Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

INSTITUTO OSWALDO CRUZ

Pós-Graduação em Biologia Celular e Molecular

MARIELA MARTÍNEZ GÓMEZ

DIVERSIDADE GENÉTICA DOS ROTAVIRUS DA ESPÉCIE A ANTES E APÓS A INTRODUÇÃO DA VACINA MONOVALENTE NO BRASIL

Tese apresentada ao curso de Pós-Graduação em Biologia Celular e Molecular do Instituto Oswaldo Cruz, FIOCRUZ, como parte dos requisitos para obtenção do título de Doutor em Ciências. Área de Concentração: Virologia

Orientador: Dr. José Paulo Gagliardi Leite

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Rio de Janeiro, 31 de Janeiro de 2014

“Cuando en tierras extrañas miro triste la lejanía azul del horizonte,
siento clarito al Olimar que pasa y la brisa me trae olor a monte...
Este cielo no es el cielo de mi tierra, y esta luna no brilla como aquella,
como aquella que alumbró mis sueños altos,
más altos que el temblor de las estrellas...
Tantas voces y miradas tan queridas ya no están en el boliche,
en los asados, otros vagan sin consuelo por el mundo...
Ay, paisito mi corazón ta' llorando...”

Ta' llorando. Los Olimareños.

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SUMÁRIO

LISTA DE SIGLAS E ABREVIATURAS

Aa – Aminoácido

ADN – Ácido desoxirribonucléico

AG – Do inglês: *Acute gastroenteritis*

ARN – Ácido ribonucleico

ARN(+) – Ácido ribonucleico de porlaridade positiva

ARN(-) – Ácido ribonucleico de polaridade negativa

ARNdf – Ácido ribonucleico de fita dupla

ARNm – Ácido ribonucleico mensageiro

ARNsf – Ácido ribonucleico de fita simple

ARNt -Ácido ribonucleico de transferência

Bases: A – Adenina; C – Citosina; G – Guanina; U - Uracila

Ca^{2+} – Íon cálcio

Cl^{-} – Íon cloro

CpG - Do inglês: *Cytosine-phosphate-Guanine*

DLPs – Do inglês: *Doble Layer Particles* – Partículas virais de camada dupla

eIF-4G1 - Do inglês: *Eukaryotic translation initiation factor 4-gamma 1*

EGPA – Eletroforese em gel de poliacrilamida

ENC - Do inglês: *Effective number of códon*

EUA – Estados Unidos de América

FIOCRUZ – Fundação Oswaldo Cruz

GA – Gastroenterite Aguda

GAVI – Do inglês: *Global Alliance for Vaccines and Immunisation* - Aliança Mundial para Vacinas e Imunização

GpC - Do inglês: *Guanine–phosphate-Cytosine*

HAV - Do inglês: *Hepatitis A virus* - Vírus da hepatite A

HIV-1 - Do inglês: *Human immunodeficiency vírus type* - Vírus da imunodeficiência humana tipo 1

HSP - Do inglês: *Heat Shock Proteins* – Proteínas do choque térmico

INF – Do inglês: *Interferon* - Interferon

ICTV – Do inglês: *International Committee on Taxonomy of Viruses* – Comitê Internacional de Taxonomia dos vírus

IOC – Instituto Oswaldo Cruz

IRFs – Do inglês: *Interferon regulatory factors* - Fatores reguladores do Interferon

LRRR - Laboratório de Referencia Regional para Rotavírus

LVCA – Laboratório de Virologia Comparada e Ambiental

ME – Microscopia eletrônica

MS – Ministério da Saúde

NDPkinase - Do inglês: *Nucleoside diphosphate kinase*

NF κβ – Do inglês: *Nuclear factor κβ* - fator nuclear κβ

NIP – Do inglês: *National Immunization Program*

nm – Nanômetros

NSPs – Do inglês: *Non structural proteins* – Proteínas não-estruturais

Nt – Nucleotídeo

NTPase - Do inglês: *Nucleoside triphosphatase*

NTPs - Do inglês: *Nucleoside triphosphates* – Nuclesídeo trifosfato

OMS – Organização Mundial da Saúde

OPAS – Organização Pan Americana da Saúde

ORF – Do inglês: *Open reading frame* - Fase de leitura aberta

PABP- Do inglês: *Poly(A)-binding protein* - Proteína de união à cauda Poli A

PAHO – Do inglês: *Pan American Health Organization*

pb – Pares de bases

PCs - Do inglês: *Polimerase complexes* - Complexos da polimerase viral

PNI – Programa Nacional de Imunização

RCWG – Do inglês: *Rotavirus Classification Working Group*

RE – Retículo endoplasmático

+RNAs - Do inglês: *Ribonucleic acid positive strand* – ARN simple fita de polaridade positiva

RTPase - Do inglês: *Ribonucleic acid triphosphatase*

RV – Rotavírus

RVA – Rotavírus da espécie A

RVE – Rotavírus da espécia E

RVF – Rotavírus da espécie F

RVG – Rotavírus da espécie G

RV1 – Do inglês: *Rotarix vaccine* - Vacina monovalente (G1P[8]) Rotarix®

RV5 – Do inglês: *Rotateq vaccine* - Vacina pentavalente (G1-4, P[8]) Rotateq®

SA11 – Do inglês: *Simian rotavírus A 11* – Rotavírus A símio

SAGE – Do inglês: *Strategic Advisory Group of Experts on Immunization*

- Grupo de Assessoria Estratégica de Especialistas em Imunização

Tc – Do inglês: *Tissue culture*

TLR9 – Do inglês: *Toll-like 9*

VPs – Do inglês: *Viral structural proteins* - Proteínas estruturais

VLPs – Do inglês: *Virus-like Particles* - Partículas semelhantes a vírus

WHO – Do inglês: *World Health Organization*

Wt – Do inglês: *Wild type*

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Mariela Martínez Gómez

RESUMO

Os Rotavírus da espécie A (RVA) são os principais agentes etiológicos causadores de gastroenterite aguda (GA) em crianças ≤5 anos. Após a introdução, em março de 2006, da vacina monovalente G1P[8] (Rotarix® - RV1) no Programa Nacional de Imunizações (PNI) pelo Ministério da Saúde (MS) do Brasil, observou-se uma mudança na epidemiologia dos genótipos de RVA circulantes na população. Apesar desta variação dos genótipos circulantes, observou-se no Brasil uma redução de 22-28% de mortes e de 21-25% de hospitalizações em crianças <2 anos de idade, particularmente nas regiões Norte e Nordeste. Provavelmente a variação dos genótipos de RVA, tanto no tempo quando nas regiões geográficas, esteja relacionada a diversos fatores. O fato da vacina RV1 apresentar uma constelação genética (11 genes) *Wa-like* pode estar influenciando na eficácia da mesma frente aos RVA que apresentem constelações diferentes, como vírus *DS-1-like* e *AU-1-like*. Neste estudo foi analisada a diversidade genética de RVA de diversos genótipos detectados no Brasil antes e após a introdução da vacina RV1 pelo PNI, tanto em crianças vacinadas quanto não vacinadas, com o intuito de: i) identificar e caracterizar variantes de RVA emergentes e reemergentes; ii) contribuir para um melhor entendimento da dinâmica evolutiva deste vírus; iii) identificar mutações pontuais e genes que foram introduzidos mediante reestruturação genética, que eventualmente posam estar vinculados ao fato de RVA causarem GA inclusive em crianças vacinadas. Deve-se ressaltar que este estudo engloba amostras de diferentes regiões geográficas do Brasil, visto que dados epidemiológicos sugerem diversidades genotípicas regionais. Todas as cepas G2P[4] para as quais foram analisados os 11 segmentos gênicos apresentaram uma constelação gênica *DS-1-like*: I2-R2-C2-M2-A2-N2-T2-E2-H2, apesar de que diversas variantes virais circularam no período estudado. Não foram observadas diferenças nos sítios antigênicos das proteínas VP8* e VP7 entre crianças vacinadas e não vacinadas. As cepas G1P[6] analisadas apresentaram uma constelação gênica *Wa-like*: I1-R1-C1-M1-A1-N1-T1-E1-H1. As cepas G12P[9] apresentaram uma constelação gênica *AU-1-like*: I3-R3-C3-M3-A3-N3-T3-E3-H6, enquanto as cepas G12P[8] revelaram uma constelação gênica *Wa-like*: I1-R1-C1-M1-A1-N1-T1-E1-H1. Além disso, a análise do viéis no uso de códons de RVA genótipo G2P[4] revelou que existe uma correlação entre o viéis no uso de códons e a composição de bases, o que sugere que a pressão mutacional é um fator importante na determinação do viéis no uso de códons em RVA humanos.

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GENETIC DIVERSITY OF SPECIE A ROTAVIRUSES BEFORE AND AFTER MONOVALENT VACCINE INTRODUCCION IN BRAZIL

Mariela Martínez Gómez

ABSTRACT

Specie A rotaviruses (RVA) are the main etiological agents of acute gastroenteritis (AG) in children ≤ 5 years old. After the introduction of monovalent vaccine G1P[8] (Rotarix® - RV1) in the National Immunization Program (NIP) in March 2006, a change in the epidemiology of RVA circulating genotypes in the population was observed in Brazil. Despite this variation, a reduction of 22-28% of deaths and 21-25% of hospital admissions in children < 2 years of age, particularly in the North and Northeast regions, occurred after RV1 introduction. RVA genotypes variation along time and in the different geographical regions might be related to several factors. Since the RV1 vaccine has a Wa-like genetic constellation the effectiveness of this vaccine against RVA showing different genetic constellations, such as DS-1-like and AU-1-like viruses, it might be influenced. In this study we analyzed the genetic diversity of different RVA genotypes detected in Brazil, before and after the introduction of the RV1 vaccine by the NIP, in vaccinated and unvaccinated children in order to: i) identify and characterize RVA emerging and reemerging variants; ii) contribute to a better understanding of the evolutionary dynamics of this virus; iii) identify point mutations and genes that have been introduced by reassortment events and that could eventually be linked to the fact that some RVA are capable to cause AG in vaccinated children. It should be emphasized that this study includes different geographical regions of Brazil, as epidemiological data suggests regional genotypic diversity. All G2P[4] strains analyzed revealed a DS-1-like genome constellation: I2-R2-C2-M2-A2-N2-T2-E2-H2. However, several viral variants circulated during the study period. No differences were observed inside antigenic sites of VP7 and VP8* proteins between vaccinated and unvaccinated children. The G1P[6] strains analyzed showed a Wa-like genome constellation: I1-R1-C1-M1-A1-N1-T1-E1-H1. The G12P[9] strains showed a AU-1-like genome constellation: I3-R3-C3-A3-M3-N3-T3-E3-H6, while G12P[8] strains showed a Wa-like genome constellation: I1-R1-C1-M1-A1-N1-T1-E1-H1. Furthermore, analysis of codon usage bias of RVA G2P[4] genotype revealed that a correlation between the codon usage bias and base composition exists, suggesting that the mutational pressure is the main factor in determining the codon usage bias in human RVA.

CAPITULO I

OS ROTAVÍRUS DA ESPÉCIE A (RVA)

1. Introdução

Antes da identificação dos agentes virais causadores da Gastroenterite Aguda (GA) mais de 80% dos episódios de gastroenterites não tinham agente etiológico identificado. Só após a utilização da microscopia eletrônica em amostras fecais foi possível a identificação de alguns destes agentes. Assim, Bishop e colaboradores (1973) descreveram a presença de vesículas citoplasmáticas com partículas virais semelhantes aos *Orbivirus* e por este motivo receberam o nome de *Orbivirus-like agent*. Partículas virais semelhantes foram também detectadas por Flewett e colaboradores (1973). Em 1978 foi proposto que estes vírus constituíssem um novo gênero, *Rotavirus* (RV), dentro da família *Reoviridae* (Flewett & Wood, 1978). No Brasil os RV foram detectados pela primeira vez por Linhares e colaboradores (1977). Atualmente, de acordo com o Comitê Internacional para a Taxonomia dos Vírus (*ICTV*, do inglês *International Committee on Taxonomy of Viruses*) os Rotavírus se dividem em cinco espécies, de A a E (fonte: http://www.ictvonline.org/virusTaxonomy.asp. Acesso em 10/11/2013 às 19:00hs)

Os Rotavírus da espécie A (RVA) são os principais agentes etiológicos causadores de GA em crianças e jovens, estima-se que ocorrem aproximadamente 453,000 mortes por ano mundialmente (Tate et al., 2013). Em 2006 e 2007 a Organização Panamericana de Saúde (OPAS) e o Grupo de Assessoria Estratégica de Especialistas em Imunização (*SAGE*, do inglês: *Strategic Advisory Group of Experts on Immunization*) da Organização Mundial de Saúde (OMS), declararam que a introdução de uma vacina era prioridade nas Américas com a meta de prevenir as mortes e hospitalizações causadas por este vírus (WHO, 2009). Duas vacinas foram recomendadas pela Organização Mundial da Saúde: i) a vacina monovalente Rotarix® (RV1) (*GlaxoSmithKline* [GSK], Rixensart, Bélgica); ii) a vacina pentavalente Rotateq® (RV5) (Merck, North Wales, PE, EUA).

Assim como o Brasil, outros 26 países tem incorporado a vacina RV1 no Programa Nacional de Imunizações (PNI). Estudos realizados no Brasil mostraram uma redução de 17% e 22% nas hospitalizações e taxas de mortalidade, respectivamente, em 2009. Esta redução foi mais acentuada nas regiões Norte e Nordeste do país, onde as condições sociais e económicas são mais precárias do que em outras regiões, demonstrando a importância da vacinação anti-RVA (do Carmo et al., 2011).

1.1. Estrutura do vírion

A partícula viral infecciosa (vírion) apresenta aproximadamente 100 nanômetros (nm) de diâmetro, simetria icosaédrica e é constituída por três camadas proteicas, sendo um vírus não envelopado (Figura 1). A camada proteica interna, ou cerne viral, é composto por 120 copias da proteína VP2. A região terminal da proteína VP2 está livre e acredita-se que esta região está em contato com o complexo da polimerase viral composto pelas proteínas VP1 (ARN polimerase – ARN dependente) e VP3 (enzima responsável pela adição de um CAP no ARN) (McClain et al., 2010). A camada intermediaria é constituída por 260 trímeros da proteína VP6 que permitem estabilidade ao cerne interno; além de funcionar como um adaptador para a camada externa que é essencial para a adsorção e entrada do vírus na célula. Esta última camada é composta por 260 trímeros da glicoproteína VP7, diretamente posicionados sobre os trímeros da proteína VP6. Os trímeros de VP7 são dependentes de íons cálcio (Ca^{2+}), os quais mantêm a estabilidade destes. Se estendendo da camada formada pela proteína VP7, encontram-se 60 trímeros formados pela proteína VP4, que apresenta diferentes mudanças conformacionais durante a penetração na membrana da célula hospedeira (Patton, 2012).

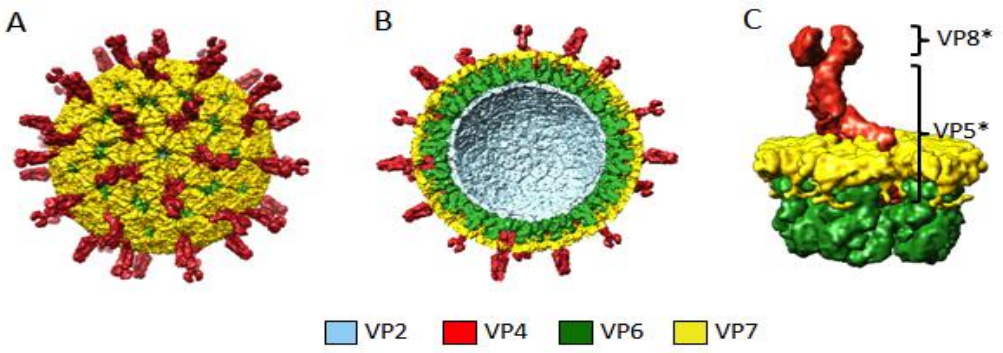

Figura 1. Estrutura do vírion dos rotavírus da espécie A. A) Esquema da estrutura do vírion; B) Camada proteica tripla; C) Esquema da estrutura da proteína VP4. Figura adaptada de Patton, 2012.

1.2 Organização do genoma

O genoma dos RVA consiste de 11 segmentos de Ácido ribonucleico (ARN) de dupla fita (ARNdf) que variam em tamanho de 667 a 3302 pares de bases (pb), codificando 6 proteínas estruturais (VP1-4, VP6-7) e 5 ou 6 proteínas não estruturais (NSP1-6) (Figura 2) (Estes & Kapikian, 2007).

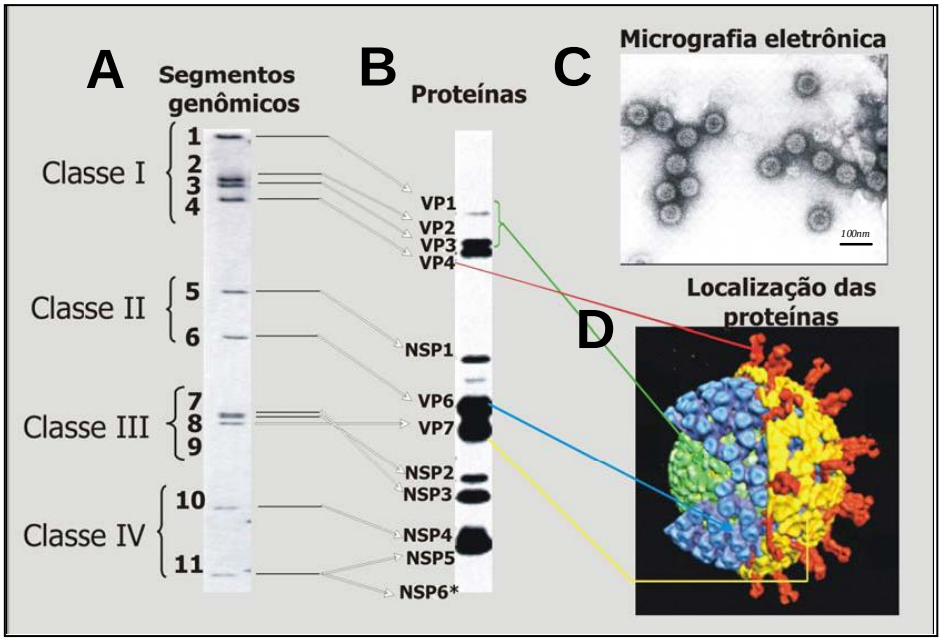

Figura 2. Esquema estrutural do genoma e partículas virais dos rotavírus. A: Eletroforese em gel de poliacrilamida (EGPA) dos 11 segmentos do genoma do rotavírus A símio (SA-11). B: Gel de poliacrilamida mostrando a separação das proteínas estruturais (NSP1 – NSP6) e não estruturais (VP1 – VP4, VP6 – VP7) sintetizadas pelo rotavírus A SA-11. C: Micrografia eletrônica de rotavírus A. D: Reconstrução em 3D do vírion de SA-11, mostrando a localização das proteínas estruturais. Figura adaptada de Conner & Ramig, 1997.

A fase de leitura aberta (*ORF*, do inglês *Open Reading Frame*) esta flanqueada pelas regiões não codificantes 3´ e 5´ que podem variar em comprimento mas que apresentam em comum pequenas sequencias nucleotídicas, que são importantes sinais para a transcrição, transporte de ARN, replicação, montagem ou empacotamento dos segmentos genômicos (Patton & Spencer, 2000; Estes & Kapikian, 2007) (Figura 3). Os ARNdf estão pareados do começo ao fim e a fita positiva contem na região 5´ a sequencia CAP. Todos os 11 segmentos devem apresentar os mesmos sinais de ativação em *cis* já que são todos replicados pela

mesma polimerase. Além disso, cada segmento deve conter um sinal único porque estes devem ser distinguidos um dos outros durante o empacotamento (Estes & Greenberg, 2013).

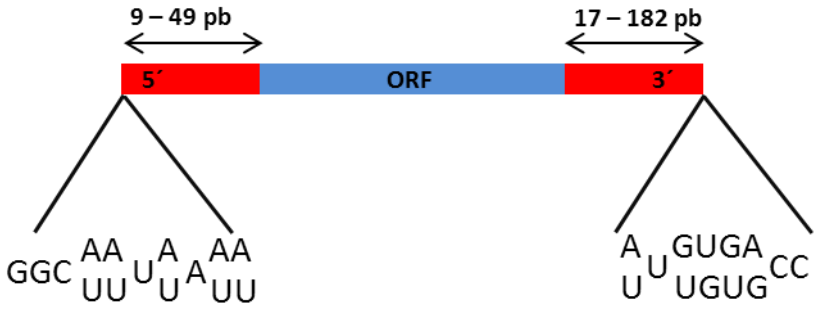

Figura 3. Esquema da organização geral dos segmentos gênicos de rotavírus da espécie A. Abreviações: A, Adenina; C, Citosina; G, Guanina; *ORF*, do inglês *Open Reading Frame*; pb, pares de bases; U, Uracilo.

1.3 Proteínas estruturais

Estudos forneceram evidencias de que a proteína estrutural VP1 é a ARN polimerase dependente de ARN que atua como replicase e transcriptase. A estrutura da VP1 contém quatro canais diferentes que levam ao centro catalítico da mesma. Acredita-se que os quatro canais estejam envolvidos em: a) a entrada dos ARN de polaridade (+) (ARN(+)) e ARN de polaridade (-) (ARN(-)); b) entrada dos Nucleotídeo trifosfato (*NTPs*, do inglês: *nucleoside triphosphates)*; c) saída dos ARNdf e ARN(-); d) saída dos ARN(+) (Estes & Greenberg, 2013).

A VP2 é a proteína mais abundante do cerne viral. Faz parte do complexo de replicação e liga-se tanto à VP1 quanto à VP3, através de um domínio na sua porção N-terminal (Arnoldi et al., 2007). Estudos recentes demostraram que para a ativação da VP1 é necessária a interação do domínio principal da proteína VP2 com a mesma. A porção N terminal da VP2 forma um canal em cada um dos cinco axis da partícula viral que é fundamental para que ocorra a encapsidação das proteínas VP1 e VP3, interações com o ARN e síntese do ARNdf (McDonald & Patton, 2011). Além disso, VP2 também interage com a proteína VP6 nos estágios iniciais da morfogênese viral. A interação entre estas duas proteínas (VP2 e VP6) é fundamental para a formação de partículas imaturas ou incompletas,

ou seja, aquelas que possuem apenas duplo capsídeo proteico (*DLPs*, do inglês: *double layer particles*) (Estes & Kapikian, 2007).

A proteína VP3 apresenta atividades guanidil e metiltransferase e é a enzima responsável pela adição da CAP (Patton, 1995; Subodh et al., 2006; McDonald & Patton, 2011). Dessa forma, VP3 é responsável por modificar a extremidade 5' da molécula de ARN viral e gerar uma estrutura de CAP similar a encontrada no àcido ribonucleico mensageiro (ARNm) de eucariotos (McDonald & Patton, 2011).

A proteína VP4 é não glicosilada e tem papel essencial no ciclo replicativo viral, não só pela adesão e internalização à célula, mas também hemaglutinação, neutralização e virulência (Dunn et al., 1995; Ludert et al., 1996). É susceptível à proteólise, o que resulta na exposição de sítios ativos que proporcionam a penetração do vírus na célula (Arias et al., 1996; Estes & Kapikian, 2007). A ativação da proteína VP4 requer clivagem proteolítica da mesma, gerando dois peptídeos VP5* e VP8*, os quais permanecem associados ao vírion. O peptídeo VP5* tem atividade de neutralização cruzada entre os diferentes tipos de VP4 e, possivelmente, possui os epítopos responsáveis pela adsorção do vírus à célula (Kirkwood et al., 1996). Foi demonstrado que algumas estirpes de RVA interagem com integrinas ou proteínas de choque térmico (*HSP*, do inglés *Heat Shock Proteins*) antes ou após a ligação do RVA à célula hospedeira (Guerrero et al., 2000; Zárate et al., 2003). O peptídeo VP8* contém a maioria dos epítopos associados às reações tipo-específicas, dependentes da ligação deste com ácido siálico (Isa et al., 1997). Cinco epítopos de neutralização foram mapeados em VP5* (5-1 a 5-5) e quatro em VP8* (8-1, 8-4) (Figura 4) (Estes & Kapikian, 2007).

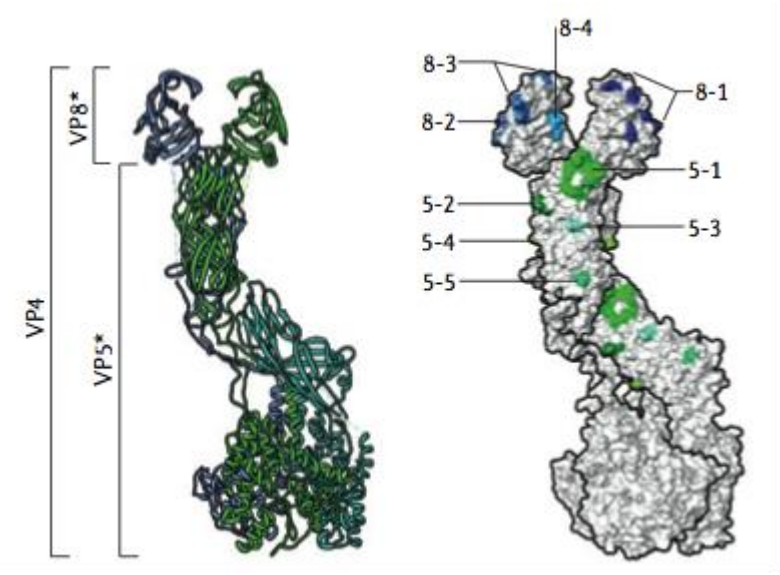

Figura 4. Representação esquemática dos sítios antigénicos presentes na proteína VP4. Figura adaptada de Trask et al., 2012.

A proteína VP6 é a proteína mais abundante dos RVA. Representando na partícula viral aproximadamente 51%. Provavelmente esta seja a causa pela qual a maioria dos anticorpos gerados durante a infecção dos RVA seja contra esta proteína (Svensson et al., 1987). A proteína VP6 é formada por dois domínios: um que interage com VP7 e VP4 do capsídeo externo e outro que interage com VP2 do cerne. Assim, VP6 participa em duas funções importantes para os RVA: a) adsorção e penetração na célula; b) transcrição do ARNdf (Heiman et al., 2008).

A glicoproteína VP7 modula a atividade de VP4 no processo de adsorção e entrada dos RVA na célula, interagindo com proteínas da família das integrinas, na superfície celular, após o processo de adsorção mediado pela proteína VP4 (Guerrero et al., 2000). Íons Ca^{2+}, em concentrações apropriadas, são necessários para estabilidade de VP7. Na sua ausência, os trímeros da proteína encontram-se dissociados, promovendo a liberação da VP7 do vírion. Essa mudança conformacional de VP7 reflete em um arranjo distinto para a proteína VP4, que possibilita a entrada do vírion na célula. Aoki e colaboradores (2009) demonstraram que anticorpos neutralizantes contra a proteína VP7 estabilizam o trímero formado pela proteína, inibindo as alterações em VP4, necessárias para permitir a entrada do vírus na célula hospedeira. Cinco epítopos foram descritos na proteína VP7, entretanto só dois são alvos de anticorpos neutralizantes: 7-1 (7-1a e 7-1b) e 7-2 (Aoki et al., 2009) (Figura 5).

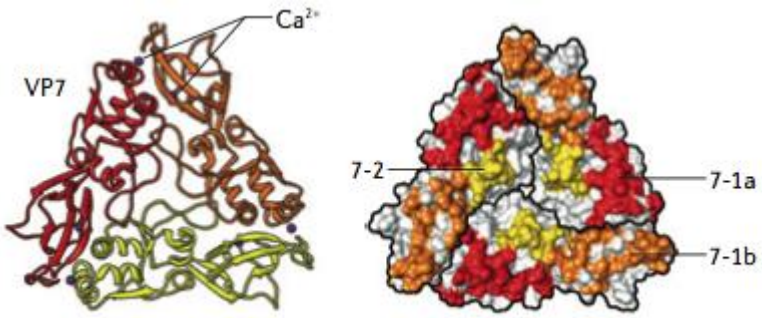

Figura 5. Representação esquemática dos epítopos 7-1 e 7-2 da proteína VP7. Figura adaptada de Trask et al., 2012.

1.4 Proteínas não estruturais

A proteína NSP1, codificada pelo segmento genômico 5, é a proteína viral menos conservada (Dunn et al., 1995). Na morfogênese viral, encontra-se associada ao citoesqueleto celular favorecendo a ligação vírus-célula (James et al., 1999). Esta proteína é capaz de modular a ativação de interferon (*IFN*, do inglês *Interferon*) tipo I. A produção de *IFN* é crítica para encadear uma resposta imune inespecífica em casos de infecção viral. NSP1 antagoniza a resposta imune, via indução da degradação de um ou mais componentes necessários para expressão do *IFN* tipo I, via fatores regulatórios do *IFN* 3, 5 e 7 (*IFRs*, do inglês *Interferon regulatory factors*) ou via fatores nucleares κβ (*NF κβ*, do inglês *Nuclear Factor κβ*) (Barro & Patton, 2005; Graff et al., 2009). Estudos demonstram que dependendo da estirpe de RVA, o mecanismo efetor de NSP1 é diferente (Arnold & Patton, 2011).

A proteína NSP2 é frequentemente encontrada associada à NSP5, ambas formam estruturas conhecidas como viroplasmas. NSP2 é altamente conservada sendo expressa em altas concentrações nas células infectadas, provavelmente porque é uma proteína com diversas funções fundamentais para a formação da partícula viral (Estes & Kapikian, 2007). NSP2 é capaz de se unir ao ARN de fita simple (ARNsf) e tem atividade de helicase, *NTPase* (do inglês *nucleoside triphosphatase*), *NDPkinase* (do inglês *Nucleoside diphosphate kinase*) e *RTPase* (*do inglês Ribonucleic acid triphosphatase*) (Taraporewala et al., 1999, 2001; Kumar et al., 2007; Caprio et al., 2006). A NSP2 participa de várias funções: transcrição, tradução, replicação e empacotamento do RNAdf (Estes & Kapikian, 2007).

O segmento 7 codifica a proteína NSP3, e a mesma tem três domínios, o domínio N terminal de união ao ARN (Aminoácido (Aa) 1 – 170); o domínio de interação com eIF-4G1 (Do inglês *Eukaryotic translation initiation factor 4-gamma 1*), formado pelos últimos 107 resíduos da região C terminal e o domínio de dimerização (Aa 150 – 206) (Piron et al., 1999). Estudos demostraram que a proteína NSP3 compete na célula hospedeira com a proteína de união à cauda Poli A (*PABP*, do inglês *Poly(A)-binding protein*) o que levou a crer que a proteína facilitaria a tradução do ARNm e ao silenciamento da síntese proteica da célula hospedeira. Porém, estudos demostraram que a NSP3 não é necessariamente requerida para a tradução do ARN viral (Piron et al., 1998; Padilla-Noriega, et al., 2002; Varani & Allain, 2002; Montero et al., 2006) Além disso, observou-se que a mesma estaria implicada no espalhamento extra-intestinal do vírus no hospedeiro infectado. Entretanto, ainda não foi elucidado o mecanismo que estaria envolvido (Mossel & Ramig, 2002, 2003).

A proteína NSP4, codificada pelo segmento 10, foi a primeira enterotoxina viral descrita (Ball et al., 1996). Esta proteína se localiza em diversos sítios dentro da célula e contribui na morfogênese, replicação e patogênese dos RVA. Na célula encontra-se em diversas formas: a proteína inteira se localiza ancorada no reticulo endoplasmático (RE) atraves do domínio hidrofóbico N terminal; enquanto que a região C terminal se orienta ao citoplasma. A região que vai do Aa 45 ao 175 constitui a região citoplasmática e retém todas as propriedades biológicas importantes associadas a esta proteína, incluindo: mobilização do Ca^{+2} intracelular, permeabilizacão da membrana; união ao Ca^{2+} e a VP4, união às partículas semelhantes a vírus (*VLPs*, do inglês *Virus-like Particles*) e indução da diarreia em camundongos. Foi observado que uma forma secretada desta proteína contendo os Aa 112 a 175 é capaz de induzir diarreia assim como a proteína completa (Zhang et al., 2000).

O segmento 11 tem duas *ORFs*. A maior codifica para a proteína NSP5 que possui atividade autoquinase e em células infectadas apresenta formas hipo e hiper fosforiladas (Taraporewala & Patton, 2004). A sua fosforilação é modulada mediante interação com a proteína NSP2 (Afrikanova et al., 1998). A outra *ORF* codifica para a proteína NSP6. Algumas variantes de RVA não codificam esta proteína; outras apresentam baixos níveis de expressão, que indicam que NSP6 apresenta papel regulatório não essencial na replicação viral (Taraporewala & Patton, 2004; López et al., 2005).

Durante o processo de replicação, NSP2, NSP5 e NSP6 estão associadas à formação do viroplasmas (Estes & Kapikian, 2007). Na ausência de outras proteínas virais, a interação entre NSP2 e NSP5 resulta em partículas defectivas semelhantes às *VLPs in vitro*. A NSP5 também interage com a VP1 e a VP2. Estudos baseados no silenciamento do gene que

codifica para esta proteína demostraram que a mesma é essencial para a replicação e encapsidação do vírus (Campagna et al., 2005; López et al., 2005).

1.5 Replicação viral

A replicação dos RVA ocorre no citoplasma das células absortivas diferenciadas, que se encontram na porção apical das vilosidades do intestino delgado (Estes & Kapikian, 2007). Proteases do trato gastrintestinal clivam a proteína VP4 em VP8* e VP5*. A proteína VP8* seria a responsável pela adsorção do vírus à célula. A maioria dos anticorpos neutralizantes é dirigida contra esta proteína. Em alguns casos observou-se que esta proteína interage com o ácido siálico na superfície celular, mas acredita-se que outras moléculas na superfície celular também atuam como receptores destes vírus. O vírus é endocitado e transformado em vesículas endossomicas, onde a concentração reduzida de Ca^{+2} desencadeia a perda da proteína VP7 da partícula viral e a penetração da membrana endossomal mediada pela VP5*. O mecanismo de penetração viral ainda não foi totalmente esclarecido. Entretanto, são propostos os seguintes mecanismos: i) endocitose mediada por receptor; ii) penetração direta por meio de *rafts* lipídicos, que são microdomínios lipídicos com alta densidade de glicoesfingolipídeos e um conjunto específico de proteínas, tais como *HSPs* e integrinas. Possivelmente, mais de um mecanismo de penetração viral atuem nos RVA (Isa et al., 2004). A liberação da camada proteica externa ativa o complexo interno da polimerase (VP1 e VP3) e os ARN(+) capeados são transcritos. Estes ARN(+) servem tanto como ARNm na síntese das proteínas virais assim como moldes para produzir ARN(-) durante a replicação viral. As proteínas sintetizadas e o ARNfs viral são reunidos no citoplasma da célula infectada, constituindo um material amorfo denominado viroplasma. O empacotamento do genoma se inicia quando a VP1 une-se ao extremo 3´ do ARN(+) viral. Acredita-se que ocorra uma interação entre os 11 segmentos genômicos, e que a proteína VP2 se organize em torno dos mesmos e desta forma ative a VP1. A proteína VP6 reconhece o core sintetizado formando as *DLPs*. A NSP4 possui domínio citoplasmático que ancora as *DLPs*, por intermédio de VP6 e VP4, propiciando o brotamento das *DLPs* para o interior do RE. Pouco depois ocorre a montagem das partículas com capsídeo externo, resultando em partículas virais maduras com diâmetro aproximado de 80 nm. Após a liberação do vírion da célula, as proteases do trato gastrointestinal clivam a proteína VP4 em VP8* e VP5*, o que finalmente resulta no vírion completo infeccioso (Figura 6) (Estes & Kapikian, 2007; McDonald & Patton, 2011). Estudos *in vitro* indicaram que o vírus pode sair da célula através de lise da célula ou através da

utilização de uma via secretora (Musalem & Espejo, 1985; Jourdan et al., 1997; Trask et al., 2012).

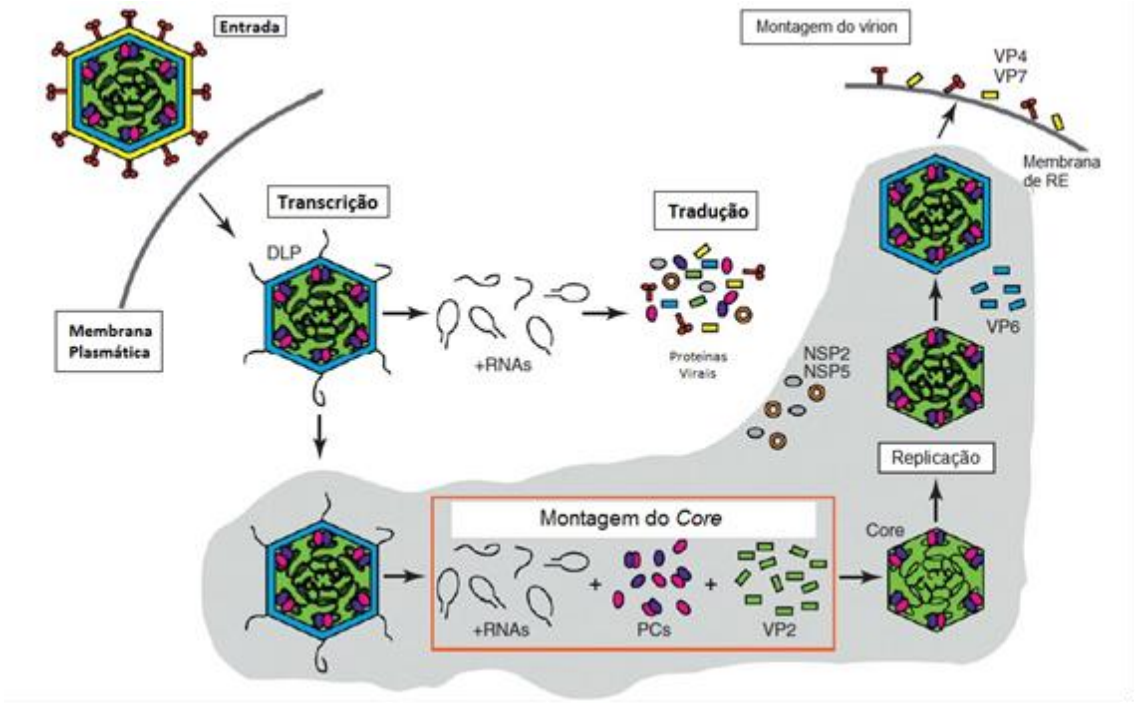

Figura 6. Esquema da biossíntese dos rotavírus da espécie A. Figura adaptada de McDonald & Patton, 2011. Abreviações: *DLPs*: do inglês *Double layer particles* - partículas de dupla camada; *NSPs*: do inglês *non structural proteins* - proteínas nâo estruturais; *PCs*: do inglês *Polimerase complexes* - Complexos da polimerase viral; *+RNAs*: do inglês *Ribonucleic acid positive strand* - ARN simple fita de polaridade positiva; RE: reticulo endoplasmático; *VPs*: do inglês *Viral structural proteins* - proteínas estruturais.

1.6 Classificação dos rotavírus da espécie A

Tradicionalmente a classificação dos vírus tem se baseado em características morfológicas e sorológicas. No entanto as técnicas moleculares tem se tornado um padrão para a detecção, caracterização e classificação viral. O *ICTV* reconhece oficialmente cinco espécies de rotavírus (RVA até Rotavirus E-RVE), existem mais duas espécies, Rotavirus F-RVF e Rotavirus G-RVG, que já foram propostas, mas ainda não foram reconhecidas pelo *ICTV* (Kindler et al., 2013).

Os RVA têm sido classificados com base em: i) proteínas de superfície, sorotipos G (VP7) e P (VP4); ii) padrão de migração dos segmentos de ARN quando submetidos a eletroforese em gel de poliacrilamida (EGPA) (eletroferotipos), podendo apresentar perfil curto, longo, super curto, ou atípico; iii) padrões de hibridização de ARN do genoma completo (genogrupos); e iv) com base na análise das sequencias dos genes que codificam para as proteínas VP7 e VP4, em genótipos G e P, respetivamente. Mais recentemente, um novo sistema de classificação baseada na análise das sequencias nucleotídicas dos 11 genes dos RVA foi estabelecido pelo *Rotavirus Classification Working Group* (*RCWG*) (Tabela 1) (Matthijnssens et al., 2011). Este novo sistema de classificação recomenda nomear a sequência viral obtida considerando-se os seguintes parametros: i) a espécie de RV; ii) a origem do vírus (humano ou animal), identificando também se a sequencia foi obtida a partir de vírus salvagem (*wt*, do inglês *wild type*) ou vírus em cultura celular (*tc*, do inglês *tissue culture*); iii) o país em que foi detectado o vírus, utilizando o código padrão de 3 letras; iv) o nome comum dado pelo pesquisador; v) o ano de detecção; vi) os genótipos G e P da seguinte forma: GXP[X]. Com base nesta nomenclatura, foram descritos até o momento 27 G-, 37 P-, 17 I-, 9 R-, 9C-, 8 M-, 16 A-, 10 N-, 12 T-, 15 E-, e 11 H genótipos. Cada letra maiúscula corresponde, respectivamente, aos genes que codificam para as proteínas VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 (Matthijnssen et al., 2011). No caso do vírus apresentar uma constelação genômica I1-R1-C1-M1-A1-N1-T1-E1-H1, I2-R2-C2-M2-A2-N2-T2-E2-H2, ou I3-R3-C3-M3-A3-N3-T3-E3-H3 se classifica como pertencente ao genótipo 1 (ou *Wa-like*), 2 (ou *DS-1-like*), ou *AU-1-like*, respectivamente (Matthijnssen & Van Ranst, 2012).

Tabela 1. Valores de *cut-off* de percentagem de identidade nucleotídica que definem os diferentes genótipos de rotavírus da espécie A, considerando-se os 11 segmentos genômicos. Tabela adaptada de Matthijnssens et al., 2011.

Gene	Valor de *cut-off* de identidade nucleotídica (%)	Genótipos	Designação dos nomes dos genótipos
VP7	80	27G	Glicoproteína
VP4	80	37P	Sensível a Protease
VP6	85	17I	Capsídeo Interno
VP1	83	9R	ARN Polimerase Dependente de ARN
VP2	84	9C	Proteína do cerne
VP3	81	8M	Metiltransferase
NSP1	79	16A	Antagonista do Interferon
NSP2	85	10N	NTPase
NSP3	85	12T	Promotor Traducional
NSP4	85	15E	Enterotoxina
NSP5	91	11H	Fosfoproteína

1.7 Diversidade genética

Existe uma grande diversidade de cepas de RVA que circulam na população humana, gerada através de diversos mecanismos: i) acumulação de mutações pontuais (*genetic drift*); ii) reestruturações dos segmentos gênicos (*genetic shift*); iii) transmissão direta de vírus animais para humanos; iv) rearranjos genéticos (deleções, inserções e duplicações) (Kirkwood, 2010).

A ocorrência de mutações pontuais é frequente nos RVA, assim como ocorre com outros vírus ARN, e podem produzir mudanças detectáveis de fenótipo (Taniguchi & Urasawa, 1995). Para os genótipos G9 e G12 a taxa de mutação do gene VP7 foi calculada em $1.87x10^{-3}$ e $1.66x10^{-3}$, valores semelhantes aos obtidos com outros vírus ARNs (Matthijnssen et al., 2010, Ghosh & Kobayashi, 2011).

