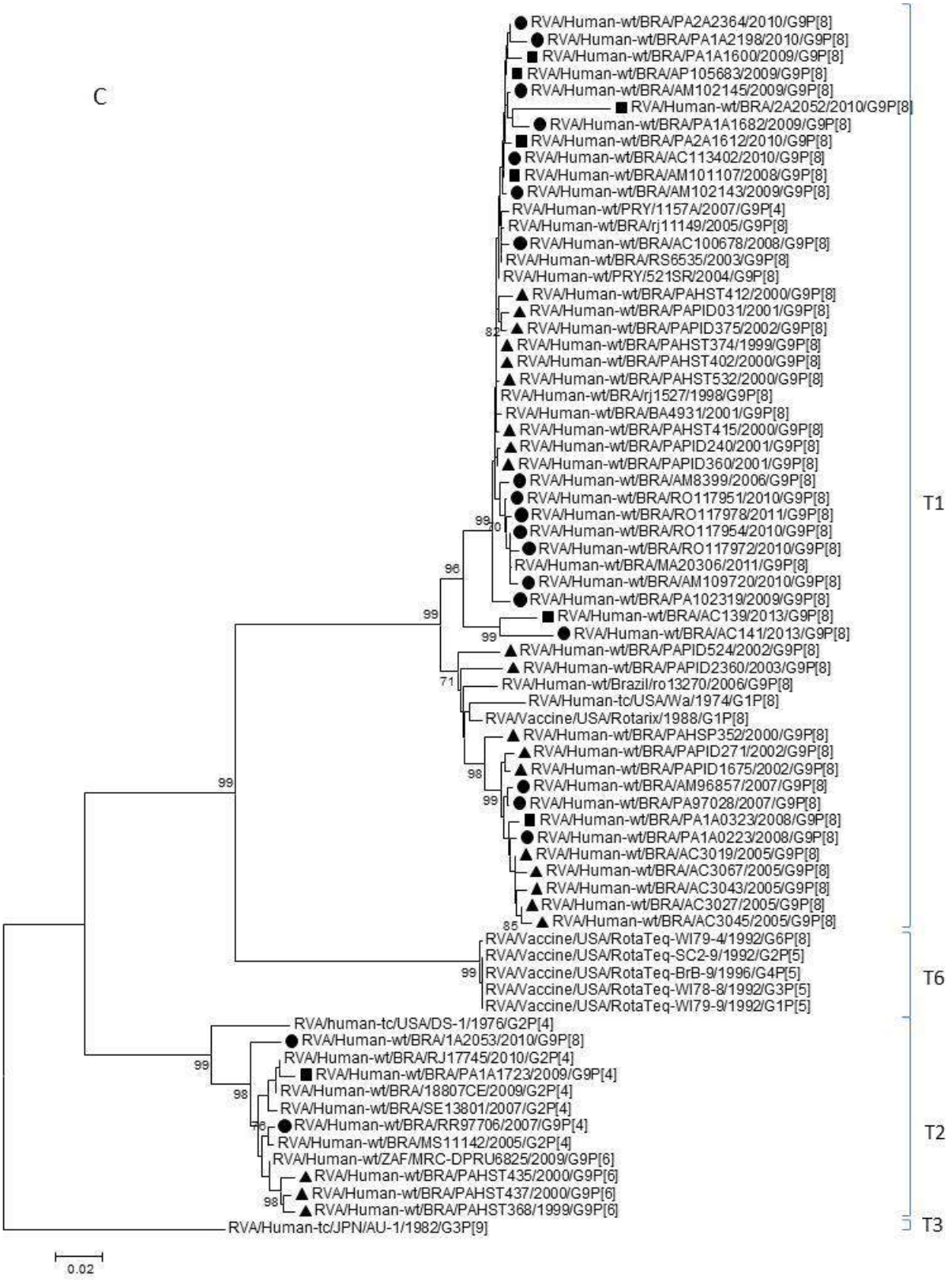
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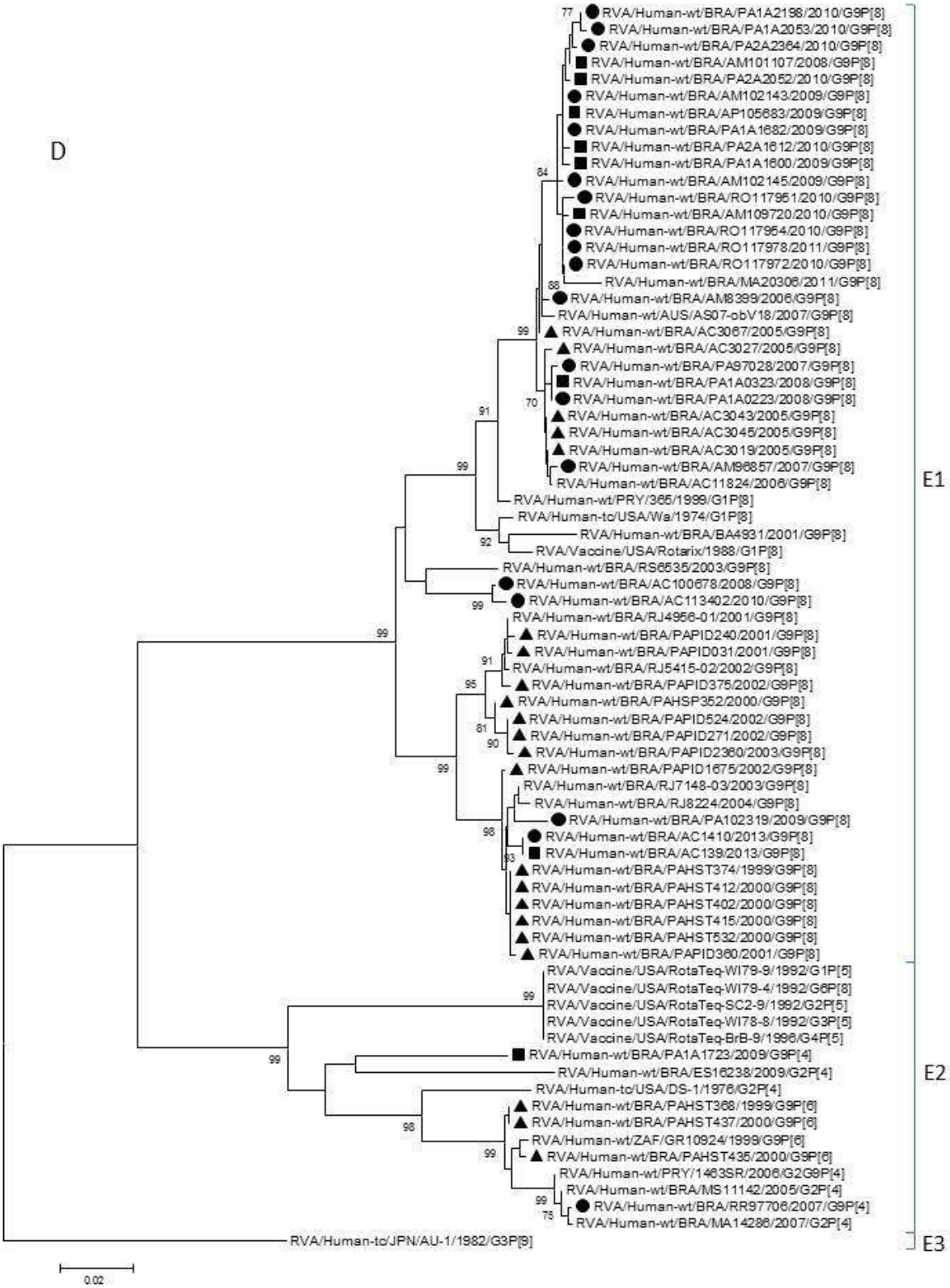
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RVA/Human-wt/BRA/PAHST412/2000/G9P[8]
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