

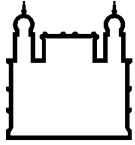
MINISTÉRIO DA SAÚDE
FUNDAÇÃO OSWALDO CRUZ
INSTITUTO OSWALDO CRUZ

Doutorado em Programa de Pós-Graduação em Biologia Parasitária

**ROTAVÍRUS A E NOROVÍRUS EM CRIANÇAS DA COMUNIDADE DE
MANGUINHOS, RIO DE JANEIRO: SUSCEPTIBILIDADE DO
HOSPEDEIRO, MONITORAMENTO DO VÍRUS VACINAL RV1 E
GENÓTIPOS CIRCULANTES**

CARINA CANTELLI PACHECO DE OLIVEIRA

Rio de Janeiro
Novembro de 2019



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INSTITUTO OSWALDO CRUZ
Programa de Pós-Graduação em Biologia Parasitária

CARINA CANTELLI PACHECO DE OLIVEIRA

Rotavírus A e norovírus em crianças da comunidade de Manguinhos, Rio de Janeiro: susceptibilidade do hospedeiro, monitoramento do vírus vacinal RV1 e genótipos circulantes

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Ciências.

Orientador: Prof. Dr. José Paulo Gagliardi Leite

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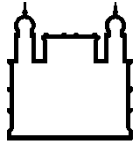
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MONITORAMENTO DO VÍRUS VACINAL RV1 E GENÓTIPOS
CIRCULANTES**

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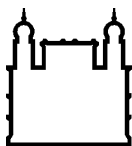
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Rio de Janeiro, 28 de Novembro de 2019

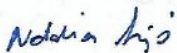


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Ata da defesa de tese de doutorado em Biologia Parasitária de **Carina Cantelli Pacheco de Oliveira**, sob orientação do Dr. José Paulo Gagliardi Leite. Ao vigésimo oitavo dia do mês de novembro de dois mil e dezenove, realizou-se às dez horas, na Sala 06 - Pavilhão Cardoso Fontes, o exame da tese de doutorado intitulada: **“Rotavírus A e Norovírus em crianças da comunidade de Manguinhos, Rio de Janeiro: susceptibilidade do hospedeiro, monitoramento do vírus vacinal RV1 e genótipos circulantes”**, no programa de Pós-graduação em Biologia Parasitária do Instituto Oswaldo Cruz, como parte dos requisitos para obtenção do título de Doutora em Ciências - área de concentração: Genética e Bioquímica, na linha de pesquisa: Estudos Bioquímicos e Moleculares de Parasita, Vetores e Hospedeiros.. A banca examinadora foi constituída pelos Professores: Dr^a. Flávia Barreto dos Santos - IOC/FIOCRUZ (Presidente), Dr^a. Natália Motta de Araújo - IOC/FIOCRUZ, Dr^a. Rita de Cássia Nasser Cubel Garcia - UFF/RJ e como suplentes: Dr^a. Elena Cristina Caride – BIO-MANGUINHOS/FIOCRUZ e Dr. Rafael Brandão Varella – UFF/RJ. Após arguir a candidata e considerando que a mesma demonstrou capacidade no trato do tema escolhido e sistematização da apresentação dos dados, a banca examinadora pronunciou-se pela APROVAÇÃO da defesa da tese de doutorado. De acordo com o regulamento do Curso de Pós-Graduação em Biologia Parasitária do Instituto Oswaldo Cruz, a outorga do título de Doutora em Ciências está condicionada à emissão de documento comprobatório de conclusão do curso. Uma vez encerrado o exame, o Coordenador do Programa, Dr. Rafael Maciel de Freitas, assinou a presente ata tomando ciência da decisão dos membros da banca examinadora. Rio de Janeiro, 28 de novembro de 2019.

Dr^a. Flávia Barreto dos Santos (Presidente da Banca): 

Dr^a. Natália Motta de Araújo (Membro da Banca): 

Dr^a. Rita de Cássia Nasser Cubel Garcia (Membro da Banca): 

Dr. Rafael Maciel de Freitas (Coordenador do Programa): 

Dedico esta tese as pessoas mais importantes em minha vida: meus queridos pais, Ricardo Luiz (*in memoriam*) e Elfrida, meu amado filho, José Ricardo (em meu ventre) e meu marido, Paulo Vinícius.

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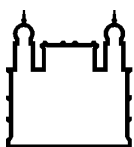
Aos meus amados pais Ricardo Luiz Pacheco de Oliveira (*in memoriam*) e Elfrida Cantelli Pacheco de Oliveira, pela dedicação e ao amor incondicional dispensado durante todas as fases de minha vida.

Ao meu marido, amigo e companheiro, Paulo Vinícius Rabha de Souza, pelo seu amor e paciência nesta caminhada.

Ao meu amado filho, em meu ventre, José Ricardo, milagre de Deus em minha vida.

A todos os pais e crianças residentes de Manguinhos participantes deste estudo.

*“Cada descoberta nova da ciência é
uma porta nova pela qual encontro
mais uma vez Deus, o autor dela”.*
Albert Einstein



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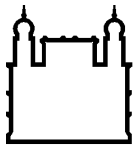
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RESUMO

TESE DE DOUTORADO EM BIOLOGIA PARASITÁRIA

Carina Cantelli Pacheco de Oliveira

Os rotavírus A (RVA) e os norovírus são os principais vírus associados à etiologia da gastroenterite aguda (GA) em <5 anos. A partir de 2006, com a introdução das vacinas de RVA, observou-se um declínio expressivo da morbidade/mortalidade por estes vírus, em detrimento a crescente importância das infecções pelos norovírus nesta faixa etária. Antígenos do grupo histosanguíneo humano (HBGAs), presentes na mucosa intestinal, têm sido descritos como potenciais ligantes e/ou cofatores requeridos nos estágios da patogênese das infecções por estes vírus, influenciando na epidemiologia molecular e evolução em diferentes populações. Este estudo teve como objetivo associar o perfil de susceptibilidade (HBGA/Secretor/Lewis) de crianças residentes na comunidade de Manguinhos, no Rio de Janeiro, à eficácia da resposta a vacina RV1 (Rotarix®/G1P[8]), assim como às infecções naturais pelos norovírus. Com este propósito realizou-se o monitoramento das infecções por RVA e norovírus em uma coorte de crianças de 0-11 meses, pela detecção e caracterização molecular destes vírus em amostras de fezes de crianças sintomáticas ou não. No período de 2014 a 2018, 132 crianças foram incluídas neste estudo, disponibilizando o mesmo número de amostras de saliva para determinação do perfil de susceptibilidade por ELISA e 569 amostras de fezes para investigação de ambos os vírus por métodos moleculares. O vírus vacinal excretado foi caracterizado em 78% (85/109) das amostras RVA. A presença da mutação F167L (RV1), demonstrada pelo sequenciamento do gene VP8*, foi observada em 20,1% das crianças. Quatro casos de rotavirose pelos genótipos G3P[8], G12P[8] e G3P[9] foram observados. Os norovírus foram detectados em 21,2% (28/132) das crianças, com incidência de 5,8 infecções em 100 criança-meses. A razão de detecção foi de 5,6% (17,1% dos casos diarreicos e 4,7% dos assintomáticos), sendo seis diferentes genótipos detectados (GII.4_Sydney_2012[P31], GII.4_Sydney_2012[P16], GII.4_Sydney_2012[P4_New_Orleans_2009], GII.2[P16], GII.6[P7] e GI.3[P13]). Em relação ao perfil HBGA, observou-se 80,3% das crianças com o *status* secretor. A caracterização do gene *FUT2* (Se) em crianças secretoras Le (a+b+) e não secretoras, e do gene *FUT3* (Le) em Le (a-b-), possibilitou a identificação de novas mutações nesta população. A mutação F167L na RV1 excretada foi identificada em 86,4% das crianças que apresentaram o fenótipo secretor Le (a+b+), indicando que os HBGAs poderiam ser importantes marcadores na avaliação da RV1; significativa associação entre infecção sintomática pelos norovírus e o *status* secretor (Le^b) também foi observada. A ocorrência de norovírus na coorte e a sua crescente importância epidemiológica, determinou a ampliação deste estudo, com a análise de 61 amostras fecais oriundas de 49 crianças na faixa etária de 1-4 anos residentes em Manguinhos. Os norovírus foram detectados em 47,5% (29/61) das amostras (46,7% dos casos diarreicos e 50% dos assintomáticos) e quatro novos genótipos foram identificados, sendo o recombinante GII.2[P16] detectado pela primeira vez no Brasil. Os dados de vigilância epidemiológica deste estudo comprovam a importância do monitoramento dos principais vírus responsáveis pela gastroenterite infantil aguda, evidenciando a diversidade genética e a dinâmica destes vírus considerando novas abordagens como o perfil HBGA, que trouxe uma importante contribuição em relação à avaliação da eficácia da RV1 na coorte estudada.