Os rearranjos representam alterações na sequência do segmento genômico, algumas vezes na forma de deleção ou como duplicação. Estes eventos geralmente são evidenciados através da visualização do perfil eletroforético dos RVA em EGPA. Os rearranjos resultam de erros de transcrição de um único segmento e possuem nada mais do que a duplicação parcial de um gene. Provavelmente, no momento da transcrição, por uma falha da ARN polimerase dependente de ARN, ela retorne a sua fita molde, reiniciando a transcrição a partir de diferentes estágios (Desselberger, 1996).

O evento de recombinação genética em RVA ainda é pouco conhecido (Phan et al., 2007). As recombinações intra genicas não tem sido descritas com frequência, mas existem relatos de recombinação entre genes de VP7 de diferentes genótipos G e entre linhagens do mesmo G (Parra et al., 2004; Phan et al., 2007; Martínez-Laso et al., 2009).

As reestruturações de segmentos genômicos ocorrem quando duas cepas de RVA distintas infectam a mesma célula. Foram descritos pela primeira vez por Matsuno e colaboradores (1980) que realizou a coinfecção em cultura celular de RV bovino (Lincoln) e RV símio (SA-11) obtendo assim um vírus reestruturado. A ocorrência de reestruturações parece ser um fenômeno frequente nos RVA. Porem, estudos mostraram que a maioria das variantes virais originadas por este mecanismo são substituídas no decorrer do tempo por cepas que apresentam uma constelação genômica que por algum motivo é dominante na população viral (McDonald et al., 2009). A incidência das reestruturações esta influenciada pela frequência de coinfecções na população e a diversidade genética dos RVA circulantes (Ghosh & Kobayashi, 2011). A ocorrência de reestruturações parece ser mais frequente nos países em desenvolvimento, fato provavelmente vinculado às precárias condições de saneamento básico e higiene, defesas imunológicas limitadas, coinfecções com parasitas, desnutrição, além do estreito relacionamento entre o homem, animais domésticos e outros animais. Todas as possibilidades facilitam a ocorrência de infecções mistas e, consequentemente, aumenta a possibilidade dos vírus sofrerem reestruturações genéticas (Desselberger, 1996).

1.8 Uso de códons

Com o objetivo de entender melhor sobre a dinâmica evolutiva dos vírus e em particular, dos RVA, faz-se necessário o uso de ferramentas e/ou métodos importantes na elucidação dos processos evolutivos destes agentes, suas implicações na expressão de proteínas e na manutenção de variantes genéticas de importância epidemiológica.

Os códons sinônimos não são utilizados de forma randômica e, em diversos organismos, a seleção natural parece gerar um viéis no uso de códons e, neste caso, a favor do uso de códons chamados de "códons ótimos", sobretudo em genes que são altamente expressos. Diversos fatores como expressão gênica, empacotamento do ARN viral, formação da estutura das proteínas e o uso de nucleotídeos podem influenciar no uso de códons (Stoletzki & Eyre-Walker, 2007).

Têm sido propostos dois modelos para explicar o viéis no uso de códons: i) modelo associado à tradução (*Translational related model* ou *Selective model*), postula que existe uma coadaptação no uso dos códons sinônimos e na abundancia dos ARNt (Ácido ribonucleico de transferência) para optimizar a eficácia da tradução. Neste modelo espere-se uma correlação entre o uso de códons e a expressão do gene; ii) modelo mutacional (*Mutational model* ou *Neutral model*), postula que o viéis no uso de códons é o resultado dos viéis mutacionais (Duret, 2002).

Um estudo baseado na análise do viéis no uso de códons e composição nucleotídica de diferentes vírus ARN, demostrou que o viéis observado nestes vírus sería em maior parte produto de pressões mutacionais. Além disso, observou-se que os genes que codificam para a ARN polimerase apresentavam um viéis maior no uso de códons, mesmo que os genes estruturais sejam de forma geral expressos em maiores níveis que os genes não estruturais (Jenkins & Holmes, 2003). Tem sido reportado que existe um viéis maior no uso de códons nos vírus de RNA segmentado quando comparado com os vírus com genoma não segmentados, associado provavelmente ao fato dos mesmos sofrerem reestruturações gênicas (Jenkins & Holmes, 2003).

O viéis no uso de codon é geralmente estimado pelo uso do valor *ENC* (do inglês: *Effective Number of codons*), este valor pode variar de 20 quando só um códon é utilizado para cada Aa até 61, quando todos os códons sao utilizados. Em genomas onde o uso de códons é inteiramente devido à tendências mutacionais o valor do *ENC* varía entre 31 e 61 (Wright, 1990). Para os RVA este valor foi estimado em 52.5 para a região codificante inteira do genoma, sugerindo que a pressão mutacional seria a principal força atuando no viéis no uso de códons no genoma deste vírus (Jenkins & Holmes, 2003).

1.9 Patogênese

Os RVA apresentam caraterísticas que os torna agentes altamente infecciosos e adaptados ao hospedeiro, estes vírus podem infectar igualmente o homem e animais (Franco et al., 2006). A transmissão deste vírus é feita pela via fecal-oral. Após um período de

incubação de 2 a 4 dias, os sintomas geralmente começam abruptamente com febre e vômitos, seguidos de diarreia aquosa que pode durar dentre 3 a 8 dias (Staat et al., 2002; Lee et al., 2008). A doença provocada pelos RVA é mais frequente em crianças entre 3 e 36 meses de idade. Acredita-se que múltiplas infecções ocorrem ao longo da vida, e que por causa da imunidade adquirida nestes episódios as crianças com idade mais avançada e os adultos sofrem de episódios menos graves e/ou assintomáticos (Anderson & Weber, 2004).

Os enterócitos do intestino delgado dividem-se em dois tipos: enterócitos propriamente ditos e células da cripta. Os enterócitos das velocidades são enterócitos maduros que se diferenciam em cumprir funções de absorção e de digestão. Os que cumprem funções de absorção sintetizam diversas dissacaridases, peptidases, e outras enzimas que são expressas na superfície apical onde cumprem suas funções digestivas. A absorção ocorre por mecanismos passivos, difusão de solutos através de gradientes eletroquímicos ou osmóticos, e transporte ativo. As células da cripta são as progenitoras dos enterócitos das velocidades, as mesmas secretam ativamente íons Cl^- ao lume intestinal. Durante o funcionamento normal do intestino a atividade combinada dos enterócitos e das células da cripta garante o fluxo bidirecional constante de eletrólitos e agua através do epitélio (Ramig, 2004). Os RVA se replicam nos enterócitos maduros perto dos estremos das vilosidades, sugerindo assim que estes enterócitos expressam fatores que são necessários para a replicação do vírus (Conner & Ramig, 1997).

A diarreia causada pelos RVA é multifatorial e, portanto diversos mecanismos estão envolvidos (Quadro 1).

Quadro 1. Resumo apresentando os mecanismos envolvidos na geração de diarreia pelos rotavírus. Quadro adaptado de Franco et al., 2006.

Mecanismos	Comentários
A ação da toxina NSP4 induz diarreia secretória	Só demonstrado em camundongos; ocorre durante o início da infecção, antes da morte celular
Estimulação do sistema nervoso entérico (SNE) induz diarreia secretória e aumenta a motilidade intestinal	Drogas que inibem o SNE são efetivas para tratar a diarreia causada pelos RVs; ocorre durante o início da infecção, antes da morte celular
A alteração no metabolismo das dissacaridases e outras proteínas de membrana dos enterócitos induz a diarreia osmótica e mal absortiva	Ocorre durante o início da infecção, antes da morte celular
A morte dos enterócitos contribui para a ocorrência da diarreia osmótica e mal absortiva	Mecanismos tardios

Após a internalização do vírus na célula, eventos intracelulares causam a liberação de íons Ca^{2+} do RE desencadeando uma série de processos celulares, incluindo a disjunção do citoesqueleto, diminuição da expressão de dissacaridases e outras enzimas presentes na superfície apical, inibição do sistema de co-transporte de íons Na^{+} e necrose. Estes eventos levam a dificuldade na absorção através da diminuição na capacidade absortiva do epitélio (Ramig, 2004). Um fragmento da proteína NSP4 é secretado da célula e é capaz de induzir o aumento de Ca^{2+} intracelular em células vizinhas amplificando o efeito da infecção causada pelo vírus (Zhang et al., 2000; Tafazoli et al., 2001).

O Sistema Nervoso Entérico (SNE) se localiza imediatamente embaixo do epitélio das vilosidades, e está situado para receber estímulos do epitélio danificado pela infecção por RVs. Lundgren e colaboradores (2000) demostraram que o SNE está envolvido na diarreia causada pelos RVs. Mais tarde foi demonstrado que a ativação de SNE ocorre pela liberação de prostaglandinas, peptídeos sinalizadores, citoquinas e da enterotoxina viral NSP4, ocasionando o aumento da motilidade intestinal (Boshuizen et al., 2004).

1.10 Tratamento e prevenção

O tratamento da diarreia causada pelos RVA tem como base repor as perdas de fluidos e eletrólitos. Para a reidratação da criança pode-se utilizar a fórmula preconizada pela OMS ou ainda outra fórmula comercial disponível. Estas fórmulas tem se mostrado efetivas para crianças com desidratação moderada. Nos casos de diarreia grave é recomendável a utilização de fluidos intravenosos, ou no caso de que a criança tenha dificuldade de deglutição devido à intensidade dos vômitos. A terapia nutricional e a assistência médica são extremammente importantes, ajudando a reduzir a morbidade e a mortalidade causada pelos RVA. Não existem medicamentos antivirais disponíveis para o tratamento da infecção pelos RVA. Entretanto, diversos estudos tem demostrado a atividade antiviral de algumas drogas frente aos RVA (Rossignol et al., 2006; Teran et al., 2009; La Frazia et al., 2013; Shen et al., 2013). Inclusive, um estudo realizado recentemente no Brasil demostrou a atividade antiviral de plantas medicinais contra o RVA (Cecílio et al., 2012).

Somente as melhorias das condições sanitárias, bem como a destinação adequada de dejetos de humanos e de animais, não são suficientes no controle e na prevenção das infecções por RVA. A infecção natural não confere proteção completa contra reinfecção e a prevenção da GA. Durante os dois primeiros anos de vida a prevenção é fundamental, pois é neste período que ela é mais grave. Assim, a utilização de uma vacina segura e eficaz representa uma boa estratégia para diminuir as formas mais graves da doença (Anderson, 2010).

Em 2009, a OMS recomendou a inclusão de vacinas contra RVA nos programas nacionais de imunização de todos os países, e atualmente recomenda a introdução de vacinas em países onde as mortes por GA são responsáveis por mais de 10% da mortalidade infantil (< 5 anos de idade). Algumas vacinas já foram desenvolvidas ou estão em desenvolvimento. Entretanto, somente duas vacinas (RV1 e RV5) estão licenciadas para uso em vários países, incluindo o Brasil (O'Ryan et al., 2011). A RV1 é uma vacina oral monovalente (G1P[8]), atenuada, originada a partir de uma cepa humana. A RV5 é uma vacina oral, pentavalente (G1, G2, G3, G4 e P[8]) de vírus reestruturado (WC3 - bovino) (Ruiz-Palacios et al., 2006, Vesikari et al., 2006). As duas vacinas, protegem contra os 5 genótipos mais comuns de RVA que circulam mundialmente (G1-G4, G9) e o impacto positivo da introdução destas vacinas já foi demonstrado em alguns países europeus, africanos e americanos, inclusive o Brasil (Chandran et al., 2010; Carvalho-Costa et al., 2011; Linhares et al., 2011; Patel et al., 2011).

O MS brasileiro introduziu a vacina monovalente no PNI em 2006, mas a vacina pentavalente pode ser encontrada em clínicas particulares. Do Carmo e colaboradores (2011)

demonstraram que três anos após a introdução da vacina RV1 no Brasil houve uma redução de 22-28% de mortes e de 21-25% de hospitalizações em crianças <2 anos de idade.

1.11 Epidemiologia dos rotavírus da espécie A

A distribuição dos genótipos de RVA ao longo do tempo pode variar de região em região e múltiplos genótipos G e P podem cocircular na população, inclusive dentro da mesma região geográfica. Entretanto, a maioria dos estudos epidemiológicos tem demonstrado que amostras de RVA, de genótipos G1P[8], G2P[4], G3P[8], G4P[8] e G9P[8], são responsáveis pela maioria das infecções em humanos (92%), sendo importantes alvos de estudos para o desenvolvimento de vacinas (Kirkwood, 2010). Contudo o genótipo G1P[8] é o mais prevalente mundialmente, sendo responsável por mais de 70% das infecções por RVA na América do Norte, Europa e Austrália. Porém, este genótipo representa somente 30% das infecções por RVA na América do Sul e Ásia, e 23% na África (Tate et al., 2010).

No Brasil estes genótipos representam 75% dos RVA detectados (Linhares et al., 2011). Os genótipos G1-G4 foram os mais prevalentes no período de 1982 a 1995; de 1996 a 2005 o genótipo G9 emergiu e, neste período, o genótipo G2P[4] foi pouco detectado. Em 2005 o genótipo G2P[4] reemergiu e tornou-se o genótipo mais prevalente no Brasil no período de 2006 a 2011 (Figura 7) (Leite et al., 2008; Linhares et al., 2011; Carvalho-Costa et al., 2011; Carvalho-Costa et al., manuscrito em preparação). Deve-se destacar que no Sul do país o genótipo mais prevalente em 2009 foi o G3P[8]. Dados recentes demostram que em 2012 o genótipo G2P[4] deixou de ser o mais prevalente no Brasil e observou-se um aumento na prevalência do genótipo G3P[8] / G3P[X] (Carvalho-Costa et al., manuscrito em preparação).

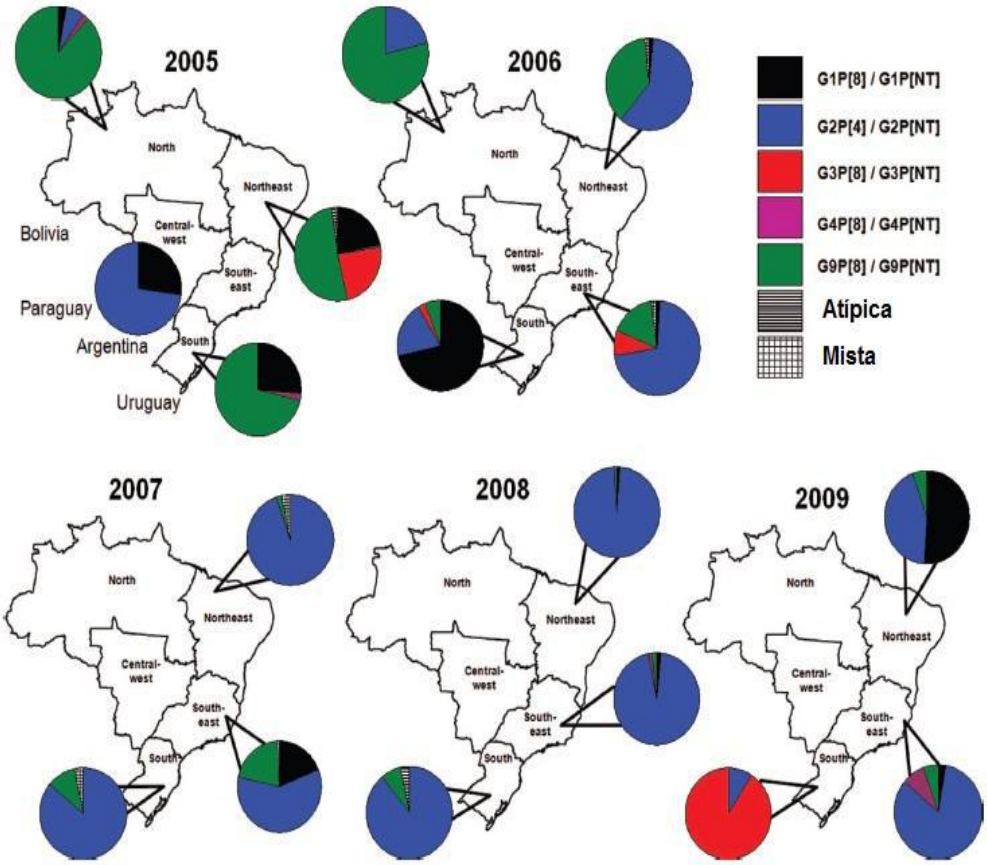

Figura 7. Distribuição dos genótipos de rotavírus da espécie A no Brasil entre 2005 e 2009. Figura adaptada de Carvalho-Costa et al., 2011.

Um surto de GA no estado do Acre no ano de 2005 foi atribuído ao RVA genótipo G9P[8], sendo descritas 12,145 hospitalizações e oito mortes na municipalidade de Rio Branco (Siqueira et al., 2010). Análises filogenéticas de amostras detectadas nesse surto revelaram a circulação de uma nova variante do genótipo G9P[8] que até o momento só tem sido detectada no Brasil (Acre) (Tort et al., 2010). Após introdução da vacina RV1 pelo PNI, a detecção do genótipo G9 tem diminuído consideravelmente no país (Carvalho-Costa et al., 2011). Na Argentina, este genótipo foi o mais prevalente entre 2006-2008, sendo que em 2009 a detecção deste genótico teve uma drastica redução e concomitantemente ocorreu o aumento dos genótipos G3P[8] e G12P[8] (Stupka et al., 2009). O genótipo G9P[8] emergiu em 1995 em diversos países do mundo e rapidamente tornou-se um dos cinco genótipos de RVA mais prevalentes (Rahman et al., 2005; Matthijnssens et al., 2010).

Como mencionado anteriormente, um aumento significativo na ocorrência do genótipo G2P[4] foi verificado a partir de 2005 (Leite et al., 2008). No período de 2006 a 2007,

primeiro ano de implementação da vacina RV1, G2 foi detectado nos estados do Rio de Janeiro, Sergipe, Pernambuco, Piauí e Minas Gerais (Leite et al., 2008). Na região Nordeste do Brasil, diferentes estudos mostraram uma prevalência de 100% para o genótipo G2P[4] no ano de 2007 (Gurgel et al., 2007, Nakagomi et al., 2008). Estudos realizados na região Norte do Brasil demostraram que a re-emergência de G2 alcançou taxas de até 90% (de Oliveira et al., 2008). Um aumento na detecção deste genótipo também foi reportado em diferentes países tais como: Honduras (Ferrera et al., 2007), Argentina, Paraguai (Amarilla et al., 2007), El Salvador e Guatemala (Patel et al., 2008).

Recentemente, outros genótipos de RVA estão sendo descritos e considerados como emergentes epidemiologicamente no mundo. Este é o caso de G5, em países da África e da Ásia; assim como G8, G10 e G12 mundialmente. Além disso, combinações atípicas em amostras humanas são detectadas em diversos países, sendo a maioria destas nos países da África, América Latina e Ásia (Matthijnssens et al., 2010; Iturriza-Gómara et al., 2011). No Brasil os genótipos G2P[8]; G3P[4]; G4P[6]/P[9]; G5P[6]/P[8]/G5P[NT]; G8P[4]/P[8]; G9P[4]/P[6]; G10P[9]; G12P[9]/P[8]/P[6] e G6 tem sido detectados esporadicamente (Araújo et al., 2001; Mascarenhas et al., 2002; Pietruchinski et al., 2006; Domingues et al., 2008; Leite et al., 2008; Soares et al., 2011; Gómez et al., 2013; Gómez et al. (artigo submetido)).

Com relação à sazonalidade, é possível observar um padrão apenas nas regiões de clima temperado, com surtos e epidemias nos meses mais frios e secos do ano. Em geral, nas regiões de clima tropical as infecções por RVA ocorrem ao longo de todo o ano (Estes & Kapikian, 2007).

2 Justificativa do trabalho

Após a introdução, em março de 2006, da vacina RV1 pelo PNI no Brasil observou-se uma mudança na epidemiologia dos RVA circulantes na população, tornando-se o genótipo G2P[4] (*DS-1 like*) o mais prevalente até 2011 (Leite et al., 2008; Carvalho-Costa et al., 2011; Carvalho-Costa et al., manuscrito em preparação). A emergência deste genótipo reflete um fenômeno continental, provavelmente relacionado a vários fatores. Dados recentes obtidos no LVCA, sugerem que diminuiu a detecção do genótipo G2 após 2010, e que esta diminuição coincidiu com o aumento na prevalência do genótipo G3P[8] (Carvalho-Costa et al., em preparação). Além disso, a detecção do genótipo G9 tem diminuído consideravelmente no país após introdução da vacina (Carvalho-Costa et al., 2011) e os genótipos G1P[6] e G12 tem sido detectados ocasionalmente (Pietruchinski et al., 2006; Leite et al., 2008; Carvalho-Costa et al., 2009, 2011; Gómez et al., 2011, 2013; Fumian et al., 2011; Linhares et al., 2011; Soares et al., 2011; Gómez et al., 2013; Gómez et al., submetido; Carvalho-Costa et al., em preparação).

Os estudos de fase III relacionados à proteção conferida pela vacina RV1 antes da implementação da mesma pelo PNI no Brasil não puderam ser adequadamente avaliados, pois os genótipos G2P[4], P[6], G12, entre outros, não se encontravam entre os mais prevalentes. A vacina RV1 apresenta uma constelação genética *Wa-like* e uma importante questão que se coloca é sobre a eficácia da mesma frente a cepas que apresentem constelações diferentes, como *DS-1-like* e *AU-1-like:* a pergunta é se poderão ocorrer mecanismos de reestruturações geneticas intra ou entre genogrupos de RVA que possam prejudicar a eficácia da RV1? Ocorrerão reestruturacoes geneticas entre amostras selvagens e a RV1? e caso afirmativo, ira variar a eficácia da vacina frente a estas novas variantes virais?

Dois estudos realizados com amostras brasileiras de crianças hospitalizadas e vacinadas com RV1 demonstraram a ocorrência destes reordenamentos genéticos (Gómez et al., 2013; Rose et al., 2013).

No inicio do desenvolvimento deste projeto não existia na literatura nenhum trabalho descrevendo a caracterização genética dos 11 genes de RVA de amostras detectadas no Brasil e, alguns poucos de outros países, sendo a maioria em paises desenvolvidos. Na literatura, a maioria dos estudos estavam relacionados com a caracterização dos genes que codificam para as proteínas VP7, VP8*, VP6, e NSP4, principalmente para VP7 e VP8*. Embora os dados sobre a diversidade genética do gene que codifica para a VP7 e/ou o que codifica para a VP8* são importantes do ponto de vista da imunidade do hospedeiro contra a doença causada pelo RVA, informações sobre esses genes não seriam suficientes para obter dados conclusivos

sobre a dinâmica evolutiva destes vírus. O fato dos RVA apresentarem um genoma segmentado resulta numa grande variedade genética destes vírus, já que diferentes mecanismos evolutivos podem atuar ao mesmo tempo, como anteriormente apresentados e discutidos: mutação pontual, rearranjo, recombinação ou reestruturação genética. Portanto, estudos que descrevam a analise dos 11 genes (constelação gênica) dos RVA poderão contribuir para melhor avaliar a diversidade genética e a evolução destes vírus em dois períodos: pré e pos introdução da vacina RV1.

Neste estudo foi analisada a diversidade genética de RVA de diversos genótipos detectados no Brasil antes e após a introdução da vacina RV1 pelo PNI com o intuito de identificar e caracterizar variantes de RVA emergentes e reemergentes no Brasil. Deve-se ressaltar que este estudo compreende diferentes regiões geográficas do Brasil, pois dados epidemiológicos sugerem diversidades genotípicas regionais (Leite et al., 2008; Carvalho-Costa et al., manuscrito em preparação).

Uma outra abordagem deste estudo foi o uso de códons, pois pouco é conhecido sobre o viéis no uso de códons pelos RVA. Estudar as causas do víéis no uso de códons é essencial para entender a interfase entre os vírus e a resposta do sistema imune, além de ajudar no desenho de vacinas, já que uma expressão eficiente das proteínas virais é fundamental para gerar imunidade (Shackelton et al., 2006). Buscando contribuir para um melhor entendimento deste mecanismo, foram estudadas as causas do víeis no uso de códons dos RVA analisando os genes que codificam para as proteínas VP7 e VP8* de amostras genótipo G2P[4] detectadas no Brasil no período de 1996-2009, assim como genomas inteiros disponíveis no banco de dados do *GenBank* (url: http://www.ncbi.nlm.nih.gov/genbank/).

3 Objetivos

3.1 Objetivo Geral

Estudar a diversidade genética de RVA de cepas pertencentes a diferentes genótipos, detectadas em diversas regiões do país, anterior e posterior a introdução da vacina RV1 pelo PNI, em março de 2006.

3.2 Objetivos Específicos

1) Amplificar, sequenciar e realizar análises filognéticas dos genes que codificam para as proteínas VP7 e VP8* de amostras de RVA detectadas entre os anos 2005 e 2011 de diferentes regiões do Brasil;

2) Analisar as sequencias aminoacídicas das proteínas VP7 e VP8*, e comparar os sitios antigênicos com a sequencias disponíveis da vacina RV1;

3) Amplificar, sequenciar, e analisar a constelação genética (11 genes) de RVA de amostras pertencentes a diferentes genótipos detectadas após 2004 no Brasil;

4) Analisar o viéis no uso de códons dos genes que codificam para as proteínas VP7 e VP8* de amostras de RVA G2P[4] detectadas em diferentes regiões do Brasil entre 1996 e 2009;

4. Metodologias e resultados

As seções “Metodologias” e “Resultados” deste manuscrito de tese serão apresentados sob a forma de artigos submetidos a publicação, aceitos ou em fase final de confecção.

CAPITULO II

DIVERSIDADE GENÉTICA DO GENÓTIPO G2P[4] ANTES E APÓS INTRODUÇÃO DA VACINA MONOVALENTE (RV1) NO BRASIL (Artigos 1 e 2)

Artigo 1: Rotavirus A Genotype P[4]G2: Genetic Diversity and Reassortment Events Among Strains Circulating in Brazil Between 2005 and 2009 (Artigo publicado).

Este artigo esta relacionado ao objetivo 1.

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Rotavirus A Genotype P[4]G2: Genetic Diversity and Reassortment Events Among Strains Circulating in Brazil Between 2005 and 2009

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Group A rotaviruses (RV-A) are the leading cause of severe gastroenteritis in infants and young children worldwide. Due to the epidemiologic complexity of RV-A, especially in developing countries, it is important to determine which genotypes are circulating, principally after the introduction in March 2006 of the monovalent (P[8]G1) Rotarix® vaccine in Brazil by the National Immunization Program. In Phase III trials with Rotarix®, the prevalence of genotype P[4]G2 was extremely low, and therefore, evaluation of heterotypic immunization against this genotype was performed by meta-analysis statistics tests. Different studies have shown the re-emergence of genotype P[4]G2 in Brazil, since 2005, as well as in other countries, suggesting that it could be a continental phenomenon related to the temporal variability in the genotype's naturally occurring distribution. It is important to note that genotype P[4]G2 does not share VP4 or VP7 antigens with the vaccine strain. Therefore, we performed a phylogenetic analysis based on VP4 (VP8*), VP7, VP6, and NSP4 genes of RV-A genotype P[4]G2 samples isolated from the five regions of Brazil between 2005 and 2009. This study revealed that different genetic variants of RV-A genotype P[4]G2 circulated in Brazil between 2005 and 2009, and that this variability is determined mainly by: occurrence of point mutations; reassortment events; and widespread global gene flow. The results obtained in this study are important to our understanding of the epidemiology and evolution of RV-A genotype P[4]G2 and demonstrate the importance of continuous monitoring and molecular characterization of RV-A strains circulating in human and animal populations. ***J. Med. Virol. 83:1093–1106, 2011.*** © 2011 Wiley-Liss, Inc.

KEY WORDS: rotavirus A; P[4]G2 genotype; genetic variability; reassortment; phylogenetic analysis

INTRODUCTION

Group A rotaviruses (RV-A) are the leading cause of severe diarrhea in children under 5 years of age worldwide, accounting for 611,000 deaths annually [Parashar et al., 2006]. RV-A belongs to the *Reoviridae* family, genus *Rotavirus*, consisting of a genome with 11 segments of double-stranded RNA (dsRNA) enclosed in a three layer protein capsid [Estes and Kapikian, 2007]. The genome encodes six structural proteins (VPs) and six non-structural proteins (NSPs). VP4 and VP7 proteins form the outer capsid and, both are involved in the virus entrance to the cell and inducing neutralizing antibodies [Estes and Kapikian, 2007]. Based on these two proteins, RV-A have been

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classified into P[ ] and G genotypes; being P[8]G1, P[4]G2, P[8]G3, P[8]G4, and P[8]G9 the most prevalent genotypes worldwide [Santos and Hoshino, 2005; Leite et al., 2008; Iturriza-Gómara et al., 2009]. Until now, at least 23 G-genotypes and 32 P-genotypes have been described [Matthijnssens et al., 2009]. The VP6 protein forms the intermediate capsid; based on this protein, rotaviruses (RVs) are classified into seven groups (A–G). Among them, RV-A is recognized as the most significant group because it has the highest prevalence and pathogenesis in humans and various animal species [Estes and Kapikian, 2007]. The NSP4 protein was the first viral enterotoxin described and has been studied extensively because it is a multifunctional protein, involved in the morphogenesis and pathogenesis of the virus [Ball et al., 1996; Horie et al., 1999; Estes and Kapikian, 2007].

Immunity related to RV-A is considered to be homotypic and heterotypic [Estes and Kapikian, 2007]. The VP4 and VP7 proteins are important for development of immunity against RVs infection, VP6 and NSP4 proteins may also be involved [Ball et al., 1996; Burns et al., 1996; Estes and Kapikian, 2007]. Genotype P[4]G2 does not contain either the VP4 or VP7 antigen of the Rotarix® (GlaxoSmithKline Biologicals, Rixensart, Belgium) vaccine strain. A study conducted by Correia et al. [2010] revealed that the Rotarix® vaccine provides high level of protection for the first year of life against severe diarrhea caused by fully heterotypic P[4]G2 strains. This genotype reemerged in Brazil in 2005, and since then it has become predominant in this country [Gurgel et al., 2007; Leite et al., 2008; Nakagomi et al., 2008; de Oliveira et al., 2008; Carvalho-Costa et al., 2009; Munford et al., 2009]. Several studies have shown that this genotype also increased in other countries inside and outside the American continent [Amarilla et al., 2007; Ferrera et al., 2007; Patel et al., 2008; Kirkwood et al., 2009; Esteban et al., 2010]. Some of these countries do not include an anti-RV-A vaccine in their immunization programs, suggesting that the increase of this genotype could represent a natural phenomenon [Santos and Hoshino, 2005; Desselberger et al., 2006; Leite et al., 2008; Carvalho-Costa et al., 2009]. Furthermore, the possibility that this genotype fluctuation is due to a natural phenomenon or is related to the introduction of the Rotarix® vaccine, or both, should not be discounted, and more studies are needed before reaching a conclusion.

The aim of this study was to determine the genetic variability present in RV-A genotype P[4]G2 strains isolated from the five regions of Brazil between 2005 and 2009, analyzing VP4 (VP8*), VP7, VP6, and NSP4 genes.

MATERIALS AND METHODS

Clinical Samples

In this study 81 samples isolated from children under 5 years old with gastroenteritis between 2005 and 2009 were used. These samples were genotyped previously as P[4]G2 RV-A and stored at the Rotavirus Reference Laboratory, Laboratory of Comparative and Environmental Virology, Oswaldo Cruz Institute—Oswaldo Cruz Foundation (LVCA, IOC—FIOCRUZ). Samples were obtained from five regions of Brazil: south: Rio Grande do Sul [RS]; southeast: Espírito Santo [ES], Minas Gerais [MG], and Rio de Janeiro [RJ]; north: Acre [AC]; northeast: Alagoas [AL], Bahia [BA], Maranhão [MA], Pernambuco [PE] and Sergipe [SE]; and centralwest: Mato Grosso do Sul [MS]. Among the 81 RV-A genotype P[4]G2 samples 63 were isolated from children that were not vaccinated for RV-A, two samples were from vaccinated children, with one and two doses (sample 15811_08SE) and for the 16 remaining samples no data related to vaccination were available (Table I).

This study was approved by the Institutional Ethics Committee (CEP/FIOCRUZ), number 311/2006.

Nucleic Acid Extraction and RT-PCR

Genomic RNA was extracted from 10% fecal specimens by the glass powder method [Boom et al., 1990]. Amplification of VP7 and VP6 genes was performed using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase Kit (Invitrogen®, Sao Paulo, Brazil) following manufacturer's instructions. Primers for VP7 gene amplification were 9Beg (1–28) and 9End (1,062–1,036) [Gouvea et al., 1990], obtaining a 1,062 bp amplicon. Primers for VP6 gene amplification were modified from Elschner et al. [2002], obtaining a 1,356 bp amplicon. Reverse transcription (RT) for VP4 (VP8*) and NSP4 genes was performed according to the RT Superscript III™ (Invitrogen®) manufacturer's instructions. For the amplification of VP4 (VP8*), the PCR protocol was modified from Gentsch et al. [1992], using the F4P4D (−2 to 22) [Gómez et al., 2010] and 4con2 (887 to 868) [Gentsch et al., 1992] primers, obtaining a 889 bp amplicon. For the NSP4 gene, PCR was performed using the following conditions: 94°C/3 min, 40 cycles: 94°C/30 sec—50°C/30 sec—72°C/30 sec, ending with 72°C/10 min. Primers used were: FN4D (5′ CGG AAA AGA TGG AAA AGC 3′) (35–52) and RN4D (5′ GGA TTG GTT AAA CGG GAT 3′) (707–690), obtaining a 672 bp amplicon. Amplicons obtained from the RT-PCR reactions were resolved by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and visualized under long-wave UV light.

Distilled Milli-Q water was used as a negative control in all steps, and recommended manipulations for PCR procedures were carried out as a precaution to avoid false-positive results.

Sequencing and Sequence Analysis

The same primers used for the PCR were used for sequencing the amplicons of the genes studied. For VP6 gene sequencing an internal primer designed in

TABLE I. Origins of the Brazilian Rotavirus A Genotype P[4]G2 Strains, Vaccine Information, and Accession Numbers Corresponding to the NPS4, VP4 (VP8*), VP6, and VP7 Nucleotide Sequences Obtained in This Study

Isolate number	Year	State	Region	Vaccine information	Accession numbers			
					NSP4	VP4	VP6	VP7
11143_05MS	2005	Mato Grosso do Sul	Central west		HM066160	HM066011		HM066066
11257_05MS	2005	Mate Grosso do Sul	Central west	NV[a]	HM066161	HM066013	HM066126	HM066067
11531_05AC	2005	Acre	North	NV	HM066162		HM066129	HM066068
11580_05AC	2005	Acre	North	NV	HM066163	HM066012	HM066128	HM066070
11581_05AC	2005	Acre	North	NV	HM123847		HM123816	
11782_05RJ	2005	Rio de Janeiro	Southeast	NV	HM123845		HM123817	
11830_06AC	2006	Acre	North	NV	HM123846		HM123818	
11837_06AC	2006	Acre	North	NV	HM066164		HM066130	HM066069
11860_06RJ	2006	Rio de Janeiro	Southeast	NV	HM066165	HM066014	HM066127	HM066071
12220_06RJ	2006	Rio de Janeiro	Southeast	NV	HM066166	HM066015	HM123819	HM066082
12224_06RJ	2006	Rio de Janeiro	Southeast	NV	HM123848			HM066089
12287_06BA	2006	Bahia	Northeast	NV	HM066167	HM066016	HM066131	HM066073
12301_06BA	2006	Bahia	Northeast	NV			HM123820	HM066079
12343_06RJ	2006	Rio de Janeiro	Southeast	NV	HM123849		HM123821	HM066088
12389_06RJ	2006	Rio de Janeiro	Southeast	NV	HM123850		HM123822	HM066077
12482_06BA	2006	Bahia	Northeast	NV	HM066168	HM066017	HM066132	HM066072
12522_06BA	2006	Bahia	Northeast	NV	HM123851		HM123823	
12540_06RJ	2006	Rio de Janeiro	Southeast	NV	HM123852			HM066076
12549_06RJ	2006	Rio de Janeiro	Southeast	NV	HM066169	HM066018	HM066133	HM066090
12585_06ES	2006	Espirito Santo	Southeast	NV	HM123853		HM123824	
12589_06RJ	2006	Rio de Janeiro	Southeast	NV	HM066170	HM066019	HM123825	HM066091
12647_06RJ	2006	Rio de Janeiro	Southeast	NV	HM123854	HM066020	HM123826	
12684_06RJ	2006	Rio de Janeiro	Southeast	NV		HM066021		
12774_06RJ	2006	Rio de Janeiro	Southeast	NV	HM123855		HM123827	HM066081
12775_06RJ	2006	Rio de Janeiro	Southeast	NV	HM123856			
12777_06BA	2006	Bahia	Northeast	NV		HM066022		
12840_06ES	2006	Espirito Santo	Southeast	NA[b]	HM066171	HM066023	HM066134	
12842_06ES	2006	Espirito Santo	Southeast	NV	HM066172	HM066024		HM066074
12916_06BA	2006	Bahia	Northeast	NV	HM123857			
13039_06RS	2006	Rio Grande do Sul	South	NV		HM066025	HM066135	HM066078
13079_06RJ	2006	Rio de Janeiro	Southeast	NV		HM066026		
13081_06RJ	2006	Rio de Janeiro	Southeast	NV		HM066027		
13151_06RJ	2006	Rio de Janeiro	Southeast	NV	HM066173	HM066028	HM123828	HM066083
13158_06ES	2006	Espirito Santo	Southeast	NV	HM066174	HM066029	HM066136	HM066084
13438_07RJ	2007	Rio de Janeiro	Southeast	V[c] (1 dose)	HM123858			
13514_07ES	2007	Espirito Santo	Southeast	NV	HM123859			
13663_07AL	2007	Alagoas	Northeast	NA	HM123860		HM123829	
13668_07AL	2007	Alagoas	Northeast	NV	HM066175	HM066030	HM066137	HM066085
13780_07RJ	2007	Rio de Janeiro	Southeast	NV	HM123861		HM123830	HM066092
13788_07SE	2007	Sergipe	Northeast	NA		HM066031	HM066138	HM066086
13793_07SE	2007	Sergipe	Northeast	NV		HM066032	HM123831	HM066087
13891_07RJ	2007	Ric de Janeiro	Southeast	NV	HM123862		HM123832	HM066093
14255_07RJ	2007	Rio de Janeiro	Southeast	NV	HM123863			
14263_07RS	2007	Rio Grande do Sul	South	NV			HM123833	
14303_07MG	2007	Minas Gerais	Southeast	NV	HM066176	HM066033	HM066139	HM066094
14322_07MG	2007	Minas Gerais	Southeast	NV	HM123864		HM123834	
14344_07RS	2007	Rio Grande do Sul	South	NV			HM123835	
14397_07MA	2007	Maranhão	Northeast	NV	HM123865		HM123836	
14422_07RS	2007	Rio Grande do Sul	South	NV	HM066177	HM066034		HM066095
14426_07ES	2007	Espirito Santo	Southeast	NA	HM123866	HM066035		
14919_08RS	2008	Rio Grande do Sul	South	NV	HM066178	HM066036	HM066140	HM066096
15311_08RJ	2008	Rio de Janeiro	Southeast	NV	HM066179	HM066043	HM066141	HM066097
15382_08BA	2008	Bahia	Northeast	NA	HM123867	HM066037		HM066098
15385_08BA	2008	Bahia	Northeast	NA	HM066180	HM066038	HM066154	HM066099
15593_08AL	2008	Alagoas	Northeast	NV	HM123868	HM066039		HM066100
15771_08PE	2008	Pernambuco	Northeast	NV	HM066181	HM066061	HM066142	HM066101
15774_08PE	2008	Pernambuco	Northeast	NV	HM066182	HM066052		HM066102
15777_08PE	2008	Pernambuco	Northeast	NV		HM066044	HM123837	HM066103
15782_08MG	2008	Minas Gerais	Southeast	NA	HM066183	HM066053	HM066143	HM066104
15786_08ES	2008	Espirito Santo	Southeast	NV	HM066184	HM066045	HM066144	HM066105
15811_08SE	2008	Sergipe	Northeast	V (2 doses)	HM066185	HM066062	HM066145	HM066106
15830_08RS	2008	Ric Grande do Sul	South	NV		HM066054		
15836_08RS	2008	Rio Grande do Sul	South	NV	HM123869	HM066055	HM123838	HM066107
15840_08RS	2008	Rio Grande do Sul	South	NV	HM066186	HM066056	HM066146	HM066108

(Continued)

Table I. (*Continued*)

Isolate number	Year	State	Region	Vaccine information	Accession numbers			
					NSP4	VP4	VP6	VP7
15859_08MG	2008	Minas Gerais	Southeast	NV	HM066187	HM066057	HM066147	HM066109
15860_08RJ	2008	Rio de Janeiro	Southeast	NV	HM066188	HM066058	HM066148	HM066110
15863_08MA	2008	Maranhão	Northeast	NA	HM066189	HM066046	HM066156	HM066111
15894_08ES	2008	Espirito Santo	Southeast	NV	HM123870		HM123839	HM066112
15898_08MG	2008	Minas Gerais	Southeast	NA	HM066190	HM066059	HM066149	HM066113
15900_08MG	2008	Minas Gerais	Southeast	NV	HM066191	HM066065	HM066150	HM066114
15953_08RS	2008	Rio Grande do Sul	South	NA	HM123871	HM066047	HM123840	HM066115
15958_08RS	2008	Rio Grande do Sul	South	NV	HM066192	HM066048	HM066151	HM066116
15983_08BA	2008	Bahia	Northeast	NV	HM066193	HM066049	HM066152	HM066117
15988_08BA	2008	Bahia	Northeast	NV	HM066194	HM066063	HM066153	HM066118
15990_08BA	2008	Bahia	Northeast	NV			HM123841	HM066119
16054_09MA	2009	Maranhão	Northeast	NA		HM066050		HM066120
16056_09MA	2009	Maranhão	Northeast	NA	HM066195	HM066051		HM066121
16064_09MA	2009	Maranhão	Northeast	NA	HM066196	HM066040	HM066157	HM066122
16099_09ES	2009	Espirito Santo	Southeast	NA	HM066197	HM066064	HM066155	HM066123
16100_09ES	2009	Espirito Santo	Southeast	NA	HM066198	HM066041	HM066158	HM066124
16101_09ES	2009	Espirito Santo	Southeast	NA	HM066199	HM066042	HM066159	HM066125

[a]Non-vaccinated children (NV).
[b]Non-information available about vaccination (NA).
[c]Vaccinated children (V).

this study was used: 5′ GAC GGV GCR ACT ACA TGG T 3′ (747–766). The sequencing reaction was performed for both strands and was carried out using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit® and an ABI Prism 3730 Genetic Analyzer (both from Applied Biosystems, Foster City, CA) using the Genomic Platform for DNA sequencing (PDTIS/FIOCRUZ) [Otto et al., 2008]. All reactions were repeated at least once for the accuracy of the study.