RVA/Vaccine/USA/RotaTeq-BrB-9/1996/G4P[5]
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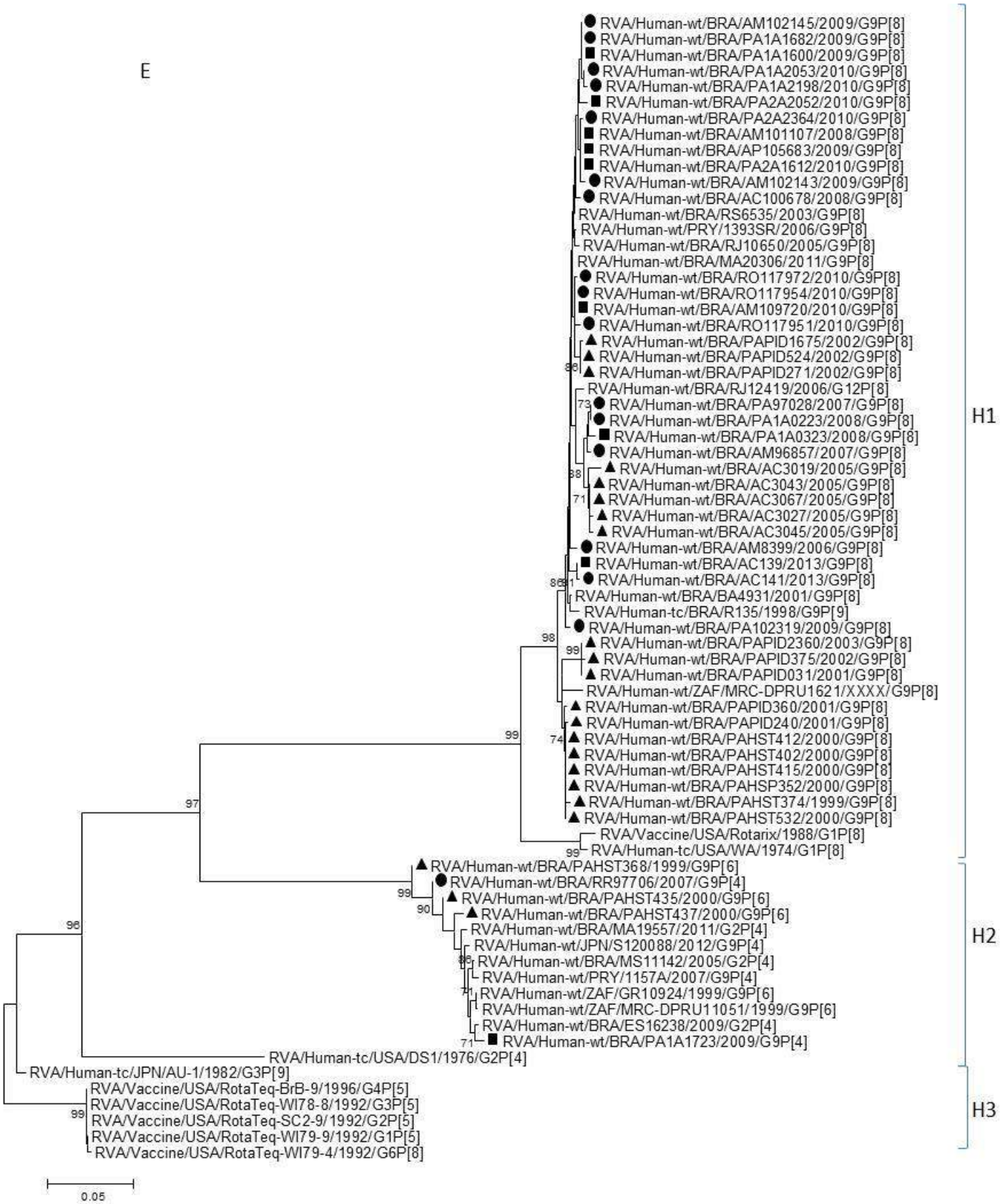
E1
E2
E3
0.02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RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P[8]
0.05
H1
H2
H3