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ROTAVIRUS A AND NOROVIRUS IN CHILDREN FROM MANGUINHOS COMMUNITY, RIO DE JANEIRO: HOST SUSCEPTIBILITY, VACINAL VIRUS (RV1) AND CIRCULATING GENOTYPES

ABSTRACT

PHD THESIS IN BIOLOGIA PARASITÁRIA

Carina Cantelli Pacheco de Oliveira

Rotavirus A (RVA) and norovirus are the major viruses associated with the etiology of acute gastroenteritis (GA) in <5 years-old. Since 2006, with the introduction of RVA vaccines, a significant decline in morbidity/mortality due to those viruses was observed, undermining the growing importance of norovirus infections in this age group. Human histo blood group antigens (HBGAs) present in the intestinal mucosa have been described as potential ligands and/or cofactors required in the pathogenic stages of infections by these viruses, influencing the molecular epidemiology and evolution in different populations. This study aimed to associate the susceptibility profile (HBGA/Secretor/Lewis) of children living in the community of Manguinhos, Rio de Janeiro, with the efficacy of the RV1 vaccine response (Rotarix[®]/G1P[8]), as well as with natural norovirus infections. For this purpose, RVA and norovirus infections were monitored in a cohort of children aged 0-11 months old by the detection and molecular characterization of these viruses in stool samples of symptomatic or asymptomatic children. From 2014 to 2018, 132 children were included in this study, providing the same number of saliva samples to determine the susceptibility profile by ELISA, and 569 stool samples for investigation of both viruses by molecular methods. The vaccine virus shedding was characterized in 78% (85/109) of RVA samples. The presence of the F167L mutation (RV1), demonstrated by sequencing of the gene VP8*, was observed in 20.1% of children. Four cases of rotavirus by genotypes G3P[8], G12P[8] and G3P[9] were observed. Noroviruses were detected in 21.2% (28/132) of children, with an incidence of 5.8 infections in 100 child-months. The detection rate was 5.6% (17.1% of diarrheal cases and 4.7% of asymptomatic cases), with six different genotypes detected (GII.4_Sydney_2012[P31], GII.4_Sydney_2012[P16], GII.4_Sydney_2012[P4_New_Orleans_2009], GII.2[P16], GII.6[P7] and GI.3[P13]). Regarding the HBGA profile, 80.3% of the children with secretor status were observed. The characterization of the *FUT2* (*Se*) gene in Le (a+b+) and non-secretor children, and the *FUT3* (*Le*) gene in Le (a-b-), allowed the identification of new mutations in this population. The F167L mutation in RV1 shedding was identified in 86.4% of children who presented the Le (a+b+) secretor phenotype, indicating that HBGA could be important marker in RV1 assessment; significant association between symptomatic norovirus infection and secretor status (Le^b) was also observed. The occurrence of norovirus in the cohort and its increasing epidemiological importance determined the expansion of this study by analyzing 61 stool samples from 49 children aged 1-4 years living in Manguinhos. Noroviruses were detected in 47.5% (29/61) of the samples (46.7% of diarrheal cases and 50% of asymptomatic cases) and four new genotypes were identified, with the recombinant GII.2[P16] detected for the first time in Brazil. The epidemiological surveillance data from this study showed the importance of monitoring the main viruses responsible for acute childhood gastroenteritis, highlighting the genetic diversity and dynamics of these viruses considering new approaches such as the HBGA profile, which made an important contribution regarding the evaluation of RV1 efficacy in the cohort studied.

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LISTA DE SIGLAS E ABREVIATURAS

aa - aminoácido

DDA – doença diarréica aguda

ELISA - Do inglês: 'Enzyme-Linked Immunosorbent Assay' – ensaio de imunoabsorção enzimática

EUA – Estados Unidos da América

F167L – substituição do aa fenilalanina pelo aa leucina na posição 167 na proteína VP8*/VP4

Fiocruz – Fundação Oswaldo Cruz

G – genogrupo dos norovírus

GA – gastroenterite aguda

GAPPD - Do inglês: 'Integrated Global Action Plan for the Prevention and Control of Pneumonia and Diarrhoea' – Plano Integrado de Ação Global para Prevenção e Controle de Pneumonia e Diarréia

HBGA - Do inglês: 'Histo-blood group antigens' – antígenos do grupo histosanguíneo

IOC – Instituto Oswaldo Cruz

Leu – leucina

LRRR - Laboratório de Referência Regional em Rotavirose (LRRR)

LVCA - Laboratório de Virologia Comparada e Ambiental

nm - nanômetro

nt - nucleotídeo

NSP - Do inglês: 'non structural protein' – proteína não estrutural

NTPase – proteína não estrutural do norovírus

OMS - Organização Mundial da Saúde

OPAS - Organização Pan Americana da Saúde

ORF - Do inglês: 'open read frame' – fase aberta de leitura

P2 - Do inglês: 'protruding domain 2' – domínio protuberante 2

PAGE – Do inglês: 'polyacrilamide gel electrophoresis' - eletroforese em gel de poliacrilamida.

PCR - Do inglês: 'Polymerase Chain Reaction' – reação em cadeia da polimerase

Phe - fenilalanina

PNI - Programa Nacional de Imunização

RdRp - Do inglês: 'RNA-dependent RNA polymerase' – RNA polimerase dependente de RNA

RNA - Do inglês: 'ribonucleic acid' – ácido ribonucleico

RT-PCR - Do inglês: 'Reverse Transcription Polymerase Chain Reaction'- reação em cadeia da polimerase após transcrição reversa

RT-qPCR - Do inglês: 'Reverse Transcription quantitative Polymerase Chain Reaction' - reação quantitativa em cadeia da polimerase após transcrição reversa

RVA - Rotavírus da espécie A

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

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

S - Do inglês: 'shell domain' – domínio em concha

SNP – Do inglês: 'single nucleotide polymorphism' – polimorfismo de um único nucleotídeo

VLP - Do inglês: 'virus-like particles' – partículas semelhantes a vírus

VP - Do inglês: 'viral protein' – proteína viral

WHO - Do inglês: 'World Health Organization'

INTRODUÇÃO

1.1 A gastroenterite aguda

A gastroenterite aguda (GA) é uma importante causa de morbimortalidade, sendo a morbidade de etiologia viral similar em países desenvolvidos e em desenvolvimento, enquanto a mortalidade é elevada nestes últimos, sendo a quinta causa em <5 anos de idade, com uma estimativa de 446.000 mortes/ano (GBD, 2018).

A diarreia é um dos principais sintomas da infecção do trato intestinal, definida como o aumento do número de evacuações (≥ 3) e alteração de consistência (aquosa ou semi aquosa), podendo ser causada por uma variedade de agentes (bacterianos, virais ou parasitológicos) (WHO, 2017).

Dentre os agentes etiológicos virais, os rotavírus e os norovírus são os mais importantes, em termos de saúde pública mundial (Patel et al, 2008, Tate et al, 2016, Bányai et al, 2018). É uma doença prevenível e tratável, e esforços globais vêm sendo conduzidos na estratégia de redução da gravidade da doença e assim, diminuição dos índices de mortalidade. Dentre estas ações, o plano em vigor *GAPPD* (do inglês: *'Integrated Global Action Plan for the Prevention and Control of Pneumonia and Diarrhoea'*) visa o alcance de < 1 morte por diarreia a cada 1.000 nascimentos, até o ano de 2025 (WHO, 2019).

1.2 Rotavírus A (RVA)

Os rotavírus pertencem à família *Reoviridae*, gênero *Rotavirus* sendo uma população geneticamente diversa de vírus de RNA fita dupla (RNAfd). As partículas virais são esféricas, não envelopadas, com aproximadamente 100 nm de diâmetro (Figura 1A) e capsídeo com simetria icosaédrica, sendo formado por uma tripla camada protéica (Figura 1B) (Estes & Greenberg, 2013).

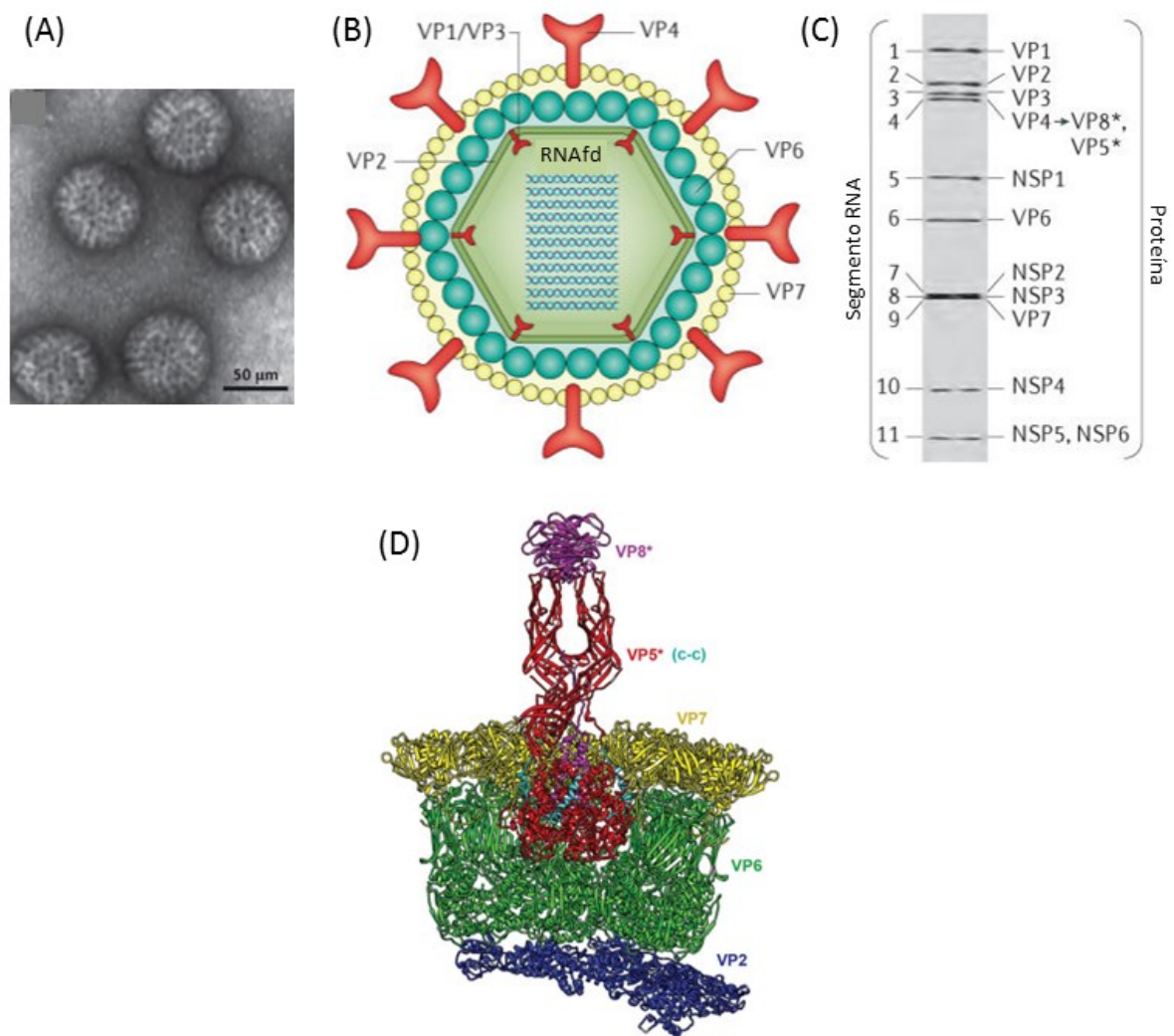


Figura 1: Estrutura dos rotavírus. (A) Partícula viral visualizada através da técnica de microscopia eletrônica. (B) Representação esquematizada do vírion, com a tripla camada protéica, apresentando as duas proteínas do capsídeo externo, VP4 (espícula viral, em vermelho) e VP7 (glicoproteína, em amarelo) e o genoma segmentado, localizado internamente. (C) Migração eletroforética (PAGE) dos segmentos do RNAfd do rotavírus símio (SA11) e proteínas codificadas. (D) Interações da proteína VP4 com as demais proteínas. O subdomínio VP8* (VP4) é a região hipervariável, que interage com o hospedeiro; o subdomínio VP5* está ancorado na proteína VP6 (camada intermediária, em verde) (Adaptado de Settembre et al, 2011 e Crawford et al, 2017).