A nucleotide sequence BLAST search was performed using the National Centre for Biotechnology Information (NCBI, National Institute of Health, Bethesda, MD), Basic Local Alignment Tool (BLAST) server on the GenBank database [Altschul et al., 1990]. The nucleotide sequences were aligned using the CLUSTAL W program [Thompson et al., 1994] with corresponding gene sequences of selected RV-A strains available from the GenBank database. Once aligned, phylogenetic trees were generated by the Neighbor-joining method under a matrix of genetic distances established under the Kimura-two parameter model [Felsenstein, 1993], using the MEGA v. 4.0 program [Tamura et al., 2007]. The robustness of each node was assessed by bootstrap resampling (2,000 replicates). The lack of different genes sequences corresponding to the same isolate in the GenBank database turned out impossible to perform the phylogenetic analysis with the same isolate for the four genes studied.

The nucleotide sequences corresponding to the VP4 (VP8*) and VP7 genes from Brazilian genotype P[4]G2 strains, and strains selected from the GenBank database, were translated to amino acid sequences using MEGA v. 4.0 program [Tamura et al., 2007].

RESULTS

A total of 239 nucleotide sequences, 54 for VP4 (VP8*), 58 for VP7, 60 for VP6 and 67 for NSP4, were obtained from 81 Brazilian genotype P[4]G2 samples isolated between 2005 and 2009 (Table I). According to the classification proposed by Matthijnssens et al. [2008b], all samples revealed a consensus genotype constellation of P[4]-G2-I2-E2 considering VP4 (VP8*), VP7, VP6, and NSP4 genes, respectively.

VP4 (VP8*) Gene Analysis

Nucleotide sequences corresponding to the VP4 (VP8*) gene obtained from Brazilian samples, together with sequences obtained from the GenBank database, were used to construct the phylogenetic tree shown in Figure 1. Phylogenetic analysis showed that all sequences obtained from Brazilian samples analyzed in this study belonged to Lineage V. Two major clusters were observed, 2005–2009 and 2008–2009, containing strains from the five regions of Brazil, and from the southeast region (Rio de Janeiro, Minas Gerais and Espírito Santo), respectively (Fig. 1). Two sequences obtained from samples of Espírito Santo state in 2006 grouped in a separate branch (Fig. 1, ES 2006).

VP7 Gene Analysis

Nucleotide sequences corresponding to the VP7 gene obtained from Brazilian samples were used to construct the phylogenetic tree shown in Figure 2, together with sequences from the GenBank database. It was shown that all sequences obtained from Brazilian samples analyzed in this study belonged to

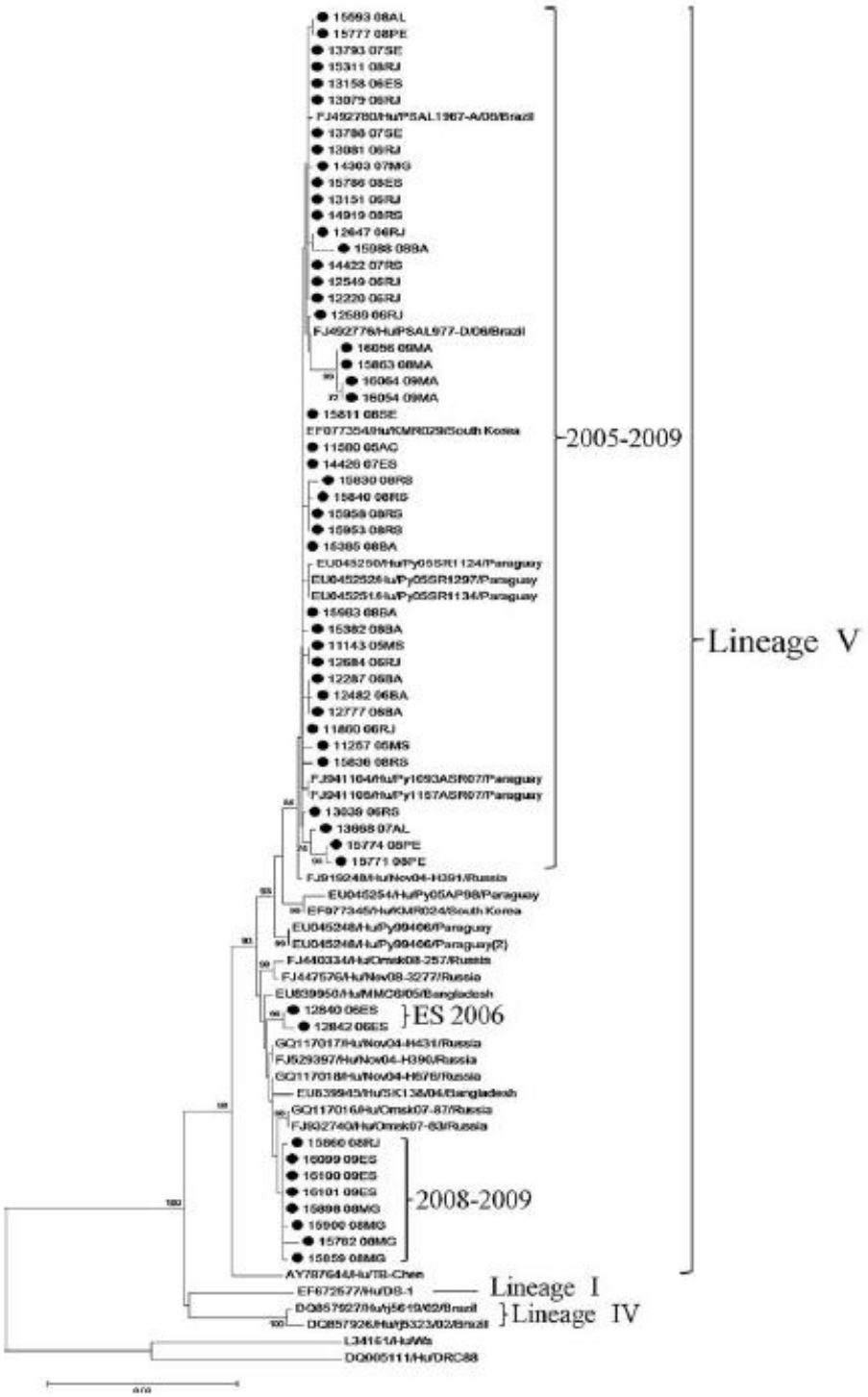

Fig. 1. Phylogenetic analysis based on VP4 (VP8*) nucleotide sequences of Brazilian rotavirus A genotype P[4]G2 strains and sequences from the GenBank database. All strains obtained from the GenBank database are named including their accession number. The Brazilian P[4]G2 strains are marked with a filled circle. The scale bar at the bottom of the tree indicates distance. Bootstrap values (2,000 replicates) are shown at the branch nodes, values lower than 70% are not shown.

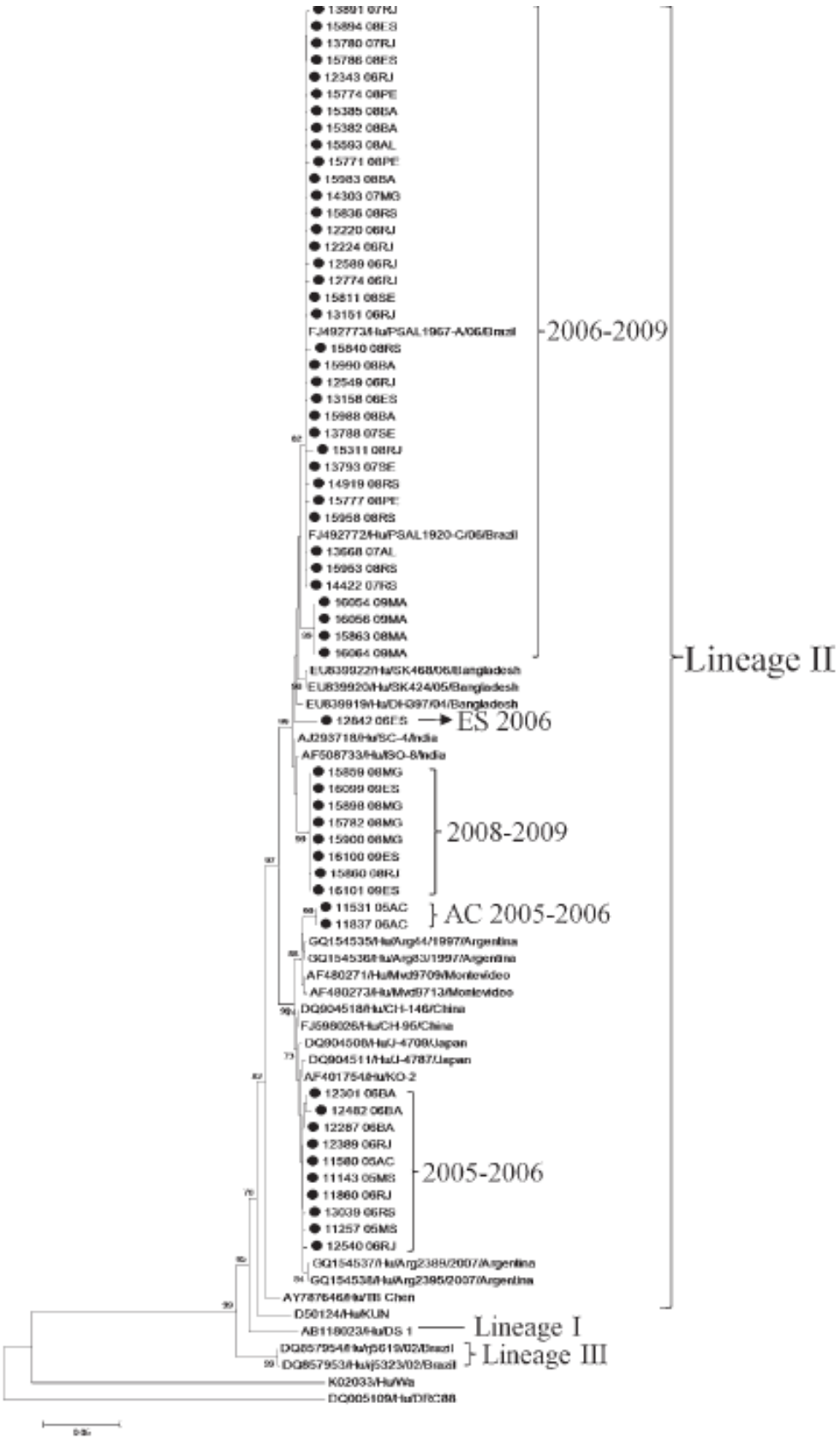

Fig. 2. Phylogenetic analysis based on VP7 nucleotide sequences of Brazilian rotavirus A genotype P[4]G2 strains and sequences from the GenBank database. All strains obtained from the GenBank database are named including their accession number. The Brazilian P[4]G2 strains are marked with a filled circle. The scale bar at the bottom of the tree indicates distance. Bootstrap values (2,000 replicates) are shown at the branch nodes, values lower than 70% are not shown.

Lineage II. Sequences obtained from samples detected between 2005 and 2006 from the five regions of Brazil grouped together (Fig. 2, 2005–2006). Sequences obtained from samples detected in Acre between 2005 and 2006 (11531_05AC and 11837_06AC) grouped separately (Fig. 2, AC 2005–2006). Sequences obtained from samples of the south, southeast, and northeast of Brazil between 2006 and 2009 grouped in a major cluster (Fig. 2, 2006–2009). Sequence corresponding to 12842_06ES sample obtained of Espírito Santo (2006), appeared in a separate branch (Fig. 2, ES 2006). Samples isolated from the southeast region between 2008 and 2009 grouped together and in a separate cluster from the other Brazilian samples (Fig. 2, 2008–2009).

Amino Acid Substitutions in VP4 (VP8*) and VP7 Proteins

Sequence alignments in Figures 3 and 4 show the amino acid substitutions observed for the P[4]G2 VP4 (VP8*) and VP7 amino acid sequences obtained from Brazilian samples when compared to DS-1 prototype strain, and sequences obtained from the GenBank database. The alignment of the deduced amino acid sequences from the VP8* gene revealed three amino acid substitutions inside the antigenic sites, at positions $64^{V\rightarrow I}$ and $120^{I\rightarrow V}$. Substitution at position 64 was found in four sequences obtained from samples of Rio Grande do Sul in 2008 (data not shown), whereas substitution at positions 120 was found in all

	32	49	64	85	89	99	120	130	133	149	160	162	166
EF672577)Hu/DS-1	S	N	V	S	N	N	I	V	M	G	S	R	M
EU839945)Hu/SK138/04/Bangladesh	N	.	.	.	D	S	V	.	S	S	N	.	.
GQ117016)Hu/Omsk07-87/Russia	N	.	.	.	D	S	V	.	S	S	N	K	.
FJ919248)Hu/Nov04-H391/Russia	N	.	.	.	D	S	V	I	S	S	N	.	.
FJ440334)Hu/Omsk08-257/Russia	N	.	.	.	D	S	V	I	S	S	N	.	.
EU045248)Hu/Py99406/Paraguay	N	.	.	.	D	S	V	.	S	S	N	.	.
EU045251)Hu/Py05SR1134/Paraguay	N	.	.	.	D	S	V	I	S	S	N	.	.
FJ492776)Hu/PSAL977-D/06/Brazil	N	.	.	.	D	S	V	I	S	S	N	.	.
FJ492780)Hu/PSAL1967-A/06/Brazil	N	.	.	.	D	S	V	I	S	S	N	.	I
FJ941105)Hu/Py1157ASR07/Paraguay	N	.	.	.	D	S	V	I	S	S	N	.	.
DQ857927)Hu/rj5619/02/Brazil	N	.	.	.	D	S	.	I	.	S	N	.	.
DQ857926)Hu/rj5323/02/Brazil	N	.	.	.	D	S	.	I	.	S	N	.	.
HM066011/Hu/11143_05MS/Brazil	N	.	.	.	D	S	V	I	S	S	N	.	.
HM066013/Hu/11257_05MS/Brazil	N	.	.	.	D	S	V	I	S	S	N	.	.
HM066012/Hu/11580_05AC/Brazil	N	.	.	.	D	S	V	I	S	S	N	.	.
HM066014/Hu/11860_06RJ/Brazil	N	.	.	.	D	S	V	I	S	S	N	.	.
HM066016/Hu/12287_06BA/Brazil	N	.	.	.	D	S	V	I	S	S	N	.	.
HM066023/Hu/12840_06ES/Brazil	N	.	.	.	D	S	V	.	S	S	N	.	.
HM066024/Hu/12842_06ES/Brazil	N	.	.	.	D	S	V	.	S	S	N	.	.
HM066030/Hu/13668_07AL/Brazil	N	D	.	.	D	S	V	I	S	S	N	.	.
HM066031/Hu/13788_07SE/Brazil	N	.	.	.	D	S	V	I	S	S	N	.	.
HM066033/Hu/14303_07MG/Brazil	N	.	.	.	D	S	V	I	S	S	N	.	.
HM066035/Hu/14426_07ES/Brazil	N	.	.	.	D	S	V	I	S	S	N	.	.
HM066061/Hu/15771_08PE/Brazil	N	D	.	.	D	S	V	I	S	S	N	.	.
HM066052/Hu/15774_08PE/Brazil	N	D	.	.	D	S	V	I	S	S	N	.	.
HM066046/Hu/15863_08MA/Brazil	N	.	.	R	D	S	V	I	S	S	N	.	T
HM066048/Hu/15958_08RS/Brazil	N	.	I	.	D	S	V	I	S	S	N	.	.
HM066063/Hu/15988_08BA/Brazil	N	.	.	.	D	S	V	I	S	S	N	.	.
HM066051/Hu/16056_09MA/Brazil	N	.	.	R	D	S	V	I	S	S	N	.	T
HM066040/Hu/16064_09MA/Brazil	N	.	.	R	D	S	V	I	S	S	N	.	T
HM066053/Hu/15782_08MG/Brazil	N	.	.	.	D	S	V	.	S	S	N	K	.
HM066057/Hu/15859_08MG/Brazil	N	.	.	.	D	S	V	.	S	S	N	K	.
HM066058/Hu/15860_08RJ/Brazil	N	.	.	.	D	S	V	.	S	S	N	K	.
HM066059/Hu/15898_08MG/Brazil	N	.	.	.	D	S	V	.	S	S	N	K	.
HM066065/Hu/15900_08MG/Brazil	N	.	.	.	D	S	V	.	S	S	N	K	.
HM066064/Hu/16099_09ES/Brazil	N	.	.	.	D	S	V	.	S	S	N	K	.
HM066041/Hu/16100_09ES/Brazil	N	.	.	.	D	S	V	.	S	S	N	K	.
HM066042/Hu/16101_09ES/Brazil	N	.	.	.	D	S	V	.	S	S	N	K	.

2008-2009 Genetic variant

Fig. 3. Deduced amino acid sequences of VP4 (VP8*) protein of rotavirus A P[4] strains. Dots indicate similarity with the DS-1 (EF672577) prototype sequence at that position. Positions indicated in gray color are situated inside antigenic sites of this protein. Accession numbers are indicated in the name of each strain. Several amino acid sequences from Brazilian samples analyzed in this study were identical, for that reason only some samples belonging to each cluster observed in the phylogenetic analysis were included in the figure.

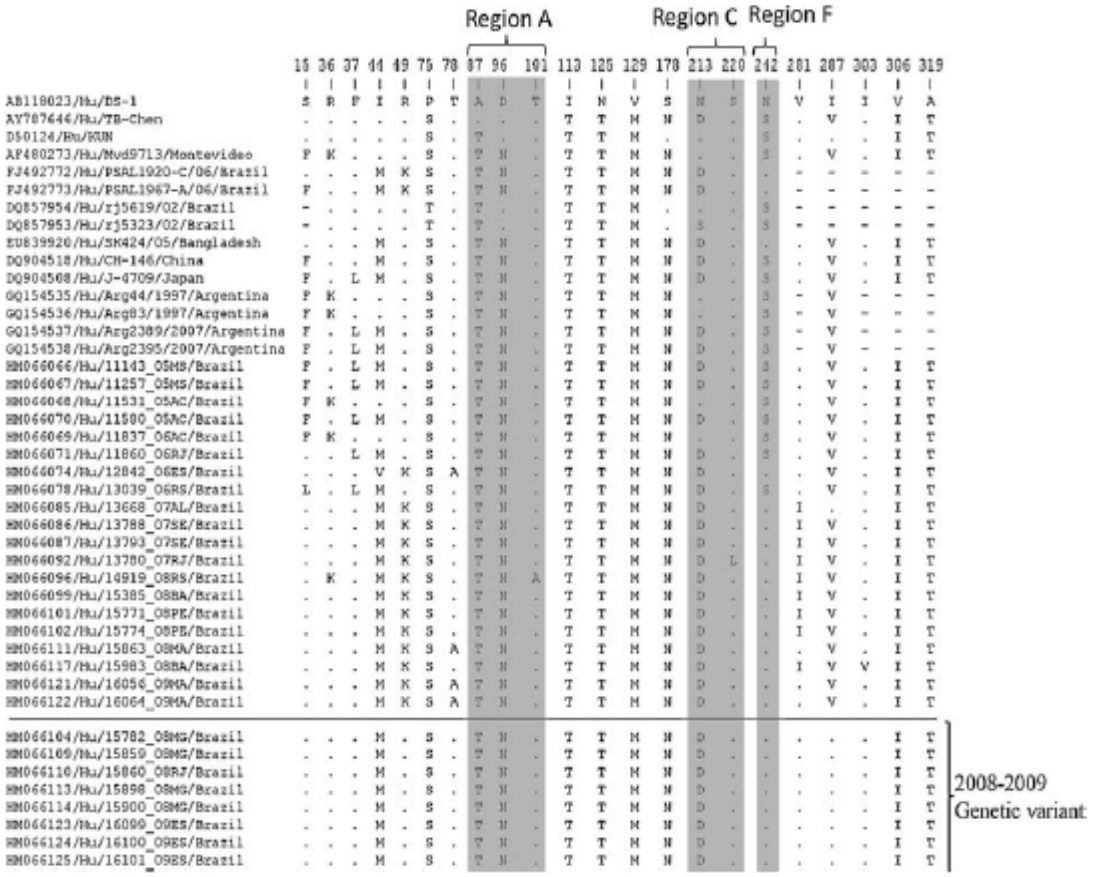

Fig. 4. Deduced amino acid sequences of VP7 protein of rotavirus A G2 strains. Dots indicate similarity with the DS-1 (AB118023) prototype sequence at that position. Positions indicated in gray color are situated inside antigenic sites of this protein (Regions A, C, and F). Accession numbers are indicated in the name of each strain. Several amino acid sequences from Brazilian samples analyzed in this study were identical, for that reason only some samples belonging to each cluster observed in the phylogenetic analysis were included in the figure.

the sequences obtained in this study, as well as substitutions at positions: $32^{S\rightarrow N}$, $89^{N\rightarrow D}$, $99^{N\rightarrow S}$, $133^{N\rightarrow S}$, $149^{G\rightarrow S}$, and $160^{S\rightarrow N}$. Three sequences (13668_07AL; 15771_08PE; and 15774_08PE) showed an amino acid substitution at position $49^{N\rightarrow D}$. All sequences that clustered inside 2008–2009 cluster showed an amino acid substitution at position $162^{R\rightarrow K}$. Brazilian sequences belonging to 2005–2009 cluster showed an amino acid substitution at position $130^{V\rightarrow I}$. Sequences obtained from samples of Maranhão, detected in 2008 and 2009, showed two amino acid substitutions at positions $85^{S\rightarrow R}$ and $166^{M\rightarrow T}$.

The alignment of the deduced amino acid sequences from the VP7 gene revealed amino acid substitutions inside the variable regions A, C, and F, which are considered important antigenic sites [Kirkwood et al., 1993], at positions $87^{A\rightarrow T}$, $96^{D\rightarrow N}$, $101^{T\rightarrow A}$, $213^{N\rightarrow D}$, $220^{S\rightarrow L}$, and $242^{N\rightarrow S}$. Substitutions at positions 87 and 96 were observed in all the Brazilian sequences. Substitution at position 101 was observed in one sequence obtained from a sample of Rio Grande do Sul in 2008 (14919_08RS), and substitution $213^{N\rightarrow D}$ was observed in all Brazilian sequences but sequences belonging to AC 2005–2006 cluster. Substitution at position 220 was observed in four samples (13780_07RJ, 13891_07RJ, 15786_08ES, and 15894_08ES) that grouped inside 2006–2009 cluster.

Finally, sequences that clustered forming 2005–2006 and AC 2005–2006 clusters showed an amino acid substitution at position 242. Amino acid substitutions at positions $75^{P\rightarrow S}$, $113^{I\rightarrow T}$, $125^{N\rightarrow T}$, $129^{V\rightarrow M}$, $178^{S\rightarrow N}$, $306^{V\rightarrow I}$, and $319^{A\rightarrow T}$ were found in all the Brazilian sequences obtained in this study. Sequences that clustered inside AC 2005–2006 showed a substitution at position $36^{R\rightarrow K}$, as well as sequence obtained from 14919_08RS sample. Sequence obtained from 12842_06ES sample showed a Valine (V) in this position.

VP6 Gene Analysis

Nucleotide sequences corresponding to the VP6 gene from Brazilian samples analyzed in this study

together with sequences available at the GenBank database were used to construct the phylogenetic tree shown in Figure 5. As can be observed in the Figure 5, sequences obtained from Brazilian samples grouped separately. Sequences obtained from samples of Acre detected in 2005 and 2006 (11531_05AC and 11837_06AC) grouped together (Fig. 5, AC 2005–2006). All other sequences obtained from Brazilian samples grouped into two separate clusters (Fig. 5, Clusters A and B). Cluster A was formed by sequences obtained from samples of the five Brazilian regions detected between 2005 and 2006, and sequences from samples of the south (Rio Grande do Sul) and northeast (Bahia, Sergipe, and Pernambuco) detected in 2008 (Fig. 5, 2005–2008). Cluster B was divided into two sub-clusters: (a) one was formed by sequences obtained from samples that circulated in different regions of Brazil between 2006 and 2009 (Fig. 5, 2006–2009); (b) the second was formed by sequences obtained from samples of the southeast region of Brazil that circulated between 2008 and 2009 (Fig. 5, 2008–2009). Sequence obtained from 12840_06ES sample grouped in a separate branch, inside Cluster B (Fig. 5, ES 2006).

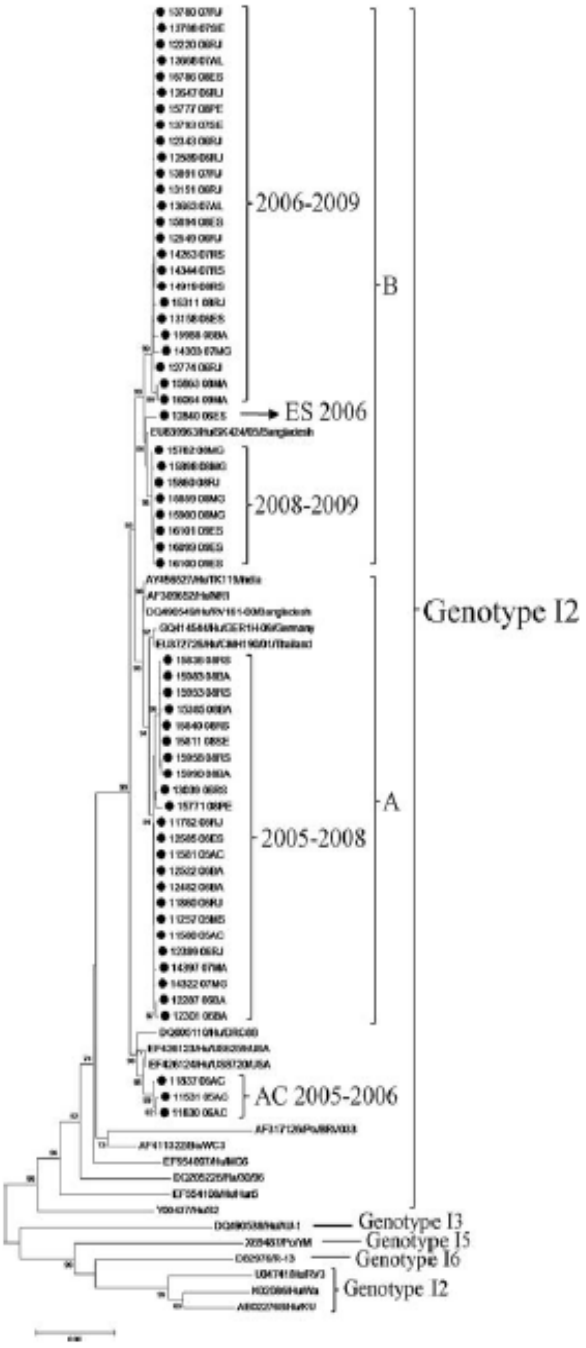

Fig. 5. Phylogenetic analysis based on VP6 nucleotide sequences of Brazilian rotavirus A genotype P[4]G2 strains and sequences from the GenBank database. All strains obtained from the GenBank database are named including their accession number. The Brazilian P[4]G2 strains are marked with a filled circle. The scale bar at the bottom of the tree indicates distance. Bootstrap values (2,000 replicates) are shown at the branch nodes, values lower than 70% are not shown.

NSP4 Gene Analysis

Nucleotide sequences corresponding to the NSP4 gene were used to construct the phylogenetic tree shown in Figure 6. All sequences obtained from Brazilian samples analyzed in this study clustered together with strains belonging to genogroup A-KUN. Sequences obtained from samples of the five regions of Brazil detected between 2006 and 2009 formed a monophyletic group (Fig. 6, 2006–2009); as well as sequences obtained from samples from the southeast region of Brazil detected between 2008 and 2009 (Fig. 6, 2008–2009). Sequences obtained from Brazilian samples detected between 2005 and 2006 clustered together with two sequences obtained from samples detected in Mina Gerais during 2007, and two sequences obtained from samples detected in Pernambuco during 2008 (Fig. 6, 2005–2006). Sequence obtained from 13158_06ES and 12389_06RJ samples clustered in a separate branch of the tree (Fig. 6, see black arrows). Sequences obtained from 12840_06ES and 12842_06ES samples, detected in Espírito Santo during 2006, clustered together (Fig. 6, ES 2006), as well as 12287_06BA and 12916_06BA samples, detected in Bahia during the same year (Fig. 6, BA 2006). Sequence obtained from 11581_05AC sample grouped in a separate branch of the tree (Fig. 6, see black arrow).

Analysis of the NSP4 deduced amino acid sequences revealed amino acid substitution within three antigenic sites described for this protein when comparing with DS-1 prototype strain [Borgan et al., 2003], the inter-species variable domain (aa 135–141) [Mohan and Atreya, 2000] and the enterotoxin domain (aa 114–135) [Ball et al., 1996; Horie et al., 1999] (data not shown).

DISCUSSION

Genetic variability of RV-A is represented by the accumulation of single site mutations (genetic drift)

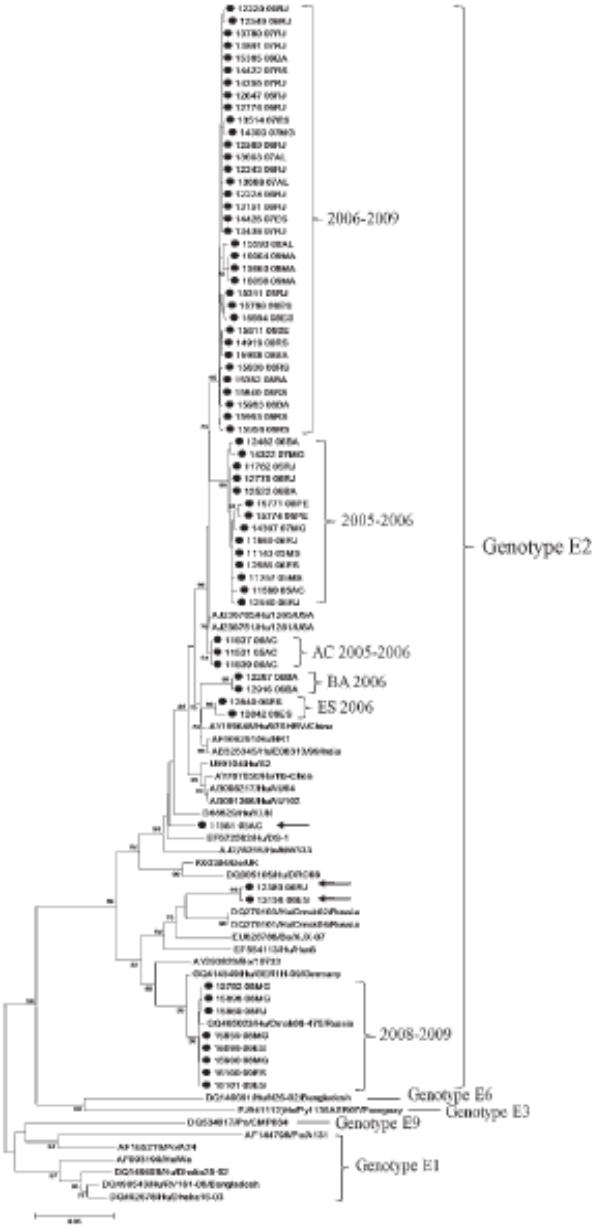

Fig. 6. Phylogenetic analysis based on NSP4 nucleotide sequences of Brazilian rotavirus A genotype P[4]G2 strains and sequences from the GenBank database. All strains obtained from the GenBank database are named including their accession number. The Brazilian P[4]G2 strains are marked with a filled circle. The scale bar at the bottom of the tree indicates distance. Bootstrap values (2,000 replicates) are shown at the branch nodes, values lower than 70% are not shown.

and by sudden changes in the RV-A genome (genetic shift) most of all represented by reassortment events [Ramig, 1997; Estes and Kapikian, 2007; Matthijnssens et al., 2008a; McDonald et al., 2009]. The increasing amount of sequences in public databases has made possible a more in depth study of RV-A evolution taking into account all RV-A genes [Matthijnssens et al., 2008a; McDonald et al., 2009]. Consequently, the number of studies reporting reassortment events among strains has increased [Steyer et al., 2008; Matthijnssens et al., 2008a, 2010a,b; McDonald et al., 2009; Stupka et al., 2009; Gómez et al., 2010].

Analysis of VP4 (VP8*) and VP7 Amino Acid Sequences

The analysis of the VP8* deduced amino acid sequences obtained from Brazilian samples revealed that two amino acid substitutions occurred inside of three antigenic sites described for this protein at positions 64 and 120 when comparing with DS-1 prototype strain [Kovacs-Nolan et al., 2003] (Fig. 3). Substitution at position 64 was only found in four sequences obtained from Brazilian samples, whereas substitution at position 120 was observed in all the sequences obtained from samples belonging to lineage V. Sequences obtained from Brazilian samples that grouped inside the 2005–2009 cluster showed an amino acid substitution at position 130, this substitution was described recently in sequences obtained from samples of Parauapebas, northern Brazil detected in 2006 and 2008 [Mascarenhas et al., 2010]. Sequences obtained from samples of Maranhão showed two amino acid substitutions that were not present in other sequences considered for the analysis. Furthermore, all of the sequences that grouped inside the 2008–2009 cluster showed an amino acid substitution at position 162, a substitution shared with a sequence obtained from a sample of Russia in 2007 (GQ117016).

All Brazilian samples examined in this study showed amino acid substitutions at positions $89^{N\rightarrow D}$, $133^{N\rightarrow S}$, and $149^{G\rightarrow S}$ (Fig. 3). Previous studies have shown that amino acids 87–89 and 148–150 are associated with limited cross-reactive neutralization, whereas amino acid residues at positions 100, 114–135, and 173–188 represents strains-specific neutralization sites in VP8* [Mackow et al., 1988].

When comparing the VP7 deduced amino acid sequences with DS-1 prototype strain many substitutions were observed, six were inside previous described antigenic sites: 87, 96, and 101 (region A), 213 and 220 (region C) and 242 (region F) (Fig. 4) [Kirkwood et al., 1993]. Some of these substitutions were observed not just in the sequences obtained from Brazilian samples but in most of the sequences available in database. Amino acid substitution observed at position 96 was previously associated with an outbreak of P[4]G2 that occurred in Taiwan in 1993 [Zao et al., 1999]. However, this study demonstrates that this substitution was observed in all sequences obtained from Brazilian samples examined in this study and in most of the sequences corresponding to genotype P[4]G2 available in the GenBank database (data not shown), suggesting that this change was selected over time and maintained in the VP7 gene variants of genotype P[4]G2. Therefore, the presence

of this substitution ($96^{D\rightarrow N}$) is more frequent and its relation to outbreaks should be better examined. Substitution at position 49 was described for RV-A samples isolated from an outbreak of diarrhea at a camp for tsunami victims at Mus village, Car Nicobar Island, India in 2005 [Sugunan et al., 2007].

The amino acid changes in the VP8* and VP7 proteins were found in almost all the Brazilian sequences studied as well as in the majority of the sequences at GenBank database, suggesting that these changes become more common in the population as a result of positive selection since they appeared. It is possible that they represent some kind of advantage for the virus to adapt. Furthermore, it is necessary to investigate the occurring mutations in order to have a better understanding of the virus evolution, and to determine the relationship of these mutations with vaccine efficacy, if any.

Phylogenetic Analysis of Brazilian RV-A Genotype P[4]G2 Strains

Phylogenetic analysis based on the VP4 (VP8*) and VP7 nucleotide sequences revealed that at least five RV-A genotype P[4]G2 genetic variants circulated in Brazil between 2005 and 2009. However, three (2005–2006; 2006–2009; and 2008–2009) were found circulating in different states; whereas the other two just appeared in Acre (AC 2005–2006) and Espírito Santo (ES 2006). Although all the Brazilian samples had the same consensus genotype constellation, different reassortment events among samples belonging to 2005–2006 and 2006–2009 RV-A genotype P[4]G2 genetic variants were observed. Phylogenetic analysis showed that sequences obtained from Brazilian samples genotype P[4]G2 detected between 2005 and 2009 belong to lineage V and II, for the VP4 (VP8*) and VP7 genes, respectively, together with sequences obtained from samples of other South American countries.

Reassortment Events Among RV-A Genotype P[4]G2 Strains

The phylogenetic analysis based on the VP4 (VP8*) nucleotide sequence showed that sequences obtained from samples detected between 2005 and 2009 from the five different regions of Brazil clustered together with sequences obtained from samples of Paraguay, South Korea, and northern Brazil in a major cluster (Fig. 1, 2005–2009) [Min et al., 2004; Espinola et al., 2008; Martínez et al., 2010; Mascarenhas et al., 2010]. However, in the phylogenetic analysis based on the VP7 and NSP4 nucleotide sequences, sequences from the same strains separate into two different monophyletic groups, 2005–2006 and 2006–2009, respectively (Figs. 2 and 6). This data suggest that a reassortment event occurred. Most likely, the RV-A genotype P[4]G2 strains that began to circulate in 2006 acquired the VP4 (P[4]) gene from the P[4]G2 strains that were already in circulation since 2005 in Brazil. For some reason, this new combination of genes acquired by P[4]G2 strains in 2006 was positively selected in the Brazilian population. Maybe this shift in the VP7 and NSP4 genes was related to the introduction of Rotarix® vaccine in March 2006. The selection of these gene variants may be related to many factors, including: antigenicity, fitness of the viral progeny, infectivity, and virulence. Further studies should be done to determine the reason for this selection.

Interestingly, sequences obtained from samples that clustered inside the 2006–2009 cluster for the VP7 and NSP4 genes, did not group as a monophyletic group but as two clusters in the phylogenetic analysis of the VP6 nucleotide sequences, 2006–2009 and 2005–2008 (Figs. 2, 5, and 6). Furthermore, in the phylogenetic analysis based on VP6 gene, sequences obtained from Brazilian samples detected in 2008 grouped together with sequences obtained from samples that grouped inside the 2005–2006 cluster in the VP7 and NSP4 phylogenetic analysis (Figs. 2, 5, and 6). This result suggests that, at some point in the past, when these P[4]G2 strains acquired the VP4 (P[4]) gene from the P[4]G2 2005–2006 genetic variant, the VP6 gene was also acquired.

Sequences obtained from Pernambuco's samples detected in 2008 (15771_08PE and 15774_08PE) grouped inside the 2005–2009, 2005–2008 (15771_08PE), and 2005–2006 clusters for the VP8*, VP6, and NSP4 genes, respectively (Figs. 1, 5, and 6). Whereas, for the VP7 gene the sequences obtained from the same samples grouped inside the 2006–2009 cluster suggesting that these samples also originated from reassortment events between human samples (Fig. 2).

The phylogenetic analysis based on the NSP4 gene revealed that the sequence obtained from 12389_06RJ and 13158_06ES samples, detected in Rio de Janeiro and Espírito Santo, respectively, during 2006, showed a close genetic relationship with sample XJX-07 of bovine origin and clustered distantly from the other sequences obtained from Brazilian samples (Fig. 6, see black arrows) [Mulherin et al., 2008]. When analyzing the VP4 (VP8*), VP7 and VP6 genes, sequences obtained from 13158_06ES sample clustered inside the 2005–2009, 2006–2009 and 2006–2009 genetic variants observed for these genes, respectively (Figs. 1, 2, and 5). Analysis of the VP7 and VP6 gene sequences from sample 12389_06RJ revealed that belonged to the 2005–2006 and 2005–2008 genetic variants (Figs. 2 and 5). Together these data suggest that both samples originated from reassortment events. These results are in agreement with the common origin described for DS-1-like and bovine strains by Matthijnssens et al. [2008a].

RV-A Genotype P[4]G2 Strains With Distinctive VP4 (VP8*), VP7, VP6, and NSP4 Genes

In this study different reassortment events were observed involving samples that belonged to the

2005–2006 and 2006–2009 P[4]G2 genetic variants. Whereas, for AC 2005–2006, ES 2006, and 2008–2009 RV-A P[4]G2 genetic variants, phylogenetic analysis showed that samples representing such variants clustered forming monophyletic groups, or in a separate branch (ES 2006) showing a distinctive constellation of genes regarding the four genes studied (except for AC 2005–2006).

Sequences obtained from 12840_06ES and 12842_06ES samples detected in Espirito Santo during 2006 grouped separately from the others Brazilian samples analyzed in this study for the four genes studied (Figs. 1 and 2 [12842_06ES], Fig. 5 [12840_06ES] and Fig. 6), indicating that probably these strains just appeared in this state and did not spread. Furthermore, sequences obtained from samples of Acre during 2005 and 2006 also grouped separately from other Brazilian samples for the VP7, VP6, and NSP4 genes (Figs. 2, 5, and 6). It was not possible to amplify the VP4 (VP8*) gene of these samples. It is worth mentioning that these strains correspond to an outbreak in Acre in 2005 caused by RV-A genotype G9, that was the prevalent genotype representing 70% of single infections, and genotype G2, that represented only 6% [Siqueira et al., 2010]. A study conducted by Tort et al. [2010] showed that the G9 genetic variant that circulated in Acre in 2005 did not circulate in other Brazilian states, and these samples were characterized as a new G9 genetic variant. Additionally, the results obtained in the current study revealed that this outbreak was also characterized by a RV-A G2 genetic variant that was only found in this state. Sample 11581_05AC that clustered inside 2005–2008 genetic variant in the VP6 phylogenetic analysis, in the NSP4 analysis clustered in a separate branch distantly from the other Brazilian samples (Figs. 5 and 6).

Since 2008 a new P[4]G2 genetic variant started circulating in Brazil in the southeast region (Espírito Santo, Minas Gerais and Rio de Janeiro) (Figs. 1, 2, 5, and 6, 2008–2009). Phylogenetic analysis based on the NSP4 gene revealed that Brazilian samples corresponding to 2008–2009 genetic variant showed close genetic relationship with a sample of bovine origin: 10733 [Martella et al., 2003] (Fig. 6). Phylogenetic analysis suggests that this variant is distantly related, mostly for VP4 (VP8*) and NSP4 genes, from the other P[4]G2 genetic variants described in this study, and indicates that this variant was probably introduced in Brazil recently.

Gene Flow Occurring Into and Out of Brazilian Population

The VP4 (VP8*) and VP7 phylogenetic analysis revealed that sequences corresponding to Brazilian samples detected from 2005 to 2009 did not form a monophyletic group, but showed close phylogenetic relationship with samples isolated worldwide (Figs. 1 and 2). The phylogenetic pattern observed for VP4 (VP8*) and VP7 genes showed the gene flow occurring into and out of Brazil. In the case of the RV-A genotype P[4]G2, results obtained in this study suggest that the estimate for the number of migration events could be better determined by VP7 gene analysis, as the VP4 (VP8*) gene was maintained over P[4]G2 Brazilian samples. However, it should be considered that there are not sufficient sequences in the database in order to undertake such analysis with other genes, and the background of VP4 (VP8*) and VP7 sequences for RV-A genotype P[4]G2 existing in databases is not representative of global isolates.