7 - DISCUSSÃO

O genótipo G9 emergiu/reemergiu na epidemiologia molecular do RVA, sendo, posteriormente, considerado um dos genótipos mais detectados em todo o mundo, por isso denominado usual (VOLOTÃO et al., 2005; CARMONA et al., 2006; MONTENEGRO et al., 2007; RIBEIRO et al., 2008).

Como descrito em vários estudos, este genótipo circulava antes da introdução da vacina em diversas localidades e, após a introdução da vacina com a continua vigilância das cepas circulantes preconizado pela OMS, o G9 continuou sendo detectado, não sendo erradicado da epidemiologia molecular do RVA (LEITE; CARVALHO-COSTA; LINHARES, 2008; CARVALHO-COSTA et al., 2011).

Dito como um genótipo “promíscuo”, consegue se adaptar facilmente a diversos genótipos P sendo detectado em diferentes combinações binárias, além de ser descrito acometendo animais, principalmente suínos, conferindo ao G9 uma relevância epidemiológica que reflete em diversos estudos abordando sua caracterização (HOSHINO et al., 2004; PAGE et al., 2010; MATTHIJNSSENS; VAN RANST, 2012; GHOSH et al., 2012; PATTON, 2012; ESONA et al., 2013; LEWIS et al., 2014).