Seu genoma, segmentado, é constituído por 11 genes, podendo codificar seis proteínas estruturais VP1-6 (*VP*, do inglês: 'viral protein') e de cinco a seis proteínas não estruturais NSP1-5/6 (*NSP*, do inglês: 'non structural

protein) (Figura 1C), devido ao 11^o segmento ser bicistrônico (podendo codificar até duas proteínas, NSP5/NSP6) (Mattion et al, 1991). Com base na antigenicidade da proteína VP6 (presente no capsídeo intermediário), oito espécies de rotavírus têm sido descritas (A-H), e duas novas espécies recentemente identificadas em cães e morcegos (I e J, respectivamente) têm sido propostas (Mihalov-Kovács et al, 2015, Bányai et al, 2017). Dentre elas, a espécie A (RVA) é a de maior importância epidemiológica em humanos e animais (Dóro et al, 2015).

Os rotavírus acometem uma diversidade de hospedeiros, dentre eles aves e mamíferos, incluindo o homem, sendo a transmissão interespecífica importante contribuição para diversidade e evolução (Estes & Greenberg, 2013, Steyer et al, 2013, Soma et al, 2013). Os principais mecanismos evolutivos descritos são: a acumulação de mutações pontuais (*drift*), que podem ocorrer continuamente em todos os segmentos genômicos, devido à atividade da RNA polimerase (Blackhall et al, 1996) e aos *reassortments* genéticos, evento que acontece nas coinfeções (Iturriza-Gómara et al, 2001).

As principais classificações dos rotavírus têm como base, principalmente, as sequências nucleotídicas dos genes que codificam para as duas proteínas do capsídeo externo viral, além da análise das sequências dos demais genes. A primeira delas, conhecida como classificação binária, é a predominantemente descrita em estudos epidemiológicos, utilizando para isto a caracterização dos genes que expressam as proteínas VP7 e VP4, em genótipos G (Glicoproteína) e P (sensível a Protease), respectivamente. Esta última proteína é composta pelos subdomínios VP8* e VP5* (Figura 1D), sendo proteoliticamente clivada no trato gastrointestinal, o que aumenta a infectividade da partícula viral, permitindo a interação inicial e penetração na célula do hospedeiro. Ambas as proteínas do capsídeo externo contêm epítomos neutralizantes, induzindo a produção de anticorpos em seus hospedeiros (Estes & Greenberg, 2013).

A segunda baseia-se na caracterização nucleotídica do genoma completo dos RVA, denominada 'constelação gênica' (VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/NSP6). Cada letra atribui um segmento genômico e o número arábico o atual genótipo caracterizado, sendo até o

momento, os seguintes genótipos descritos: 36G, 51P, 26I, 22R, 20C, 20M, 31A, 22N, 22T, 27E e 22H (RCWG, 2019). Com base nesta classificação, temos os seguintes genogrupos descritos: 1 (*Wa-like*): I1-R1-C1-M1-A1-N1-T1-E1-H1, incluindo a maioria dos genótipos humanos G1/G3/G4/G9/G12-P[8]; 2 (*DS-1-like*): G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2; e 3 (*AU-1-like*): G3-P[9]-I3-R3-C3-M3-A3-N3-T3-E3-H3 (Matthijnssens et al, 2008, 2011).

Uma característica fundamental na epidemiologia dos RVA é seu padrão marcadamente sazonal; em climas temperados, a infecção por RVA ocorre nos meses mais frios e secos do ano (outono e inverno) e, em climas tropicais, as taxas tendem a ser igualmente distribuídas ao longo do ano. No Brasil, a sazonalidade do RVA é variável, com aumento da incidência entre os meses de maio a setembro (período mais frio e seco) nos Estados das regiões Central, Sul e Sudeste; nas Regiões Norte e Nordeste do país, a ocorrência se distribui de maneira uniforme durante todo o ano (Luchs & Timenetsky, 2016).

Infecções por RVA são adquiridas principalmente por via fecal-oral, incluindo fômites e contato pessoa a pessoa ou com objetos contaminados; a transmissão pelo consumo de água ou alimentos contaminados pode ocorrer, mas é rara (Estes & Greenberg, 2013).

O espectro clínico da rotavirose é amplo, variando de um quadro assintomático até uma diarreia grave e vômito causando desidratação, desequilíbrio eletrolítico, acidose metabólica, choque e morte (ou seja, múltiplos mecanismos fisiopatológicos estão envolvidos no processo). Em casos típicos, após o período de incubação de 1 a 3 dias, a doença tem um início abrupto, com febre ($\geq 39^{\circ}\text{C}$) e vômito (< 24 horas) seguidos de diarreia aquosa profusa não sanguinolenta. Estudos indicam que a enterotoxina viral dos rotavírus (NSP4), poderia desencadear a diarreia caracterizada como secretória (pelo efluxo exacerbado para o lúmen intestinal dos íons cloreto e de água) (Linhares et al, 2015, Santos & Soares, 2015).

Não existem antivirais específicos contra a infecção pelos RVA; a terapia de reidratação oral e/ou intravenosa para manutenção do equilíbrio osmótico e de eletrólitos permanece como base no tratamento. Os sintomas gastrointestinais costumam desaparecer dentro de 3 a 7 dias, mas podem perdurar por até 2 a 3 semanas. Embora, na maioria dos casos a recuperação

seja completa, podem ocorrer fatalidades, principalmente em crianças ≤ 1 ano de idade (Linhares et al, 2015, Santos & Soares, 2015). Recentes evidências indicam que, em mais de 2/3 das crianças com quadro de gastroenterite grave apresentaram a presença de rotavírus no sangue (antigenemia), indicando que os rotavírus poderiam infectar sítios extraintestinais, devido ao quadro de infecção viral sistêmica (CDC, 2015, Barakat et al, 2016).

1.2.1 Vacinas de RVA

Em 2006, a Organização Pan Americana da Saúde (OPAS) e a Organização Mundial da Saúde (OMS) declararam como prioridade a introdução de uma vacina para RVA nos Programas Nacionais de Imunização (PNI) nas Américas, com a meta de prevenir mortes e hospitalizações causadas por estes vírus (WHO, 2009). Duas vacinas foram licenciadas: Rotarix[®] (RV1, GlaxoSmithKline, Rixensart, Belgium) e Rotateq[®] (RV5, Merck Inc., USA), e atualmente, já foram introduzidas em mais de 100 países no mundo. Até o final de 2018, 74 países utilizavam a RV1 em seus PNIs, 14 países a RV5, e em 9 países ambas (ROTA council, 2019).

A RV1 é uma vacina monovalente (genótipo G1P1A[8]) de vírus vivo atenuado, derivada da cepa parenteral 89-12, isolada em Cincinnati, Ohio, EUA, a partir de uma criança doente infectada pelo rotavírus em 1989. A atenuação do vírus foi realizada após diluição limitante e passagem contínua em células, resultando na vacina RIX4414 (RV1) (Bernstein et al, 1998). Após um grande estudo clínico de fase III (Ruiz-Palacios et al, 2006), foi verificada a eficácia vacinal em relação à gravidade das diarreias, propiciando proteção tanto homo- como heterotípica (Vesikari et al, 2007). No Brasil, a RV1 foi introduzida no calendário nacional de imunizações em março de 2006, sendo administrada em duas doses, aos 2 e 4 meses de idade.

A segunda vacina, RV5, é pentavalente, originada a partir de reestruturações genéticas entre amostras humana (P[8]) e bovina (cepa WC3 G6P7[5]), contendo os híbridos G1P[5], G2P[5], G3P[5], G4P[5] e G6P[8] (Vesikari et al, 2006, O'Ryan et al, 2015). A RV5 também apresentou

resultados satisfatórios de eficácia em estudos clínicos de fase III, sendo administrada em três doses, aos 2, 4 e 6 meses de idade (Vesikari et al, 2006). A RV5 está licenciada no Brasil, estando disponível na rede particular de vacinação.

No Brasil, o número de internações por diarreias pelos RVA (CID10A080) (Figura 2) e a taxa de mortalidade infantil após a introdução da RV1 foram significativamente reduzidos (Leite et al, 2008, do Carmo et al, 2011, Linhares & Justino, 2014, DATASUS, 2018), assim como evidenciado em outros países do mundo, com ambas as vacinas (RV1/RV5) (Sánchez-Uribe et al, 2016, Troeger et al, 2018, Burke et al, 2019, Mwenda et al, 2019). A eficácia vacinal pode ser comprovada em crianças < 1 ano nos países da América Latina e Caribe, variando de 75,4-81,8% para a RV1 e 76,1-88,8% para a RV5 (de Oliveira et al, 2015).

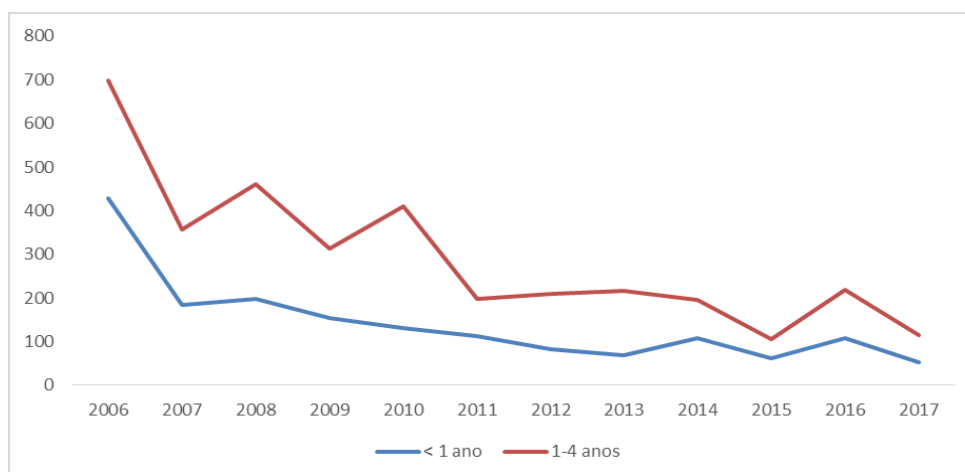


Figura 2: Internações por diarreia associadas ao RVA (CID10A080) em crianças <5 anos no Brasil, de 2006 a 2017 (DATASUS, 2018).