The increased prevalence of RV-A genotype P[4]G2 reported in different continents, including countries where the vaccine Rotarix® is being administered, as well as in countries where no vaccine is used, showed the importance of continuing RV-A genotypes surveillance [Amarilla et al., 2007; Ferrera et al., 2007; Leite et al., 2008; Patel et al., 2008; Antunes et al., 2009]. There is insufficient information to establish whether the increase in the prevalence of this genotype is related to the introduction of the Rotarix® vaccine in Brazil, which reduced the incidence of other genotypes, or if it is due to natural genotypic variations. In the current study only one sample belonged to a vaccinated child with two doses, sample 15811_08SE (Table I). Phylogenetic analysis showed that this sample clustered together with samples corresponding to non-vaccinated children. Hence, further studies to compare RV-A genotype P[4]G2 samples isolated from vaccinated and unvaccinated children are being conducted. Finally, data obtained in this study showed that many gene combinations are present in Brazilian samples and that different RV-A genotype P[4]G2 genetic variants circulated in Brazil between 2005 and 2009. Which of these combinations of genes are best for the virus will probably determine which of these genetic variants will continue to circulate in the coming years. In this context, natural selection may limit the extent of the observed genetic shift that occurs during RV-A evolution. Rotaviruses (RVs) must balance the advantages of gene reassortment with the disadvantages of unlinking preferred genes/protein combinations [McDonald et al., 2009]. In order to obtain more information, additional sequences of the RV-A genotype P[4]G2, as well as from other RV-A genotypes, should be examined in respect of all RV-A genes.

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Artigo 2: Prevalence and genomic characterization of G2P[4] genotype after monovalent vaccine introduction in Brazil. (Artigo em fase final de confecção)

Este artigo esta relacionado aos objetivos 1, 2, e 3.

Prevalence and genomic characterization of G2P[4] genotype after monovalent vaccine introduction in Brazil.

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Running Head: Genetic characterization of Brazilian G2P[4] RVA strains

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Abstract

A relationship between Rotarix® (RV1) introduction and the increased prevalence of group A rotavirus (RVA) genotype G2P[4] in Brazil has been proposed. This study aims to: i) estimate the prevalence of G2P[4] in Brazil between 2005 – 2011; ii) perform phylogenetic analyses of G2P[4] strains detected in five Brazilian regions, and iii) assess the full genome genetic background of selected strains. RVA surveillance was performed with fecal samples obtained from patients with acute gastroenteritis. RVA was detected through polyacrylamide gel electrophoresis and/or ELISA and genotyped through multiplex RT-PCR. G2P[4] strains obtained from vaccinated and unvaccinated children were analyzed for the VP7 and VP8* genes through genome sequencing, the complete genome characterization of selected strains being also performed. G2P[4] detection rate among RVA-positive samples was 112/227 (49%) in 2006, 139/211 (66%) in 2007, 240/284 (85%) in 2008, 66/176 (37,5%) in 2009, 367/422 (87%) in 2010 and 75/149 (50%) in 2011. For both the VP7 and VP8* encoding genes, 53 sequences (20 from vaccinated children) were analyzed and shared up to 99% identity with other G2P[4] strains detected worldwide, different genetic variants being observed. Most changes in antigenic epitopes in VP7 and VP8* have been maintained in the Brazilian strains along the years, and all were present before RV1 introduction. Eleven strains (4 from vaccinated, 7 from unvaccinated children) were characterized as G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2, sharing up to 99% of nucleotide identity with strains isolated worldwide. Reassortments between human strains analyzed were observed. Data obtained in the current study support the idea that other factors than RV1 introduction are responsible for the increase prevalence of G2P[4] in Brazil.

Introduction

Group A rotaviruses (RVA) are responsible for approximately 453.000 deaths worldwide each year among children under 5 years old (Tate et al., 2011, Wazny et al., 2013). RVA belongs to the *Reoviridae* family and possesses segmented dsRNA genome (11 gene segments) encoding six structural proteins (VP1-4, VP6-7) and six non-structural proteins (NSP1-6). Based on their two outer capsid proteins, VP7 and VP4, RVA have been classified into G (Glycoprotein) and P (Protease-sensitive) genotypes. Up-to-date there are 27 G types and 37 P types reported (Matthijnssens et al., 2011; Trojnar et al., 2012). Although VP7 and VP4 can segregate independently and theoretically lead a large number of G and P combinations, six combinations (G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8]) are more frequent in human population representing more than 90% of the global burden of rotavirus disease (Leite et al., 2008; Iturriza-Gómara et al., 2009; Banyai et al., 2012). However, intra-genotype diversity and variation can be observed in both space and time dimensions; in addition, periodic emergences of novel strains and/or uncommon combinations have also been reported (Santos & Hoshino, 2005; Leite et al., 2008; Matthijnssens & Van Ranst, 2012).

The binary genotype classification system has been extended to the entire genome to better characterize RVA strains specifying the genotype of all 11 genome segments (Matthijnssens et al., 2011). Based on this classification, most of the human RVA detected worldwide possess either the Wa-like genotype constellation (I1-R1-C1-M1-A1-N1-T1-E1-H1) or the DS-1-like genotype constellation (I2-R2-C2-M2-A2-N2-T2-E2-H2) also called as genotype 1 and 2, respectively (Heiman et al., 2008; Matthijnssens et al., 2008; Mc Donald et al., 2009; Matthijnssens & Van Ranst, 2012).

In April 2009, the World Health Organization (WHO) provided the recommendation for introduction of anti-RVA vaccines in national immunization programs of developing countries worldwide. Universal vaccination against rotavirus has been considered strategic in order to reduce both mortality and hospitalization due to diarrheal diseases, along with other

measures such as oral rehydration, breastfeeding, zinc administration and improvement of sanitation in developing countries (WHO 2013). Two live oral rotavirus vaccines, Rotarix® (RV1; GlaxoSmithKline) and Rotateq® (RV5; Merck), with proven efficacy against severe rotavirus disease are currently licensed. In Brazil, RV1, an attenuated human monovalent (G1P[8]) vaccine was already included in the National Immunization Program since March 2006. An increase in the relative frequency of G2P[4] RVA occurred in Brazil in the immediate post-vaccine years (Leite et al., 2008; Carvalho-Costa et al., 2011; Correia et al., 2010; Dulgheroff et al., 2012). Some studies argued that both events, vaccine introduction and increase prevalence of G2P[4], were associated suggesting that the selective pressure generated by RV1 would somehow select this heterotypic strain among others (Nakagomi et al., 2008; Gurgel et al., 2009). However, G2P[4] emerged in Brazil in 2005, shortly before the beginning of universal mass vaccination with RV1. In addition, Latin American countries that had not yet introduced universal vaccination also reported increased detection of G2P[4] at that time (Amarilla et al., 2007; Ferrera et al., 2007; Patel et al., 2008). Despite these shifts in genotype distribution, a significant reduction in the number of hospitalizations due to acute diarrhea as well as in the frequency of RVA detection in hospitalized children with diarrhea has been observed after the introduction of RV1 in Brazil (Sáfadi et al. 2010; Carvalho-Costa et al. 2011).

The evolving prevalence rates of different RVA genotypes during and after vaccine introduction, as well as their epidemiological characteristics are important issues and represent a challenge to rotavirus vaccination programs. In this context, monitoring the emergence of genotypes and escaping strains associated to breakthrough infections is considered strategic in order to assess the impact of universal vaccination. In the current study the prevalence of RVA genotype G2P[4] in Brazil between 2005 – 2011 have been assessed. Furthermore, G2P[4] strains detected in the five regions of Brazil from 2005 to 2011 have been analyzed for the VP7 and VP8* encoding genes, and compared with RVA strains

available in the Genbank database, including the RV1 vaccine. The complete genome characterization of eleven G2P[4] Brazilian selected strains was also performed.

Material and Methods

Laboratory based RVA surveillance and specimen collection

Laboratory based RVA surveillance was performed with fecal samples obtained from patients with acute gastroenteritis. Samples are sent to the Regional Rotavirus Reference Laboratory - Laboratory of Comparative and Environmental Virology (RRRL-LVCA) by a network of state public health laboratories. These labs, in turn, receive samples of health centers and hospitals in the National Health System. We studied 9,073 fecal samples, from which 1,813 were RVA-positive. Among RVA-positive patients, 361 (19.9%) were fully (two doses) vaccinated children. Table 1 presents the year distribution, RVA detection rates and prevalence of G2P[4] among RVA-positive patients.

Group A rotavirus detection and G- and P- genotyping

In order to detect RVA in stool samples, polyacrylamide gel electrophoresis and enzyme immunoassay kits (EIARA, Biomanguinhos; Premier Rotaclone, Meridian Bioscience, Inc.; Ridascreen, R-Biopharm), according to the manufacture's protocols, were used in 10% fecal suspensions in phosphate-buffered saline pH 7.4. Nucleic acid was extracted from 10% fecal suspensions by the glass powder method described by Boom et al. (1990), including modifications as described by Gómez et al. (2013) and the QIAamp Viral RNA mini kit (Qiagen/Westburg, Leusden, The Netherlands) according to the manufacturer's instructions. In RVA-positive samples, the extracted RNA was reverse transcribed and G- and P- genotyping was performed using semi-nested multiplex PCRs as previously described (WHO/IVB/08.17, 2008). G and P genotypes were confirmed by sequencing for 57 samples

(23 were obtained from vaccinated children with RV1, and 34 from unvaccinated children) and eleven selected strains representative from the surveillance period (2005-2011) were investigated by whole genome analysis. The study strains were named according to the guidelines of the Rotavirus Classification Working Group (RCWG) (Matthijnssens et al., 2011).

Genome segments amplification and sequencing

The amplification of the eleven genome segments from selected strains were performed using a OneStep RT-PCR Kit (QIAGEN) following manufacturer's instructions and amplifications conditions as described by Matthijnssens et al. (2010). The cDNA products were resolved in agarose gels electrophoresis and purified using the Expo-SAP-IT PCR Product Cleanup Kit (Affymetrix, Miles Rd, Cleveland, OH, USA). Sequencing was performed with an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit™ on an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the Instituto de Tecnologia em Imunobiológicos (Bio-Manguinhos / FIOCRUZ), and an ABI Prism 3130 automated sequencer (Applied Biosystems, Foster City, CA, USA) at the Rega Institute of Medical Research (University of Leuven, Belgium).

Sequence and phylogenetic analysis

Sequences obtained in the current study were deposited in the GenBank database under the following accession numbers: XXXXX–XXXXX. Nucleotide blast analyses were performed with obtained sequences and multiple sequence alignments were carried out using the ClustalW program (Thompson et al., 1994). Phylogenetic analyses were constructed using the Neighbor-Joining method with the Kimura-two parameter model in MEGA5.0 (Tamura et al., 2011). The statistical significance of the branch was assessed by bootstrap resampling analysis (2000 replicates).

Deduced amino acid sequences of VP8* and VP7 proteins of Brazilian G2P[4] RVA strains were compared with the RV1 strain, and RV5 strain (just VP7) using the Bioedit v.7.2.3 softwear (Hall, 1999).

Results

RVA Surveillance and G2P[4] prevalence

After 2004, the first G2P[4] RVA sample has been detect in August, 2005 in far western Brazil (Mato Grosso do Sul State), bordering Paraguay. During August and September 2005, more 22 G2P[4] samples were characterized in the same Brazilian region. During September, 2005, seven G2P[4] samples were characterized in the State of Acre, also in far western Brazil, bordering Bolivia. In December, 2005, one RVA positive sample was characterized in Rio de Janeiro, the second largest Brazilian city, which attracts people from all states of Brazil. Throughout 2005, the RRRL detected RVA positive fecal samples in 33 other municipalities, with no identification of G2P[4], except those described above. Frequency of detection of G2P[4] in 2005 was therefore 31/344 (9%). (Figure 1).

In the post vaccination era, detection rates of G2P[4] among RVA-positive children with acute diarrheal disease were as follows: 112/227 (49%) in 2006, 139/211 (66%) in 2007, 240/284 (85%) in 2008, 66/176 (37,5%) in 2009, 367/422 (87%) in 2010 and 75/149 (50%) in 2011. Additionally, G2P[4] has spread in the surveyed municipalities during the studied period: the rate of municipalities with G2P[4] detection were as follows: 8 G2P[4] positive municipalities / 98 surveyed municipalities (8%) in 2005, 24/164 (15%) in 2006, 35/137 (26%) in 2007, 70/173 (40%) in 2008, 22/146 (15%) in 2009, 86/222 (39%) in 2010 and 23/168 (14%) in 2011.

VP7 and VP8* sequence and phylogenetic analysis

For the VP8* encoding gene, it was possible to obtain 53 sequences (20 from vaccinated children). Five RVA-positive samples were studied in 2005, five in 2006, seven in 2007, four in 2008, four in 2009, 22 in 2010, and six in 2011. Brazilian G2P[4] strains shared up to 99% nucleotide sequence identity with other G2P[4] strains detected worldwide, including strains previously reported in Brazil (Gómez et al., 2011). The phylogenetic analysis revealed that all studied strains belonged to lineage V (Figure 2). Furthermore, Brazilian G2P[4] strains grouped into several clusters formed by: a) strains detected between 2005 and 2011; b) strains detected in 2007 in Rio Grande do Sul state (South); c) strains detected between 2008 and 2011; d) strains detected in 2010 (Figure 2).

Similarly, 53 sequences were obtained for the VP7 encoding gene (20 from vaccinated children): six in 2005, five in 2006, seven in 2007, four in 2008, four in 2009, twenty-one in 2010, and six in 2011. Again, Brazilian strains shared up to 99% identity with other G2P[4] strains detected worldwide, including previous reported G2P[4] strains (Gómez et al., 2011). The phylogenetic analysis revealed that all analyzed strains belonged to lineage II (Figure 3), and grouped in several clusters: a) strains detected between 2005 and 2007, b) strains detected in 2007 in Rio Grande do Sul state (Southern Brazil), c) strains detected between 2008 and 2011, d) strains detected between 2008 and 2010, and e) strains detected between 2006 and 2011 (Figure 3).

Comparison of the deduced amino acid sequences of the RV1 VP8* antigenic epitope and the Brazilian G2P[4] strains showed that strains grouped inside cluster “a”, “b”, and “c” shared 46%, and strains grouped inside cluster “d” shared 54% of amino acid identity (Figure 4). Regarding VP7 protein, comparison of the deduced amino acid sequences inside antigenic epitopes revealed that Brazilian G2P[4] strains shared 38% of identity with RV1 strain, and 93% with RV5 G2 strain (Figure 4). Vaccinated and non vaccinated children shared the same amino acid changes for both proteins analyzed.

Whole-genome characterization of G2P[4] Brazilian strains

Eleven G2P[4] Brazilian strains, representative of the different clusters observed when analyzing the VP7 and VP8* encoding genes, were selected to perform complete genome analysis. Four of these strains were isolated from vaccinated children, and seven from unvaccinated children, all hospitalized with acute gastroenteritis between 2005 and 2011.

The genotypes for each of the 11 RVA genome segments was assigned and all strains belonged to G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2 genetic background (Table 2; Supplementary material Figures 1-9). The VP2 sequence of strain RVA/Human-wt/BRA/SE19300/2010/G2P[4] could not be obtained. Although all strains have displayed the same genomic constellation, it was possible to observed different genetic variants of G2P[4] circulating during the study period in Brazil (Figures 2 and 3).

Analysis of NSP1-5, VP1-3, and VP6 sequences showed that G2P[4] Brazilian strains shared between 87,3 - 100%, and 93 - 100% of nucleotide and amino acid identity values, respectively. Nucleotide blast analysis revealed that Brazilian strains shared up to 99% of nucleotide identity with strains isolated worldwide in the last decade.

Discussion

G2P[4] has been the most common genotype detected in Brazil after the onset of mass vaccination (Gurgel et al., 2007, de Oliveira et al., 2008; Nakagomi et al., 2008; Leite et al., 2008; Carvalho-Costa et al., 2009, 2011; da Silva Soares et al., 2013). The emergence of this genotype has motivated a great discussion and a relationship between this phenomenon and vaccination has been proposed (Gurgel et al., 2007; Nakagomi et al., 2008). The main reason is the fact that the efficiency and effectiveness of the monovalent vaccine against G2P[4] is relatively lower than against P[8] genotypes which are VP4-homotypic and belong to the same genomic constellation (Wa-like) (Correia et al., 2010). In this context, it has been

proposed that universal vaccination would have exerted selective pressure on circulating strains of RVA, selecting G2P[4] (Gurgel et al., 2007; Nakagomi et al., 2008).

The results of this study demonstrate that the detection rate of G2P[4] in RVA-positive samples has been high in the years after the introduction of mass RV1 vaccination. With the exception of 2009 this rate has been above 50%, reaching 87% in 2010. G2P[4] has also been frequently detected in children vaccinated with RV1.

In this study, we examine in more detail the geographical location of G2P[4] positive samples, trying to identify a route of spread of this genotype during its process of emergence in Brazil. The frequency of G2P[4] detection between 2001 and 2003 was very low, so only three positive samples could be identified in the RRRL monitoring data. In 2004, G2P[4] was not characterized in any of the 651 RVA-positive fecal samples obtained from 2.554 children with gastroenteritis from 11 Brazilian states. In the present survey, we confirmed that G2P[4] was absent in the samples studied in the first semester of 2005. In this year, the first cities in which G2P[4] was detected are located in western Brazil, on the border with Paraguay. Throughout 2005, G2P[4] continued to be detected in this region and in Amazonian Brazil, bordering Peru and Bolivia, being absent from the most populous Brazilian regions of the Atlantic coast. Only in December 2005, G2P[4] was detected in the main Brazilian cities, specifically in Rio de Janeiro. Universal vaccination against RVA began in March 2006 and, starting this year, G2P[4] was early detected in all monitored regions, in a phase in which the cohort of vaccinated children was still very limited.

It has been suggested that specific vaccines may select escape genotypes, including long-term effects of weak selective pressures (Pérez-Sautu et al., 2011). If so, emerging strains resulting from evolutionary pressure imposed by vaccines may gradually substitute previous genotypes (Streck et al., 2013). In this scenario, the vaccine can present reduced performance. In this context, one would expect that the phenomenon of selection of strains would be accompanied by a reduction in the genetic variability of RVA and that a

genetic distance of circulating strains in the selection scenario in relation to other regions would arise (Hoffmann et al. 2008). The analysis of the genetic and antigenic backgrounds of G2P[4] strains identified throughout the process of implementation of mass immunization with RV1 may be useful to clarify its potential impact on the distribution of genotypes which occurred between 2005 and 2011 in Brazil.

Our data suggest that the G2P[4] strains circulating in Brazil during implementation of mass immunization with RV1 are not separated into genetic clusters defined by pre and post vaccination periods and maintains a close relationship with G2P[4] strains circulating in other countries that had not yet implemented universal vaccination against RVA. Similarly, G2P[4] strains identified in vaccinated and unvaccinated children grouped together in the same genetic clusters, with no significant differences in the sequences of the genes encoding VP7 and VP8*. Moreover, phylogenetic analysis revealed that contemporary G2P[4] strains (circulating from 2005 to 2011) belonged to a novel lineage constellation, distinct from the reference strain DS-1 and the SC2-9 G2-reassortant strain of RV5, as previously reported (Doan et al., 2011; Gómez et al., 2011; Do et al., 2013; Giammanco et al., 2013). Analysis of the genomic background of Brazilian G2P[4] RVA strains revealed a complete DS-1-like background, and different genetic variants were observed circulating among Brazilian regions (Supplementary material Figures 1-9).

In addition, comparison of the RV1 amino acid sequences inside previous described antigenic epitopes in VP7 and VP8*, with Brazilian G2P[4] strains revealed that most of the changes observed have been maintained in the Brazilian strains along the years, and all were present before RV1 introduction.

In conclusion, data support the idea that other factors than RV1 introduction are responsible for the increase prevalence of G2P[4] in Brazil. It has been proposed that G2P[4] reemerges periodically in 10-years cycles (Bishop et al., 1991). Leite et al. (2008) reported a wave of G2P[4] circulation in the beginning of the 1990s, near 10 years before reemergence

of this genotype in 2005. In adition, the long period of little or no circulation of this genotype previous to RV1 introduction would have create favorable conditions for the accumulation of immunological susceptible individuals (Carvalho-Costa et al., 2009; Assis et al., 2013). This fact, together with the genetic diversity found in different geographical regions throughout the years before, during and after the introduction of the vaccine might explained the high prevalence of genotype G2P[4] in Brazil since 2005.

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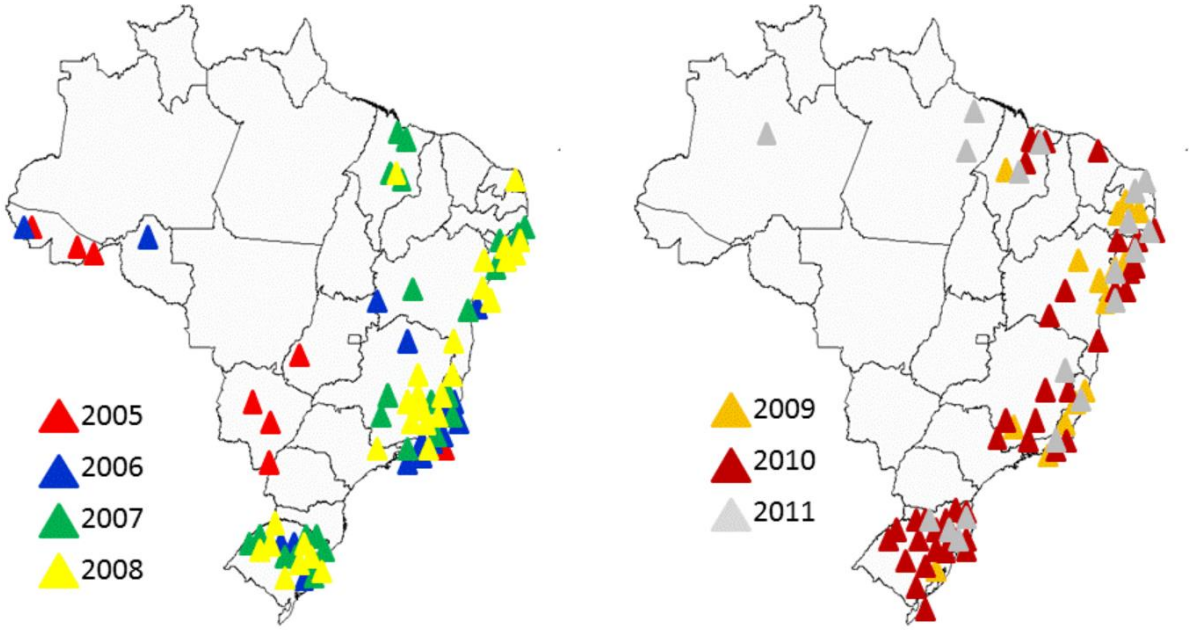
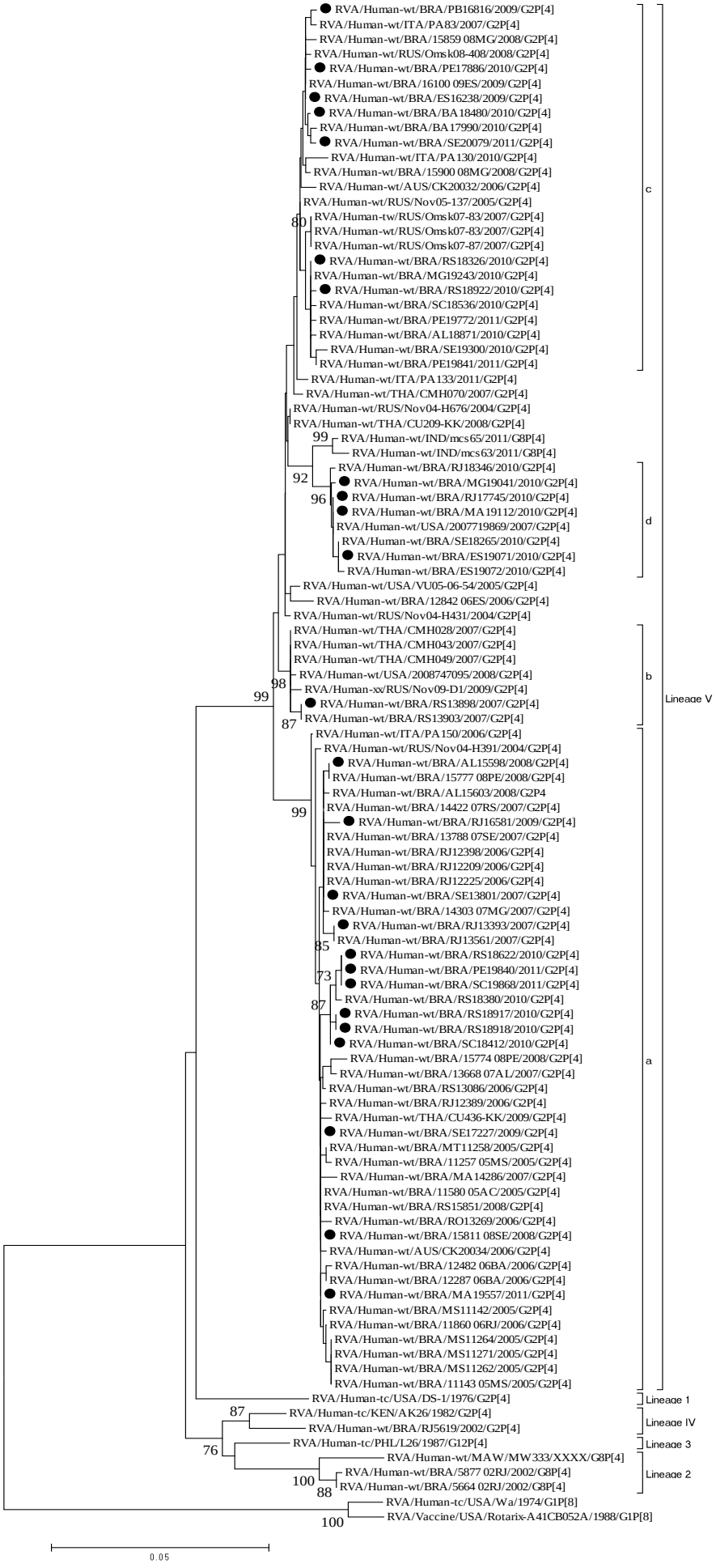


Figure 1. Spatial spread of G2P[4] rotavirus A in Brazil. The triangules indicate the cities where G2P[4] was detected. First G2P[4] strains were detected in August/September 2005 (6 months before the implementation of universal vaccination) in western states and in Dezember/2005 in Rio de Janeiro city (red triangules). In the following years, G2P[4] spread in vast áreas in many surveyed states.


RVA/Human-wt/BRA/PB16816/2009/G2P[4]
RVA/Human-wt/ITA/PA83/2007/G2P[4]
RVA/Human-wt/BRA/15859 08MG/2008/G2P[4]
RVA/Human-wt/RUS/Omsk08-408/2008/G2P[4]
RVA/Human-wt/BRA/PE17886/2010/G2P[4]
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RVA/Human-tc/KEN/AK26/1982/G2P[4]
RVA/Human-wt/BRA/RJ5619/2002/G2P[4]
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RVA/Human-wt/MAW/MW333/XXXX/G8P[4]
RVA/Human-wt/BRA/5877 02RJ/2002/G8P[4]
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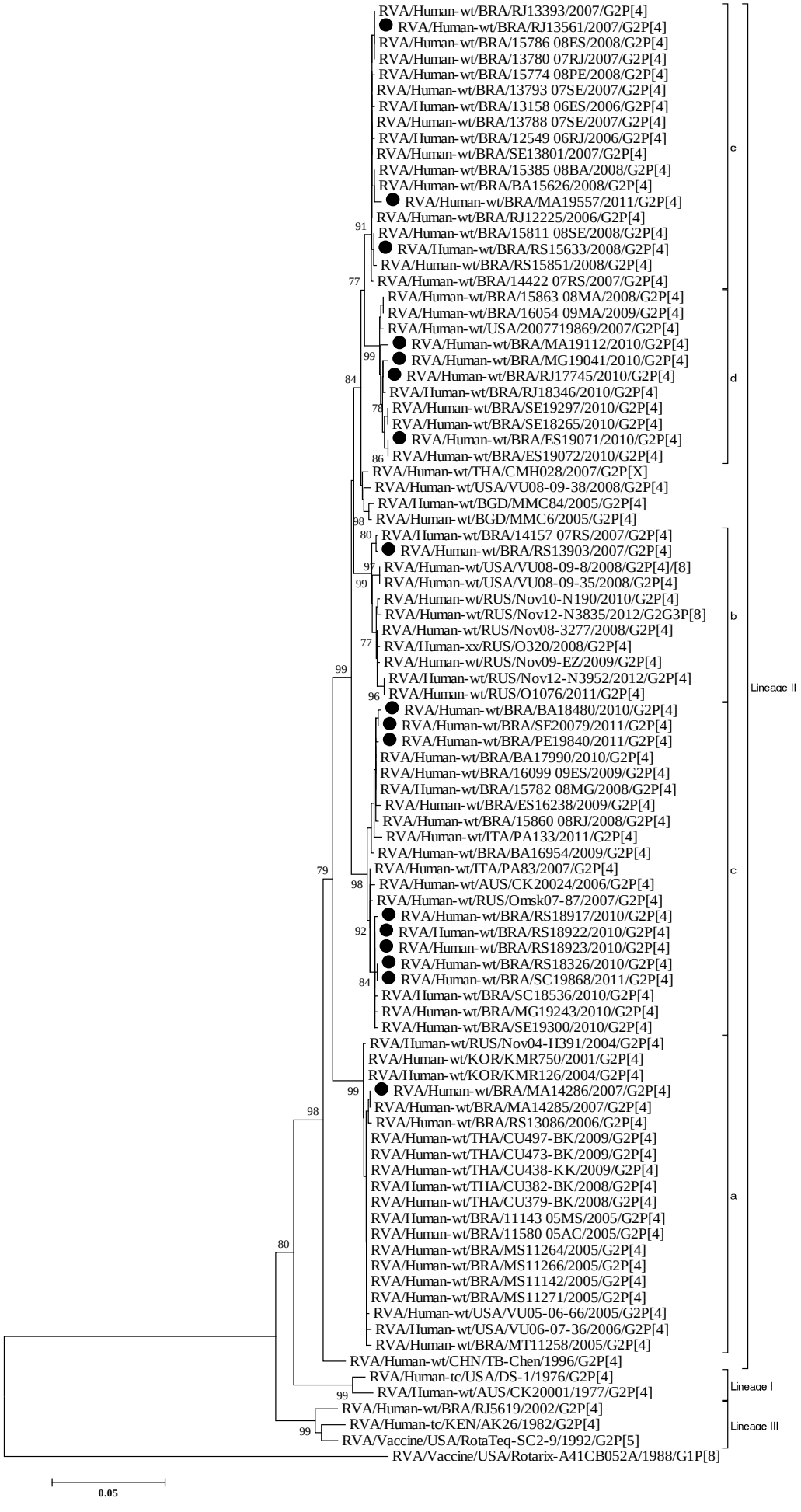
RVA/Human-tc/USA/Wa/1974/G1P[8]
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P[8]
c
d
b
a
Lineage V
Lineage 1
Lineage IV
Lineage 3
Lineage 2
80
92
96
98
99
87
99
85
73
87
87
76
100
88
100
0.05

Figure 2. Phylogenetic analysis based on VP8* nucleotide sequences from Brazilian G2P[4] strains detected between 2005 and 2011, and rotavirus A strains available at the GenBank database. Strains marked with a filled circle represent vaccinated RVA strains. Boostrap values under 70% are not shown. G2 Lineages are shown at the right side.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RVA/Human-wt/BRA/15786 08ES/2008/G2P[4]
RVA/Human-wt/BRA/13780 07RJ/2007/G2P[4]
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RVA/Human-wt/BRA/13158 06ES/2006/G2P[4]
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RVA/Human-wt/BRA/12549 06RJ/2006/G2P[4]
RVA/Human-wt/BRA/SE13801/2007/G2P[4]
RVA/Human-wt/BRA/15385 08BA/2008/G2P[4]
RVA/Human-wt/BRA/BA15626/2008/G2P[4]
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RVA/Human-wt/BRA/15863 08MA/2008/G2P[4]
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RVA/Human-wt/USA/2007719869/2007/G2P[4]
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RVA/Human-wt/BRA/MG19041/2010/G2P[4]
RVA/Human-wt/BRA/RJ17745/2010/G2P[4]
RVA/Human-wt/BRA/RJ18346/2010/G2P[4]
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RVA/Human-wt/BRA/SE18265/2010/G2P[4]
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RVA/Human-wt/BGD/MMC84/2005/G2P[4]
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RVA/Human-wt/BRA/11580 05AC/2005/G2P[4]
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RVA/Human-wt/AUS/CK20001/1977/G2P[4]
RVA/Human-wt/BRA/RJ5619/2002/G2P[4]
RVA/Human-tc/KEN/AK26/1982/G2P[4]
RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P[5]
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P[8]
e
d
b
c
a
Lineage II
Lineage I
Lineage III
0.05

Figure 3. Phylogenetic analysis based on VP7 nucleotide sequences from Brazilian G2P[4] strains detected between 2005 and 2011, and rotavirus A strains available at the GenBank database. Strains marked with a filled circle represent vaccinated RVA strains. Boostrap values under 70% are not shown. G2 Lineages are shown at the right side.

Antigenic residues in VP8* protein

	8-1										8-2		8-3									8-4		
	100	146	148	150	188	190	193	194	195	196	180	183	113	114	115	116	125	131	132	133	135	87	88	89
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P[8]	**D**	**S**	**S**	**N**	**S**	**S**	**N**	**L**	**N**	**N**	**E**	**R**	**N**	**P**	**V**	**D**	**S**	**S**	**N**	**D**	**N**	**N**	**T**	**N**
RVA/Human-tc/USA/DS-1/1976/G2P[4]	.	G	.	G	.	N	D	.	.	.	.	.	S	Q	T	N	N	E	.	N	D	.	.	.
G2P[4] Brazilian strains: clusters a, b, and c	.	G	S/G	S	.	N	D	.	.	.	.	.	N/S	Q	T	N	N	E	.	S	D	.	.	D
G2P[4] Brazilian strains: cluster d	.	G	.	S	.	.	D	.	.	.	.	.	S	.	T	N	N	E	.	S	D	.	.	D

Antigenic residues in VP7 protein

	7-1a														7-1b						7-2								
	87	91	94	96	97	98	99	100	104	123	125	129	130	291	201	211	212	213	238	242	143	145	146	147	148	190	217	221	264
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P[8]	**T**	**T**	**N**	**G**	**E**	**W**	**K**	**D**	**Q**	**S**	**V**	**V**	**D**	**K**	**Q**	**N**	**V**	**D**	**N**	**T**	**K**	**D**	**Q**	**N**	**L**	**S**	**M**	**N**	**G**
RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P[5]	A	N	S	D	.	.	E	N	.	D	T	M	N	.	.	D	.	S	.	S	R	.	N	T	S	D	I	S	.
RVA/Human-tc/USA/DS-1/1976/G2P[4]	A	N	S	D	.	.	E	N	.	D	N	.	N	.	.	D	.	N	.	N	R	.	N	T	S	D	I	S	.
G2P[4] Brazilian strains	.	N	S	N	.	.	E	N	.	D	T	M	N	.	.	D	.	.	.	S/N	R	.	N	T	S	D	I	S/A	.

Figure 4. Aligment of the deduced amino acid sequences inside antigenic epitopes of VP8* and VP7 proteins of Brazilian G2P[4] strains analyzed compared with RV1 strain and RV5 G2 strain (VP7). Amino acid changes are indicated with a grey shadow.

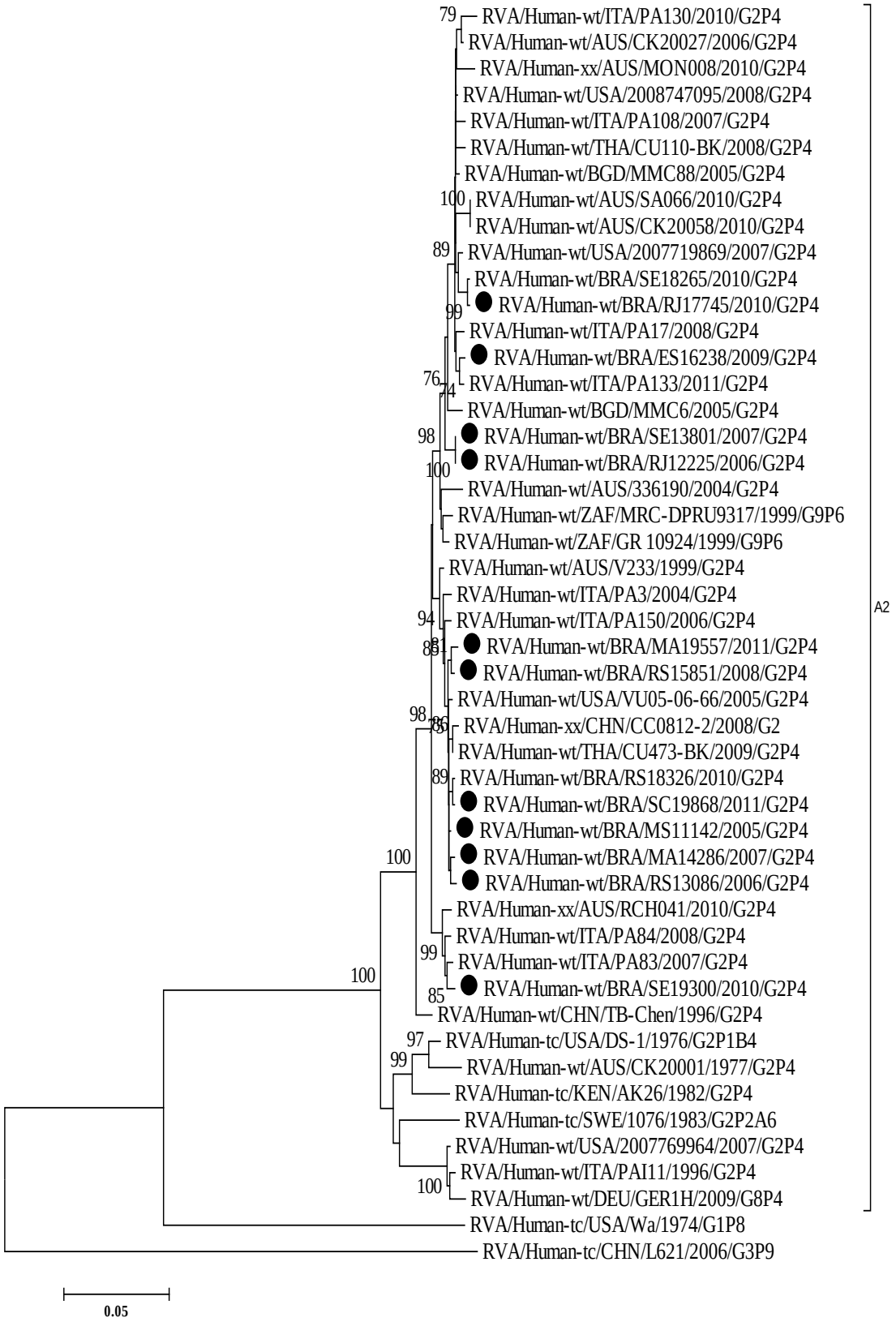
Table 1. Rate of specie A rotavirus (RVA) detection and G2P[4] prevalence among RVA-positive samples.

Year	Samples Studied (n)	RVA-positive samples, n (%)	G2P[4] rate of detection among RVA-positive samples, n (%)
2005	1292	344 (26.6)	31 (9)
2006	1576	227 (14.4)	112 (49)
2007	1232	211 (17.1)	139 (66)
2008	1013	284 (28)	240 (85)
2009	980	176 (17.9)	66 (37,5)
2010	1863	422 (22.6)	367 (87)
2011	1117	149 (13.3)	75 (50)

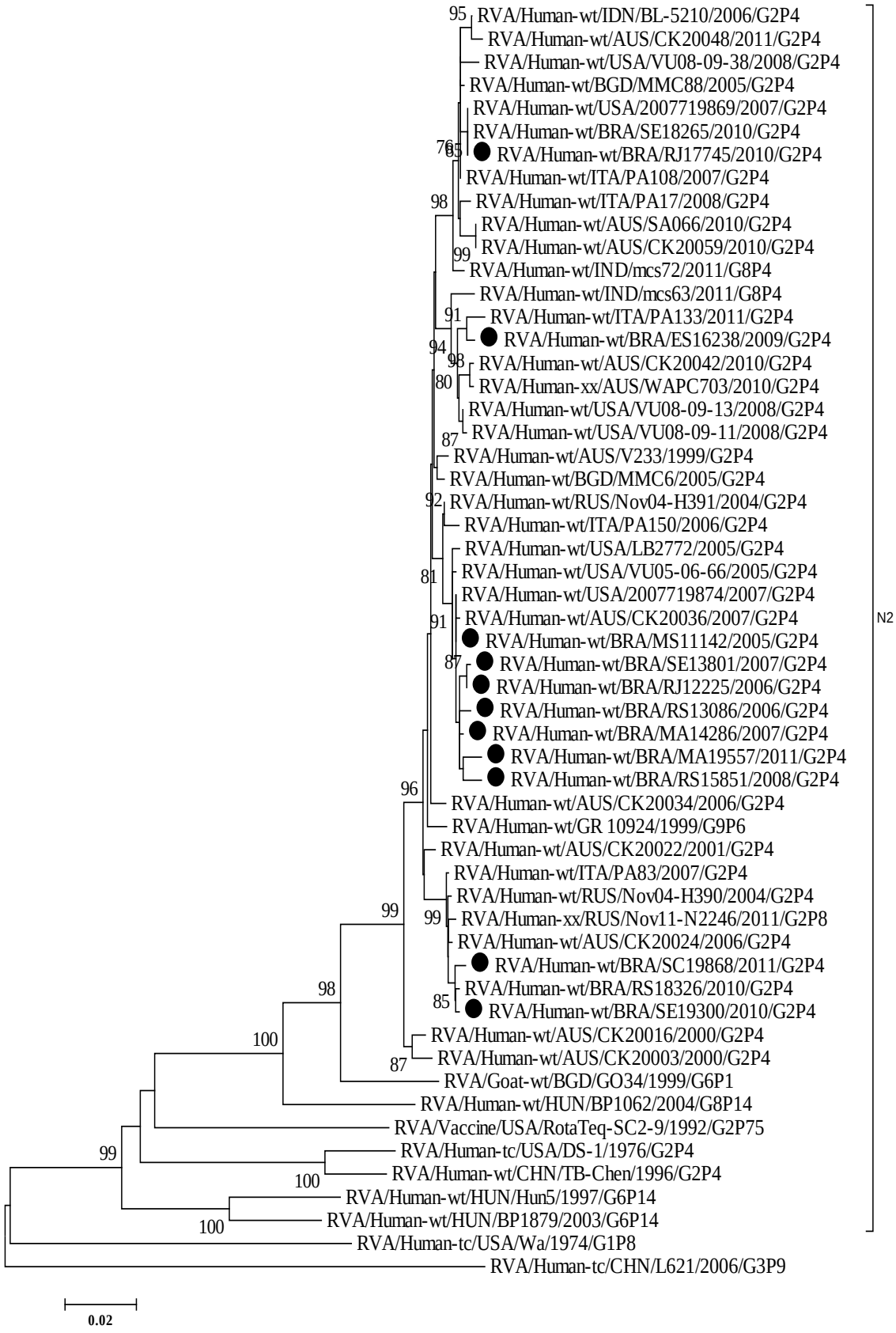
Table 2. Genomic background of RV1, RV5, and G2P[4] Brazilian rotavirus A strains. Name of G2P[4] vaccinated strains are in italic, as well as genotypes obtained with partial sequences.