7.1 - FREQUÊNCIA DO GENÓTIPO G9 NA REGIÃO NORTE

O genótipo G9 foi detecado pela primeira vez na região Norte na década de 90, assim como em diversas localidades em escala global, sendo por isso denominado genótipo emergente (RAMACHANDRAM et al., 1998; BON et al., 2000; CUNLIFFE et al, 2001; ARMAH et al., 2003; KIRKWOOD et al., 2004; SANTOS; HOSHINO, 2005; LEITE; CARVALHO-COSTA; LINHARES, 2008; WHO, 2008)

Desde então, o G9 foi continuamente detectado, tal como observado no presente estudo, principalmente associado ao tipo P[8], sendo por isso considerado uma das combinações usuais de RVA (SANTOS; HOSHINO, 2005; LEITE; CARVALHO-COSTA; LINHARES, 2008; WHO, 2011; PATTON, 2012).

No período antes da introdução da vacina, observou-se a frequência do genótipo G9 em até 43% na região, no ano de 2005, o que corrobora com dados expostos por Carvalho-Costa et al. (2011), o qual relatou a frequência deste genótipo em 52% no ano de 2005 no Brasil, enfatizando a elevada circulação do genótipo G9 em todo país.

No período pós vacina, devido ao monitoramento dos tipos de RVA circulantes, observou-se que em países que adotaram a vacina Rotarix® , houve maior detecção dos tipos G9 e G2, tal como relatado por Kirkwood et al (2009). No Brasil, Leite, Carvalho-Costa e Linhares (2008) demonstraram a circulação do G9 em 11% das infecções por RVA no período pós-vacina, sendo apenas superado pelo tipo G2 enquanto que, na presente análise, o G9 foi continuamente detectado no período pós-vacina observando-se sua maior frequência de 12,5% no período de 2008 a 2010, contudo não foi o tipo mais prevalente, sendo os tipos G1, G2, G3 e G12 os mais frequentemente associados à infecções na região.

Apesar do G9 não pertencer à composição das vacinas contra RVA mais utilizadas, tais imunizantes apresentaram resultados satisfatórios combatendo a evolução mais grave em infecções por este genótipo (LINHARES et al., 2006a; ARAÚJO et al., 2007a; VESIKARI et al., 2006; 2007; O'RYAN; LINHARES, 2009; JUSTINO et al., 2012).

A combinação binária de G9 mais frequentemente detectada em todo o mundo é o G9P[8], tal como descrito por Santos e Hoshino (2005) e relatado em diversos estudos, tal como em Leite; Carvalho-Costa e Linhares (2008), Tort et al (2010), Rahman et al (2011), Afrad et al (2014), Kiulia et al (2014) e Zhang et al (2016), sendo tal realidade refletida na região Norte.

Outras combinações binárias como G9P[4] e G9P[6] foram caracterizadas no presente e foram relatadas sendo detectadas em outras localidades, com menor frequência que o G9P[8], contudo revelando significativo impacto na saúde coletiva como relatado por Yen et al (2011) no México com o G9P[4] (SANTOS et al, 2001; LEITE; CARVALHO-COSTA; LINHARES, 2008; KIULIA et al., 2014; LUCHS et al., 2015).

7.2 – ANÁLISE FILOGENÉTICA DO GENÓTIPO G9 DA REGIÃO NORTE

O genótipo G9 já foi relatado associado a diferentes tipos P, revelando sua capacidade de adaptação, sendo denominado como o tipo de RVA mais “promíscuo” descrito (HOSHINO et al., 2004; GHOSH et al., 2012; PATTON, 2012; ESONA et al., 2013; LEWIS et al., 2014). Apesar de tal característica, a linhagem circulante de G9 é predominantemente uma, a linhagem III, tal como afirmado por Matthijnssens et al (2010).