Mundialmente, são seis os genótipos de RVA prevalentes: G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] e G12P[8] (Doró et al, 2014). Após o advento de ambas as vacinas, o genótipo G2P[4] reemergiu e tornou-se o genótipo prevalente no período de 2006 a 2011; quando foi substituído pelos genótipos G3P[8] e G9P[8]. Em 2014, o genótipo G12P[8] emergiu mundialmente, sendo

o principal genótipo detectado em crianças e adultos com GA, incluindo o Brasil (Figura 3) e países que tenham ou não introduzido uma das vacinas em seus PNI (Carvalho-Costa et al, 2009, Cilla et al, 2012, Stupka et al, 2012, Luchs & Timenetsky, 2016, Luchs et al, 2016, da Silva et al, 2017, Carvalho-Costa et al, 2019).

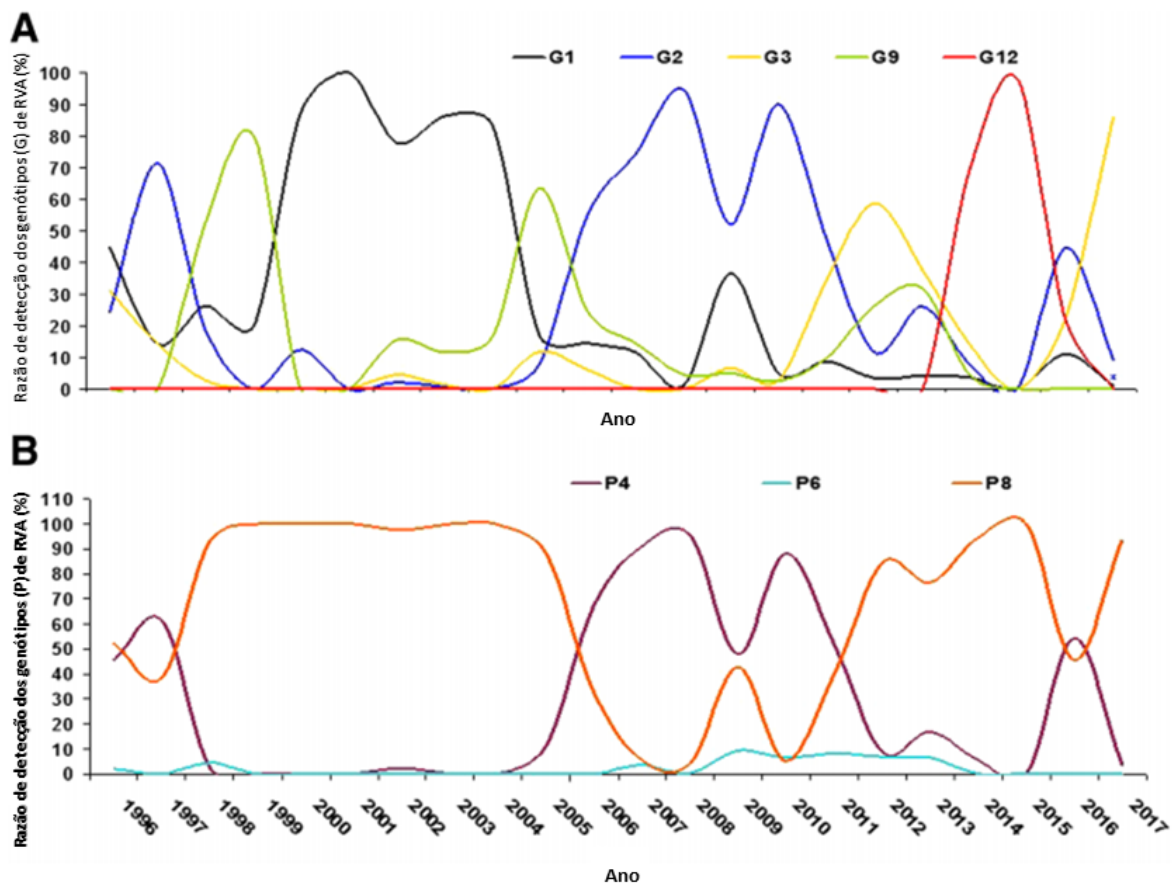


Figura 3: Gráficos das frequências dos genótipos G (A) e P (B) durante os 20 anos de vigilância dos RVA (período de 1996 a 2016, incluindo as eras pré/pós vacina RVA), em 22 Estados do Brasil. Dados do monitoramento realizado pelo Laboratório de Referência Regional em Rotavírus (LRRR)/Laboratório de Virologia Comparada e Ambiental (LVCA) do Instituto Oswaldo Cruz (IOC) (Adaptado de Carvalho-Costa et al, 2019).

Rearranjo genético entre cepas selvagem e vacinal foram descritos para G1P[8]/RV5 na Nicarágua (Bucardo et al, 2012) e Finlândia (Hemming & Vesikari, 2014), e de G1P[8]/RV1 no Brasil (Rose et al, 2013). Até o momento não existem evidências de que as vacinas estejam relacionadas com a flutuação de genótipos de RVA, ou seja, favorecendo uma pressão seletiva

(Dóro et al, 2014). Contudo, especula-se que as vacinas atenuadas orais (RV1/RV5) com constelação gênica *Wa-like*, poderiam influenciar na dinâmica das constelações *DS-1-like* e *AU-1-like* (Leite et al, 2008, Heylen et al, 2014).

Recentemente duas vacinas orais atenuadas foram pré-qualificadas pela OMS, em 2018, e licenciadas para uso exclusivo na Índia: Rotavac[®] (Bharat Biotech, Hyderabad, Índia) e Rotasiil[®] (Serum Institute of India, Pune, Índia). A Rotavac[®] é uma vacina monovalente de genótipo G9P[11], derivada da cepa 11E6 neonatal humana, enquanto a Rotasiil[®] é uma vacina pentavalente, liofilizada, originada do *reassortment* humano-bovino (G1-4 e G9), sendo administrada em 3 doses, em 6, 10 e 14 semanas de vida. Outras duas vacinas orais nacionalmente licenciadas são a Rotavin-M1 (POLYVAC), genótipo G1P[8] (cepa HK0118-2003), comercialmente vendida no Vietnã e a LLR-85 'Lanzhou Lamb Rotavirus' (Lanzhou Institute of Biological Products, Lanzhou, China), de vírus vivo atenuado, derivada de uma cepa de cordeiro de genótipo G10P[15], isolada em 1985, está sendo utilizada na China. Contudo ainda não há dados disponíveis sobre a eficácia destas últimas vacinas. Além disso, atualmente alguns protótipos vacinais de uso parenteral, compostos de subunidades não replicantes (VLPs, *do inglês: 'virus-like particles'*), ou por vírus inativado, estão em desenvolvimento (Burke et al, 2019).

1.3 Norovírus

Os norovírus pertencem à família *Caliciviridae*, gênero *Norovirus*, sendo constituídos por um genoma de RNA fita simples linear de polaridade positiva. São vírus não envelopados, de simetria icosaédrica e com aproximadamente 27-40 nm de diâmetro (Figura 4A e 4B). Seu genoma é dividido em três fases abertas de leitura (ORF *do inglês: 'open read frame'*) (Figura 4C), sendo a ORF1 responsável por codificar uma poliproteína que, após clivagem, dará origem às proteínas não estruturais (NS1-2/p48, NS3-NTPase, NS4-p22, NS5-VPg, NS6-Protease e NS7-RdRp); a ORF2 expressará a proteína estrutural principal VP1 (responsável pela formação do capsídeo viral), composta pelos subdomínios S (*do inglês: 'shell'*), P1 e P2, sendo este último altamente

variável, e ORF3, a proteína estrutural secundária VP2, que se localiza na região mais interna do capsídeo viral (Green, 2013).

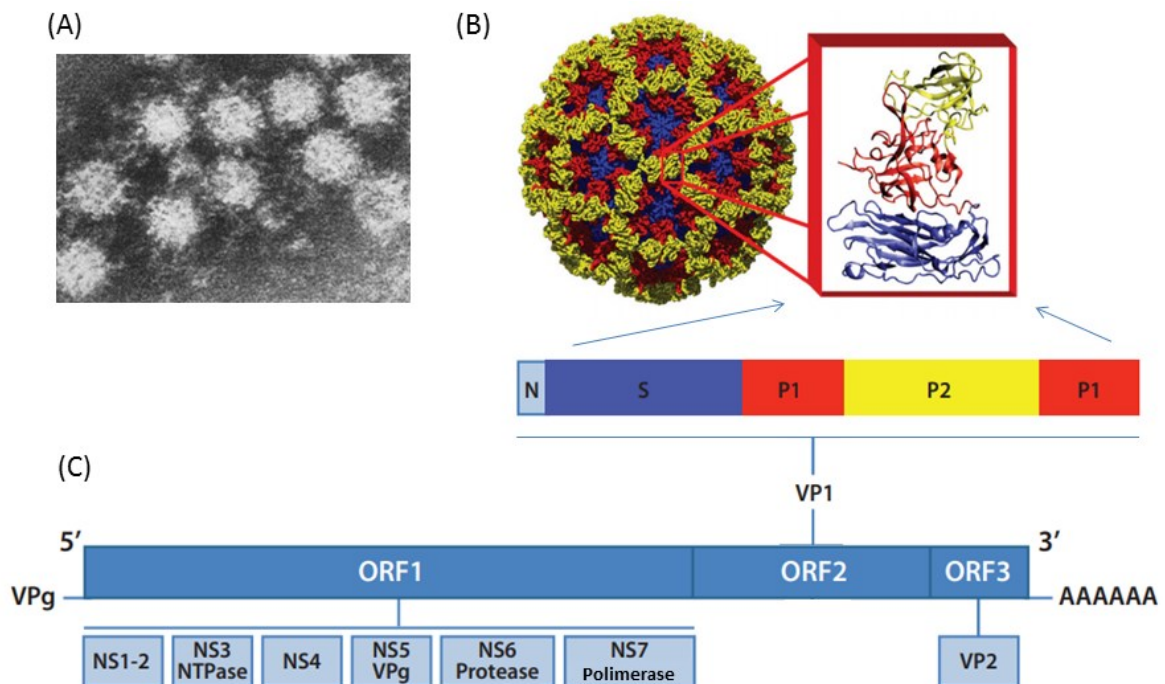


Figura 4: Estrutura dos norovírus humanos. (A) Partícula viral visualizada através da técnica de microscopia eletrônica. (B) As proteínas VP1 estão organizadas em simetria icosaédrica para formação do capsídeo viral dos norovírus. O monômero da VP1 está ampliado, apresentando os subdomínios: P2 (em amarelo, região mais externa e hipervariável), P1 (vermelho) e S (azul). (C) Organização do genoma e proteínas não estruturais (NS) e estruturais codificadas (VP1/2) (Adaptado de Kapikian et al, 1972, Robilotti, 2015 e Lopman et al, 2015).