Strain	RVA Vaccines Genomic Background										
	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P[8]	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P[5]	G1	P[5]	I2	R2	C2	M1	A3	N2	T6	E2	H3
RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P[5]	G2	P[5]	I2	R2	C2	M1	A3	N2	T6	E2	H3
RVA/Vaccine/USA/RotaTeq-WI78-8/1992/G3P[5]	G3	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Vaccine/USA/RotaTeq-BrB-9/1996/G4P[5]	G4	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P[8]	G6	P[8]	I2	R2	C2	M2	A3	N2	T6	E2	H3
	Brazilian G2P[4] Genomic Background										
	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Human-wt/BRA/MA19557/2011/G2P[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/SC19868/2011/G2P[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/SE19300/2010/G2P[4]	G2	P[4]	I2	*R2*	-	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/RJ17745/2010/G2P[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/ES16238/2009/G2P[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/RS15851/2008/G2P[4]	G2	P[4]	I2	*R2*	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/MA14286/2007/G2P[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/SE13801/2007/G2P[4]	G2	P[4]	I2	*R2*	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/RJ12225/2006/G2P[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/RS13086/2006/G2P[4]	G2	P[4]	I2	*R2*	C2	M2	A2	N2	T2	E2	H2
RVA/Human-wt/BRA/MS11142/2005/G2P[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2

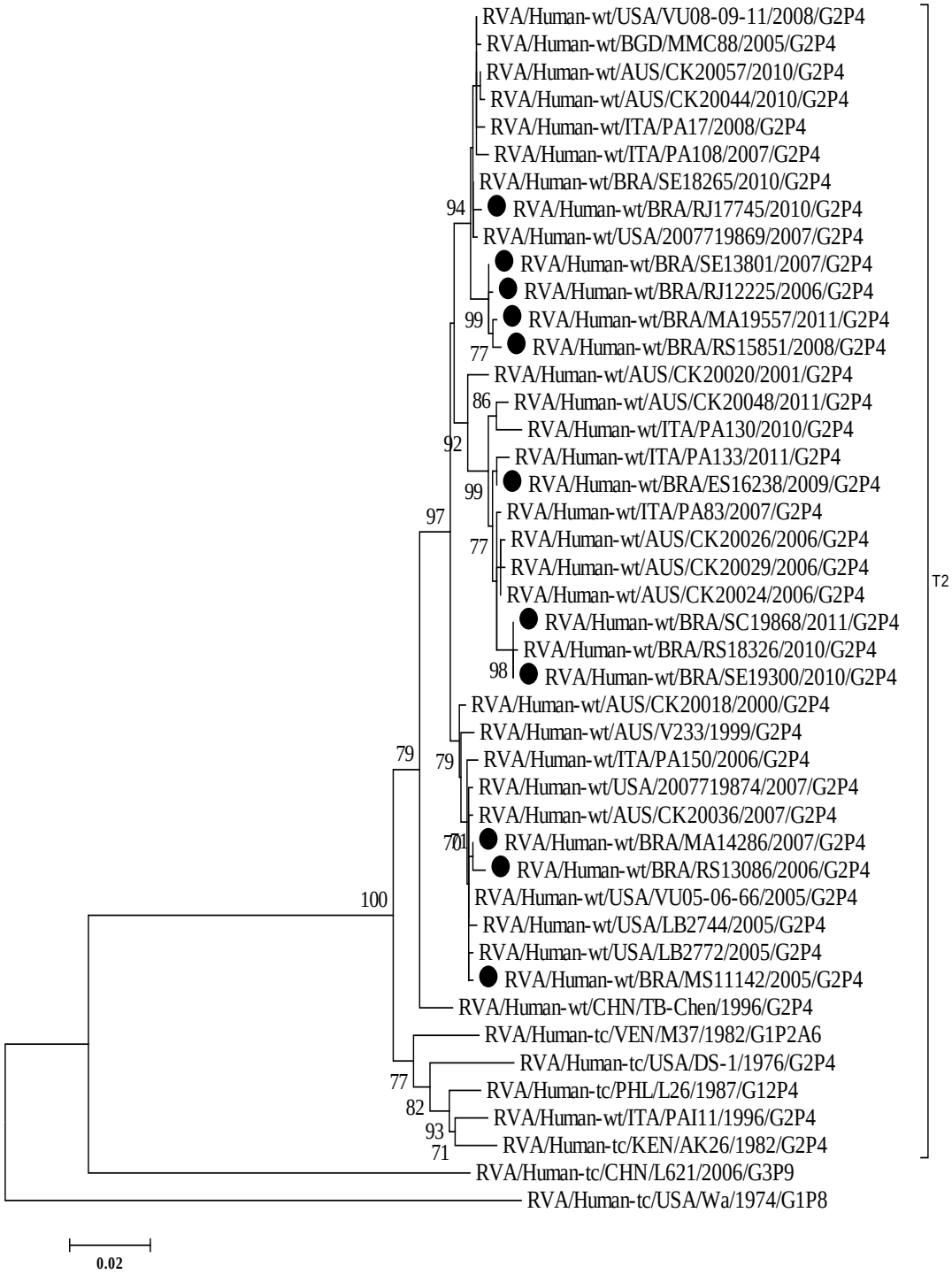
Supplementary material



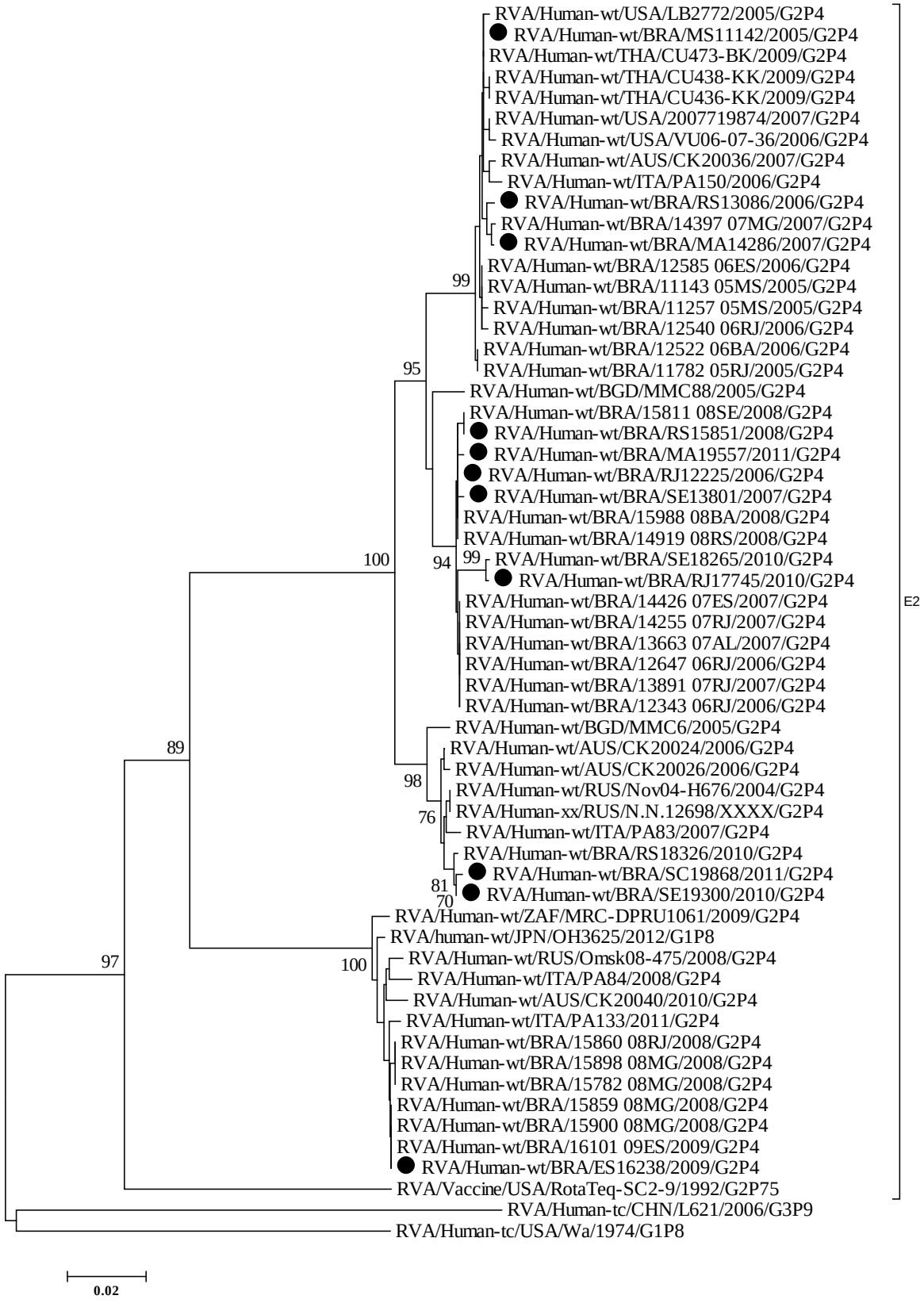
Supplementary material Figure 1. Phylogenetic analysis based on NSP1 nucleotide sequences. Brazilian G2P[4] strains are marked with a filled circle. Boostrap values under 70% are not shown.



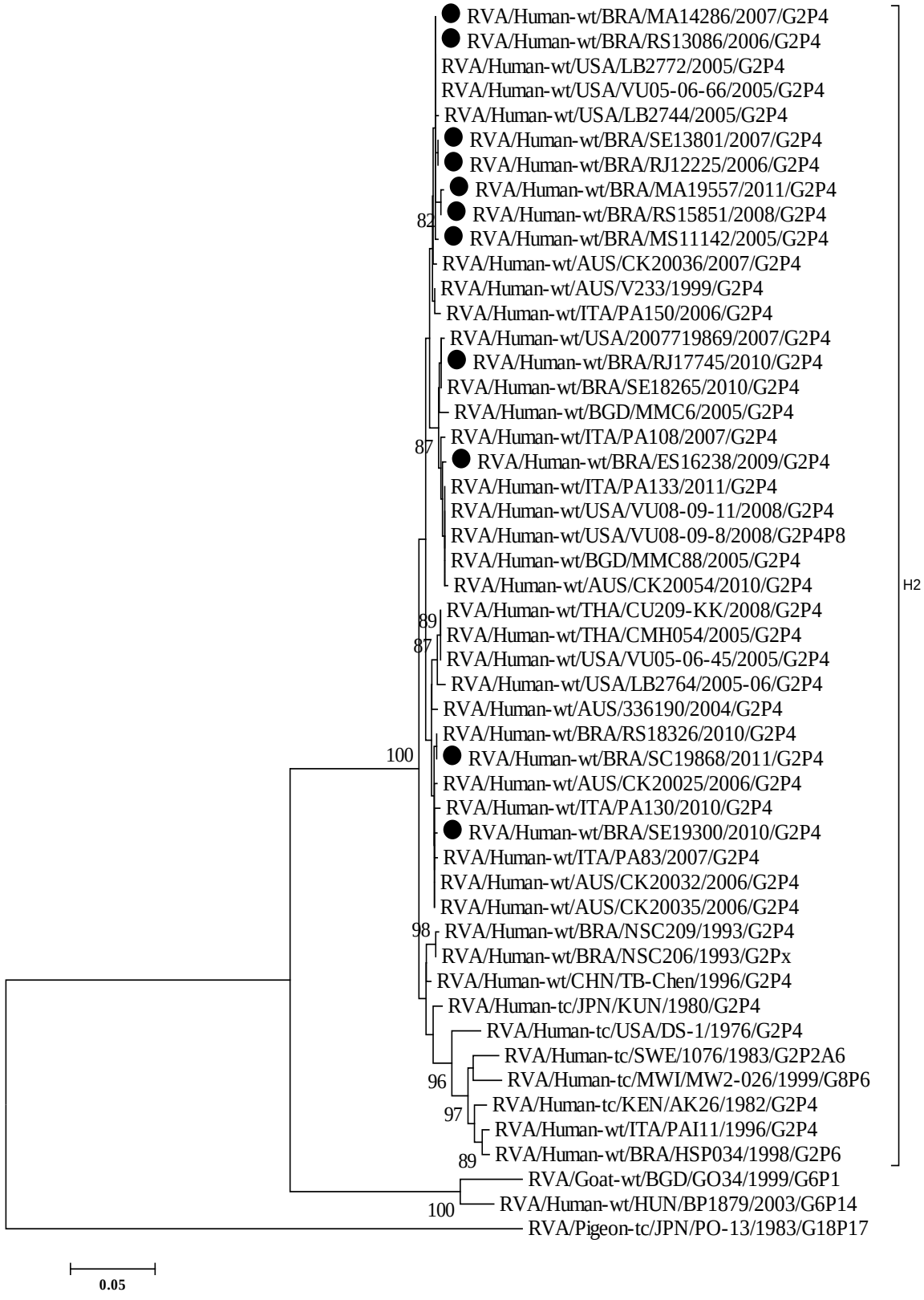
Supplementary material Figure 2. Phylogenetic analysis based on NSP2 nucleotide sequences. Brazilian G2P[4] strains are marked with a filled circle. Boostrap values under 70% are not shown.



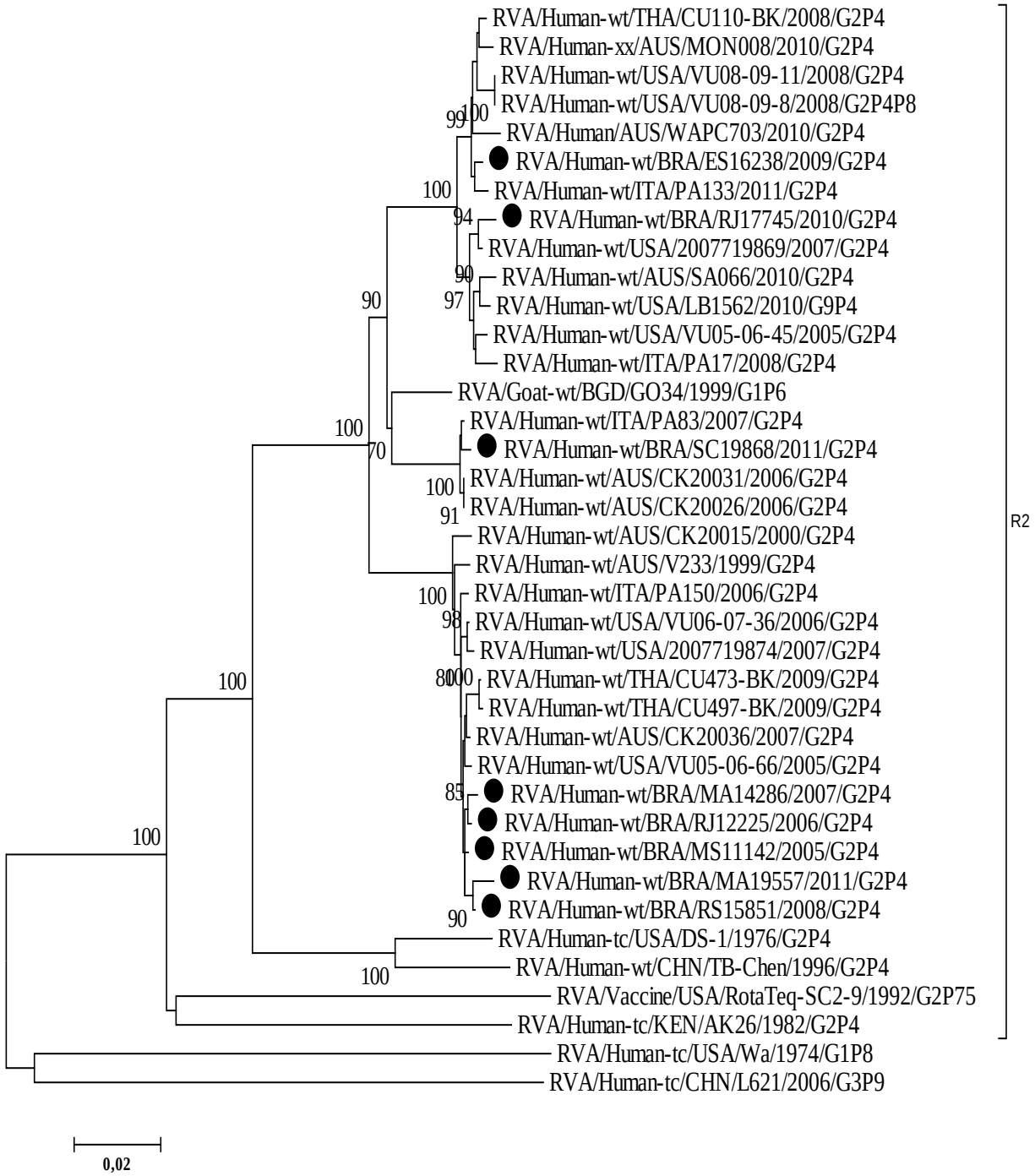
Supplementary material Figure 3. Phylogenetic analysis based on NSP3 nucleotide sequences. Brazilian G2P[4] strains are marked with a filled circle. Boostrap values under 70% are not shown.



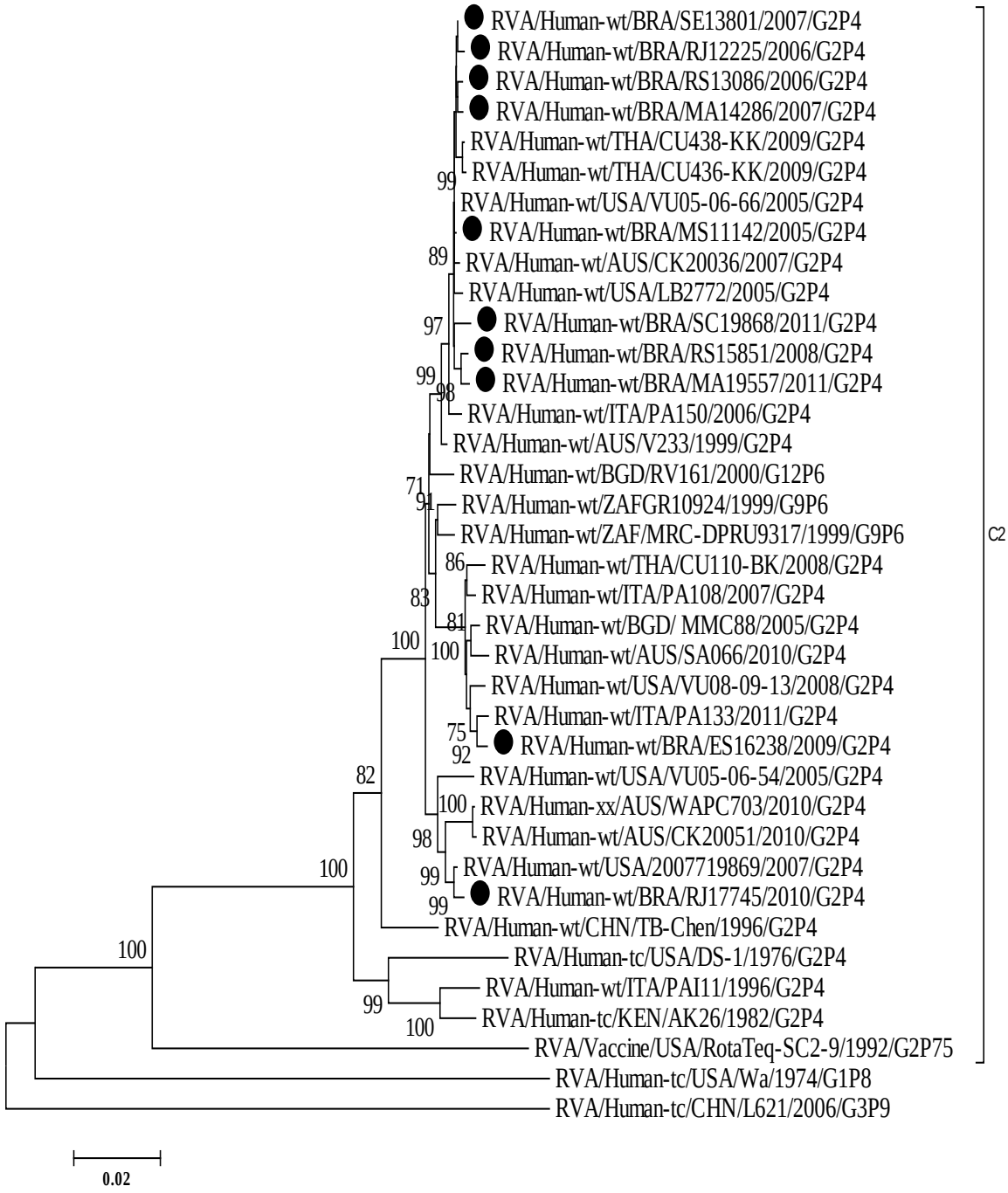
Supplementary material Figure 4. Phylogenetic analysis based on NSP4 nucleotide sequences. Brazilian G2P[4] strains are marked with a filled circle. Boostrap values under 70% are not shown.



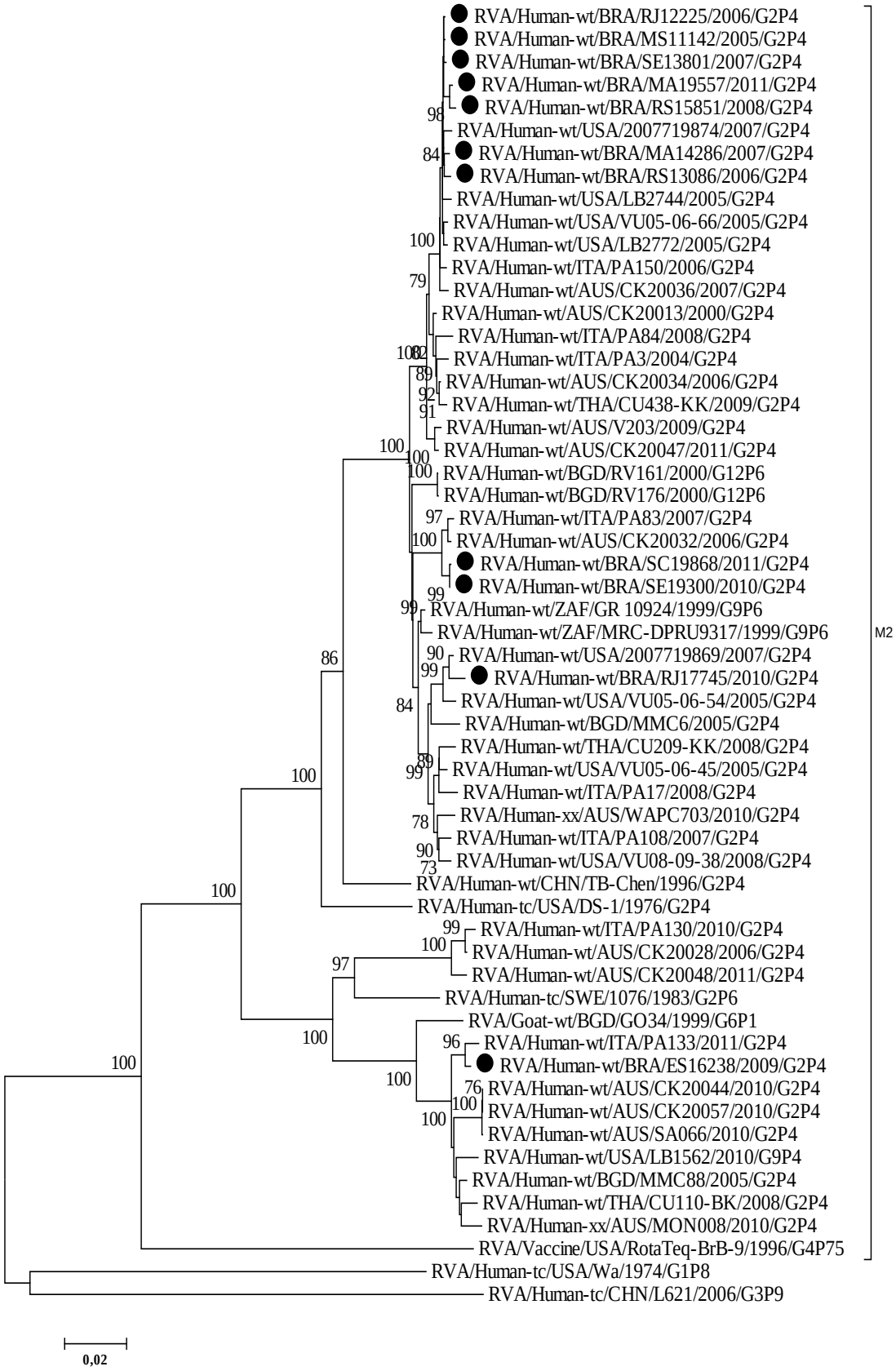
Supplementary material Figure 5. Phylogenetic analysis based on NSP5 nucleotide sequences. Brazilian G2P[4] strains are marked with a filled circle. Boostrap values under 70% are not shown.



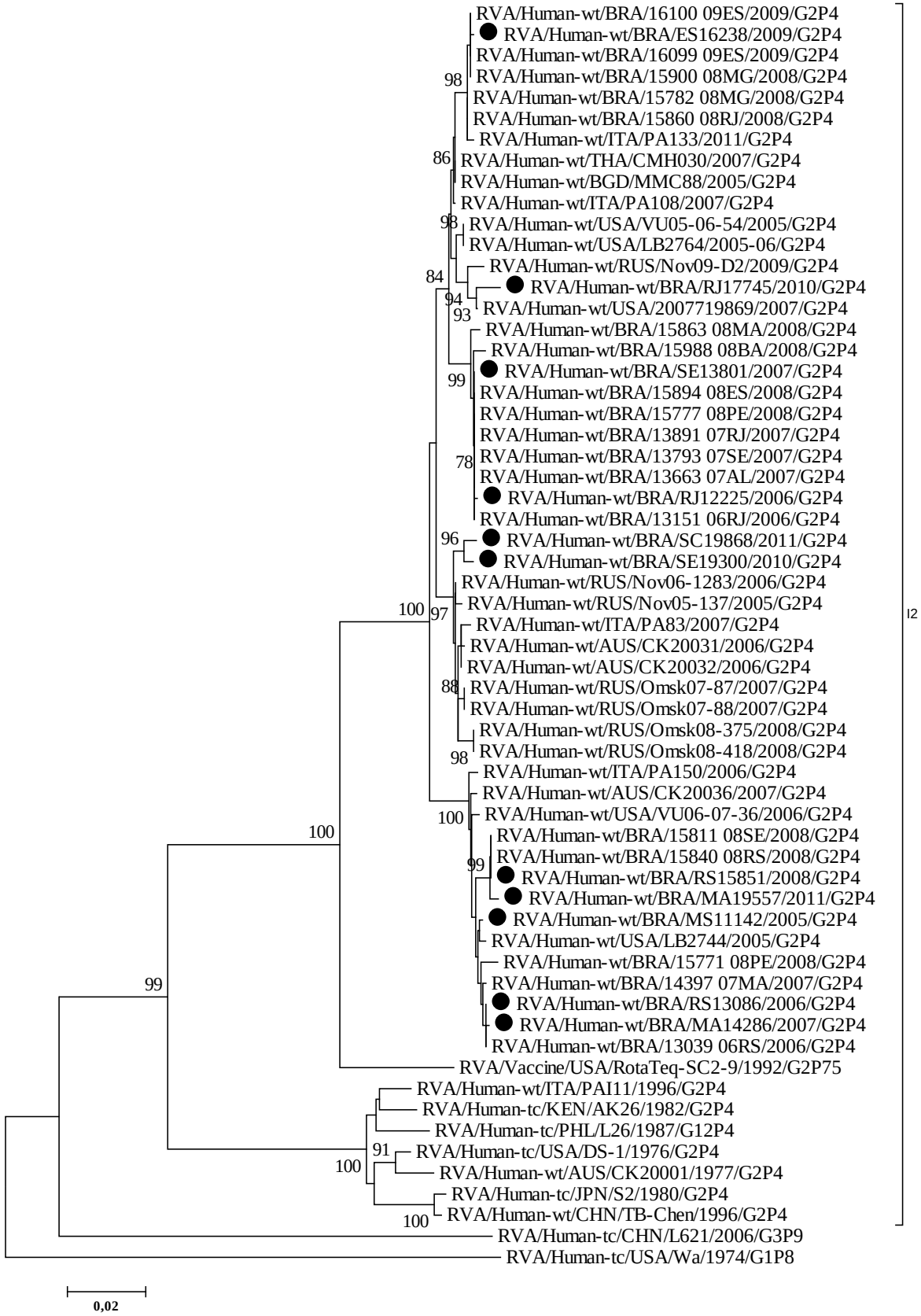
Supplementary material Figure 6. Phylogenetic analysis based on VP1 nucleotide sequences. Brazilian G2P[4] strains are marked with a filled circle. Boostrap values under 70% are not shown.



Supplementary material Figure 7. Phylogenetic analysis based on VP2 nucleotide sequences. Brazilian G2P[4] strains are marked with a filled circle. Boostrap values under 70% are not shown.



Supplementary material Figure 8. Phylogenetic analysis based on VP3 nucleotide sequences. Brazilian G2P[4] strains are marked with a filled circle. Boostrap values under 70% are not shown.



Supplementary material Figure 9. Phylogenetic analysis based on VP6 nucleotide sequences. Brazilian G2P[4] strains are marked with a filled circle. Boostrap values under 70% are not shown.

CAPITULO III

ANÁLISE FILOGENÉTICA DE RVA GENÓTIPO G1P[6] DETECTADO EM CRIANÇAS VACINADAS COM RV1 NO BRASIL (Artigo 3)

Artigo 3: Phylogenetic analysis of G1P[6] group A rotavirus strains detected in Northeast Brazilian children fully vaccinated with Rotarix™

Este artigo esta associado aos objetivos 1, 2, e 3.

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Phylogenetic analysis of G1P[6] group A rotavirus strains detected in Northeast Brazilian children fully vaccinated with Rotarix™

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ABSTRACT

In 2009 the World Health Organization recommended the use of group A rotavirus (RVA) vaccines in all national immunization programs (NIPs) in order to control severe RVA gastroenteritis disease. In Brazil, Rotarix™ was introduced in the NIP in March 2006, and a significant reduction in mortality rates among children ⩽5 years old was observed, especially in the Northern and Northeastern Brazil. In the current study the 11 gene segments of six Brazilian G1P[6] RVA strains, isolated in 2009 and 2010 from vaccinated children, were analyzed in order to investigate if the genetic composition of these strains might help to elucidate why they were able to cause acute gastroenteritis in vaccinated children. All six Brazilian RVA strains revealed a complete Wa-like genotype constellation: G1-P[6]-I1-R1-C1-M1-A1-N1-T1-E1-H1. Phylogenetic analysis showed that all six strains were nearly identical and showed a close genetic relationship with contemporary typical human Wa-like RVA strains. These results suggests that the fact that these strains were able to cause acute gastroenteritis in vaccinated children is likely not due to the genetic background of the strains, but rather to other factors such as host relating factors, co-infecting pathogens or vaccine efficacy. P[6] RVA strains are detected rather occasionally in humans in most regions of the world, except for South Asia and Sub-Saharan Africa. However, recently two studies conducted in Brazil showed the circulation of G12P[6] and G2P[6]. This is the first report on the detection and complete genome analyses of G1P[6] RVA strains in Brazil. Surveillance studies will be crucial to further investigate the prevalence of this genotype in the Brazilian population, and the efficacy of current licensed vaccines, which do not contain the P[6] genotype.

1. Introduction

Group A Rotaviruses (RVAs) belongs to the family *Reoviridae* and possesses a segmented double-stranded RNA genome composed of 11 segments encoding five (or six) nonstructural proteins (NSPs) and six structural proteins (VPs) (Estes and Kapikian, 2007). Two viral surface proteins, VP7 and VP4, are used to classify RVA strains into G- (Glycolysated) and P-types (Protease sensitive), respectively. An extended classification system for RVA strains based on all the 11 gene segments was developed by the Rotavirus Classification Working Group (RCWG). This system defines the following genotypes: Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, based on nucleotide similarities cut off values for the VP7, VP4, VP6, VP1-3, NSP1-5/6 encoding genome segments, respectively. Currently, 27 G-, 37 P-, 17 I-, 9 R-, 9C-, 8 M-, 16 A-, 10 N-, 12 T-, 15 E-, and 11 H-genotypes have been described (Guo et al., 2012; Matthijnssens et al., 2011; Papp et al., 2012; Trojnar et al., 2012). Worldwide the majority of human RVA strains possess either the Wa-like genotype constellation (I1-R1-C1-M1-A1-N1-T1-E1-H1) or the DS-1-like genotype constellation (I2-R2-C2-M2-A2-N2-T2-E2-H2) (Heiman et al., 2008; Matthijnssens et al., 2008; McDonald et al., 2009; Matthijnssens and Van Ranst, 2012). Until now, only one Brazilian RVA strain, RVA/Human-wt/BRA/IAL28/1992/G5P[8], has been completely characterized to date, and possesses a Wa-like genome constellation (Heiman et al., 2008).

Annually RVA gastroenteritis accounts for approximately one third of the total diarrheal deaths worldwide (Black et al., 2010). In developing countries improving sanitary conditions and access to a safe water supply alone will not be sufficient to prevent RVA

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gastroenteritis. Malnourished children with poor health are more vulnerable to serious infections causing acute diarrhea and suffer multiple episodes of acute diarrhea every year (United Nations Children's Fund (UNICEF)/World Health Organization (WHO), 2009). Therefore, vaccination is considered the best alternative among public health measures to reduce and prevent the global burden caused by RVA infections. In Brazil, the monovalent (G1P[8]) Rotarix™ vaccine (GlaxoSmithKline, Rixensart, Belgium) was introduced in the NIP in March, 2006, and vaccine coverage have been estimated at 83% in 2010 (Centers for Disease Control, 2011). Recent studies have shown a significant reduction in morbidity and mortality rates among children younger than 5 years old, especially in the Northern and Northeastern regions of Brazil where the rates of mortality are higher than in other Brazilian regions (do Carmo et al., 2011; Carvalho-Costa et al., 2011).

RVA strains bearing the P[6] genotype have been detected in combination with a wide range of G-genotypes (G1-G6, G8-G12, and G25) worldwide, and in combination with both Wa-like and DS-1 like genotype constellations (Kirkwood et al., 1999; Linhares et al., 2002; Rahman et al., 2007a; Heiman et al., 2008; Esona et al., 2010; Mwenda et al., 2010; Rippinger et al., 2010; Le et al., 2011). In industrialized countries P[6] RVA strains are rarely detected in humans. However, human P[6] RVA strains have been described as one of the most prevalent genotypes in South Asia and Sub-Saharan Africa (Ramani, 2007; Armah et al., 2010; Todd et al., 2010). In Brazil, the P[6] genotype has been sporadically detected in association with G1–5, G8, and G9, in four out of five Brazilian regions: North, Northeast, Southeast, and West Central (Santos et al., 1994; Timenetsky et al., 1994; Leite et al., 1996; Araujo et al., 2001; Linhares et al., 2002; Mascarenhas et al., 2002, 2006, 2007; Volotao et al., 2006; Gurgel et al., 2008; Leite et al., 2008; Carvalho-Costa et al., 2011; Soares et al., 2012).

A limited number of human P[6] RVA strains have been completely characterized to date, none of them in combination with G1 (Rahman et al., 2007b; Heiman et al., 2008; Matthijnssens et al., 2006, 2008; Pietsch and Liebert, 2009; Rippinger et al., 2010; Wang et al., 2010; Jere et al., 2011; Than et al., 2011; Heylen et al., 2012; Zeller et al., 2012a). The main objective of the current study was to analyze six G1P[6] RVA strains isolated from vaccinated children that were hospitalized with acute gastroenteritis in Northeastern, Brazil, in order to investigate if the genetic composition of these strains might help to understand why these strains were able to cause acute gastroenteritis despite the fact that these children were vaccinated.

2. Materials and methods

2.1. Clinical samples

Six stool samples collected from hospitalized children with acute gastroenteritis vaccinated with two doses of Rotarix™ were collected from northeastern Brazilian states: Bahia (RVA/Human-wt/BRA/BA17290/2009/G1P[6]), Ceará (RVA/Human-wt/BRA/CE17436/2010/G1P[6]), Alagoas (RVA/Human-wt/BRA/AL18874/2010/G1P[6]), and Pernambuco (RVA/Human-wt/BRA/PE18948/2010/G1P[6]; RVA/Human-wt/BRA/PE18949/2010/G1P[6] and RVA/Human-wt/BRA/PE18963/2010/G1P[6]) (Table 1).

2.2. Nucleic acid extraction and RT-PCR

Nucleic acid was extracted from 200 μl of 10% fecal suspensions by the glass powder method described by Boom et al. (1990), including the following modifications: 200 μl of 10% of fecal suspensions were added to 500 μl of L6 buffer, vortexed for 5 s and kept at room temperature for 5 min. Subsequently, 7.5 μl of silica solution was added and the tubes were placed in an orbital shaker for 20 min. After centrifugation at 16,000*g* for 30 s the supernatant was discarded and the silica pellet was washed with 500 μl of L2 buffer, 500 μl of 70% ethanol, and 500 μl of acetone. After each wash, the sample was centrifuged for 30 s at 16,000*g*, and the supernatants were discarded. Tubes were dried at 56 °C for 15 min. The pellet was dissolved in 60 μl of RNAse-DNAse free water, vortexed, and incubated for 15 min at 56 °C with the lid closed. Afterwards, the tube was vortexed and centrifuged at 16,000*g* for 3 min. Finally, the nucleic acid-containing supernatant was recovered in a new tube and stored at −20 °C.

Amplification of the VP6 and VP7 gene segments were performed using the SuperScript™ III One-Step RT-PCR System with the Platinum™ Taq DNA Polymerase Kit (Invitrogen™, Brazil) using the following temperature profile: 55 °C for 1 h, 94 °C for 5 min, 40 cycles of 94 °C/1 min, 55 °C/1 min, and 72 °C/3 min, with a final extension of 72 °C for 7 min. To amplify the NSP1–3 and NSP5 genes the SuperScript™ III One-Step RT-PCR System with the Platinum™ Taq DNA Polymerase Kit (Invitrogen™, Brazil) was used as described in previous studies (Nakagomi and Kaga, 1995; Matthijnssens et al., 2006). Reverse transcription (RT) for the NSP4, VP1-3, and VP4 (VP8*) gene segments were performed with the High Capacity cDNA Reverse Transcription Kit™ (Applied Biosystems, Brazil) according to the manufacturer's instructions. The PCR protocol used to amplify VP8* was described by Gentsch et al. (1992), with modifications from Gómez et al. (2010). For NSP4 the protocol described by Gómez et al. (2011) was used. The VP1 gene segment was partially amplified according to the protocol of Varghese and colleagues (2006), and the PCR for the amplification of VP2 and VP3 was carried out as follows: 94 °C for 2 min, 40 cycles of amplification (30 s at 94 °C, 30 s at 50 °C, and 1.5 min at 72 °C, with a final extension of 7 min at 72 °C. All primers used in the current study, and the lengths of the obtained fragments are shown in Supplementary material Table 1.

2.3. Sequencing and phylogenetic analyses

Sequencing was performed with an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit™ and an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the Instituto de Tecnologia em Imunobiológicos (Bio-Manguinhos), FIOCRUZ. The same set of primers used in the RT-PCR was used for

Table 1
Available patient information for G1P[6] rotavirus A strains analyzed in this study, including: Brazilian states were the samples were collected; date of birth; age of the patient at the time of sample collection; vaccine doses administration dates.

Sample	Brazilian State	Date of birth	Age	1st Rotarix™ Dose	2nd Rotarix™ Dose	Sample collection date
RVA/Human-wt/BRA/BA17290/2009/G1P[6]	Bahia	–	2 years			30-Sep-2009
RVA/Human-wt/BRA/CE17436/2010/G1P[6]	Ceará	April 4, 2006	3 years and 9 months	6-May-2006	4-Aug-2006	14-Jan-2010
RVA/Human-wt/BRA/AL18874/2010/G1P[6]	Alagoas	January 30, 2009	1 year and 6 months	4-Jan-2009	1-Jun-2009	26-Aug-2010
RVA/Human-wt/BRA/PE18948/2010/G1P[6]	Pernambuco	March 13, 2007	3 years and 6 months	15-May-2007	17-Jul-2007	5-Oct-2010
RVA/Human-wt/BRA/PE18949/2010/G1P[6]	Pernambuco	February 1, 2007	3 years and 9 months	4-Mar-2007	2-Jul-2007	5-Oct-2010
RVA/Human-wt/BRA/PE18963/2010/G1P[6]	Pernambuco	September 18, 2009	1 year	19-Nov-2009	10-Feb-2010	5-Oct-2010

sequencing, plus two internal primers for the NSP3 encoding gene (Tort et al., 2010). All reactions were repeated at least twice for the accuracy of the study.

Sequences obtained in the current study were deposited in GenBank database under the following accession numbers: JN869248–JN869289; JX455052–JX455075.

Multiple sequence alignments were carried out using the ClustalW program (Thompson et al., 1994). Phylogenetic analyses were performed using the Neighbor-Joining method with the Kimura-two parameter model in MEGA5.0 (Tamura et al., 2011). The statistical significance of the branch was assessed by bootstrap resampling analysis (2000 replicates).

3. Results

3.1. Genome constellation of Brazilian G1P[6] RVA strains

Analysis of the 11 RVA genome segments revealed that all six Brazilian RVA samples detected from vaccinated children possessed the Wa-like genotype, and showed the following genotype constellation: G1-P[6]-I1-R1-C1-M1-A1-N1-T1-E1-H1. The same genotype constellation has been previously described for human P[6] strains in combination with G3, G4, G11, and G12 (Table 2). The six analyzed strains were nearly identical across their entire genome both at the nucleotide and amino acid level, with identity values ranging from: 99.2–100% and 98.6–100%, respectively.

3.2. Phylogenetic and sequence analyses of VP8*(VP4) and VP7

Phylogenetic analysis based on the VP8* (VP4) encoding gene showed a close genetic relationship between the Brazilian P[6] strains and a G2P[6] RVA strain detected in the USA in 2006 (RVA/Human-wt/USA/06-242/2006/G2P[6]) and four recently described P[6] strains that were found in combination with G1 or G6 and were detected in Burkina Faso (RVA/Human-wt/BFA/267-BF/2010/G1P[6]; RVA/Human-wt/BFA/225-BF/2010/G1P[6]; RVA/Human-wt/BFA/259-BF/2010/G1P[6]; RVA/Human-wt/BFA/281-BF/2010/G1G6P[6]) inside P[6]-I lineage (Fig. 1A). Nucleotide and amino acid identity values among these strains and Brazilian G1P[6] strains ranged between 98.0–98.7% and 97.4–98.5%, respectively. Previously detected P[6] strains in combination with G2, G4 and G9 in Brazil also belonged to P[6]-I lineage but clustered separately. Seventeen amino acid differences were observed in previous described antigenic sites when compared with the Rotarix™ vaccine strain, which belongs to the P[8] genotype (Supplementary material Table 2).