Ao analisar o gene VP7, observou-se a predominância da linhagem III de G9, denominada de cosmopolita, sendo esta a variante genética circulante desde sua emergência na década de 90, tal como descrito em diversos estudos conduzidos (KIRKWOOD et al., 2003; HOSHINO et al., 2005; PARRA et al., 2005; LIN et al., 2006; ESONA et al. 2013). Em estudos desenvolvidos com G9 RVA detectadas no Brasil, observou-se a predominância da linhagem III-G9 tanto no periodo antes quanto após introdução da vacina no calendário nacional de imunização tal como descrito por Araújo et al., (2007); Tort et al. (2010) e Gómez et al., (2014).

A análise dos epítopos de neutralização do gene VP7 demonstrou a substituição de resíduos aminoacídicos das amostras G9 RVA do presente estudo quando comparados aos genes VP7 das cepas vacinais. Tais modificações foram observadas nas amostras coletadas pré e pós introdução da vacina, sendo descritas em estudos conduzidos por Page et al (2010), Zeller et al (2012), Esona et al (2013), Than et al (2013) and Gómez et al (2014) em diferentes localidades, demonstrando a existência de tais modificações antes da implantação dos imunizantes em todo mundo.

A análise do gene VP4 demonstrou que todas as amostras P[8] agruparam na linhagem III-P[8] e, apesar de formar grupos distintos, apresentaram elevada similaridade de nucleotídeos e aminoácidos. Da mesma forma que na presente análise, a linhagem III-P[8] foi associada ao G9 em estudos realizados por Espínola et al. (2008), Paul et al. (2008) Rahman et al (2008), Donato et al. (2012) and Ianiro et al. (2013). Araújo et al (2007), Tort et al. (2010) and Gómez et al (2014), os quais também observaram a formação de distintos grupos dentro da

linhagem III-P[8], demonstrando a circulação de diferentes variantes genéticas associadas ao G9. Embora o P[8] vacinal agrupe em outras linhagens (P[8]-I e P[8]-II), estudos conduzidos antes da implantação da vacina demonstrou resultados satisfatórios contra as infecções por G9 RVA (DENNEHY, 2008; LANZIERI et al., 2010; LINHARES; JUSTINO, 2014).

A análise dos epítopos de neutralização do gene VP4 P[8] revelou mais substituições aminoacídicas quando comparadas à Rotarix^{TM}, imunizante adotado na rede pública de saúde. Tais modificações foram também relatadas por Zeller et al (2012) em amostras G9P[8] na Bélgica e por Gómez et al (2014) com G9P[8] coletados no Brasil.

O gene VP4 P[4] e VP4 P[6] associados ao G9 agruparam nas linhagens P[4]-V e P[6]-I, respectivamente. Tal como observado por Lewis et al (2014), o VP4-P[4] associado ao G9 é muito similar ao VP4-P[4] associado ao G2 no período pós introdução da vacina. Tal fato pode ser associado à elevada prevalência do tipo G2P[4] de RVA após a introdução do imunizante contra RV em todo o mundo, sendo a linhagem P[4]-V detectada em combinação com G2 em estudo conduzido com amostras do Brasil por Gómez et al (2014).

O genótipo P[6], muito associado a infecções neonatais sintomáticas e assintomáticas, assim como a RV de origem animal, principalmente suíno, como relatado por Mascarenhas et al (2007), foi descrito no presente estudo, sendo similar a amostras humanas descritas por Kirkwwod et al (1999) no EUA e Page et al (2010) na África.

A análise filogenética das demais proteínas estruturais e não estruturais das amostras G9 demonstrou a associação de G9P[8] aos genogrupo 1 (R1 [VP1], C1 [VP2], M1 [VP3], I1 [VP6], A1 [NSP1], N1 [NSP2], T1 [NSP3], E1 [NSP4] and H1 [NSP5]), enquanto que G9P[4] e G9P[6] foram associados ao genogrupo 2 (R2 [VP1], C2 [VP2], M2 [VP3], I2 [VP6], A2 [NSP1], N2 [NSP2], T2 [NSP3], E2 [NSP4] and H2 [NSP5]), apresentando variada similaridade devido algumas amostras possuírem maior similaridade com genes caracterizados de outros tipos G “não-G9”, fato este também observado por Esona et al (2013) em amostras G9P[8] na África, indicando a elevada variabilidade genética obtida com o reestruturação genômica.