Com base na sequência nucleotídica do gene que codifica a proteína VP1, o gênero *Norovirus* possui dez genogrupos (GI-GX) (Figura 5), sendo GI e GII os responsáveis pela maioria das doenças em humanos (Kroneman et al, 2013, Vinjé, 2015, Chhabra et al, 2019, Parra, 2019). A classificação binária dos norovírus é dada de acordo com a caracterização molecular da polimerase (RdRp, 3' ORF1, genótipo tipo P) e do capsídeo viral (região S, 5' ORF2) (Kroneman et al, 2013) (Figura 6). De acordo com esta classificação, até o momento já foram descritos 60 genótipos tipo-P e 49 genótipos do capsídeo (Figura 7) (Chhabra et al, 2019).

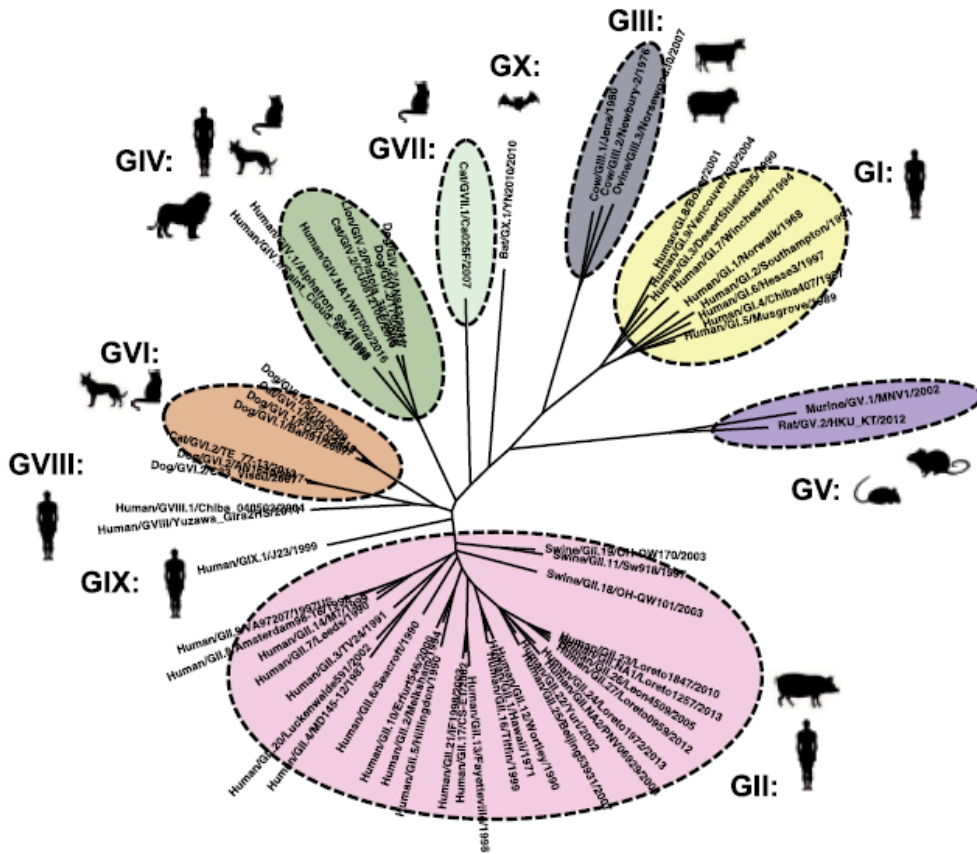


Figura 5: Classificação filogenética dos norovírus, apresentando os 10 genogrupos caracterizados, baseado nas sequências aminoacídicas de VP1. (Adaptado de Parra, 2019).

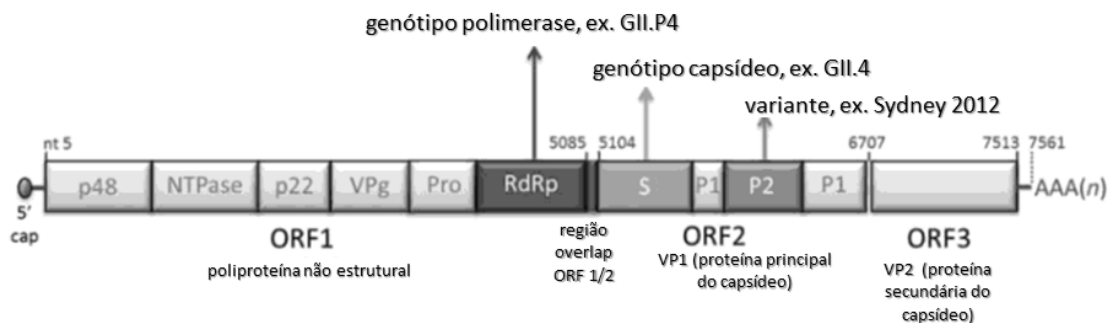


Figura 6: Representação do genoma dos norovírus, com as regiões de genotipagem em destaque: RdRp (polimerase)/ORF1, região S (shell) e P2/ORF2 (Adaptado de Brown & Breuer, 2017).

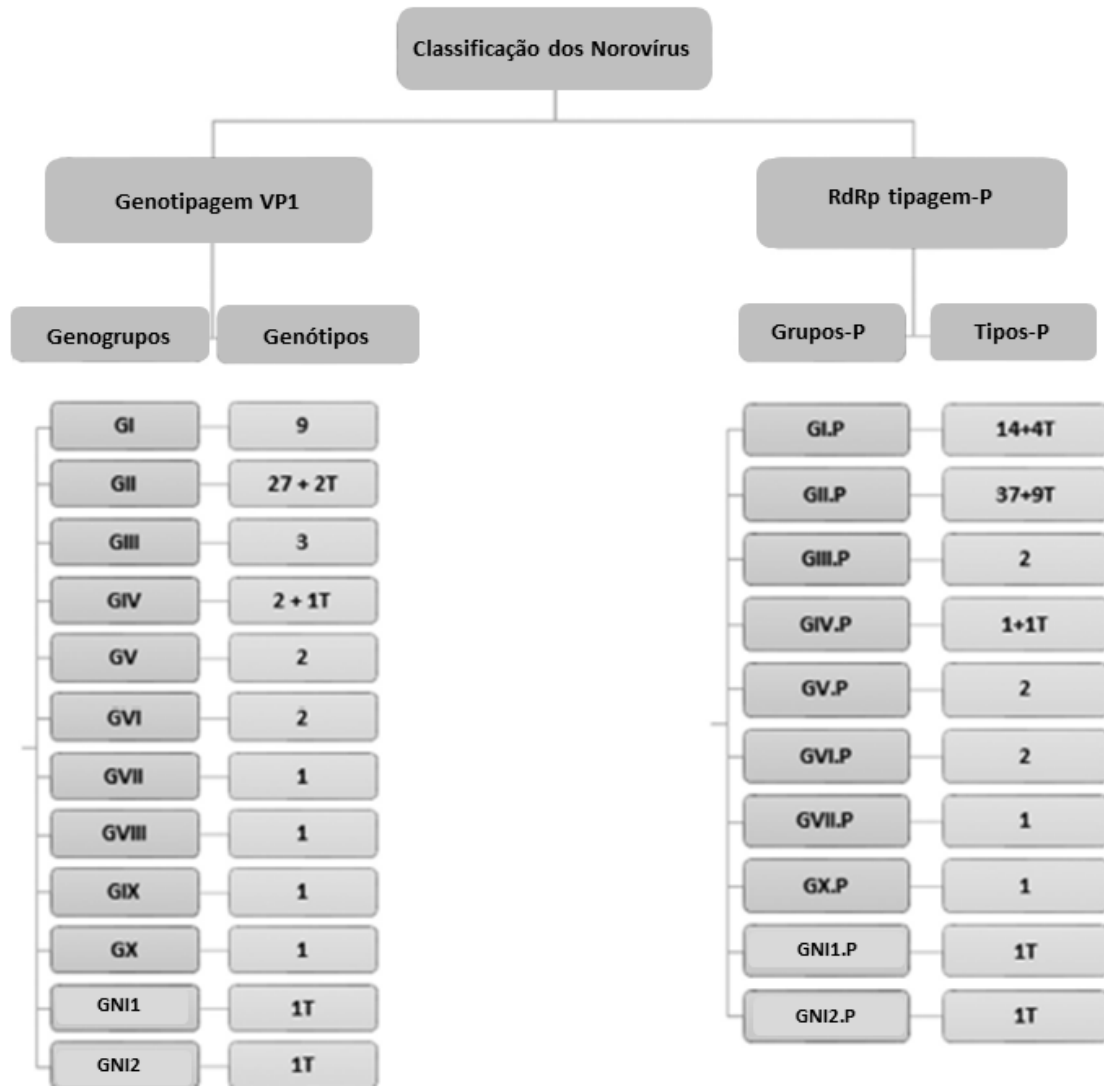


Figura 7: Atual classificação dos norovírus baseada nas sequências aminoacídicas de VP1 (genogrupos/genótipos) e região parcial da polimerase (RdRp) (Grupos-P/Tipos-P) (GNI= Genogrupos/Grupos-P não identificados) (Adaptado de Chhabra et al, 2019).