Phylogenetic analysis revealed a close genetic relationship between the VP7 gene segments of G1P[6] and G1P[8] RVA strains detected worldwide between 2006 and 2010, all clustering in lineage G1-I (Fig. 1B). The most closely related strains were G1P[8] strains from USA (RVA/Human-wt/USA/2007719739/2007/G1P[8]), Belgium (RVA/Human-wt/BEL/BE00039/2008/G1P[8]) and Nicaragua (RVA/Human-wt/NCA/28 J/2010/G1P[8]), showing 98.9–99.1%–99.2–100% of nucleotide and amino acid identity values, respectively. Other Brazilian G1P[8] samples from the same geographical region (Northeast Brazil), detected from vaccinated children in Sergipe and Pernambuco in 2009 also clustered relatively close to the G1P[6] Brazilian strains (97.7–98.6%–98.1–99.6%, nucleotide and amino acid identity values, respectively). The VP7 gene of Rotarix™ grouped in lineage G1-II together with Brazilian samples detected between 1998 and 2005, and revealed 93.4–93.6% and 94.2–94.5% of nucleotide and amino acid identity. Analysis of the amino acid sequences, revealed that the Brazilian G1P[6] strains carried four amino acid substitutions inside previous described antigenic sites when compared with Rotarix™, at positions: N94S, S123N, K291R and M217T (Supplementary material Table 3).

3.3. Phylogenetic analyses of the NSP1–5 genome segments

Phylogenetic analysis based on NSP1–4 nucleotide sequences, shown that all six Brazilian G1P[6] strains belonged to a monophyletic cluster, showing a close genetic relationship with contemporary typical human Wa-like RVA strains detected worldwide (Australia, Bangladesh, Belgium, Brazil, India, Nicaragua, South Africa and USA) belonging to different G- (G1, G9, G11, G12) and P-genotypes (P[4], P[6], P[8], P[25]) (Fig. 2).

The most closely related RVA strains for NSP1 were: two G12 strains from South Africa, RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and RVA/Human-wt/ZAF/3176WC/2009/G12P[6] (Fig. 2A), with identity values ranging from 98.9–99.1% and 98.5–98.9% for nucleotide and amino acid sequences, respectively; for NSP2, two G1P[8] strains from Australia(RVA/Human-wt/AUS/CK00001/2004/G1P[8] and RVA/Human-wt/AUS/CK00009/2004/

Table 2
Genotype constellation comparison. The six Brazilian G1P[6] RVA strains analyzed in the current study are represented by RVA/Human-wt/BRA/BA17290/2009/G1P[6] strain. Green and red indicate Wa-like and DS-1-like gene segments, respectively. The P[6] VP4 genotype is colored yellow, and blue is used to indicate a gene segment of porcine origin. Brazilian strains are shown in bold.

	Strain	Origin	Genotypes										
			VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
Wa-like	RVA/Human-tc/USA/Wa/1974/G1P[8]	Human	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/USA/Rotarix-A41CB052A/1988/G1P[8]	Human	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/BGD/Dhaka12/2003/G12P[6]	Human	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/ZAF/3176WC/2009/G12P[6]	Human	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/BGD/Matlab13/2003/G12P[6]	Human	G12	P[6]	I1	R1	C1	M1	A1	N1	T2	E1	H1
	RVA/Human-tc/KOR/CAU195/200X/G12P[6]	Human	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/DEU/GER172/2008/G12P[6]	Human	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/ECU/EC2184/200x/G11P[6]	Human	G11	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-tc/GBR/ST3/1975/G4P2A[6]	Human	G4	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-tc/AUS/RV3/1977/G3P[6]	Human	G3	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/BRA/BA17290/2009/G1P[6]	Human	G1	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
DS-1-like	RVA/Human-tc/USA/DS-1/1976/G2P1B[4]	Human	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
	RVA/Human-wt/BEL/F01322/2009/G3P[6]	Human	G3	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2
	RVA/Human-wt/COD/DRC86/2003/G8P[6]	Human	G8	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2
	RVA/Human-wt/BGD/N26/2002/G12P[6]	Human	G12	P[6]	I2	R2	C2	M2	A2	N1	T2	E6	H2
	RVA/Human-wt/BGD/RV161/2000/G12P[6]	Human	G12	P[6]	I2	R2	C2	M2	A2	N2	T2	E1	H2
	RVA/Human-wt/BGD/RV176/2000/G12P[6]	Human	G12	P[6]	I2	R2	C2	M2	A2	N2	T2	E6	H2
	RVA/Human-wt/USA/06-242/2006/G2P[6]	Human	G2	P[6]	I2	R2	C2	M2	A2	N2	T2	E2	H2

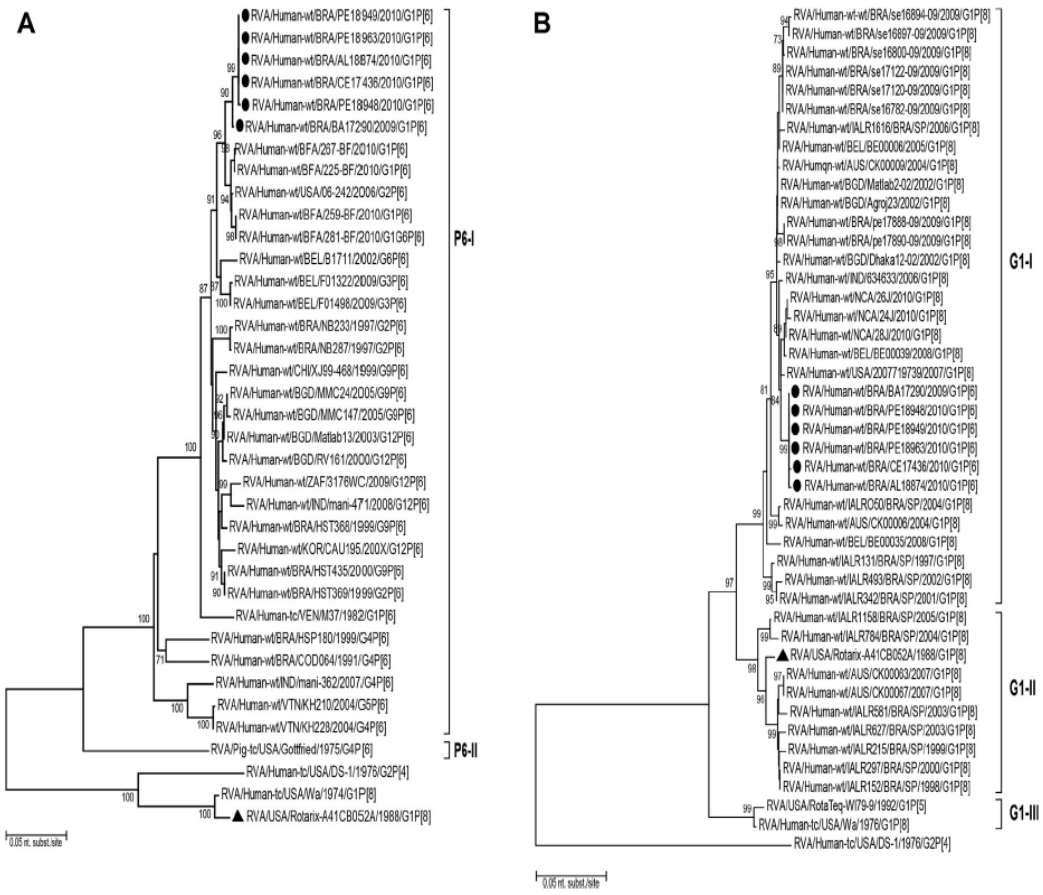

Fig. 1. Phylogenetic analysis based on the VP8* (A) and VP7 (B) gene nucleotide sequences of Brazilian rotavirus A G1P[6] strains, and sequences from the GenBank database. Numbers at the nodes indicate Bootstrap values, 2000 re-sampled datasets; only values above 70% are shown. The scale bar at the bottom represents 0.05 substitutions per nucleotide position (nt.subst./site). Brazilian G1P[6] strains are marked with a filled circle. Rotarix™ vaccine strain is marked with a filled triangle.

G1P[8]) and two from Bangladesh (RVA/Human-wt/BGD/Matlab36/2002/G11P[8] and RVA/Human-xx/BGD/MMC71/2005/G1P[8]), shared the highest identity values ranging from 98.6–98.8% at the nucleotide and 99–99.3% at the amino acid level (Fig. 2B); for NSP3, three G9P[8] strains from Rio de Janeiro (Southeast, Brazil), RVA/Human-wt/BRA/rj1528/1998/G9P[8], RVA/Human-wt/BRA/rj1527/1998/G9P[8] and RVA/Human-wt/BRA/rj1538/1998/G9P[8] (Fig. 2C), identity values ranged from 99.4–99.5% on the nucleotide level, and on the amino acid level strains were 100% identical; and for NSP4: two strains from the USA detected in 2007–2008, RVA/Human-wt/USA/2007719739/2007/G1P[8] and RVA/Human-wt/USA/VU08–09-39/2008/G12P[8] (Fig. 2D), showing 99.0–99.4% identity for nucleotide sequences and they were 100% identical in the amino acid level.

In the case of NSP5, phylogenetic analysis showed that the Brazilian G1P[6] strains clustered together with two G1P[8] Brazilian strains, also from vaccinated children, detected in Pernambuco, Northeastern, in 2009 (RVA/Human-wt/BRA/pe17888/2009/G1P[8] and RVA/Human-wt/BRA/pe17890/2009/G1P[8]), and two samples from South Africa (Fig. 2E). Identity values among Brazilian G1P[6] strains and G1P[8] RVA strains from Pernambuco ranged from 99.8–100% and 99.4–100% on the nt and aa level, respectively. Two strains from South Africa (RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and RVA/Human-wt/ZAF/3176WC/2009/G12P[6]) also clustered nearby and showed 99.6–99.8% and 99.4–100% nucleotide and amino acid identity values comparing with the Brazilian G1P[6] strains.

3.4. Phylogenetic analyses of the VP1–3 and VP6 genome segments

As observed for the NSP coding genes, the VP1–3 and VP6 encoding gene segments of the G1P[6] Brazilian strain showed a close genetic relationship with Wa-like strains detected worldwide (Argentina, Australia, Bangladesh, Belgium, Brazil, India, Nicaragua, South Africa, Thailand and USA) possessing the G1, G9, G11, G12 and P[4], P[6], P[8], P[25] genotypes (Fig. 3).

For the VP1 and VP2 encoding genes, Brazilian samples grouped in a monophyletic cluster and showed the closest genetic relationship with strains from Australia (VP1: RVA/Human-wt/AUS/CK00095/2010/G1P[8]; VP2: RVA/Human-wt/AUS/CK00006/2004/G1P[8]) and USA (VP1: RVA/Human-wt/USA/VU06-07-21/2006/G3P[8]; VP2: RVA/Human-wt/USA/2007719739/2007/G1P[8]) (Fig. 3A and B). Nucleotide and amino acid identity among Brazilian strains and closely related strains was 98.7–99.1%–100%, and 95.6–99.2%–98.6–99.6%, for VP1 and VP2, respectively. The Brazilian G1P[6] strains also clustered in a monophyletic cluster when analyzing the VP3 encoding gene (Fig. 3C). The most closely related strain was RVA/Human-wt/AUS/CK00006/2004/G1P[8], showing 98.6–98.7% and 99.0–99.3% of nucleotide and amino acid identity values, respectively. The VP6 nucleotide sequences of the Brazilian G1P[6] strains formed a monophyletic cluster (Fig. 3D). The most closely related strains were one strain from Australia (RVA/Human-wt/AUS/CK00088/2009/G1P[8]) and one strain from India (RVA/Human-xx/IND/ISO94/2005/G9PX), showing 99.1–99.5% and 99.4–99.7% of nucleotide and amino acid identity values, respectively, compared with Brazilian G1P[6] strains.

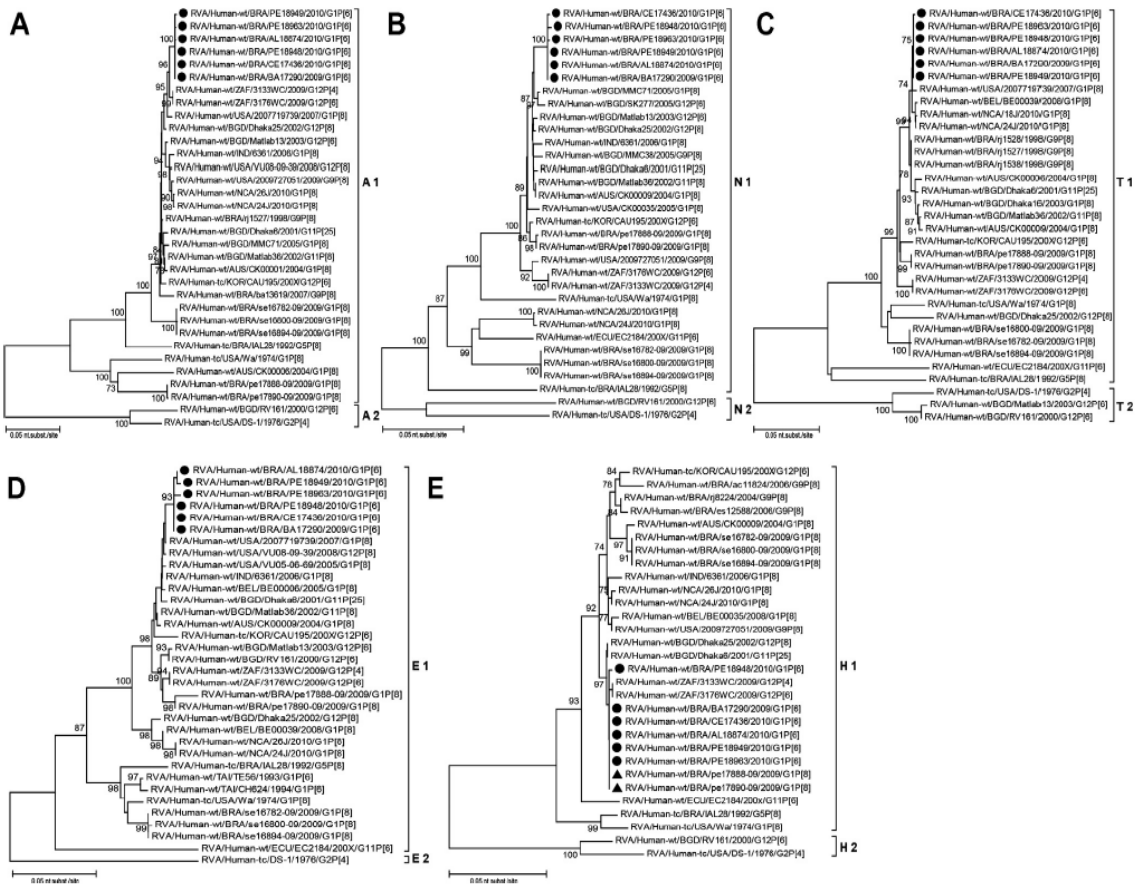

Fig. 2. Phylogenetic analysis based on NSP1 (A), NSP2 (B), NSP3 (C), NSP4 (D) and NSP5 (E) nucleotide sequences of Brazilian rotavirus A G1P[6] strains, and sequences from the GenBank database. Numbers at the nodes indicate Bootstrap values, 2000 re-sampled datasets; only values above 70% are shown. The scale bar at the bottom represents 0.05 substitutions per nucleotide position (nt.subst./site). Brazilian G1P[6] strains are marked with a filled circle.

4. Discussion

In this study, six G1P[6] RVA strains have been identified from children vaccinated with two doses of Rotarix™ vaccine in the Northeastern region of Brazil. Current licensed RVA vaccines target common antigens (G1–G4 and P[8]) considered important in eliciting neutralizing antibody responses. Studies regarding the efficacy of the Rotarix™ vaccine against circulating genotypes did not include P[6] strains, since this genotype was not a prevalent P-genotype during the clinical trials. Recently, two studies conducted in Brazil showed the circulation of P[6] strains: (a) Fumian and colleagues (2011) revealed the circulation of P[6] genotypes in Rio de Janeiro after introduction of the Rotarix™ vaccine, accounting for 25% of the P-genotypes detected from sewage influent samples, in combination with genotype G2; (b) Soares and colleagues (2012) detected G12P[6] RVA strains in the Northern region. Unfortunately, only partial NSP4 sequences are available from the P[6] detected in Rio de Janeiro, and only VP7 sequences are available from the G12P[6] strains detected in the Northern region. Together these results suggests that in recent years the P[6] genotype circulated in some parts of Brazil in combination with at least three different G-genotypes (G1, G2 and G12). Further studies should be conducted in order to investigate the prevalence of P[6] genotype in Brazil.

Results obtained in the current study showed that the VP8* sequences from P[6] Brazilian strains were closely related to African strains, as well as with one G2 strain from the USA (Heylen et al., 2012; Nordgren et al., 2012) (Fig. 1A). These strains all shared an amino acid change at position N135S, when compared with Rotarix™ strain, that was not present in other P[6] samples analyzed (Supplementary material Table 2). In total, seventeen amino acid changes were observed in the VP8* protein antigenic regions among Brazilian P[6] strains and the Rotarix™ vaccine strain (Supplementary material Table 2), showing that they are highly diverse as previously observed by Zeller et al. (2012b). Analysis of the VP7 encoding gene showed that the most recently detected G1 strains in Brazil belonged to a distinct G1 lineage (G1-I) when comparing with the Rotarix™ vaccine strain (genotype G1-II) (Fig. 1B). Four amino acid substitutions inside previous described antigenic sites were observed when compared Brazilian strains with the Rotarix™ vaccine at positions N94S, S123N, M217T, and K291R (Supplementary material Table 3). The fact that these substitutions were observed in almost all G1-I strains may suggests that they have been positive selected over time and might be related to some kind of advantage regarding viral fitness. It has been previously suggested that G1-I strains that accumulated mutations in these amino acid residues, could evolve over time into a variant that may be able to (partially) escape the immune response elicited by the vaccine, potentially causing a vaccine breakthrough (Maranhao et al., 2012). It is important to emphasize that the samples analyzed in the current study did not correspond to an outbreak.

All six G1P[6] Brazilian strains analyzed in this study possessed a Wa-like genotype constellation (Table 2), and showed a close genetic relationship among each other and with contemporary RVA strains from all over the world, with different G- and P-genotype combinations, and also for the other RVA genes (Figs. 1A and B, 2

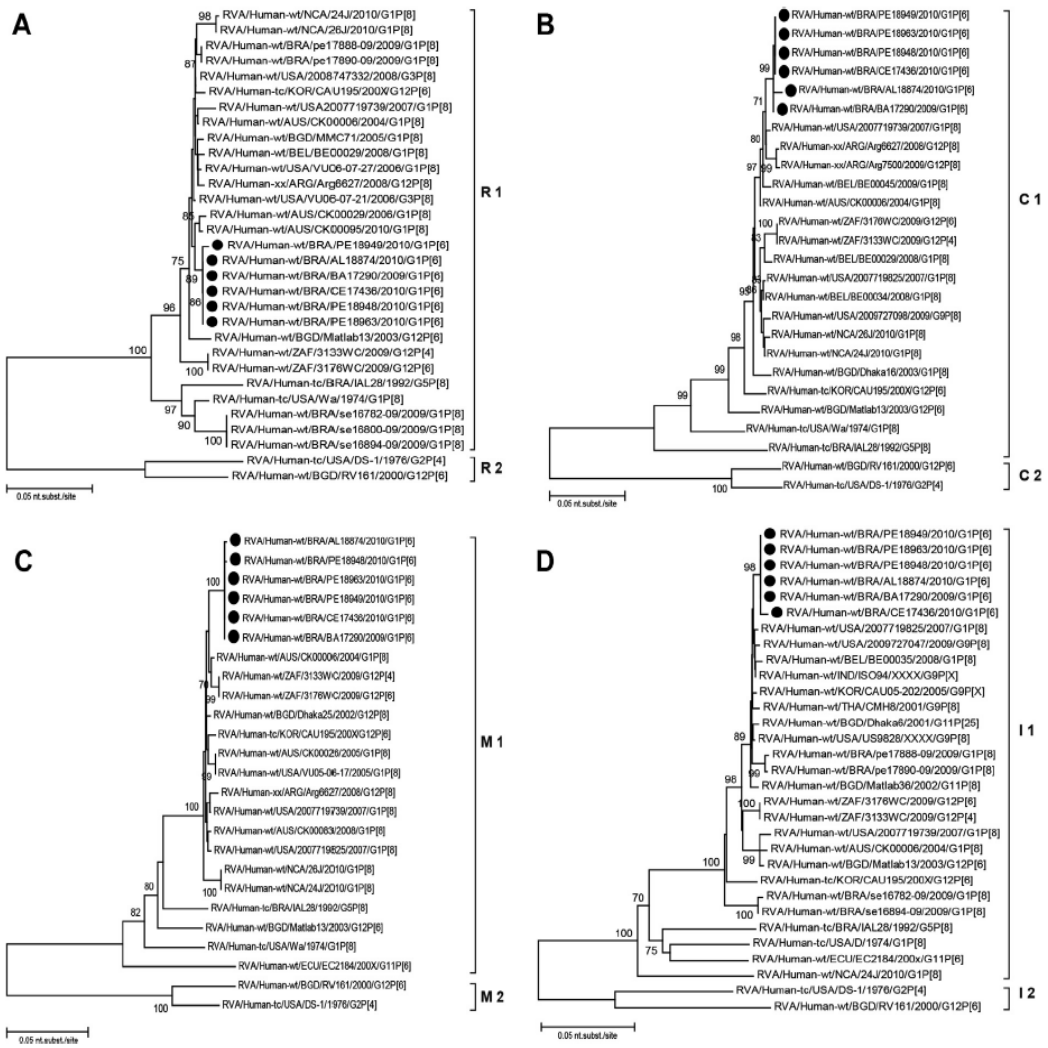

Fig. 3. Phylogenetic analysis based on VP1 (A), VP2 (B), VP3 (C) and VP6 (D) nucleotide sequences of Brazilian rotavirus A G1P[6] strains, and sequences from the GenBank database. Numbers at the nodes indicate Bootstrap values, 2000 re-sampled datasets; only values above 70% are shown. The scale bar at the bottom represents 0.05 substitutions per nucleotide position (nt.subst./site). Brazilian G1P[6] strains are marked with a filled circle.

and 3). Phylogenetic analysis of the NSP5 encoding gene suggests the occurrence of reassortment events between P[8] and P[6] co-circulating strains in Brazil (Fig. 2E). The fact that these strains just shared 1 out of the 11 RVA encoding genes could suggest that the P[6] strains analyzed in the current study were imported from elsewhere or that they are related to yet unknown RVA strains circulating in Brazil.

In addition, Brazilian G1P[6] strains analyzed cannot be considered as heterotypic strains when compared with Rotarix™ strain since they possess a Wa-like genome constellation and only the VP8* (VP4) encoding gene belonged to a different P-genotype (Matthijnssens and Van Ranst, 2012). These results suggests that most likely the reason of the breakthrough cases was not due to the genetic background of the analyzed strains, but rather due to other factors. Whereas VP4 and VP7 play an important role by stimulating the production of neutralizing antibodies, other factors such as high titers of rotavirus-specific antibodies in breast milk and levels of RV-specific IgA antibodies in the gut lumen have been described as possibly interfering with the efficacy of orally administered RVA vaccines. However further studies are needed to be carried out for a better understanding of the mechanisms associated to RVA protection (Desselberger and Huppertz, 2011).

The six RVA genomes reported in this paper represents the first report of 11 RVA genome segments from G1P[6] strains. As previously mentioned, to date only one strain from Brazil has been completely characterized (Heiman et al., 2008). Sequence data from all 11 RVA genome segments from circulating strains may help to assess the impact of RVA vaccines in strain diversity and evolution, as well as in identifying RVA strains that might challenge the efficacy of currently licensed vaccines (Leite et al., 2008; Carvalho-Costa et al., 2011; Yen et al., 2011).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2013.03.028.

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Supplementary material

Supplementary material table 1. Primers used in the amplification and sequencing of the 11 rotavirus A genome segments of Brazilian G1P[6] strains analyzed in the current study.

Gene Segment	Primer	Sequence 5'-3'	Nucleotide position	Fragment lenght (bp)	Reference
1 (VP1)	VP1F	GGCTATTAAAGCTGTACAATGGG	1 – 23	686	Varghese et al., 2006
	VP1R	TAATCCTCATGAGAAAACACTGAC	686 – 663		Varghese et al., 2006
2 (VP2)	GEN-VP2F	GGCTATTRAAGGYTCAATGG	1-20	942	Matthijnssens et al., 2006
	V2Rint1	GGTCTAATATATCGRGCTGTT	942-922		Mijatovic-Rustempasic et al., 2011
3 (VP3)	K-VP3F	GGCTATTAAAGCAGTACYAGT	1-21	977	Mijatovic-Rustempasic et al., 2011
	V3Rint1	ATGGATCCCATGTCTCAAATG	977-957		Mijatovic-Rustempasic et al., 2011
4 (VP4)	F4P4D	GCATGGCTTCACTCATTTATAGAC	2 - 22	889	Gomez et al., 2010
	4Con2	ATTTCGGACCATTTATAACC	887 - 868		Gentsch et al., 1992
5 (NSP1)	NSP1F	GGGCTTTTTTTTGAAAAGTC	1 - 20	1566	Nakagomi & Kaga, 1995
	NSP1R	GGTCACATTTTATGCTGCCTA	1566 - 1546		Nakagomi & Kaga, 1995
6 (VP6)	FVP6	GGCTTTTAAACGAAGTCTTC	1 - 20	1356	Chinsangaram et al., 1993
	RVP6	GGTCACATCCTCTCACTACA	1356-1337		Modified from Chinsangaram et al., 1993
7 (NSP3)	GEN-NSP3F	GGCTTTTAATGCTTTTCAGTG	1 - 21	1069	Matthijnssens et al., 2006
	GEN-NSP3R	ACATAACGCCCCTATAGC	1069 - 1052		Matthijnssens et al., 2006
	NSP3FSeq	TACGTTGAWGARARRATGGA	497 - 516		Tort et al., 2010
	NSP3RSeq	TCAACTTCCATYYTYTCWTCAA	522 - 501		Tort et al., 2010
8 (NSP2)	VF3F	GGCTTTTAAAGCGTCTCAGTC	1 - 21	1058	Varghese et al., 2004
	PCRg8R3GG	GGTCACATAAGCGCTTTCTAT	1058 - 1038		Patton et al., 1996
9 (VP7)	Beg9	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1 - 28	1062	Gouvea et al., 1990
	End9	GGTCACATCATACAATTCTAATCTAAG	1062 - 1036		Gouvea et al., 1990
10 (NSP4)	FN4D	CGGAAAAGATGGAAAAGC	35 - 52	673	Gomez et al., 2011
	RN4D	GGATTGGTTAAACGGGAT	707 - 690		Gomez et al., 2011
11 (NSP5)	GEN-NSP5F	GGCTTTTAAAGCGCTACAG	1 - 19	700	Matthijnssens et al., 2006
	GEN-NSP5R	GGTCACAAAACGGGAGT	700 - 684		Matthijnssens et al., 2006

Supplementary material table 2. Alignment of VP8* (VP4) antigenic residues between Rotarix™ strain and rotavirus A strains detected in Brazil and worldwide. Strains are shown by name at the left side of the figure. Conserved amino acid residues are indicated by dots. Antigenic residues are divided in four epitopes, 8-1, 8-2, 8-3, and 8-4. Numbers at the top denote amino acid positions based on the Rhesus rotavirus (RRV) strain. Shaded residues indicate amino acid that differ between G1P[6] Brazilian strains and the Rotarix™ strain.

		Antigenic residues in VP8* protein																							
		8-1										8-2		8-3									8-4		
		100	146	148	150	188	190	193	194	195	196	180	183	113	114	115	116	125	131	132	133	135	87	88	89
	RVA/USA/Rotarix-A41CB052A/1988/G1P[8]	**D**	**S**	**S**	**N**	**S**	**S**	**N**	**L**	**N**	**N**	**E**	**R**	**N**	**P**	**V**	**D**	**S**	**S**	**N**	**D**	**N**	**N**	**T**	**N**
	RVA/Human-tc/USA/Wa/1974/G1P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	RVA/Human-tc/GBR/ST3/1975/G4	.	.	V	S	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	.	T	N	Q
	RVA/Human-tc/AUS/RV3/1977/G3	.	.	V	A	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	.	T	N	Q
	RVA/Human-wt/BRA/NB233/1997/G2	.	G	V	A	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	.	T	N	Q
	RVA/Human-wt/BRA/HST368/1999/G9	.	.	V	A	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	.	T	N	Q
	RVA/Human-wt/BRA/HST369/1999/G2	.	.	V	A	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	.	T	N	Q
	RVA/Human-wt/BRA/HST435/2000/G9	.	.	V	A	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	.	T	N	Q
	RVA/Human-wt/BGD/RV161/2000/G12	.	.	V	A	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	.	T	N	Q
	RVA/Human-wt/BGD/RV176/2000/G12	.	.	V	A	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	.	T	N	Q
	RVA/Human-wt/KOR/CAU195/200X/G12	.	.	V	A	Y	.	.	.	S	E	.	H	T	N	Q	S	M	E	.	N	.	T	N	Q
	RVA/Human-wt/ECU/EC2184/200X/G11	.	.	V	A	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	.	T	N	Q
P6-I	RVA/Human-wt/BEL/B1711/2002/G6	.	G	V	G	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	.	T	N	Q
	RVA/Human-wt/BGD/Matlab13/2003/G12	.	.	V	A	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	.	T	N	Q
	RVA/Human-wt/BGD/MMC24/2005/G9	.	.	V	A	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	.	S	N	Q
	RVA/Human-wt/USA/06-242/2006/G2	.	.	V	A	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	S	T	N	Q
	RVA/Human-wt/ZAF/3176WC/2009/G12	.	N	V	A	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	.	T	N	Q
	RVA/Human-wt/BEL/F01498/2009/G3	.	.	V	A	Y	.	.	.	S	E	.	N	T	N	Q	S	T	E	.	N	.	T	N	Q
	RVA/Human-wt/BRA/BA17290/2009/G1	.	.	V	A	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	S	T	N	Q
	RVA/Human-wt/BFA/267-BF/2010/G1	.	.	V	A	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	S	T	N	Q
	RVA/Human-wt/BFA/225-BF/2010/G1	.	.	V	A	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	S	T	N	Q
	RVA/Human-wt/BFA/259-BF/2010/G1	.	.	G	A	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	S	T	N	Q
	RVA/Human-wt/BFA/281-BF/2010/G1G6	.	.	G	A	Y	.	.	.	S	E	.	H	T	N	Q	S	T	E	.	N	S	T	N	Q
P6-II	RVA/Pig-tc/USA/Gottfried/1983/G4	.	N	A	I	Y	T	.	.	P	D	.	H	P	S	Q	.	V	E	.	S	D	I	N	K
	RVA/Human-tc/USA/DS-1/1976/G2P[4]	.	G	.	G	.	N	D	.	.	.	.	.	S	Q	T	N	N	E	.	N	D	.	.	.

Supplementary material table 3. Alignment of the VP7 antigenic residues between Rotarix™ strain and rotavirus A strains detected in Brazil and worldwide. Strains are shown by name at the left side of the figure. Conserved amino acid residues are indicated by dots. Antigenic residues are divided in three epitopes, 7-1a, 7-1b, and 7-2. Numbers at the top denote amino acid positions. Shaded residues indicate amino acid that differ between G1P[6] Brazilian strains and the Rotarix™ strain.

		Antigenic residues in VP7 protein																														
		7-1a														7-1b						7-2										
		87	91	94	96	97	98	99	100	104	123	125	129	130	291	201	211	212	213	238	242	143	145	146	147	148	190	217	221	264		
	RVA/USA/Rotarix-A41CB052A/1988/G1P[8]	**T**	**T**	**N**	**G**	**E**	**W**	**K**	**D**	**Q**	**S**	**V**	**V**	**D**	**K**	**Q**	**N**	**V**	**D**	**N**	**T**	**K**	**D**	**Q**	**N**	**L**	**S**	**M**	**N**	**G**		
	RVA/Human-wt/BRA/IALR152/1998/G1P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	RVA/Human-wt/BRA/IALR215/1999/G1P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
G1-II	RVA/Human-wt/BRA/IALR297/2000/G1P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	RVA/Human-wt/BRA/IALR627/2003/G1P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	RVA/Human-wt/BRA/IALR784/2004/G1P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.		
	RVA/Human-wt/BRA/IALR1138/2005/G1P[8]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.		
G1-III	RVA/Human-tc/USA/Wa/1976/G1P[8]	.	.	.	.	D	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.		
	RVA/Human-wt/BRA/IALR131/1997/G1P[8]	.	.	S	.	.	.	.	.	.	N	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.		
	RVA/Human-wt/BRA/IALR342/2001/G1P[8]	I	.	S	.	.	.	.	.	.	N	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.		
	RVA/Human-wt/BGD/Matlab2/2002/G1P[8]	.	.	S	.	.	.	.	.	.	N	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.		
	RVA/Human-wt/AUS/CK00006/2004/G1P[8]	.	.	S	.	.	.	.	.	.	N	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.		
	RVA/Human-wt/BRA/IALR1202/2005/G1P[8]	.	.	S	.	.	.	.	.	.	N	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.		
	RVA/Human-wt/BEL/BE00006/2005/G1P[8]	.	.	S	.	.	.	.	.	.	N	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.		
	RVA/Human-wt/IND/634633/2006/G1P[8]	.	.	.	.	.	.	.	.	.	N	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.		
G1-I	RVA/Human-wt/BRA/IALR1393/2006/G1P[8]	.	.	S	.	.	.	.	.	.	N	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.		
	RVA/Human-wt/IND/6365/2006/G1P[8]	.	.	S	.	.	.	.	.	.	N	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.		
	RVA/Human-wt/USA/2007719739/2007/G1P[8]	.	.	S	.	.	.	.	.	.	N	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.		
	RVA/Human-wt/BEL/BE00039/2008/G1P[8]	.	.	S	.	.	.	.	.	.	N	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.		
	RVA/Human-wt/BRA/se16782-09/2009/G1P[8]	.	.	S	.	.	.	.	.	.	N	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.		
	RVA/Human-wt/BRA/pe17890-09/2009/G1P[8]	.	.	.	.	.	.	.	.	.	N	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	F	T	.	.		
	RVA/Human-wt/BRA/BA17290/2009/G1P[6]	.	.	S	.	.	.	.	.	.	N	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.		
	RVA/Human-wt/NCA/26J/2010/G1P[8]	.	.	S	.	.	.	.	.	.	N	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.		
G2	RVA/Human-tc/USA/DS-1/1976/G2P[4]	A	N	S	D	.	.	E	N	.	D	N	.	N	.	.	D	.	N	.	N	R	.	N	T	S	D	I	S	.		

CAPITULO IV

CONSTELAÇÃO GÊNICA DE RVA GENÓTIPO G12P[9] E G12P[8] DETECTADOS NO BRASIL (Artigo 4)

Artigo 5: Distinct evolutionary origins of G12P[8] and G12P[9] group A rotavirus strains circulating in Brazil. (Artigo aceito: 08/04/2014)

Este artigo esta associado aos objetivos 1 e 3.

MEEGID - Infection, Genetics and Evolution 6:19 AM (4 hours ago)

to me, michel.tibayre.

Ms. Ref. No.: MEEGID-D-14-00129R1
Title: Distinct evolutionary origins of G12P[8] and G12P[9] group A rotavirus strains circulating in Brazil.
Infection, Genetics and Evolution

Dear Ms. Gomez,

I am pleased to confirm that your paper "Distinct evolutionary origins of G12P[8] and G12P[9] group A rotavirus strains circulating in Brazil." has been accepted for publication in Infection, Genetics and Evolution.

When your paper is published on ScienceDirect, you want to make sure it gets the attention it deserves. To help you get your message across, Elsevier has developed a new, free service called AudioSlides: brief, webcast-style presentations that are shown (publicly available) next to your published article. This format gives you the opportunity to explain your research in your own words and attract interest. You will receive an invitation email to create an AudioSlides presentation shortly. For more information and examples, please visit http://www.elsevier.com/audioslides.

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With kind regards,

Daniel Cadar, PhD
Editor
Infection, Genetics and Evolution

Distinct evolutionary origins of G12P[8] and G12P[9] group A rotavirus strains circulating in Brazil.

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Running Head: Genetic characterization of Brazilian G12 RVA

Abstract. G12 group A rotavirus (RVA) are currently recognized as a globally emerging genotype and have been described in combination with several P-types. In Brazil, G12 RVA strains have been described in the Southern (2003) and Northern (2008 – 2010) regions, in combination with the P[9] and P[6] genotype, respectively. To date, few complete genomes of G12 RVA strains have been described (none from Brazilian strains), considering G12P[9] genotype just one strain, RVA/Human-tc/THA/T152/1998/G12P[9], has their 11 gene segments characterized. This study aims to determine the genomic constellation of G12P[9] and G12P[8] RVA strains detected in Brazil between 2006 and 2011. Therefore, the eleven gene segments of five Brazilian G12 RVA strains were amplified and sequenced, and the genotype of each gene segment was assigned using phylogenetic analysis. Complete genome analyses of G12 RVA strain circulating between 2006 and 2011 in Brazil revealed a conserved Wa-like genomic constellation for three G12P[8] RVA strains; whereas the two G12P[9] strains possessed distinct reassorted AU-1-like genomic constellations, closely related to the reference strain RVA/Human-tc/THA/T152/1998/G12P[9] in most genes. The results obtained in the current study suggest that G12P[9] (AU-1-like) and G12P[8] (Wa-like) strains detected in different regions of Brazil do not share a common origin. Moreover, while Brazilian G12P[8] RVA strains showed a complete Wa-like human constellation, both G12P[9] strains possessed an NSP1 gene of bovine origin (NSP1), and RVA/Human-wt/BRA/PE18974/2010/G12P[9] also possessed a VP3 gene of canine/feline origin.

Keywords: G12 rotavirus, genome constellation, reassortments.

1. Introduction

Group A rotavirus (RVA) genotype G12 was first detected in children with diarrhea in the Philippines in 1987 (Taniguchi *et al.*, 1990). Recently, this genotype has been described in several studies conducted worldwide, and their global spread was demonstrated in the last decade (Rahman *et al.*, 2007; Matthijnssens *et al.*, 2010a). In Brazil, G12 RVA strains have been described in the Southern (2003) and Northern (2008 – 2010) regions, in combination with the P[9] and P[6] genotypes, respectively (Pietruchinski *et al.*, 2006; Soares *et al.*, 2011). However, only information about the VP7 encoding gene of these Brazilian G12 strains is available in the GenBank database. Although data on the genetic diversity of the VP7 and/or VP4 genes are important from the point of view of host immunity against RVA disease, information on these genes is not enough to obtain conclusive data on the evolutionary dynamics of RVA strains. Therefore, it is important to perform whole genome analysis of RVA strains to better understand the genetic diversity of these viruses (Ghosh & Kobayashi, 2011).

This work reports the complete genotype constellation of two G12P[9] RVA strains detected in 2008 (RVA/Human-wt/BRA/PE15776/2008/G12P[9]) and 2010 (RVA/Human-wt/BRA/PE18974/2010/G12P[9]) in Pernambuco (PE) state (Northeastern Brazil), and three G12P[8] strains, one detected in Rio de Janeiro (RJ) (Southeastern Brazil) in 2006 (RVA/Human-wt/BRA/RJ12419/2006/G12P[8]) and two in Bahia (BA) (Northeastern Brazil) in 2011 (RVA/Human-wt/BRA/BA20142/2011/G12P[8] and RVA/Human-wt/BRA/BA20144/2011/G12P[8]).

2. Material and Methods

All strains were recovered from children hospitalized with acute gastroenteritis, and strains RVA/Human-wt/BRA/BA20142/2011/G12P[8] and RVA/Human-wt/BRA/PE18974/2010/G12P[9] were collected from children vaccinated with one and two doses of RV1 vaccine, respectively. Nucleic acids extraction and amplicon synthesis were performed using a QIAamp Viral RNA Mini Kit and OneStep RT-PCR Kit (QIAGEN®) following manufacturer's instructions and amplifications conditions as described by Matthijnssens *et al.* (2010b). PCR amplicons were purified using the QIAquick PCR purification kit (QIAGEN®) and sequenced with the ABI Prism BigDye terminator cycle sequencing reaction kit (Applied Biosystems®) on an ABI Prism 3130 automated sequencer (Applied Biosystems®) (Matthijnssens *et al.*, 2010b).

Phylogenetic analyses were performed using the neighbor-joining method (Kimura-two parameter model, 2,000 bootstrap replicates) in MEGA 5.0 (Tamura *et al.*, 2011). Other

methods as GTR: General Time Reversible plus Gamma (VP3-4, NSP1) and TVM: Transversion Model plus Gamma (VP7) were also used (data not shown). Sequences obtained in the current study were deposited in the GenBank database under the following accession numbers: KF907251 to KF907305.

This research was approved by the Fiocruz Ethical Research Committee (N^{o}311/06).

3. Results

G12P[9] strains clustered together with other G12P[9] strains detected worldwide inside lineage II for the VP7 gene (Fig. 1a), and showed up to 99% similarity with strains detected in South America and Eastern/Southeastern Asia. G12 strains in combination with P[8] grouped in a separate cluster together with the majority of the G12P[8] and G12P[6] strains described worldwide (99% similarity) inside lineage III (Fig. 1a).

Phylogenetic analysis based on VP4 gene revealed that G12P[9] Brazilian strains clustered inside lineage II and were closely related to RVA/Human-tc/THA/T152/1998/G12P[9] strain (99% similarity) (Fig. 1b). Brazilian G12P[8] strains clustered inside lineage P[8]-III. Strain detected in 2006 was closely related to contemporary G1P[8] and G12P[8] strains detected worldwide (2002-2008), whereas the two strains detected in 2011 were closely related to strains detected in recent years (2009-2010) in the USA, Australia, and Nicaragua (99% similarity) (Fig. 1b).

G12P[9] and G12P[8] Brazilian strains revealed a distinct genomic constellation: AU-1-like and Wa-like, respectively (Table 1). Phylogenetic analyses of the NSP2-5, VP1, VP2, and VP6 encoding genes of the G12P[9] Brazilian strains showed a very close genetic relationship with RVA/Human-tc/THA/T152/1998/G12P[9] (data not shown). For the VP3 encoding gene, RVA/Human-wt/BRA/PE18974/2010/G12P[9] strain was closely related to canine/feline P[9] strains, or human strains that are believed to be of canine/feline origin (Fig. 2a). The NSP1 gene of Brazilian G12P[9] strains belonged to A3 genotype clustering with several bovine strains, or strains that are believed to be of bovine origin (Fig. 2b).

Brazilian strain RVA/Human-wt/BRA/RJ12419/2006/G12P[8] clustered together with G1P[8] strains detected in USA (2005), while RVA/Human-wt/BRA/BA20142/2011/G12P[8] and RVA/Human-wt/BRA/BA20144/2011/G12P[8] were nearly identical and clustered with G12P[8] strains detected in Thailand (2009) for most of the RVA genes (data not shown).

4. Discussion

G12 RVA strains have been found in combination with each of the three major human genotype constellations: Wa-like, DS-1-like, and AU-1-like (Rahman *et al.*, 2007; Heiman *et*

al., 2008; Ghosh *et al.*, 2010; Jere *et al.*, 2011; Stupka *et al.*, 2012). However, the majority of G12 strains possess the Wa-like genotype constellation (Freeman *et al.*, 2009; Matthijnssens & Van Ranst, 2012), probably because they were found in combination with the P[8] genotype.

In the current study G12P[8] Brazilian strains analyzed revealed a human Wa-like genomic constellation, whereas the G12P[9] revealed a reassorted AU-1-like genomic constellation. While all G12P[9] strains previously described worldwide possessed a rare A12 genotype (Castello *et al.*, 2009), the NSP1 gene of Brazilian G12P[9] strains belonged to the A3 genotype. It has been suggested that G12P[9] strains previously detected in Argentina, Brazil, and Paraguay originated from Eastern Asia (Castello *et al.*, 2009). In this study all genes, except for NSP1 and VP3 (RVA/Human-wt/BRA/PE18974/2010/G12P[9]), were shown to possess a close genetic relationship with strains detected in Eastern and Southeastern Asia. However, it is important to notice that just few G12P[9] strains have been detected and characterized to date.