A amostra RVA/Human-wt/BRA/PA1A2053/2010/G9P[8], apesar de possuir todos os genes do genogrupo 1, apresentou a NSP3 pertencente ao genogrupo 2 (T2). Esta reestruturação genômico pode ocorrer em um ou mais genes em um infecção com diferentes tipos de RVA já foi relatado por Nyaga et al (2013) com amostras G9P[6] e G9P[8] na África; Dóro et al. (2014) com G9P[8] na Hungria; Lewis et al. (2014) com G9P[4] no EUA; Komoto et al (2015) com amostras G1P[8] na Tailândia; Silva et al (2015) com G1P[8] no Brasil; Arana et al (2016) com G3P[8] na Espanha e Cowley et al (2016) com amostras G3P[8] detectadas na Austrália, demonstrando que tal fenômeno ocorre com vários genótipos em diferentes localidades, proporcionando elevada variabilidade genética ao RVA.

No presente estudo, observou-se a associação de amostras G9P[8] à constelação *Wa-like*, enquanto que os tipos G9P[4] e G9P[6] foram associados à constelação *DS1-like*. Tais associações foram previamente descritos por Jere et al. (2011) com G9P[6], Ianiro et al (2013) com G9P[8], Esona et al (2013) com G9P[8], Nyaga et al (2013) em amostras G9P[6] e G9P[8]. Gómez et al (2014) relataram a circulação no Brasil, antes e após a introdução da vacina, de G9P[8] associado à constelação *Wa-like*, tanto em crianças vacinadas quanto em não vacinadas, tal como abordado no presente estudo.

Poucos estudos relatam a caracterização de amostras G9P[4], devido ser considerado um tipo não usual de RVA, entretanto, as amostras G9P[4] do presente estudo demonstraram elevada similaridade com a constelação *DS1-like* de amostras G2P[4], sendo tal fato observado no periodo pós introdução da vacina com a elevada circulação do genótipo G2 em escala global, inclusive no Brasil (LEITE et al., 2008; Patel et al., 2008; CARVALHO-COSTA et al., 2009; KIRKWOOD et al., 2009; MASCARENHAS et al., 2010; ESTEBAN et al, 2010; OLIVEIRA et al., 2012; JUSTINO et al., 2012).

8 - CONCLUSÕES

A partir da introdução do genótipo G9 na região Norte na década de 90, consolidou sua permanência na epidemiologia molecular local, sendo detectado continuamente desde então com diferentes tipos P, podendo ser considerado um tipo de RVA usual na região.

Apesar dos imunizantes vigentes conferirem proteção cruzada frente as infecções causadas pelo genótipo G9, o mesmo foi detectado circulando na região tanto em crianças vacinadas quanto não vacinadas, contudo, em frequência menor após a implantação deste imunizante.

A baixa detecção do genótipo G9 pode ser associada à sua elevada similaridade genética entre as amostras circulantes antes e após a introdução da vacina no calendário de imunizações.

Houve a detecção de reestruturação genômicas em amostras G9, demonstrando o mecanismo de evolução do RVA neste genótipo, enfatizando a necessidade da contínua monitorização dos tipos virais circulantes e que possam alterar a epidemiologia molecular local e as estratégias vacinais na região.

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ANEXO A – Parecer de aprovação do Comitê de Ética em Pesquisa

MS - SECRETARIA DE VIGILÂNCIA EM SAÚDE
INSTITUTO EVANDRO CHAGAS

Parecer de Aprovação nº 0047/2011
Protocolo CEP/IEC - Nº 0017/2011
CAAE: 0016.0.072.000-11

Ananindeua/PA, 24 de novembro de 2011.

COMITÊ DE ÉTICA EM PESQUISA COM HUMANOS

Projeto: "Análise molecular de rotavírus tipo G9 de crianças na região Norte do Brasil"

Pesquisador Responsável: Joana D' Arc Pereira Mascarenhas

Conforme decisão do Comitê de Ética em Pesquisa do Instituto Evandro Chagas/SVS/MS, cientificamos que o projeto em epígrafe foi considerado **aprovado.**

Recomendamos que a coordenação mantenha atualizados todos os documentos pertinentes ao projeto.

Este CEP se incumbirá dos procedimentos de acompanhamento preconizados pela Resolução 196/96 e suas complementares, do Conselho Nacional de Saúde/Ministério da Saúde.

Deverá ser encaminhado relatório semestral e, ao final, elaborado um relatório consolidado, incluindo os resultados finais da pesquisa, em um prazo máximo de 60 (sessenta) dias, após a finalização da pesquisa.

MANOEL DO CARMO PEREIRA SOARES
Coordenador do CEP/IEC

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