Após a introdução das vacinas de RVA, as noroviroses têm emergido como principal causa das GA infantis (Figura 8) (Koo et al, 2013, Lopman et al, 2016, Kamioka et al, 2019). Esses vírus estão associados com 18% de todos os casos de GA em < 5 anos de idade, com aproximadamente 212.000 mortes, principalmente nos países em desenvolvimento (Ahmed et al, 2014, Pires et al, 2016). Além disso, a crescente melhoria nas metodologias de detecção moleculares vem propiciando avanços no sistema de vigilância global (Glass et al, 2000).

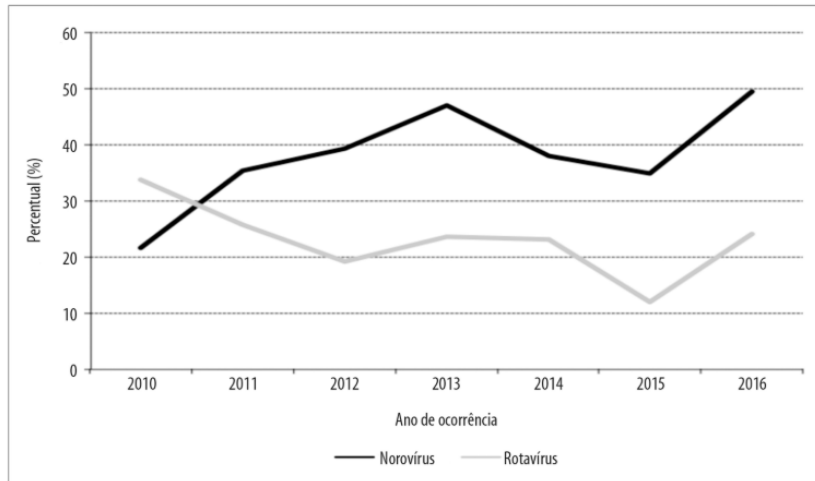


Figura 8: Vigilância das GA no município de São Paulo, durante o período de 2010-2016 (Kamioka et al, 2019).

A facilidade com que esses vírus são transmitidos, a baixa dose infectante, e a estabilidade no ambiente propiciam extensos surtos, especialmente em locais confinados. Os norovírus infectam pessoas de todas as idades, tendo um período de incubação de 24-48h. A infecção é caracterizada pelos sintomas de náuseas, vômitos (podendo ser em jatos), dores abdominais, febre e diarreia não sanguinolenta. O vômito geralmente é o sintoma mais prevalente em adultos, enquanto a diarreia frequentemente acomete crianças (Patel et al, 2009). Geralmente os sintomas se resolvem em 2-3 dias, contudo, a persistente excreção dos vírions pode durar dias, semanas ou meses, sendo esta característica relativamente comum em crianças ou em indivíduos imunocomprometidos (Robilotti et al, 2015).

1.3.1 Diversidade genética dos norovírus

Os norovírus são vírus geneticamente diversos, e mutações pontuais (*drift*) na região hipervariável do capsídeo viral (subdomínio P2) e eventos de recombinação na região *hostspot* ORF1-2 (entre vírus de mesmo genótipo ou diferentes), contribuem para sua evolução e diversidade genética (Eden et al, 2013, Kroneman et al, 2013, White, 2014, Vinjé, 2015).

O GII.4 têm sido o genótipo predominante, circulando por mais de duas décadas em casos epidêmicos e/ou esporádicos de GA no Brasil (Fioretti et al, 2011, Fioretti et al, 2014, Costa et al, 2017) e no mundo (Siebenga et al, 2009, Vinjé, 2015, Cannon et al, 2017). Em média, a cada 2-7 anos, novas variantes GII.4 surgem e desde 2012, a variante dominante é a GII.4 Sydney (Siebenga et al, 2007, Kroneman et al, 2013). Contudo, outros genótipos incomuns, como GII.17 e GII.2, têm emergido recentemente e ocasionado surtos em diversos países (da Silva et al, 2017, Andrade et al, 2017, Tohma et al, 2017, da Silva Ribeiro de Andrade et al, 2018, Sang & Yang, 2018).

Estudos conduzidos nesta última década no Brasil apontam a diversidade de genótipos circulando nas diferentes regiões do país, sendo comumente detectados, além dos genótipos anteriormente citados, também GII.6, GII.3 e GII.12 (Sá et al, 2015, Fumian et al, 2016, Costa et al, 2017, Santos et al, 2017, Gondin et al, 2018, Dábilla et al, 2019).

Os norovírus GII são os prevalentes em todo mundo, enquanto GI é detectado associado na maioria dos casos assintomáticos (Fioretti et al, 2011, Galeano et al, 2013, de Andrade et al, 2014, Vega et al, 2014). Atualmente, não existem vacinas licenciadas para os norovírus, contudo alguns diferentes protótipos estão em desenvolvimento. Neste contexto, o conhecimento epidemiológico sobre a GA associada ao norovírus, bem como a diversidade viral e os mecanismos de evolução são importantes para direcionar o desenvolvimento e introdução de uma vacina eficaz (Bartsch et al, 2016, Riddle & Walker, 2016, Tohma et al, 2017). Recentemente, um estudo de Fase IIb da vacina de norovírus bivalente (GI.1 e GII.4), composta por VLPs, está em andamento (Mattison et al, 2018).

1.4 Epidemiologia dos RVA e norovírus no Rio de Janeiro

Em relação à epidemiologia dos RVA e norovírus no Rio de Janeiro, ao longo das últimas décadas, diversos trabalhos descrevem o monitoramento através de estudos clínicos e ambientais.

Alguns destes estudos contemplaram um panorama de vigilância dos RVA em hospitais sentinela no Rio de Janeiro, com avaliação de amostras

diarreicas coletadas antes de março de 2006 (período pré-vacina RVA). Dentre eles, o estudo de vigilância dos RVA conduzido por Santos e colaboradores (2003), no período de 1997-1999, apontou a prevalência dos genótipos G1P[8], G2P[4], G3P[8] e G4P[8], além de combinações incomuns, como G9P[8], G9P[6] e G9P[4]. Araújo e colaboradores (2002), no período de 1996-1998 também detectaram os genótipos G2P[4], G1P[8] e G3P[8], confirmando a prevalência destes no mesmo período. O estudo de Volotão e colaboradores (2007), relativo ao período de 2000-2004 evidenciou os genótipos G1P[8], G4P[8], G9P[8] como os mais prevalentes, em concordância ao estudo de Carvalho-Costa e colaboradores (2006), com amostras coletadas em 2004, descrevendo a prevalência do G1P[8] e G9P[8].

Amostras clínicas coletadas no período de fevereiro de 2005 a dezembro de 2007 (períodos pré/pós-vacina) apontaram a reemergência do genótipo G2P[4] (perfil *DS-1-like*), logo após a introdução da RV1 no estudo de Carvalho-Costa e colaboradores (2009). O monitoramento de efluentes de uma estação de tratamento no Rio de Janeiro, realizado no estudo de Fumian e colaboradores (2011), entre 2009-2010, indicou a detecção dos RVA em 100% do esgoto bruto e 71% do esgoto tratado, e predominância do genótipo G2P[4], de acordo com o estudo clínico apresentado por Carvalho-Costa e colaboradores (2009). Em seu último trabalho, Carvalho-Costa e colaboradores (2019) descreveram a vigilância dos RVA realizada no Laboratório de Referência Regional em Rotavíruses (LRRR)/ Laboratório de Virologia Comparada e Ambiental (LVCA), com dados de 22 Estados do Brasil, incluindo Rio de Janeiro, do período de 1996-2016 (Figura 3), corroborando ao panorama mundial de flutuação de genótipos durante as eras pré/pós-vacina RVA.

Em relação aos norovírus, estudos de vigilância em hospitais sentinela, centros de saúde e em creche também foram realizados no Rio de Janeiro (Victoria et al, 2007, Soares et al, 2007, Ferreira et al, 2008, 2010 e 2012) descrevendo a detecção dos norovírus em quadros diarreicos, variando entre 14,5-66% no período de 1994 a 2008. Dentre eles, os trabalhos que realizaram a caracterização molecular da região VP1 dos norovírus, já destacavam a importância da circulação do genótipo GII.4 e suas variantes. Além disso,

Ferreira e colaboradores (2012) descreveram uma grande variabilidade genética (GI.2, GI.3, GI.8 GII.2, GII.3, GII.4 e variantes 2001 e 2006b, GII.6, GII.7, GII.12 e GII.17) dos norovírus circulando nos surtos monitorados durante os 15 anos de vigilância na creche.

Recentemente, Fioretti e colaboradores (2018) e Fumian e colaboradores (2019) descreveram estudos de monitoramento clínico e ambiental (análise de esgoto bruto e tratado) no Rio de Janeiro, entre os períodos de 2012 e 2014. O estudo de Fumian e colaboradores (2019) evidenciou a dominância do genótipo GII.4 Sydney 2012 em amostras de águas residuais (68,5%) e em casos clínicos (71%), além da detecção dos genótipos do capsídeo GII.2, GII.3 e GII.1; contudo, somente os genótipos GII.17 e GII.5 foram detectados em amostras de esgoto bruto. Fioretti e colaboradores (2018) demonstraram a ocorrência do norovírus GIV circulando em amostras clínicas e de esgoto bruto/tratado na região metropolitana do Rio de Janeiro. Estes resultados destacam a importância do monitoramento ambiental como ferramenta estratégica na complementação aos dados clínicos na vigilância epidemiológica destes vírus (Prado & Miagostovich, 2014, Fioretti et al, 2018, Fumian et al, 2019).

1.5 Susceptibilidade genética do hospedeiro às rota- e norovirose

Estudos têm indicado a importância dos fatores genéticos do hospedeiro em relação à susceptibilidade a determinadas infecções ou doenças (Cooling, 2015, Heggelund et al, 2017). Em relação às rota- e norovirose, têm-se verificado esta premissa, mostrando que o grau de susceptibilidade (ou resistência) pode diferir entre os indivíduos (Lindesmith et al, 2003, Tan & Jiang, 2014, Cooling, 2015, Franchini & Bonfani, 2015).