At the time Brazil introduced the RV1 vaccine in the National Immunization Program (March 2006), G12 specific vaccine effectiveness could not be demonstrated because of the absence of detection of G12 previous to vaccine introduction. Since RV1 possess a human Wa-like genomic constellation, and G12P[9] strains revealed an AU-1-like genomic constellation, a difference in vaccine efficacy would be expected.

5. Conclusion

The results obtained in the current study suggest that G12P[9] and G12P[8] strains detected in different regions of Brazil do not share a common origin. Moreover, while Brazilian G12P[8] RVA strains showed a complete Wa-like human constellation, both G12P[9] strains possessed a NSP1 gene of bovine origin, and RVA/Human-wt/BRA/PE18974/2010/G12P[9] possessed a VP3 gene of canine/feline origin. It is important to mention that the AU-l-like genotype constellation, sporadically found in humans, is believed to have a close evolutionary relationship with canine/feline RVAs (Matthijnssens *et al.*, 2008).

A more in depth analysis of G12 RVA strains detected worldwide, involving full genomic and antigenic characterization, will help to identify potential mutations and/or reassorted genes that might be correlated with the capacity of RVA strains in evade the immunity caused by RVA vaccines (Gentsch *et al.*, 2009). Availability of such data will be crucial to analyze the efficacy of RVA vaccines.

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Table 1. Genotype constellation comparison. Brazilian strains are shown in bold. Green, red and orange indicate Wa-like, DS-1-like and AU-1-like gene segments, respectively, and blue is used to indicate a gene segment of bovine origin. Gray is used to indicate canine/feline gene segment VP3 found in RVA/Human-wt/BRA/PE18974/2010/G12P[9] strain.

	Strain	Origin	Genotypes									
			VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
Wa-like	RVA/USA/Rotarix-A41CB052A/1988/G1P1A[8]	Human	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-tc/USA/Wa/1974/G1P1A[8]	Human	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/BRA/RJ12419/2006/G12P[8]	Human	G12	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/BRA/BA20142/2011/G12P[8]	Human	G12	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/BRA/BA20144/2011/G12P[8]	Human	G12	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/ZAF/3176WC/2009/G12P[6]	Human	G12	P[6]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/BGD/Matlab13/2003/G12P[6]	Human	G12	P[6]	I1	R1	C1	M1	A1	N1	T2	E1	H1
	RVA/Human-wt/ARG/6653/2008/G12P[8]	Human	G12	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Human-wt/ZAF/3133WC/2009/G12P[4]	Human	G12	P[4]	I1	R1	C1	M1	A1	N1	T1	E1	H1
	RVA/Porcine-wt/IND/RU172/2002/G12P[7]	Porcine	G12	P[7]	I5	R1	C1	M1	A1	N1	T1	E1	H1
DS-1-like	RVA/Human-tc/USA/DS-1/1976/G2P1B[4]	Human	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
	RVA/Human-wt/BGD/N26/2002/G12P[6]	Human	G12	P[6]	I2	R2	C2	M2	A2	N1	T2	E6	H2
	RVA/Human-wt/BGD/RV161/2000/G12P[6]	Human	G12	P[6]	I2	R2	C2	M2	A2	N2	T2	E1	H2
	RVA/Human-wt/BGD/RV176/2000/G12P[6]	Human	G12	P[6]	I2	R2	C2	M2	A2	N2	T2	E6	H2
	RVA/Human-tc/PHL/L26/1987/G12P[4]	Human	G12	P[4]	I2	R2	C2	M1/M2	A2	N1	T2	E2	H1
AU-1-like	RVA/Human-tc/JPN/AU-1/1982/G3P[9]	Human	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
	RVA/Human-wt/BRA/PE15776/2008/G12P[9]	Human	G12	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H6
	RVA/Human-wt/BRA/PE18974/2010/G12P[9]	Human	G12	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H6
	RVA/Human-tc/THA/T152/1998/G12P[9]	Human	G12	P[9]	I3	R3	C3	M3	A12	N3	T3	E3	H6
	RVA/Human-wt/ARG/Arg720/1999/G12P[9]	Human	G12	P[9]					A12				
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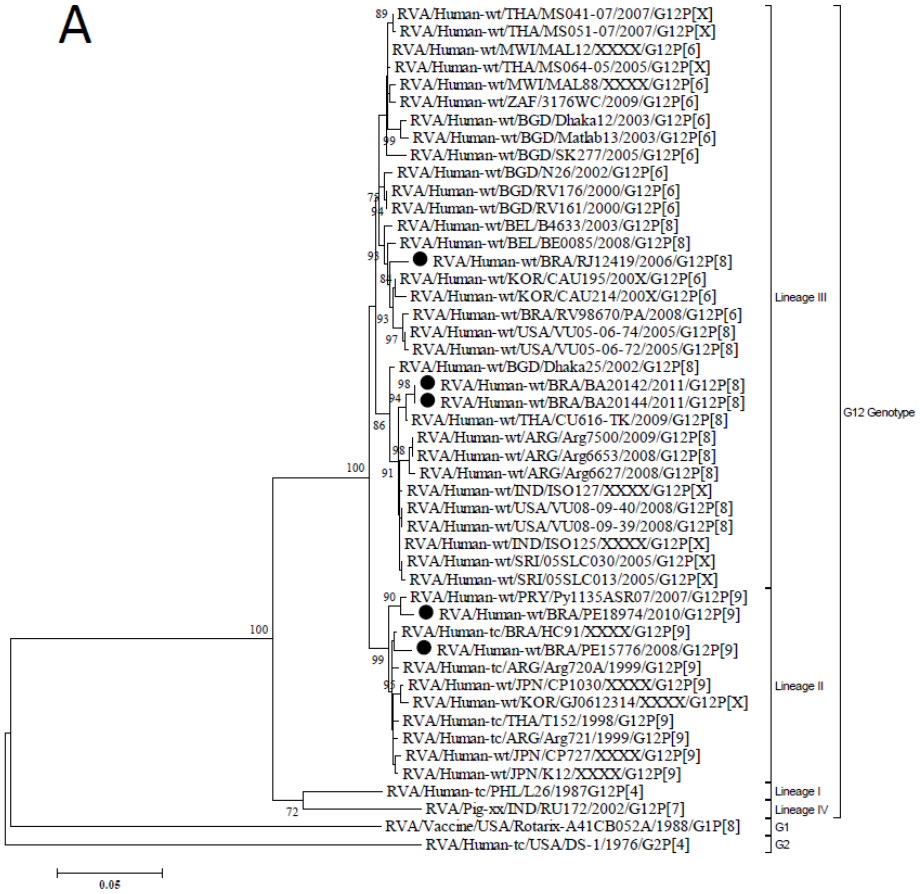
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Lineage II
Lineage I
Lineage IV
G1
G2
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P[8]-I
Lineage II
Lineage I
P[9] Genotype
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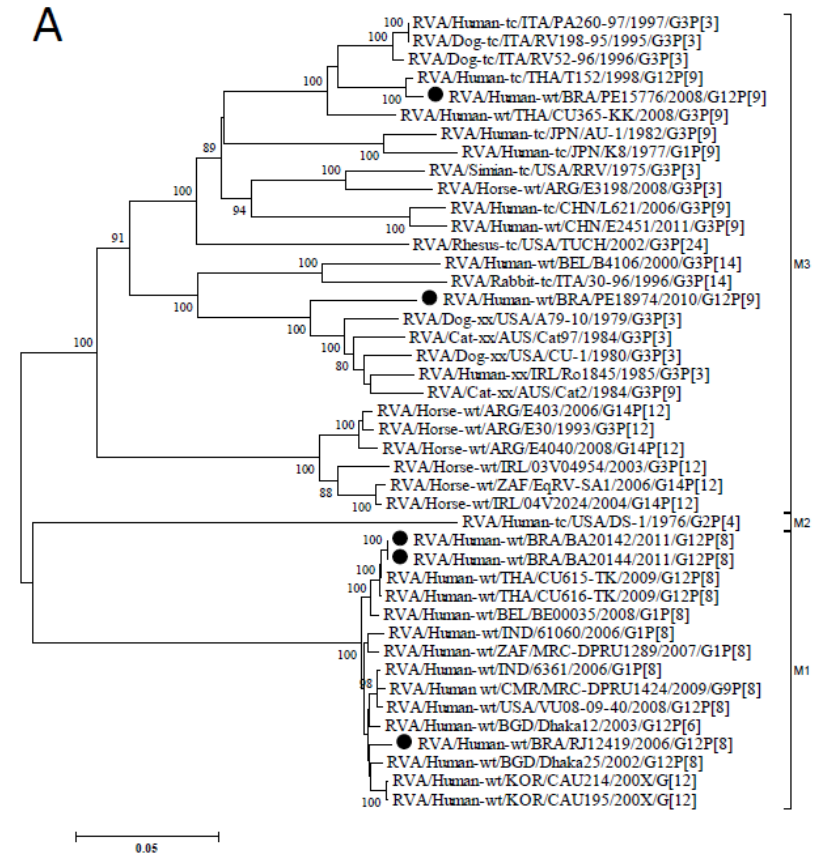
Figure 1. Phylogenetic analysis based on the VP7 (A) and VP4 (B) gene nucleotide sequences of Brazilian G12 strains, and sequences from the GenBank database. Numbers at the nodes indicate bootstrap values; only values above 70% are shown. The scale bar at the bottom represents 0.05 substitutions per nucleotide position (nt.subst./site). Brazilian G12 strains are marked with a filled circle.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M1
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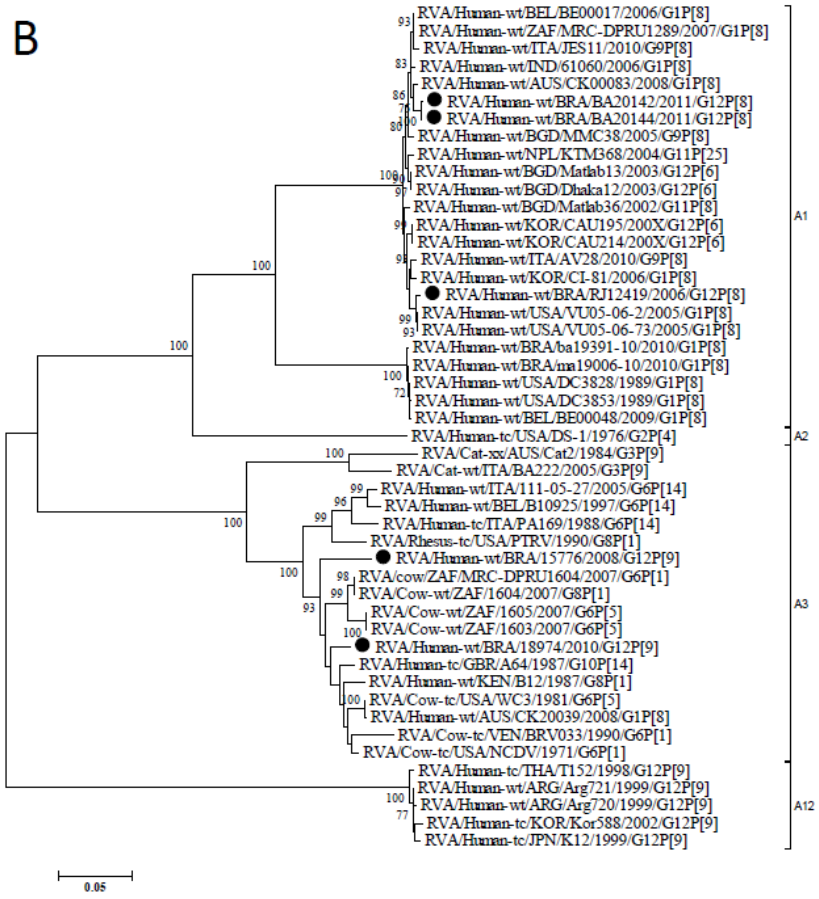
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RVA/Human-tc/USA/DS-1/1976/G2P[4]
RVA/Cat-xx/AUS/Cat2/1984/G3P[9]
RVA/Cat-wt/ITA/BA222/2005/G3P[9]
RVA/Human-wt/ITA/111-05-27/2005/G6P[14]
RVA/Human-wt/BEL/B10925/1997/G6P[14]
RVA/Human-tc/ITA/PA169/1988/G6P[14]
RVA/Rhesus-tc/USA/PTRV/1990/G8P[1]
RVA/Human-wt/BRA/15776/2008/G12P[9]
RVA/cow/ZAF/MRC-DPRU1604/2007/G6P[1]
RVA/Cow-wt/ZAF/1604/2007/G8P[1]
RVA/Cow-wt/ZAF/1605/2007/G6P[5]
RVA/Cow-wt/ZAF/1603/2007/G6P[5]
RVA/Human-wt/BRA/18974/2010/G12P[9]
RVA/Human-tc/GBR/A64/1987/G10P[14]
RVA/Human-wt/KEN/B12/1987/G8P[1]
RVA/Cow-tc/USA/WC3/1981/G6P[5]
RVA/Human-wt/AUS/CK20039/2008/G1P[8]
RVA/Cow-tc/VEN/BRV033/1990/G6P[1]
RVA/Cow-tc/USA/NCDV/1971/G6P[1]
RVA/Human-tc/THA/T152/1998/G12P[9]
RVA/Human-wt/ARG/Arg721/1999/G12P[9]
RVA/Human-wt/ARG/Arg720/1999/G12P[9]
RVA/Human-tc/KOR/Kor588/2002/G12P[9]
RVA/Human-tc/JPN/K12/1999/G12P[9]
A1
A2
A3
A12
0.05

Figure 2. Phylogenetic analysis based on the VP3 (A) and NSP1 (B) gene nucleotide sequences of Brazilian G12 strains, and sequences from the GenBank database. Numbers at the nodes indicate bootstrap values; only values above 70% are shown. The scale bar at the bottom represents 0.05 substitutions per nucleotide position (nt.subst./site). Brazilian G12 strains are marked with a filled circle.

CAPITULO V

ANALISE DO VIÉIS NO USO DE CÓDONS E RESTRIÇÕES NA COMPOSIÇÃO DOS GENES QUE CODIFICAM PARA AS PROTEÍNAS VP8* E VP7 (Artigo 5)

Artigo 5: Analysis of human P[4]G2 rotavirus strains isolated in Brazil reveals codon usage bias and strong compositional constraints (Artigo publicado)

Este artigo esta relacionado ao objetivo 4.

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Analysis of human P[4]G2 rotavirus strains isolated in Brazil reveals codon usage bias and strong compositional constraints

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ABSTRACT

The *Rotavirus* genus belongs to the family *Reoviridae* and its genome consist of 11 segments of double-stranded RNA. Group A rotaviruses (RV-A) are the main etiological agent of acute viral gastroenteritis in infants and young children worldwide. Understanding the extent and causes of biases in codon usage is essential to the understanding of viral evolution. However, the factors shaping synonymous codon usage bias and nucleotide composition in human RV-A are currently unknown. In order to gain insight into these matters, we analyzed the codon usage and base composition constraints on the two genes that codify the two outer capsid proteins (VP4 [VP8*] and VP7) of 58 P[4]G2 RV-A strains isolated in Brazil and investigated the possible key evolutionary determinants of codon usage bias. The results of these studies revealed that the frequencies of codon usage in both RV-A proteins studied are significantly different than the ones used by human cells. In order to observe if similar trends of codon usage are found when RV-A complete genomes are considered, we compare these results with results found using a dataset of 10 reference strains for whom the complete codes of the 11 segments are known. Similar results were obtained using capsid proteins or complete genomes. The general correlations found between the position of each sequence on the first axis generated by correspondence analysis and the relative dinucleotide abundances indicate that codon usage in RV-A can also be strongly influenced by underlying biases in dinucleotide frequencies. CpG and GpC containing codons are markedly suppressed. Thus, the results of this study suggest that RV-A genomic biases are the result of the evolution of genome composition in relation to host adaptation and the ability to escape antiviral cell responses.

1. Introduction

Group A rotaviruses (RV-A) are the main etiological agent of acute viral gastroenteritis in infants and young children worldwide (Aoki et al., 2009; CDC, 2008). The *Rotavirus* genus belongs to the family *Reoviridae* and its genome consist of 11 double-stranded RNA (dsRNA) gene segments encoding six structural (VP) and six non-structural proteins (NSP) (Estes and Kapikian, 2007). Based on the two genes that codify the outer neutralizing capsid proteins, VP4 and VP7, a widely used binary classification system was established for RV-A that defined G (from VP7, glycoprotein) and P (from VP4, protease-cleaved protein) genotypes (Estes and Kapikian, 2007). To date, at least 25 G and 32 P genotypes have been identified (Matthijnssens et al., 2009, 2008; Collins et al., 2010; Abe et al., 2009; Ursu et al., 2009; Esona et al., 2010). Five RV-A G genotypes (G1–G4 and G9) and two P genotypes (P[8] and P[4]) are prevalent worldwide (Santos and Hoshino, 2005; Leite et al., 2008; Iturriza-Gómara et al., 2009). Different surveillance studies with RV-A-positive samples have shown that genotype P[4]G2 reemerges in Brazil in 2005, and since then has become predominant in this country (Carvalho-Costa et al., 2006; Gurgel et al., 2007; de Oliveira et al., 2008; Leite et al., 2008; Nakagomi et al., 2008; Mascarenhas et al., 2010).

Due to the degeneracy of the genetic code, most amino acids are coded by more than one codon. Synonymous codons are not used randomly, and in several organisms natural selection seems to bias codon usage toward a certain subset of optimal codons, mainly in highly expressed genes (Stoletzki and Eyre-Walker, 2007).

Two major models have been proposed to explain codon usage, the translation related model and the mutational model (Wong et al., 2010). Translational efficiency or translational accuracy bias may be due to the relationship between local tRNA abundance and

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major codon preference, wherein a particular codon of an amino acid family pairs most optimally with the most abundant tRNA (Ikemura, 1982). The discrepancies of codon usage could also be due to genome compositional constraints and mutational biases (Sharp et al., 1986).

Understanding the extent and causes of biases in codon usage is essential to comprehend the interplay between viruses and the immune response (Shackelton et al., 2006). However, the factors shaping synonymous codon usage bias, like mutational pressure, nucleotide composition or translational selection are currently unknown for human RV-A.

In order to gain insight into these matters, we analyzed the codon usage and base composition constraints of VP4 [VP8*] and VP7 gene sequences of 72 P[4]G2 RV-A strains isolated in Brazil and investigated the possible key evolutionary determinants of codon usage bias. In order to observe if similar trends of codon usage are found when RV-A complete genomes are considered, we compared these results with the ones found using a dataset of reference strains from which the complete sequences of the 11 segments are known. The results of these studies revealed a significant codon usage bias and compositional constraints in the human RV-A strains studied.

2. Materials and methods

2.1. Fecal samples, viral RNA extraction and PCR amplification

A total of 72 diarrheic stool specimens were collected from 1996 to 2009 from children up to 5 years old hospitalized with acute diarrhea. These samples were obtained from children from the States of Acre (AC), Alagoas (AL), Bahia (BA), Espirito Santo (ES), Maranhão (MA), Mato Grosso do Sul (MS), Minas Gerais (MG), Pernambuco (PE), Rio de Janeiro (RJ), Rio Grande do Sul (RS) and Sergipe (SE), and were genotyped as P[4]G2 as previously described (Fischer et al., 2000; Das et al., 1994). The viral dsRNA was extracted by the glass powder method (Boom et al., 1990). The dsRNA was reverse transcribed (RT) and amplified by polymerase chain reaction (PCR) using a pair of consensus primers corresponding to a conserved nucleotide sequence of the VP7 (Gouvea et al., 1990; Das et al., 1994) or VP4 (VP8*) (Gentsch et al., 1992; Gómez et al., 2010) genes. Temperature and time conditions for PCR amplifications were performed as originally described (Gouvea et al., 1990; Gentsch et al., 1992). Distilled Milli-Q water was used as a negative control in all steps, and recommended manipulations for PCR procedures were carried out as a precaution to avoid false-positive results.

2.2. Sequencing

DNA sequencing was performed with an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit® and an ABI Prism 3730 Genetic Analyzer (both from Applied Biosystems, Foster City, CA, USA). Sequences of the VP4 [VP8]* and VP7 genes were obtained by using the same set of primers utilized in the RT-PCR. For strain names and accession numbers, see Supplementary Material, Table 1. From the initial 72 stool samples, a total of 58 VP4 [VP8]* and 60 VP7 sequences, 818 and 978 nucleotides in-length, respectively, were obtained.

2.3. Codon usage analyses

The relative synonymous codon usage (RSCU) values of each codon in each gene (VP8* or VP7) were determined in order to measure the synonymous codon usage bias (Sharp and Li, 1986). This was done using the CodonW program (available at: http://mobyle.pasteur.fr). The RSCU of P[4]G2 RV-A VP8* and VP7 genes were compared with corresponding values of human cells (International Human Genome Sequencing Consortium, 2001). The effective number of codons (ENC) and the frequency of use of G+C at synonymous variable third positions of codons (GC_3S) (excluding Met, Trp, and termination codons) were also calculated with CodonW. ENC was used to quantify the codon usage bias of an ORF (Wrigth, 1990; Comeron and Aguade, 1998). Similarly, the fraction of the G+C nucleotides not involved in the GC_3S fraction (GC_{12}) was also calculated. All these indices were also calculated using CodonW. Dinucleotides relative frequencies were also calculated using this program as implemented in the Mobyle server (http://mobyle.pasteur.fr).

2.4. Correspondence analysis (COA)

The relationship between variables and samples can be obtained using multivariate statistical analysis. COA is a type of multivariate analysis that allows a geometrical representation of the sets of rows and columns in a dataset (Wong et al., 2010; Greenacre, 1984). Each ORF is represented as a 59-dimensional vector and each dimension correspond to the RSCU value of one codon (excluding AUG, UGG and stop codons). Major trends within a dataset can be determined using measures of relative inertia and genes ordered according to their position along the axis of major inertia (Tao et al., 2009). COA was performed on the RSCU values of the ORFs studied using the CodonW program.

2.5. Statistical analysis

Correlation analysis was carried out using Spearman's rank correlation analysis method (Wessa, 2010; available at: www.wessa.net).

2.6. Sequence alignment

Sequences were aligned using the MUSCLE program (Edgar, 2004).

2.7. Comparative analysis

In order to observe if the codon usage bias found in the outer capsid proteins of P[4]G2 RV-A strains isolated in Brazil, can also be found in other genome regions or considering complete genome codes of human RV-A strains of different genotypes and isolated elsewhere, a new dataset composed of 10 human RV-A reference strains for whom the complete codes of the 11 genome segments are known was constructed. For strain names, genotypes, accession numbers and genomic constellations see Supplementary Material Table 3.

3. Results

In order to study the extent of codon usage bias in P[4]G2 RV-A isolated in Brazil, the RSCU values of the codons in VP4 [VP8*] and VP7 ORFs were calculated, and the figures obtained for these genes, comprising a dataset of 58 and 60 sequences, respectively, are shown in Table 1.

Interestingly, the frequencies of codon usage in both VP4 [VP8*] and VP7 P[4]G2 RV-A ORFs are significantly different in relation to human cells. Particularly, extremely high biased frequencies were found for UUU (Phe), UUA (Leu), GUU and GUA (Val), UCA (Ser), CCA (Pro), GCU (Ala), UAU (Tyr), CAU (His), CAA (Gln), AAU (Asn), AAA (Lys), GAA (Glu), UGU (Cys), AGA (Arg) and GGA (Gly) in both ORFs (see Table 1). As can be seen, highly preferred codons are all U/A ending, which strongly suggests that mutational bias is the

Table 1
Codon usage in P[4]G2 RV-A strains, displayed as RSCU values.

AA	Cod	HC	VP4	VP7	AA	Cod	HC	VP4	VP7	AA	Cod	HC	VP4	VP7	AA	Cod	HC	VP4	VP7
Phe	**UUU**	**0.92**	**1.80**	**1.86**	Ser	UCU	1.14	1.07	1.00	Tyr	**UAU**	**0.88**	**1.96**	**1.50**	**Cys**	**UGU**	**0.92**	**1.97**	**1.24**
	UUC	1.08	0.20	0.14		UCC	1.32	0.40	0.27		UAC	1.12	0.04	0.50		UGC	1.08	0.03	0.76
Leu	**UUA**	**0.48**	**2.72**	**2.33**		**UCA**	**0.90**	**1.96**	**3.56**	TER	UAA	**	**	**	TER	UGA	**	**	**
	UUG	0.78	0.46	0.94		UCG	0.30	0.55	0.59		UAG	**	**	**	Trp	UGG	1.00	1.00	1.00
	CUU	0.78	1.14	0.39	Pro	CCU	1.16	0.31	0.36	His	**CAU**	**0.84**	**1.65**	**2.00**	Arg	CGU	0.48	0.04	0.64
	CUC	1.20	0.44	0.37		CCC	1.28	0.29	0.00		CAC	1.16	0.35	0.00		*CGC*	*1.08*	*0.00*	*0.00*
	CUA	**0.42**	**0.89**	**1.27**		**CCA**	**1.12**	**3.38**	**2.91**	Gln	**CAA**	**0.54**	**1.42**	**1.81**		CGA	0.66	0.02	1.78
	CUG	2.40	0.35	0.70		CCG	0.44	0.01	0.72		CAG	1.46	0.58	0.19		CGG	1.20	0.41	0.64
Ile	AUU	1.08	1.79	1.45	Thr	**ACU**	**1.00**	**1.98**	**1.51**	Asn	**AAU**	**0.94**	**1.85**	**1.77**	Ser	AGU	0.90	1.99	0.33
	AUC	1.41	0.31	0.25		ACC	1.44	0.18	0.25		AAC	1.06	0.15	0.23		AGC	1.44	0.03	0.26
	AUA	0.51	0.90	1.30		ACA	1.12	1.47	1.42	Lys	**AAA**	**0.86**	**1.65**	**1.90**	Arg	**AGA**	**1.26**	**4.70**	**2.59**
Met	AUG	1.00	1.00	1.00		ACG	0.44	0.37	0.82		AAG	1.14	0.35	0.10		AGG	1.26	0.84	0.35
Val	**GUU**	**0.72**	**1.31**	**1.97**	Ala	**GCU**	**1.08**	**1.74**	**2.49**	Asp	GAU	0.92	1.57	1.22	Gly	GGU	0.64	2.03	0.98
	GUC	0.96	0.75	0.01		GCC	1.60	0.02	0.13		GAC	1.08	0.43	0.78		GGC	1.36	0.26	0.24
	GUA	**0.48**	**1.65**	**1.57**		GCA	0.92	1.74	1.11	Glu	**GAA**	**0.84**	**1.41**	**1.57**		**GGA**	**1.00**	**1.66**	**2.51**
	GUG	1.84	0.28	0.45		GCG	0.44	0.50	0.28		GAG	1.16	0.59	0.43		GGG	1.00	0.05	0.26

RSCU, relative synonymous codon usage; AA, amino acid; Cod, codons; HC, human cells; TER, termination codon. More frequent codons in both VP4 [VP8*] and VP7 with respect to human cells are shown in bold. Codon CGC (Arg) not used in VP4 [VP8*] and VP7 P[4]G2 RV-A isolated in Brazil are shown in italics.

main force shaping codon usage in these two genes. It is interesting to note that CGC (Arg) is not used in both ORFs.

In order to investigate if these P[4]G2 RV-A strain sequences display similar composition features, the ENC values were calculated for VP8* and VP7 ORFs. These values range from 35.21 to 40.49 for VP8* and from 38.97 to 41.88 for VP7 (mean ENCs values are 37.36 and 40.56 for VP4 [VP8*] and VP7, respectively). For results obtained for Brazilian strains enrolled in these studies, see Supplementary Material Table 2. Due to the fact that almost all ENC values are <40, the results obtained for the two ORFs studied reveal that codon usage in P[4]G2 RV-A is biased.

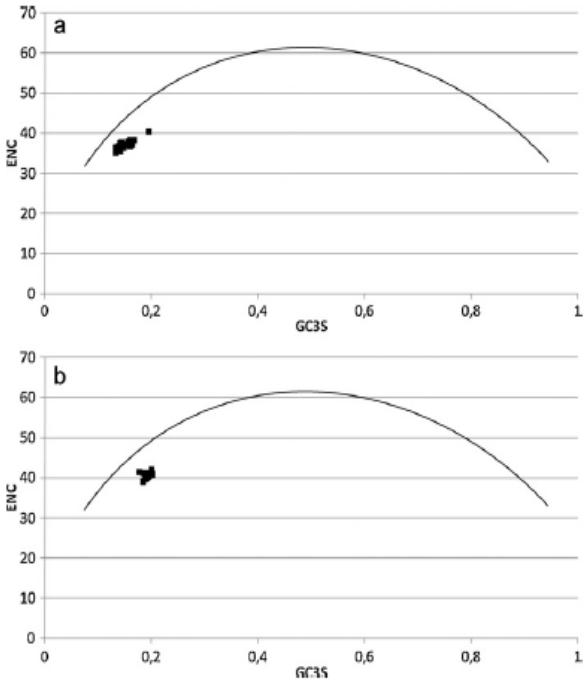

Fig. 1. Effective number of codons used in each ORF plotted against the GC_3S. The curve plots the relationship between GC_3S and ENC in absence of selection. Black square dots show the results obtained for RV-A strains. All of them lie below the expected curve. The results found for VP4 and VP7 are shown in (A) and (B), respectively.

An ENC–GC_3S plot (ENC plotted against GC_3S) can be used as a method that quantifies how far the codon usage of a gene departs from equal usage of synonymous codons (Wrigth, 1990). As shown in Fig. 1, the dotted continuous line in the plot represents a curve if codon usage is only determined by GC content at the third codon position. In other words, if GC_3S is the only determinant factor shaping the codon usage pattern, the values of ENC would fall on a continuous curve, which represents random codon usage (Jiang et al., 2007). If G+C compositional constraint influences the codon usage, then the GC_3S and ENC correlated spots would lie on or bellow the expected curve (Tsai et al., 2007). Otherwise, the codon usage bias of genes may be affected by other factors such as translational selection.

When the GC_3S values were calculated for VP4 [VP8*] and VP7 ORFs and the ENC–GC_3S plots constructed (for ENC and GC_3S values obtained for Brazilian strains enrolled in these studies, see Supplementary Material Table 2), all spots lie below and "parallel" in relation to the expected curve for both ORFs studied, indicating that the codon usage bias may be influenced by the G+C compositional constraints (see Fig. 1).

Since codon usage by its very nature is multivariate, it is necessary to analyze the data using multivariate statistical techniques (i.e. COA) in order to confirm these findings. COA is an ordination technique that identifies the major trends in the variation of the data and distributes genes along continuous axes in accordance with these trends. Moreover, it has the advantage that it does not assume that the data falls into discrete clusters and therefore can represent continuous variation accurately (Greenacre, 1984). COA creates a series of orthogonal axes to identify trends that explain the data variation, with each subsequent axis explaining a decreasing amount of the variation (Greenacre, 1984). The correlation between the position on the first axis generated by COA for each gene and the respective GC_3S values of each strain was analyzed for both VP4 [VP8*] and VP7 ORFs studied. We have found that the position of the sequences on the first axis from COA are highly correlated with the GC_3S values in both VP4 [VP8*] and VP7 ORFs ($r = 0.625$, $P < 0.0001$ and $r = -0.469$, $P < 0.001$ for VP4 [VP8] and VP7, respectively). Taking altogether, these results reveal that most of the codon usage bias is directly related to the nucleotide composition. Nevertheless, other factors may be also acting in shaping codon usage bias.

In order to analyze if the codon usage biases reported above can also be found using other genome regions or considering complete genome sequences, a new dataset was constructed composed of 10

Table 2
Codon usage in RV-A strains of different genotypes, expressed by RSCU values.

AA	Cod	HC	OC	IM	IC	NSP	Full	AA	Cod	HC	OC	IM	IC	NSP	Full
Phe	**UUU**	**0.92**	**1.53**	**1.58**	**1.52**	**1.52**	**1.51**	Ser	UCU	1.14	1.30	1.02	1.13	1.66	1.30
	UUC	1.08	0.47	0.42	0.48	0.48	0.49		UCC	1.32	0.26	0.43	0.29	0.28	0.28
Leu	**UUA**	**0.48**	**2.74**	**1.41**	**2.59**	**1.89**	**2.33**		**UCA**	**0.90**	**2.84**	**3.07**	**3.26**	**2.29**	**2.93**
	UUG	0.78	0.99	1.25	1.14	1.32	1.17		UCG	0.30	0.54	0.55	0.62	0.54	0.57
	CUU	0.78	0.47	1.37	0.60	0.92	0.70	Pro	CCU	1.16	0.61	0.26	0.43	0.90	0.54
	CUC	1.20	0.29	0.32	0.18	0.39	0.25		CCC	1.28	0.27	0.02	0.12	0.20	0.15
	CUA	**0.42**	**1.09**	**1.18**	**1.04**	**1.03**	**1.07**		**CCA**	**1.12**	**2.68**	**3.34**	**2.85**	**2.55**	**2.81**
	CUG	2.40	0.42	0.48	0.46	0.45	0.48		CCG	0.44	0.44	0.38	0.60	0.35	0.51
Ile	AUU	1.08	1.13	1.80	1.02	1.80	1.24	Thr	**ACU**	**1.00**	**1.14**	**1.35**	**1.35**	**1.64**	**1.35**
	AUC	1.41	0.22	0.30	0.23	0.27	0.24		ACC	1.44	0.24	0.12	0.24	0.24	0.23
	AUA	0.51	1.65	0.90	1.75	0.93	1.51		ACA	1.12	1.73	2.11	1.63	1.52	1.66
Met	AUG	1.00	1.00	1.00	1.00	1.00	1.00		ACG	0.44	0.89	0.43	0.78	0.61	0.76
Val	**GUU**	**0.72**	**0.95**	**1.09**	**1.36**	**1.67**	**1.33**	Ala	**GCU**	**1.08**	**1.25**	**1.57**	**1.44**	**1.34**	**1.40**
	GUC	0.96	0.33	0.71	0.38	0.32	0.37		GCC	1.60	0.26	0.32	0.33	0.17	0.28
	GUA	**0.48**	**1.77**	**1.46**	**1.46**	**1.11**	**1.43**		GCA	0.92	1.76	1.36	1.62	2.03	1.69
	GUG	1.84	0.94	0.74	0.80	0.90	0.86		GCG	0.44	0.73	0.75	0.61	0.45	0.63
Tyr	**UAU**	**0.88**	**1.46**	**1.13**	**1.47**	**1.33**	**1.43**	**Cys**	**UGU**	**0.92**	**1.35**	**1.80**	**1.39**	**1.17**	**1.31**
	UAC	1.12	0.54	0.87	0.53	0.67	0.57		UGC	1.08	0.65	0.20	0.61	0.83	0.69
TER	UAA	**	**	**	**	**	**	TER	UGA	**	**	**	**	**	**
	UAG	**	**	**	**	**	**	Trp	UGG	1.00	1.00	1.00	1.00	1.00	1.00
His	**CAU**	**0.84**	**1.69**	**1.80**	**1.66**	**1.48**	**1.62**	Arg	CGU	0.48	0.12	0.46	0.60	0.74	0.53
	CAC	1.16	0.31	0.20	0.34	0.52	0.38		*CGC*	*1.08*	*0.22*	*0.12*	*0.23*	*0.06*	*0.18*
Gln	**CAA**	**0.54**	**1.20**	**1.28**	**1.28**	**1.38**	**1.29**		CGA	0.66	0.87	0.31	0.50	0.59	0.56
	CAG	1.46	0.80	0.72	0.72	0.62	0.71		*CGG*	*1.20*	*0.29*	*0.00*	*0.15*	*0.18*	*0.18*
Asn	**AAU**	**0.94**	**1.53**	**1.38**	**1.52**	**1.56**	**1.51**	Ser	AGU	0.90	0.85	0.55	0.58	0.92	0.71
	AAC	1.06	0.47	0.62	0.48	0.44	0.49		AGC	1.44	0.22	0.39	0.11	0.31	0.21
Lys	**AAA**	**0.86**	**1.52**	**1.66**	**1.49**	**1.48**	**1.49**	Arg	**AGA**	**1.26**	**3.75**	**4.98**	**3.86**	**3.50**	**3.85**
	AAG	1.14	0.48	0.34	0.51	0.52	0.57		AGG	1.26	0.75	0.12	0.67	0.92	0.70
Asp	GAU	0.92	1.38	1.54	1.43	1.63	1.47	Gly	GGU	0.64	1.44	1.03	1.31	1.45	1.34
	GAC	1.08	0.62	0.46	0.57	0.37	0.53		GGC	1.36	0.34	0.34	0.26	0.20	0.28
Glu	**GAA**	**0.84**	**1.30**	**1.46**	**1.46**	**1.47**	**1.43**		**GGA**	**1.00**	**1.90**	**2.40**	**2.02**	**2.04**	**2.01**
	GAG	1.16	0.70	0.54	0.54	0.53	0.57		GGG	1.00	0.32	0.23	0.41	0.31	0.37

RSCU, relative synonymous codon usage; AA, amino acid; Cod, codons; HC, human cells; OC, outer capsid shell proteins; IM, intermediate protein shell; IC, inner capsid shell proteins; NSP, non-structural proteins; Full, full genome; TER, termination codon. More frequent codons with respect to human cells found in all genome regions studied are shown in bold. Frequencies sharply reduced with respect to frequencies found in human cells are shown in italics.

human RV-A reference strains, for which the complete genomes of the 11 segments are known. For strains names, genotypes, accession numbers and genomic constellations, see Supplementary Material Table 3.

By concatenation of different genome ORF's sequences, the RSCU values of the different codons were calculated for different virus regions (outer capsid shell proteins, OC, VP4+VP7; intermediate protein shell, IM, VP6; inner capid shell proteins, IC, VP1+VP2+VP3; non-structural proteins, NSP, NSP1+NSP2+NSP3+NSP4+NSP5; and full genome, VP4+VP7+VP6+VP1+VP2+VP3+NSP1+NSP2+NSP3+NSP4+NSP5, which accounts for a total of 54,318 codons). The results of these studies are shown in Table 2.

Again, the frequencies of codon usage found in different genomic regions or considering complete genomes of RV-A are significantly different in relation to human cells (see Tables 1 and 2). Highly biased frequencies were also found for the same amino acids in all genomic regions or considering full genomes (Table 2) and in agreement with the previous results found using outer capsid proteins from P[4]G2 RV-A strains isolated in Brazil. The correlation between the position on the first axis generated by COA and the respective GC_3S values of each strain was analyzed for the complete genome dataset. A high and significant correlation among the position of the sequences on the first axis of COA and the GC_3S values ($r = -0.9879$, $P < 0.01$) was also found using full, complete genomes.

It has been suggested that dinucleotide biases can affect codon bias (Tao et al., 2009). To study this possibility, the relative abundances of the 16 dinucleotides in VP8* and/or VP7 ORFs was established. The results of these studies are shown in Table 3. As can be seen, the occurrences of dinucleotides are not random and no dinucleotides is present at the expected frequencies.

In the case of VP4 [VP8*] protein, the relative abundance of CpG and GpC showed a strong deviation from the expected frequencies (i.e. 1.0) (mean ± S.D. = 0.230 ± 0.035 and 0.282 ± 0.009, respectively) and were markedly underrepresented. On the other hand, ApU and ApA are markedly over-used (mean ± S.D. = 1.951 ± 0.033 and 1.979 ± 0.04, respectively) (Table 3). Among the 16 dinucleotides, 10 are correlated with the first axis value in COA (P values <0.01, Table 3). These observations indicated that the composition of dinucleotides also determines the variation in synonymous codon usage among P[4]G2 RV-A VP4 [VP8*] ORFs. To study the possible effects of CpG and GpC under-representation on codon usage bias of VP4 [VP8*] protein, the RSCU value of the 14 codons that contain CpG and/or GpC (CCG, GCG, UCG, ACG, CGC, CGG, CGU, CGA, GCU, GCC, GCA, UGC, AGC, GGC) were analyzed. Of these triplets, 12 [CCG (mean 0.01), GCG (mean 0.50), UCG (mean 0.35), ACG (mean 0.37), CGC (mean 0.00), CGG (mean 0.41) and CGU (mean 0.04), GCC (mean 0.02), CGA (mean 0.02), UGC (mean 0.03), AGC (mean 0.03) and GGC (mean 0.26)] were markedly suppressed.

In the case of VP7 protein, again, the relative abundance of CpG and GpC showed a strong deviation from the expected frequencies (mean ± S.D. = 0.397 ± 0.014 and 0.330 ± 0.018, respectively) and were underrepresented. Interestingly, the frequencies of ApU and ApA showed a sharp deviation from the expected frequencies and again we found a markedly over-use of these dinucleotides (mean ± S.D. = 2.056 ± 0.029 and 1.948 ± 0.038, respectively) (Table 3). Among the 16 dinucleotides, seven are correlated with the position of

Table 3
Relative abundance of dinucleotides in VP4 [VP8*] and VP7 proteins from P[4]G2 RV-A Brazilian strains and summary of COA.

VP4 [VP8]		UU	UC	UA	UG	CU	CC	CA	CG
Mean ± S.D.[a]		1.490 ± 0.035	0.823 ± 0.024	1.665 ± 0.021	0.846 ± 0.020	0.610 ± 0.022	0.381 ± 0.012	1.157 ± 0.023	0.230 ± 0.035
Axis 1[b]	r	−0.261	0.384	−0.427	0.038	−0.010	0.560	0.127	0.453
	P	0.040	<0.01	<0.001	0.764	0.928	<0.0001	0.317	<0.001

VP4 [VP8]		AU	AC	AA	AG	GU	GC	GA	GG
Mean ± S.D.		1.951 ± 0.033	0.869 ± 0.034	1.979 ± 0.04	1.177 ± 0.032	0.790 ± 0.025	0.282 ± 0.009	1.166 ± 0.019	0.561 ± 0.017
Axis 1	r	−0.223	0.104	−0.469	0.584	0.422	0.422	0.628	0.191
	P	0.080	0.412	<0.001	<0.001	0.001	0.001	<0.0001	0.133

VP7		UU	UC	UA	UG	CU	CC	CA	CG
Mean ± S.D.[a]		1.547 ± 0.020	0.640 ± 0.011	1.808 ± 0.023	1.142 ± 0.013	0.755 ± 0.018	0.250 ± 0.016	0.987 ± 0.024	0.397 ± 0.014
Axis 1[b]	r	−0.249	0.209	0.227	0.180	0.345	0.781	−0.341	0.126
	P	0.054	0.105	0.080	0.164	<0.01	<0.001	<0.01	0.327

VP7		AU	AC	AA	AG	GU	GC	GA	GG
Mean ± S.D.		2.056 ± 0.029	1.167 ± 0.020	1.948 ± 0.038	0.595 ± 0.037	0.793 ± 0.025	0.330 ± 0.018	1.013 ± 0.020	0.572 ± 0.024
Axis 1	r	−0.223	−0.327	−0.025	0.285	0.419	0.093	0.101	0.320
	P	0.085	0.011	0.841	0.027	<0.01	0.471	0.429	0.013

[a] Mean values of 58 P[4]G2 RV-A strains' relative dinucleotide ratios ± standard deviation.
[b] Correlation analysis between the first axis in COA and the sixteen dinucleotides frequencies in VP4 [VP8*] and VP7 proteins is shown.