Neste cenário, diversos estudos relacionam os antígenos histosanguíneos humanos (HBGA, do inglês: '*Histo-Blood Group Antigens*') como potenciais ligantes e/ou cofatores requeridos nos estágios iniciais na patogênese das infecções pelos RVA e norovírus (Hutson et al, 2003, Huang et al, 2005, Huang et al, 2012, Liu et al, 2012, Sun et al, 2016).

Os HBGAs são complexos carboidratos (H tipo 1, Le^a, Le^b) presentes na superfície das células epiteliais e secreções biológicas como sangue, leite materno, saliva e mucosa intestinal (Daniels, 2013). Estes glicanos são catalisados por fucosil- e glicosiltransferases, pela adição sequencial de monossacarídeos a um precursor inicial. Essas transferases são codificadas pelos genes *ABO* (H), *FUT2* (Secretor) e *FUT3* (Lewis) (Marionneau et al, 2001). Diversos estudos vêm demonstrando que esta relação HBGAs-vírus é genótipo-dependente, havendo a interação dos subdomínios VP8* (da proteína VP4, espícula viral dos RVA) e P2 (da proteína VP1 do capsídeo principal dos norovírus) com estes açúcares expressos pelo hospedeiro (Hutson et al, 2003, Huang et al, 2012, Liu et al, 2012, Nordgren et al, 2013, Sun et al, 2016).

Os HBGAs são altamente polimórficos e diferem entre as populações no mundo (Marionneau et al, 2001, Ferrer-Admetlla et al, 2009). As interações epistáticas entre os genes *FUT2* (*Se*) e *FUT3* (*Le*) determinam o fenótipo Lewis (Henry et al, 1995) e, principalmente mutações *non-sense* nestes genes interferem com o nível de expressão e atividade das enzimas $\alpha(1,2)$ -fucosiltransferase e $\alpha(1,3/1,4)$ -fucosiltransferase, determinando os fenótipos 'não secretor' e 'Lewis negativo', respectivamente (Kelly et al, 1995, Koda et al, 2001, Le Pendu, 2004).

Ao todo, cinco fenótipos estão distribuídos na população: *Se/Le* (a-b+), *Se/Le* (a-b-), *Se/Le* (a+b+), secretores; enquanto *se/Le* (a-b-) e *se/Le* (a+b-), não secretores (Figura 9 e Tabela 1). Mutações nos genes *ABO*, *FUT2* e *FUT3* estão distribuídas mundialmente como polimorfismos de um único nucleotídeo (SNP do inglês: '*single nucleotide polymorphism*') podendo ser utilizadas como marcadores populacionais (étnicos) (Ferrer-Admetlla et al, 2009). Aproximadamente 20% das populações europeia e norte americana possuem o fenótipo não secretor enquanto o fenótipo Lewis negativo em certas populações da África e América Latina pode ultrapassar 30% comparados aos 4-8% na Europa (Corvelo et al, 2002, Le Pendu, 2004, Nordgren et al, 2013).

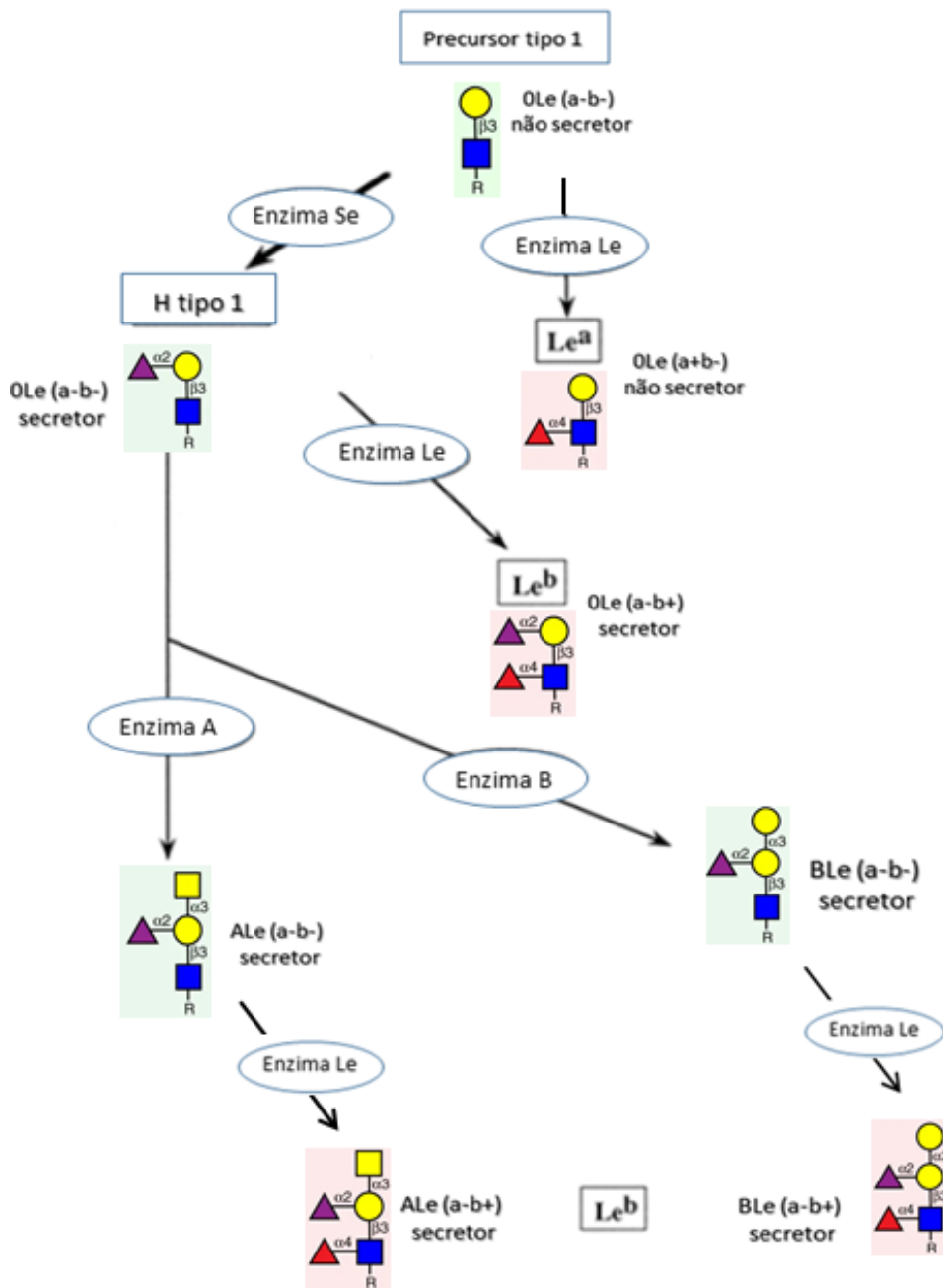


Figura 9: Biossíntese dos antígenos HBGAs/Secretor e Lewis em saliva e fenótipos. Legenda: enzima Se: $\alpha(1,2)$ -fucosiltransferase (FUT2), enzima Le: $\alpha(1,3/1,4)$ -fucosiltransferase (FUT3), enzima A: N-acetilgalactosiltransferase, enzima B: galactosiltransferase (Adaptado de Kudo et al, 1996 e Hu et al, 2018).

Tabela 1: HBGAs sintetizados através da ação combinada das enzimas FUT2 (Se) e FUT3 (Le) no trato gastrointestinal.

Enzima Se (FUT2)	Enzima Le (FUT3)	Carboidratos sintetizados	Fenótipos expressos	Status secretor
ativa* (Se>Le)	ativa	Le ^b	Le (a-b+)	secretor
ativa	ativa* (Le>Se)	Le ^a , Le ^b	Le (a+b+)	secretor
ativa	inativa	H tipo 1	Le (a-b-)	secretor
inativa	ativa	Le ^a	Le (a+b-)	não secretor
inativa	inativa	precursor tipo 1	Le (a-b-)	não secretor

Nota: * maior atividade enzimática.

Os indivíduos definidos como secretores parecem ser mais susceptíveis às infecções do que os indivíduos não secretores. A maioria dos estudos de interação HBGAs-RVA indica que os genótipos P[8] e P[4] infectam preferencialmente indivíduos secretores, enquanto não secretores seriam menos susceptíveis a estes genótipos (Huang et al, 2012, Nordgren et al, 2014, Zhang et al, 2016).

Estudos conduzidos em Burkina Faso e Nicarágua demonstraram que ambos os genes *Se* e *Le* são essenciais para a susceptibilidade às infecções pelo RVA, detectando infecções pelo genótipo P[8] em crianças com o fenótipo Lewis positivo secretor (Le^b), enquanto que o genótipo P[6] predominantemente infectou o fenótipo Lewis negativo, independente do *status* secretor (Nordgren et al, 2014, Tan & Jiang, 2014, Ramani et al, 2016). Além disso, recentes estudos têm investigado se o perfil HBGA poderia contribuir para a eficácia das vacinas orais de RVA (RV1 e RV5), sugerindo que indivíduos secretores desenvolveriam resposta mais eficaz (Kazi et al, 2017, Armah et al, 2019, Bucardo et al, 2019).

Em relação aos norovírus, estudos iniciais demonstraram que indivíduos não secretores são mais resistentes a infecção pelo genótipo GII.4 (Thorven et al, 2005), e a presença do antígeno Lewis poderia não necessariamente conferir susceptibilidade a esta variante (Carlsson et al, 2009, Nordgren et al, 2013). A interação HBGAs-norovírus difere entre genogrupos e genótipos (Huang et al, 2005), podendo afetar a epidemiologia molecular e evolução destes vírus no mundo (Jiang et al, 2017, Nordgren & Svensson, 2019). Com isso, muitas questões associadas aos HBGAs permanecem não respondidas

(Ramani et al, 2016, Cannon et al, 2019, Nordgren & Svensson, 2019), e nesta ótica, é importante que estudos clínicos e de desafio com protótipos vacinais para o norovírus, considerem o perfil secretor para o entendimento do impacto deste *status* na eficácia vacinal (Mattison et al, 2018).