Table 4
Position of codons in each of the four major axes of COA for RV-A VP4 [VP8*] and VP7 proteins.

	Axis 1			Axis 2			Axis 3			Axis 4		
	Codon	Value	Aminoacid	Codon	Value	Aminoacid	Codon	Value	Aminoacid	Codon	Value	Aminoacid
VP4	GCC	−4.183	Ala	UGC	−7.683	Cys	CCG	−6.889	Pro	GCC	−2.634	Ala
	UGC	0.660	Cys	CCG	0.898	Pro	GCC	2.071	Ala	GGG	1.967	Gly
VP7	GUC	0.610	Val	GGG	0.212	Gly	GUC	−0.960	Val	GUC	−0.100	Val
	AGG	1.488	Arg	GUC	4.447	Val	UUC	0.337	Phe	UUC	0.279	Phe

the sequences along the first axis in COA (P values <0.01, Table 3). These results indicate that the composition of dinucleotides also determines the variation in synonymous codon usage among P[4]G2 RV-A VP7 ORFs. The RSCU value for the VP7 protein of the 14 codons that contain CpG and GpC (see above) revealed that six [GCG (mean 0.28), CGC (mean 0.00), GCC (mean 0.13), GCG (mean 0.28), AGC (mean 0.26) and GGC (mean 0.24)] were markedly suppressed and five [CCG (mean 0.73), UCG (mean 0.59), CGG (mean 0.64), CGU (mean 0.64) and UGC (mean 0.75)] were slightly suppressed.

Besides, the position of each codon in each of the four major axes of COA was determined for both proteins studied. For VP4 [VP8*] ORFs, the first major axis accounted for the 28.67% of the observed variation, while the second, third and fourth axis accounted for the 21.57%, 18.56% and 12.39%, respectively. For VP7 ORFs, the first major axis accounted for the 66.00% of the observed variation; the second, third and fourth major axis accounted for the 14.82%, 8.09% and 2.40% of the observed variation, respectively. Table 4 shows the codons for which the maximum and minimum values were obtained for each of the axes studied (i.e. the most divergent codons values), indicating a strong bias in their use by both VP4 and VP7 proteins. As can be seen, the most divergent triplets tend to be GC-rich (considering the two ORFs, G+C explains 19/24 positions of these codons). Again, this can be explained in terms of a strong mutational bias.

In order to observe if the same results found using outer capsid proteins of P[4]G2 RV-A strains can be found using complete genomes, the same studies were repeated using a dataset of full complete genomes (for strains, accession numbers and genomic constellations, see Supplementary Material Table 3). The results of these studies are shown in Supplementary Material Table 4. Again, the relative abundance of CpG and GpC showed a strong deviation from the expected frequencies (i.e. 1.0) (mean ± S.D. = 0.360 ± 0.021) and (0.468 ± 0.038, respectively) and were markedly underrepresented. The frequencies of ApU and ApA also showed a sharp deviation from the expected frequencies and were markedly over-used (mean ± S.D. = 1.907 ± 0.069 and 2.089 ± 0.048, respectively). Among the 16 dinucleotides, seven are correlated with the position of the sequences along the first axis in COA (P values <0.01, Supplementary Material Table 4). Taking all these results together, it is possible to observe that the composition of dinucleotides also determines the variation in synonymous codon usage in the complete sequences of human RV-A.

4. Discussion

The results of these studies revealed that codon usage for VP4 [VP8*] and VP7 in P[4]G2 RV-A is quite different from that of human genes (see Table 1). Moreover, this is also observed considering all different genome regions or complete, full genome codes (see Table 2). This is in agreement with results found for other viruses such as human immunodeficiency virus 1 (HIV-1) (Grantham and Perrin, 1986; Kypr and Mrazek, 1987) and hepatitis A virus (Aragones et al., 2008). In other RNA viruses, like poliovirus or foot-and-mouth disease virus (FMDV) the codon usage is very

similar to that of their hosts, implying competence for tRNAs among virus and host (Sanchez et al., 2003). In these cases, competition is avoided by the induction of cellular shutoff of protein synthesis through carboxy cleavage of translation initiation factor 4G (eIF4G) by 2A and L proteases, respectively (Racaniello, 2001).

Early during the infection process RV-A also takes over the host translation machinery of the cell, causing a shutoff of cell protein synthesis, although by a different mechanism of picornaviruses. After RV-A infection, the translation initiation factor 2α (eIF2α) becomes phosphorylated and remains in this state throughout the virus replication cycle, leading to a further inhibition of cell protein synthesis (Montero et al., 2008). However, recent studies have shown that under these restrictive conditions, the viral proteins and some cellular proteins are efficiently translated (Montero et al., 2008). Whether this extremely different strategy in codon usage among RV-A and human cells is related to this fact is currently unknown, but might allow RV-A to compete successfully for translation of viral RNAs.

We analyzed synonymous codon usage and nucleotide compositional constraints in VP4 [VP8*] and VP7 genes of P[4]G2 RV-A and compare the results found with a dataset of RV-A reference strains from which the complete sequences for the 11 segments were previously known. Interestingly, in contrary to previous results found for other viruses such H5N1 influenza A Virus (mean ENC = 50.91) (Ahn et al., 2006; Zhou et al., 2005); SARS (mean ENC = 48.99) (Zhao et al., 2008); FMDV (mean ENC = 51.42) (Zhong et al., 2007); classical swine fever virus (mean ENC = 51.7) (Tao et al., 2009) and duck enteritis virus (mean ENC = 52.17) (Jia et al., 2009), the ENC values found for human P[4]G2 RV-A are comparatively low (mean ENC values of 37.36 and 40.56 for VP8* and VP7, respectively). Moreover, when the complete genomes are studied (accounting for 54,318 codons), the mean ENC value obtained is 41.60. This indicates that the overall extent of codon usage bias in RV-A genomes is significant.

We observed a general correlation between codon usage bias and base composition was observed, since all spots in the ENC–GC_3S plot lie below the curve of the predicted values (Fig. 1). Highly significant correlations between the first axis of COA and GC_3S values were obtained for both outer surface protein shells. Moreover, concatenation of complete sequences of the 11 segments of 10 reference human RV-A strains also show this significant correlation. All these results strongly suggest that mutational pressure is an important factor in determining codon usage bias in human RV-A. Nevertheless, we cannot completely discard other factors that may also account for codon usage bias.

The frequencies of dinucleotides were not random and no dinucleotides was present at the expected frequencies for both ORFs studied (VP8* and VP7, see Table 3). The same results are found using the complete genome dataset (Supplementary Material Table 4). CpG and GpC containing codons are markedly suppressed (see Tables 1 and 2). Marked CpG deficiency has been also observed in Coronaviruses (Woo et al., 2007), vertebrate-infecting members of the family *Flaviviridae* (Lobo et al., 2009), poliovirus (Rothberg and Wimmer, 1981) and other RNA viruses (Karlin et al., 1994). The CpG deficiency was proposed to be related to the immunostimulatory properties of unmethylated CpG, which were recognized by the host's innate immune system as a pathogen signature (Shackelton et al., 2006; Woo et al., 2007). This is now known to be triggered by the intracellular Pattern Recognition Receptor (PRR) Toll-like 9 (TLR9), which recognizes CpG-unmethylated DNA, and triggers several immune response pathways (Dorn and Kippenberger, 2008). Since the vertebrate immune system relies on unmethylated CpG recognition in DNA molecules as a sign of infection, and CpG under-representation in RNA viruses is exclusively observed in vertebrate viruses (Lobo et al., 2009), it is reasonable to suggest that a TLR9-like mechanism exists in the vertebrate immune system which recognizes CpG when in RNA context (such as in the genomes of RNA viruses) and triggers immune responses (Lobo et al., 2009). Moreover, recent studies on influenza A viruses, which have originated from an avian reservoir and have been infecting human hosts since 1918, were selected under strong pressure to reduce the frequency of CpG in its genome (Greenbaum et al., 2008).

The results of this work provide a basic knowledge of the mechanisms that give rise to codon usage bias in human RV-A and are also useful in understanding the processes involved in RV-A evolution. Further studies will be needed to reveal more about RV-A viral genome.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2011.01.006.

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Supplementary material

Supplementary material Table 1. Origins of the P[4]G2 RVA strains.

Name	Geographic location[a]	Year of isolation	VP4 accession number
493	Rio de Janeiro	1996	HM066010
685	Espirito Santo	1996	HM066060
5323	Rio de Janeiro	2002	DQ857926
5619	Rio de Janeiro	2002	DQ857927
11143	Mato Grosso do Sul	2005	HM066011
11257	Mato Grosso do Sul	2005	HM066013
11580	Acre	2005	HM066012
11860	Rio de Janeiro	2006	HM066014
12220	Rio de Janeiro	2006	HM066015
12287	Bahía	2006	HM066016
12482	Bahía	2006	HM066017
12549	Rio de Janeiro	2006	HM066018
12589	Rio de Janeiro	2006	HM066019
12647	Rio de Janeiro	2006	HM066020
12684	Rio de Janeiro	2006	HM066021
12777	Bahía	2006	HM066022
12840	Espírito Santo	2006	HM066023
12842	Espírito Santo	2006	HM066024
13039	Rio Grande do Sul	2006	HM066025
13079	Rio Grande do Sul	2006	HM066026
13081	Rio Grande do Sul	2006	HM066027
13151	Rio Grande do Sul	2006	HM066028
13158	Espirito Santo	2006	HM066029
13668	Alagoas	2007	HM066030
13788	Sergipe	2007	HM066031
13793	Sergipe	2007	HM066032
14303	Minas Gerais	2007	HM066033
14422	Rio Grande do Sul	2007	HM066034

14426	Espirito Santo	2007	HM066035
14919	Rio Grande do Sul	2008	HM066036
15311	Rio de Janeiro	2008	HM066043
15382	Bahía	2008	HM066037
15385	Bahía	2008	HM066038
15593	Alagoas	2008	HM066039
15771	Pernambuco	2008	HM066061
15774	Pernambuco	2008	HM066052
15777	Pernambuco	2008	HM066044
15782	Minas Gerais	2008	HM066053
15786	Espirito Santo	2008	HM066045
15811	Sergipe	2008	HM066062
15830	Rio Grande do Sul	2008	HM066054
15836	Rio Grande do Sul	2008	HM066055
15840	Rio Grande do Sul	2008	HM066056
15859	Minas Gerais	2008	HM066057
15860	Rio de Janeiro	2008	HM066058
15863	Maranhão	2008	HM066046
15898	Minas Gerais	2008	HM066059
15900	Minas Gerais	2008	HM066065
15953	Rio Grande do Sul	2008	HM066047
15958	Rio Grande do Sul	2008	HM066048
15983	Bahía	2008	HM066049
15988	Bahia	2008	HM066063
16054	Maranhão	2009	HM066050
16056	Maranhão	2009	HM066051
16064	Maranhão	2009	HM066040
16099	Espirito Santo	2009	HM066064
16100	Espirito Santo	2009	HM066041
16101	Espirito Santo	2009	HM066042

Supplementary material Table 1. Origins of the P[4]G2 RVA strains. (Cont.).

Name	Geographic location[a]	Year of isolation	VP7 accession number
11143	Mato Grosso do Sul	2005	HM066066
11257	Mato Grosso do Sul	2005	HM066067
11531	Acre	2005	HM066068
11580	Acre	2005	HM066070
11837	Acre	2006	HM066069
11860	Rio de Janeiro	2006	HM066071
12220	Rio de Janeiro	2006	HM066082
12224	Rio de Janeiro	2006	HM066089
12287	Bahía	2006	HM066073
12301	Bahía	2006	HM066079
12343	Rio de Janeiro	2006	HM066088
12389	Rio de Janeiro	2006	HM066077
12482	Bahía	2006	HM066072
12540	Rio de Janeiro	2006	HM066076
12549	Rio de Janeiro	2006	HM066090
12589	Rio de Janeiro	2006	HM066091
12774	Rio de Janeiro	2006	HM066081
12842	Espirito Santo	2006	HM066074
13039	Rio Grande do Sul	2006	HM066078
13151	Rio de Janeiro	2006	HM066083
13158	Espirito Santo	2006	HM066084
13668	Alagoas	2007	HM066085
13780	Rio de Janeiro	2007	HM066092
13788	Sergipe	2007	HM066086
13793	Sergipe	2007	HM066087
13891	Rio de Janeiro	2007	HM066093
14157	Rio de Janeiro	2007	HM066075
14303	Minas Gerais	2007	HM066094
14322	Minas Gerais	2007	HM066080

14422	Rio Grande do Sul	2007	HM066095
14919	Rio Grande do Sul	2008	HM066096
15311	Rio de Janeiro	2008	HM066097
15382	Bahía	2008	HM066098
15385	Bahía	2008	HM066099
15593	Alagoas	2008	HM066100
15771	Pernambuco	2008	HM066101
15774	Pernambuco	2008	HM066102
15777	Pernambuco	2008	HM066103
15782	Minas Gerais	2008	HM066104
15786	Sergipe	2008	HM066105
15811	Sergipe	2008	HM066106
15836	Rio Grande do Sul	2008	HM066107
15840	Rio Grande do Sul	2008	HM066108
15859	Minas Gerais	2008	HM066109
15860	Rio de Janeiro	2008	HM066110
15863	Mato Grosso	2008	HM066111
15894	Espirito Santo	2008	HM066112
15898	Minas Gerais	2008	HM066113
15900	Minas Gerais	2008	HM066114
15953	Rio Grande do Sul	2008	HM066115
15958	Rio Grande do Sul	2008	HM066116
15983	Rio Grande do Sul	2008	HM066117
15988	Bahía	2008	HM066118
15990	Bahía	2008	HM066119
16054	Maranhão	2009	HM066120
16056	Maranhão	2009	HM066121
16064	Maranhão	2009	HM066122
16099	Espirito Santo	2009	HM066123
16100	Espirito Santo	2009	HM066124
16101	Espirito Santo	2009	HM066125

[a] Brazilian states where the strains were isolated.

Supplementary material Table 2. ENC and GC_3S values for VP4[VP8*] and VP7 proteins in P[4]G2 RVA isolated in Brazil.

	VP4	
Strain	ENC	GC_3S
493_96RJ	36,97	0,144
685_96ES	36,75	0,137
5323_02RJ	37,86	0,156
5619_02RJ	37,99	0,160
11143_05MS	37,00	0,160
11257_05MS	38,32	0,167
11580_05AC	37,48	0,160
11860_06RJ	37,52	0,160
12220_06RJ	37,77	0,163
12287_06BA	37,86	0,163
12482_06BA	37,68	0,163
12549_06RJ	37,64	0,160
12589_06RJ	37,35	0,163
12647_06RJ	37,48	0,160
12684_06RJ	37,00	0,160
12777_06BA	37,86	0,163
12840_06ES	37,12	0,156
12842_06ES	40,49	0,195
13039_06RS	37,04	0,156
13079_06RS	37,77	0,163
13081_06RS	37,77	0,163
13151_06RS	37,77	0,163
13158_06ES	37,77	0,163
13668_07AL	37,50	0,160
13788_07SE	37,77	0,163
13793_07SE	37,77	0,163
14303_07MG	37,59	0,160
14422_07RS	37,77	0,163

14426_07ES	37,48	0,160
14919_08RS	37,77	0,163
15311_08RJ	37,77	0,163
15382_08BA	37,48	0,163
15385_08BA	37,48	0,160
15593_08AL	38,07	0,163
15771_08PE	36,37	0,148
15774_08PE	36,92	0,152
15777_08PE	38,07	0,163
15782_08MG	35,67	0,141
15786_08ES	37,77	0,163
15811_08SE	37,48	0,160
15830_08RS	37,22	0,163
15836_08RS	38,22	0,160
15840_08RS	37,72	0,163
15859_08MG	35,91	0,133
15860_08RJ	35,21	0,133
15863_08MA	37,96	0,163
15898_08MG	36,03	0,137
15900_08MG	36,02	0,137
15953_08RS	37,38	0,160
15958_08RS	37,38	0,160
15983_08BA	37,48	0,160
15988_08BA	36,80	0,160
16054_09MA	37,82	0,163
16056_09MA	37,96	0,163
16064_09MA	37,82	0,163
16099_09ES	36,03	0,137
16100_09ES	36,03	0,137
16101_09ES	36,03	0,137

Supplementary material Table 2. ENC and GC_3S values for VP4 [VP8*] and VP7 proteins in P[4]G2 RVA isolated in Brazil. (Cont.).

	VP7	
Strain	ENC	GC_3S
11143_05MS	40,80	0,191
11257_05MS	40,94	0,191
11531_05AC	41,42	0,177
11580_05AC	40,80	0,191
11837_06AC	41,42	0,177
11860_06RJ	41,10	0,191
12220_06RJ	40,49	0,191
12224_06RJ	40,38	0,191
12287_06BA	40,11	0,188
12301_06BA	40,16	0,191
12343_06RJ	40,38	0,191
12389_06RJ	40,10	0,188
12482_06BA	40,31	0,188
12540_06RJ	40,76	0,191
12549_06RJ	40,43	0,194
12589_06RJ	40,77	0,194
12774_06RJ	39,74	0,188
12842_06ES	41,88	0,200
13039_06RS	40,73	0,188
13151_06RS	40,46	0,191
13158_06ES	40,55	0,194
13668_07AL	40,33	0,188
13780_07RJ	40,03	0,191
13788_07SE	40,38	0,191
13793_07SE	40,38	0,191
13891_07RJ	40,20	0,191
14157_07RJ	39,29	0,184

14303_07MG	40,35	0,191
14322_07MG	40,51	0,191
14422_07RS	39,80	0,188
14919_08RS	40,65	0,191
15311_08RJ	38,97	0,184
15382_08BA	40,54	0,191
15385_08BA	40,49	0,191
15593_08AL	40,76	0,194
15771_08PE	40,82	0,194
15774_08PE	40,77	0,191
15777_08PE	40,35	0,194
15782_08MG	41,22	0,184
15786_08ES	40,03	0,191
15811_08SE	40,36	0,191
15836_08RS	39,99	0,191
15840_08RS	40,52	0,195
15859_08MG	41,22	0,184
15860_08RJ	41,24	0,185
15863_08MA	40,92	0,201
15894_08ES	40,03	0,191
15898_08MG	41,22	0,184
15900_08MG	41,22	0,184
15953_08RS	39,85	0,191
15958_08RS	40,36	0,191
15983_08BA	40,36	0,191
15988_08BA	40,42	0,194
15990_08BA	40,36	0,191
16054_09MA	40,92	0,201
16056_09MA	40,92	0,201
16064_09MA	40,92	0,201
16099_09ES	41,22	0,184
16100_09ES	41,22	0,184
16101_09ES	41,22	0,184

Supplementary material Table 3. Origins of the Human RVA full genome sequences.

Strain	Accession numbers	Genomic constellation[a]									
		VP7	**VP4**	**VP6**	**VP1**	**VP2**	**VP3**	**NSP1**	**NSP2**	**NSP3**	**NSP4**	**NSP5**
B4106	AY740731 to AY740741	G3	P[14]	I2	R2	C2	M3	A9	N2	T6	E5	H3
PA169	EF554126 to EF554136	G6	P[14]	I2	R2	C2	M2	A3	N2	T6	E2	H3
MG6	EF554093 to EF554103	G6	P[14]	I2	R2	C2	M2	A11	N2	T6	E2	H3
TB-Chen	AY787644 to AY787654	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
DS-1	EF990691 to EF990694, EF672577 to EF672583	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RV176	DQ490551 to DQ490561	G12	P[6]	I2	R2	C2	M2	A2	N2	T2	E6	H2
RV161	DQ490540 to DQ490550	G12	P[6]	I2	R2	C2	M2	A2	N2	T2	E1	H2
N26	DQ146682 to DQ146692	G12	P[6]	I2	R2	C2	M2	A2	N1	T2	E6	H2
111/05-27	EF554037 to EF554047	G6	P[14]	I2	R2	C2	M2	A3	N2	T6	E2	H3

[a] According to the recommendations of the Rotavirus Classification Working Group (Matthijnssens et al., 2008).

CAPITULO VI

DISCUSSÃO, CONCLUSÕES E PERSPECTIVAS

1. Discussão

Diversidade genética dos rotavírus da espécie A

É sabido que a distribuição de genótipos de RVA flutua tanto no decorrer do tempo quanto nas diferentes regiões geográficas. Entretanto, os mecanismos envolvidos neste fenômeno ainda não são bem conhecidos. Existem evidencias de que tanto a imunidade homotípica quanto a heterotípica contra a infecção natural do RVA desempenha um papel muito importante tanto em nível do individuo quanto da população. Existem diversos outros fatores tais como: o fluxo continuo migratório de pessoas no mundo; a emergência de variantes de RVA; a diversidade genética dos diferentes genótipos; a facilidade de transmissão e resistência do vírus; fatores relacionados ao hospedeiro (genéticos, anticorpos maternos, imunodeficiência, parasitas intestinais, má nutrição, deficiência de zinco, entre outros). Estes fatores acima mencionados poderiam também estar influenciando, em menor ou maior grau, a diversidade de genótipos de RVA mundialmente (Matthijnssens et al., 2012). Neste contexto a introdução de vacinas (mono e polivalentes) poderia representar mais um fator a ser considerado.

Atualmente, pelo menos 41 países introduziram a vacina RV1 no seu calendário de imunizações (WHO/UNICEF, 2013). Apesar dos estudos de Fase III com a RV1 terem sido satisfatórios e a OMS ter recomendado aos países inclusão de uma vacina anti-RVA em seus programas de imunização, diversas questões tem sido postuladas, tais como: i) a introdução de uma vacina monovalente poderia resultar no aumento na prevalência de genótipos que não tenham nenhum destes G ou P tipos? ii) a reestruturação gênica intra-, entre-genogrupos e entre amostras selvagens e vacinal de RVA poderiam ocorrer com maior frequência e prejudicar a eficácia da vacina RV1? Uma das maneiras de se buscar responder a estas perguntas é a analise da constelação gênica dos RVA assim como da prevalência dos genótipos circulantes, preferencialmente antes e após a introdução da vacina RV1. Duas constelações genéticas prevalecem mundialmente: *Wa-like* e *DS-1-like*. Dos genótipos mais prevalentes mundialmente só um contem uma constelação *DS-1-1ike* (G2P[4]). Os demais genótipos são *Wa-like* (G1P[8], G3P[8], G4P[8], G9P[8], e G12P[8]). A terceira constelação, *AU-1-like*, tem sido ocasionalmente encontrada em humanos (Matthijnssens e Van Ranst, 2012).

Análise da diversidade genética do genótipo G2P[4] no período de 2005 a 2011 no Brasil (Artigo 1 e 2)

G2P[4] foi o genótipo mais comumente detectado no Brasil após o início da vacinação (Gurgel et al., 2007, de Oliveira et al., 2008; Nakagomi et al., 2008; Leite et al., 2008; Carvalho -Costa et al., 2009, 2011; da Silva Soares et al., 2013). A emergência deste genótipo tem motivado uma grande discussão. Uma correlação entre o genótipo G2P[4] e a vacinação foi proposta (Gurgel et al., 2007; Nakagomi et al., 2008). Sendo a principal razão o fato de que a eficiência e eficácia da vacina monovalente contra o G2P[4] (*DS-1-like*) é relativamente menor do que contra cepas *Wa-like* (Ruiz-Palacios et al., 2006; Vesikari et al., 2007; Linhares et al., 2008; Justino et al., 2011). Neste contexto, foi proposto que a vacinação em massa teria exercido pressão seletiva sobre cepas de RVA circulantes, selecionando o G2P[4] (Nakagomi et al., 2008; Gurgel et al., 2009).

Os resultados do presente estudo demonstram que a taxa de detecção de G2P[4] em amostras RVA positivo tem sido alta nos anos após a introdução da vacinação com RV1. Com exceção de 2009, esta taxa tem sido acima de 50%, atingindo 87% em 2010. Outro fato importante é que o genótipo G2P[4] também tem sido frequentemente detectado em crianças vacinadas com RV1.

Nos artigos em questão apresentamos um estudo onde examinamos mais detalhadamente a localização geográfica de amostras positivas para G2P[4], tentando identificar uma via de propagação deste genótipo durante o processo de emergência no Brasil. A freqüência de detecção de G2P[4] entre 2001 e 2003 foi muito baixa, por isso apenas três amostras positivas puderam ser identificadas nos dados de monitoramento no Laboratório de Referencia Regional para Rotavírus (LRRR). Em 2004, G2P[4] não foi caracterizado em nenhuma das 651 amostras fecais positivas para RVA obtidas a partir de 2.554 crianças com gastroenterite de 11 estados brasileiros. Com isso confirmamos que o genótipo G2P[4] estava ausente nas amostras estudadas no primeiro semestre de 2005. Neste ano, as primeiras cidades em que o genótipo G2P[4] foi detectado estão localizadas no oeste do Brasil, na fronteira com o Paraguai. Ao longo de 2005, G2P[4] continuou a ser detectado na região amazônica e no Brasil, na fronteira com o Peru e a Bolívia, sendo ausente nas regiões brasileiras mais populosas da costa atlântica. Somente em dezembro de 2005, G2P[4] foi detectado nas principais cidades brasileiras, mais especificamente no Rio de Janeiro. A vacinação em massa contra RVA começou em março de 2006 e, a partir deste ano, o genótipo G2P[4] foi

detectado precocemente em todas as regiões monitoradas, numa fase em que o grupo de crianças vacinadas ainda era muito limitado.

Tem sido sugerido que as vacinas específicas podem selecionar genótipos de escape, incluindo os efeitos de longo prazo de pressões seletivas fracas (Pérez-Sautu et al., 2011). Neste contexto, as cepas emergentes resultantes da pressão evolutiva imposta pelas vacinas podem substituir gradualmente os genótipos anteriores (Streck et al., 2013). Como resultado, no cenário onde a seleção ocorre, uma distância genética entre as cepas circulantes surgiria em relação a outras regiões (Hoffmann et al., 2008).

A análise das origens genéticas e antigênicas de cepas G2P[4] identificadas ao longo do processo de implementação da imunização em massa com RV1 pode ser útil para esclarecer seu potencial impacto sobre a distribuição de genótipos que ocorreu entre 2005 e 2011 no Brasil. Os dados obtidos no presente estudo (artigo 1 e 2) indicam que as cepas G2P[4] circulantes no Brasil durante a implementação da vacinação em massa com RV1 não se agrupam em grupos genéticos definidos por períodos pré e pós vacinação; com isso parece existir uma estreita relação com cepas G2P[4] circulantes em outros países que ainda não tinham implementado a vacinação em massa contra RVA. Da mesma forma, cepas G2P[4] identificadas em crianças vacinadas e não vacinadas agruparam nos mesmos grupos genéticos, não havendo diferenças significativas nas sequências dos genes que codificam VP7 e VP8*. A Análise do contexto genômico de cepas brasileira G2P[4] revelou uma constelação genética *DS-1-like* completa, e diferentes variantes genéticas foram observadas circulando entre as regiões brasileiras.

Além disso, a comparação das sequências de aminoácidos dos sítios antigênicos das proteínas VP7 e VP8* da RV1 e as cepas G2P[4] brasileiras, revelou que a maioria das mudanças observadas foram mantidas nas amostras brasileiras ao longo dos anos, estando todas presentes antes da introdução da vacina RV1.

Em conclusão, os dados aqui apresentandos apoiam a ideia de que outros fatores além da introdução da RV1 seriam responsáveis pelo aumento na prevalência de G2P[4] no Brasil. Tem sido proposto que o G2P[4] reemerge periodicamente em ciclos de 10 anos (Bishop et al., 1991). Leite e colaboradores (2008) relataram uma onda de circulação do G2P[4] no início da década de 1990, perto de 10 anos antes do ressurgimento deste genótipo em 2005.

O longo período de pouca ou nenhuma circulação deste genótipo anterior à introdução da RV1 teria criado condições favoráveis para a acumulação de indivíduos imuno-susceptíveis (Carvalho-Costa et al., 2009; Assis et al., 2013). Este fato, juntamente com a diversidade genética encontrada em diferentes regiões geográficas ao longo dos anos, antes,

durante e após a introdução da vacina poderia explicar a alta prevalência do genótipo G2P[4] no Brasil desde 2005.

Análise da diversidade genética do genótipo G1P[6] em crianças vacinadas com RV1 (Artigo 3)

A circulação dos genótipos P[6] e G12, respectivamente nos estados do Rio de Janeiro e na região Norte do Brasil têm sido demonstrada (Fumian et al., 2011; Soares et al., 2012). Entretanto, apenas sequencias parciais do gene que codifica para a proteína NSP4 das amostras detectadas no Rio de Janeiro e do gene que codifica para a proteína VP7 das amostras G12 estão disponíveis no banco de dados do *GenBank*. No presente estudo seis cepas G1P[6] detectadas a partir de crianças vacinadas com duas doses da vacina RV1 que apresentaram gastroenterite aguda na região Nordeste do Brasil foram analisadas. A analise das sequencias obtidas para VP8* revelaram que as cepas Brasileiras estavam bastante relacionadas a cepas africanas, bem como com uma cepa G2P[6] detectada nos Estados Unidos (Heylen et al., 2012; Nordgren et al., 2012) (Figura 1A). Quando comparadas com a sequencia aminoacidica da RV1, estas cepas mostraram uma mudança de aminoácido na posição N135S, que não estava presente em outras amostras P[6] analisadas (Material suplementar Tabela 2). No total, foram observadas dezessete alterações de aminoácidos nas regiões antigênicas da proteína VP8* entre as amostras brasileiras e a RV1 (Material suplementar Tabela 2), mostrando a grande diversidade com relação a RV1 como observado anteriormente por Zeller e colaboradores (2012a).

A análise do gene que codifica para a proteína VP7 mostrou que as cepas genótipo G1 mais recentemente detectadas no Brasil pertencem a linhagem G1-I, distinta da vacina RV1 (G1-II) (Figura 1B). Foram observadas quatro substituições de aminoácidos no interior dos sítios antigênicos, quando comparadas as amostras brasileiras G1P[6] com a vacina RV1; posições: N94S, S123N, M217T e K291R (Material suplementar Tabela 3). Tais substituições foram observadas em quase todas as cepas G1-I e, portanto, as mesmas podem ter sido selecionadas positivamente ao longo do tempo, podendo estar relacionadas a algum tipo de vantagem em relação ao *fitness* viral. Tem sido sugerido que cepas G1-I que acumularam estas mutações pontuais poderiam evoluir, ao longo do tempo, gerando variantes capazes de escapar da resposta imune induzida pela vacina e, podendo assim, causar surtos (Maranhão et al., 2012). É importante ressaltar que as amostras G1P[6] analisadas no presente estudo não foram relacionadas a qualquer surto.

A analise da constelação gênica das cepas G1P[6] demonstrou que as mesmas pertenc em ao genótipo *Wa-like* (Tabela 2), existindo uma estreita relação com cepas contemporâneas de RVA detectadas em diferentes países, apresentando diferentes combinações de G e P (Figuras 1A, 1B, 2 e 3). A análise filogenética do gene que codifica para a NSP5 sugere a ocorrência de eventos de reestruturações genéticas entre cepas P[8] e P[6] cocirculantes no Brasil (Figura 2E). O fato das cepas G1P[6] apenas compartilharem um dos onze genes de RVA, poderia sugerir que as mesmas foram importadas de algum outro lugar ou ainda que estariam relacionadas a cepas ainda desconhecidas que circularam no Brasil.

E importante destacar que as cepas G1P[6] analisadas não podem ser consideradas heterotípicas quando comparadas com a RV1, pois possuem uma constelação genética *Wa-like*. Somente a VP8* pertence a um genótipo P diferente (Matthijnssens & Van Ranst, 2012). Estes resultados sugerem que provavelmente a razão pela qual estas cepas teriam causado gastroenterite em crianças não foi devido à constelação genética das mesmas e sim devido a outros fatores, os quais poderiam ser: i) características genéticas restritas ao hospedeiro (Hu et al., 2012; Gunaydin et al., 2013; Imbert-Marcille et al., 2013); ii) concentrações elevadas de anticorpos específicos anti-RVA no leite materno com níveis de IgA específicos de RVA no lúmen do intestino (Desselberger & Huppertz, 2011).Lembremo-nos de que a eficácia da vacina não é 100% (O´Ryan & Linhares, 2009), e sendo assim, se espera que crianças vacinadas ainda apresentem GA. Rose e colaboradores (2013), recentemente demostrou a ocorrência de reestruturações genéticas entre amostras de RVA genótipo G1P[8] (*Wa-like*) e a RV1, detectando tal genótipo em crianças vacinadas com RV1 que sofreram GA. A ocorrência de reestruturaçãoes genéticas entre amostras selvagens e vacinais também foi demostrada para a RV5 (Hemming & Vesikari, 2013)

Constelação genética dos genótipos G12P[9] e G12P[8] detectados no Brasil (Artigo 4)

O genótipo de RVA G12 foi detectado pela primeira vez em crianças com diarreia nas Filipinas, em 1987 (Taniguchi et al., 1990). Recentemente, este genótipo foi descrito em vários estudos conduzidos em diversos países e a sua expansão global tem sido demonstrada na última década (Castello et al., 2006; Bányai et al., 2007; Rahman et al., 2007; Ray et al., 2007; Alam et al., 2009; Cunliffe et al., 2009; Page et al., 2009; Pietsch & Liebert, 2009; Matthijnssens et al., 2012; Tra et al., 2011; McDonald et al., 2012; Ndze et al., 2013; Tamim et al., 2013). No Brasil, o G12 foi descrito nas regiões Sudeste (2003) e Norte (2008 - 2010), em combinação com o genótipo P[9] e P[6], respectivamente (Pietruchinski et al., 2006;

Soares et al., 2011). No entanto, apenas informações sobre o gene que codifica para a VP7 estão disponíveis no GenBank.

No artigo 4, foram analisadas as constelações genéticas de cepas de RVA genótipo G12P[9]; detectadas em 2008 e 2010 em Pernambuco (Nordeste do Brasil) e genótipo G12P[8]; detectadas no Rio de Janeiro (Sudeste do Brasil) em 2006 e Bahia (Nordeste do Brasil) em 2011. O genótipo G12 tem sido associado às constelações: *Wa-like*, *DS-1-like* e *AU-1-like* (Tabela 1) (Rahman et al., 2007; Heiman et al., 2008; Ghosh et al., 2010; Jere et al., 2011; Stupka et al., 2012), sendo que a maioria das cepas G12 descritas até o momento possuem a constelação *Wa-like* (Freeman et al., 2009; Matthijnssens et al., 2012). Esta observação pode estar relacionada ao fato de que a maioria das cepas G12 analisadas estavam associadas ao genótipo P[8]. Até o momento, apenas uma cepa G12P[9] (T152) foi analisada para os onze segmentos gênicos mostrando uma constelação genética *AU-1-like* (Rahman et al., 2007). Análises filogenéticas das sequencias nucleotidicas obtidas para NSP2-5, VP1-VP2 e VP6 das amostras brasileiras G12P[9] mostraram uma relação genética muito estreita com a cepa T152 (99% de similaridade) detectada em 1998 na Tailândia. As cepas G12P[9] anteriormente descritas pertencem ao genótipo A12 (Freeman et al., 2009). No caso da NSP1, as amostras brasileiras G12P[9], foram pertencentes ao genótipo A3, agrupando com várias cepas de RVA bovinas e humanas, sendo que as humanas possivelmente tenham origem bovina (Figura 2B).

A análise dos onze genes das cepas G12P[8] brasileiras revelou que a cepa RVA/Human-wt/BRA/RJ12419/2006/G12P[8] agrupou-se junto com cepas G1P[8] detectadas nos Estados Unidos de América (EUA) em 2005 (99% de similaridade); enquanto as cepas RVA/Human-wt/BRA/BA20142/2011/G12P[8] e RVA/Human-wt/BRA/BA20144/2011/G12P[8] foram quase idênticas e agruparam-se com cepas G12P[8] detectadas na Tailândia em 2009, para a maioria dos genes de RVA (99% similaridade). Estes resultados sugerem a circulação de diferentes variantes de G12P[8] *Wa-like* no Brasil.

Os resultados obtidos no presente estudo sugerem que as cepas brasileiras G12P[9] (*AU-1-like*) e G12P[8] (*Wa-like*) detectadas em diferentes regiões do Brasil não compartilham uma origem comum. Além disso, enquanto as G12P[8] mostraram uma constelação *Wa-like* completamente relacionada a cepas humanas; as cepas G12P[9] apresentaram um gene possivelmente de origem bovina (NSP1). Deve-se ressaltar que e a RVA/Human-wt/BRA/PE18974/2010/G12P[9] também possuiu o gene VP3 de origem canina/felina (Tabela 1). A constelação *AU-1-like* é esporadicamente mencionada em humanos. Porém acredita-se que este o genótipo *AU-1-like* tenha uma relação evolutiva próxima com cepas de RVA caninas e felinas (Nakagomi & Nakagomi, 1989; Matthijnssens et al., 2008).

Tem sido sugerido que as cepas G12P[9] previamente detectados na Argentina, Paraguai e Brasil tenham origem no Leste da Ásia (Castello et al., 2009). E neste estudo, todos os genes, exceto NSP1 e VP3 (RVA/Human-wt/BRA/PE18974/2010/G12P[9]) mostraram uma relação genética próxima com cepas detectadas no Japão, Tailândia e Coréia do Sul. Considerando-se que a Tailândia não faz parte do leste da Ásia, e que poucas cepas G12P[9] têm sido detectadas e caracterizadas até o momento, estudos complementares são necessários para a melhor compreensão da eventual origem e evolução deste genótipo.

Estudo do víeis no uso de códons de RVA genótipo G2P[4] (Artigo 5)

Os resultados obtidos neste estudo mostraram que a utilização de códons dos RVA genótipo G2P[4] para as proteínas VP8* e VP7 é bastante diferente daquele dos genes humanos (ver Tabela 1). O mesmo resultado é observado quando consideramos os 11 genes dos RVA (ver Tabela 2). Estes resultados estão em concordância com os resultados encontrados para outros vírus, tais como o vírus da imunodeficiência humana tipo 1 (HIV-1, do inglês *Human immunodeficiency vírus type* 1) (Grantham & Perrin, 1986; Kypr & Mrazek, 1987) e vírus da hepatite A (*HAV,* do inglês *Hepatitis A vírus*) (Aragones et al., 2008). No início da infecção, o RVA assume a maquinaria de tradução da célula, causando uma interrupção na síntese das proteínas celulares. O fator de iniciação da tradução 2α (eIF2α) é fosforilado, permanecendo assim durante todo o ciclo da replicação viral, e desta forma, inibe a síntese proteica celular (Montero et al., 2008). No entanto, estudos recentes têm demonstrado que nem toda a síntese de proteínas celulares é inibida (Montero et al., 2008). Se esta estratégia extremamente diferente no uso códon entre os RVA e as células humanas está relacionada a este fato é desconhecido, mas poderia permitir aos RVA competir com sucesso para a tradução de ARNm virais.

Neste estudo foi possível observar uma correlação entre o víeis no uso de códons e a composição de bases, o que sugere que a pressão mutacional é um fator importante na determinação do víeis no uso de códons em RVA humanos. Porém, não se podem descartar completamente outros fatores que podem também influenciar no viés.

As frequências de di-nucleotídeos não foram aleatórias e nenhum dos di-nucleotídeos foi observado uma frequência que sería esperada para ambas as *ORF*s estudadas (VP8* e VP7, ver Tabela 3). Os mesmos resultados foram encontrados utilizando dados do genoma completo de RVA (Tabela 4. Material Complementar). Observou-se que os di-nucleotídeos contendo *CpG* (do inglês *Cytosine-phosphate-Guanine*) e *GpC* (do inglês *Guanine–phosphate-Cytosine)* estavam marcadamente reduzidos (ver Tabelas 1 e Tabela 2). Tem sido

proposto que a deficiência en *CpG* estaria relacionada com as propriedades imuno-estimuladoras do *CpG* não metilado, que são reconhecidos pelo sistema imune-inato do hospedeiro como uma assinatura de patógeno (Shackelton et al., 2006; Woo et al., 2007). É sabido que o receptor de reconhecimento de padrões intracelular *Toll-like* 9 (*TLR9*), que reconhece os *CpG* não metilados no ADN, desencadeia várias vias da resposta imune (Dorn & Kippenberger, 2008). Nesse contexto o sistema imunológico dos vertebrados depende do reconhecimento dos *CpG* não metilados nas moléculas de ADN como um sinal de infecção. Sabe-se que os *CpG* encontram-se marcadamente reduzidos nos vírus de ARN, exclusivamente nos vírus de vertebrados (Lobo et al., 2009). Portanto, é razoável sugerir que um mecanismo vinculado ao *TLR9* existe no sistema imunológico dos vertebrados que reconhece os *CpG* no contexto do ARN, desencadeando uma resposta imune (Lobo et al., 2009) .

Estudos recentes sobre o vírus influenza A indicam que os mesmos foram selecionados sobre uma forte pressão para reduzir a frequência de *CpG* no seu genoma (Greenbaum et al., 2008).

2. Conclusões

- Todas as cepas G2P[4] para as quais foram analisados os onze segmentos gênicos apresentaram uma constelação gênica *DS-1-like*: I2-R2-C2-M2-A2-N2-T2-E2-H2, apesar de que diversas variantes virais circularam no período estudado;
- Foi evidenciada a ocorrência de reestruturações gênicas entre as variantes virais do genótipo G2P[4] que circularam no Brasil;
- Não foram observadas diferenças nos sítios antigênicos das proteínas VP8* e VP7 entre crianças vacinadas e não vacinadas;

- As seis cepas G1P[6] analisadas apresentaram uma constelação gênica *Wa-like*: I1-R1-C1-M1-A1-N1-T1-E1-H1 e, consequentemente, homotípicas quando comparadas com a RV1 (*Wa-like*);

- As duas cepas G12P[9] pertenceram ao genogrupo *AU-1-like* mostrando uma constelação gênica: I3-R3-C3-M3-A3-N3-T3-E3-H6;
- Eventos de reestruturaçõa genética foram evidenciados para os genes que codificam as proteínas NSP1 e VP3;
- Apresentaram uma relação genética muito próxima com cepas detectadas na Tailandia;

- As cepas G12P[8] revelaram uma constelação gênica *Wa-like*: I1-R1-C1-M1-A1-N1-T1-E1-H1;

- As frequências no uso de códons das duas proteínas de RVA estudadas (VP7 e VP8*) foram significativamente diferentes das observadas nas células humanas;
- Observou-se uma correlação entre o víeis no uso de códons e a composição de bases, o que sugere que a pressão mutacional é um fator importante na determinação do víeis no uso de códons em RVA humanos.

3. Perspectivas

Considerando os resultados obtidos e aqui apresentados e visando contribuir para os avanços nos estudos da variação, tanto temporal quanto geográfica, na prevalência dos diferentes genótipos de RVA circulantes após introdução da RV1; constituirão objetivos futuros:

i) Realizar análises da diversidade genéticas de diversos genótipos de RVA detectados em crianças vacinadas e não vacinadas;

ii) Investigar a ocorrência de reestruturações gênicas entre amostras selvagens e a RV1;

iii) Investigar a prevalência dos genótipos P[6], G12P[9], e G12P[8] na população brasileira.

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