1.6 Justificativa

Com o advento das vacinas RVA, a carga da DDA diminuiu consideravelmente, no Brasil e no mundo. Por outro lado, com as melhorias nas metodologias de detecção, particularmente as moleculares, os norovírus vêm assumindo importante papel como agente etiológico nas GA. A vigilância epidemiológica destes vírus foi ampliada em nível mundial, com harmonização de métodos de detecção e caracterização molecular de genótipos, gerando dados que permitiram análises de epidemiologia molecular e evolutivas destes vírus (Kroneman et al, 2011).

Recentemente, estudos têm relacionado o sistema HBGAs com susceptibilidade à infecção pelos RVA e norovírus, sendo que estudos de polimorfismos genéticos no sistema ABH/Secretor e Lewis foram majoritariamente realizados em populações caucasianas (Europa e América do Norte); portanto, é importante a realização de estudos que abranjam outras etnias (Kindberg et al, 2009, Nordgren et al, 2013, 2014, Currier et al, 2015).

O Brasil, sendo um país com dimensões continentais, diverso, de inúmeros contrastes e miscigenado, apresenta uma grande diversidade populacional (Trennepohl et al, 2014), que pode resultar em diferentes perfis de susceptibilidade genética. O geneticista Sérgio Pena e colaboradores (2000) afirmam: "*Vários autores (...) enfatizaram a natureza tri-híbrida da população brasileira, a partir dos ameríndios, europeus e africanos*". Em seu estudo ressalta que obtiveram respaldo científico a essa noção e acrescentam um importante detalhe: a contribuição europeia foi basicamente por meio de homens e a ameríndia e africana foi principalmente por meio de mulheres. A presença de 60% de matrilineagens ameríndias e africanas em brasileiros brancos é inesperadamente alta e, por isso mesmo tem grande relevância social.

Estudos sobre susceptibilidade genética (HBGAs) estão descritos no país (de Mattos et al, 2002, Corvelo et al, 2013, Moraes et al, 2019), mas poucos relacionam os RVA e/ou norovírus ao perfil de susceptibilidade do hospedeiro (Vicentini et al, 2013, Olivares et al, 2019, resultados não publicados). A realização de novos estudos que determinem a susceptibilidade genética da população brasileira frente às infecções por RVA e norovírus permitirá avaliações destes importantes agentes virais na etiologia das GA no período pós-vacina de RVA.

Considerando que a vacina RV1 para RVA foi introduzida no Brasil em março de 2006 e que diversos estudos demonstram a relevância epidemiológica dos norovírus (de Andrade et al, 2014, Fioretti et al, 2014, Santos et al, 2016, Costa et al, 2017, Gondin et al, 2018, Kamioka et al, 2019), é importante a avaliação dos HBGAs e a potencial relação com as infecções pelos RVA e norovírus. Estudos do tipo coorte referentes à vigilância dos RVA e norovírus no Brasil são raros (Linhares et al, 1989, Siqueira et al, 2017). O estudo realizado nesta tese complementa dados de vigilância epidemiológica, uma vez que permitem uma melhor avaliação dos aspectos das infecções naturais, bem como infecções em casos assintomáticos e sua transmissão, genótipos circulantes, sazonalidade, além da resposta do hospedeiro (Lopman & Kang, 2014, Lopman et al, 2015, Cannon et al, 2019), sendo pioneiro no Brasil.

Recentemente, alguns autores vêm referenciando a importância de se avaliar a relação do perfil do hospedeiro frente à resposta às vacinas de RVA (Desselberger, 2017, Kazi et al, 2017, Jiang et al, 2017, Armah et al, 2019, Bucardo et al, 2019). Especula-se que indivíduos secretores poderiam ter uma melhor resposta vacinal em relação aos não secretores. Avaliar esta relação em diferentes populações torna-se estratégico para uma melhor compreensão e monitoramento da eficácia das vacinas RV1 e RV5, uma década após a introdução das mesmas. Adicionalmente, estes estudos podem contribuir no delineamento de estratégias para o desenho de adequados protótipos vacinais para os norovírus.

Considerando-se a importância dos estudos em comunidades e contribuindo para a melhor compreensão das infecções por RVA, norovírus e

perfil do hospedeiro, este estudo foi realizado em uma coorte infantil, de 0-11 meses de idade, residindo em Manguinhos, Rio de Janeiro. Esta comunidade foi escolhida por estar localizada no entorno da Fiocruz e de acordo com o estudo de Engstrom e colaboradores (2012), apresenta um dos piores índices de desenvolvimento humano da cidade; além disso, dados demonstram um elevado (>10%) percentual de baixo peso ao nascer (<2500g), inclusive em relação a proporção do município (ou seja, alto risco de adoecimento e morte ao recém nascido), e alta taxa de mortalidade infantil (19,6/1.000 nascidos vivos), sendo maior do que a taxa do município do Rio de Janeiro. As doenças de notificação compulsória de maior ocorrência, excetuando a dengue que teve caráter epidêmico, foram: tuberculose (43%), sífilis na gestação (9%) e de Hepatite A (8%). Os casos de Hepatite A demonstram a situação de vulnerabilidade da população em relação às condições de saneamento básico, expondo crianças à doença tanto no domicílio quanto em creches e escolas.

Desta forma, este estudo contemplou a caracterização dos norovírus e os RVA excretados de crianças (0-11 meses de idade) vacinadas com a RV1 e a associação ao perfil de susceptibilidade (HBGA) nesta coorte. Em paralelo, amostras de fezes de crianças residindo em Manguinhos, na faixa etária de 1-4 anos, durante o mesmo período do estudo (2014 a 2018) foram obtidas para investigação dos genótipos de norovírus circulantes nesta faixa etária ampliada. Assim, a avaliação dos vírus vacinais (RV1) excretados e norovírus circulantes nesta comunidade e as possíveis associações com o perfil de susceptibilidade do hospedeiro contribuem com dados para avaliação da eficácia vacinal e dinâmica epidemiológica e evolutiva dos norovírus nestas eras pré-vacina de norovírus e pós-vacina RVA.

2 OBJETIVOS

2.1 Geral

Associar o perfil de susceptibilidade genética (HBGA, Secretor/Lewis) de crianças residentes na comunidade de Manguinhos, Rio de Janeiro, no período de 2014-2018, à eficácia da resposta a vacina RV1 (Rotarix[®], G1P[8]), assim como às infecções naturais por norovírus, contemplando a caracterização dos genótipos detectados nesta população.

2.2 Específicos

1. Determinar os perfis fenotípicos (secretor/não secretor, Lewis positivo/negativo) e genotípicos (identificação de polimorfismos nos genes *FUT2/FUT3*) de crianças de 0 até 11 meses de idade, de uma coorte prospectiva.
2. Caracterizar os genótipos de RVA e norovírus detectados nestas crianças.
3. Avaliar o perfil molecular dos vírus vacinais (G1P[8], RV1) excretados pela análise nucleotídica do gene VP4 (subdomínio VP8*).
4. Relacionar a susceptibilidade das crianças à ocorrência de infecções pelos norovírus e ao vírus vacinal (G1P[8]) excretado.
5. Avaliar a ocorrência e diversidade genética dos norovírus em uma faixa etária ampliada (1-4 anos) de crianças residindo em Manguinhos.

3 METODOLOGIAS E RESULTADOS

As metodologias realizadas e os resultados obtidos neste manuscrito serão apresentados sob a forma de artigos publicados e/ou em fase de submissão em revistas científicas indexadas:

Estudo 1 (artigo submetido): Rotavirus G1P[8] vaccine shedding and HBGA host genetic susceptibility in a birth community-cohort, Rio de Janeiro, Brazil, 2014-2018.

Estudo 2 (artigo em fase final de elaboração): Norovirus infection and HBGA host genetic susceptibility in a birth community-cohort, in Manguinhos, Rio de Janeiro, Brazil.

Estudo 3 (artigo publicado): High genetic diversity of noroviruses in children from a community-based study in Rio de Janeiro, Brazil, 2014-2018.

Rotavirus G1P[8] vaccine shedding and HBGA host genetic susceptibility in a birth community-cohort, Rio de Janeiro, Brazil, 2014-2018

Este estudo está relacionado aos objetivos: 1, 2, 3 e 4.

Submetido à: Scientific Reports, Nature

Resumo: Neste estudo foi realizada a avaliação molecular do gene VP8* de amostras vacinais (G1P[8], RV1) excretadas durante o período vacinal e associação ao perfil de susceptibilidade (HBGAs) de crianças de uma coorte infantil, na comunidade de Manguinhos, durante o período de novembro de 2014 a novembro de 2018. Pode-se observar uma mudança aminoacídica na posição 167 (fenilalanina substituída por uma leucina, F167L) da proteína VP8*/RV1 em 20,5% das crianças acompanhadas no estudo. Nesta coorte, verificamos uma alta frequência de indivíduos secretores, e a mutação F167L na RV1 foi detectada em 86,4% das crianças com fenótipo Le (a+b+) secretor. Nesta população foi possível determinar os genótipos de FUT2 (Secretor) em crianças Le (a+b+) secretoras e não secretoras e a detecção de duas novas mutações neste gene. O estudo destaca que os HBGAs podem ser considerados importantes marcadores para avaliação da eficácia da RV1.

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Abstract	Recent studies have investigated whether the human histo-blood group antigen (HBGAs) profile could affect the effectiveness of the oral rotavirus vaccines, suggesting secretor positive individuals develop a more robust response. We investigated the G1P[8] vaccine (RV1) shedding in association with the host susceptibility profile in children from a birth community-cohort in Rio de Janeiro, Brazil, from 2014 to 2018. A total of 132 children were followed-up between 0 to 11-month-old, stool samples were collected before/after the 1st/2nd RV1 vaccination doses and saliva samples were collected during the study. Rotavirus shedding was screened by RT-qPCR and G/P genotypes determined by multiplex RT-PCR and/or Sanger nucleotide sequencing. The sequencing indicated an F167L amino acid change in the VP8* P[8] gene in 20.5% of children in the RV1 shedding follow-up, and mutant viral subpopulations were quantified by pyrosequencing. The HBGA/secretor status was determined by enzyme immunoassay in saliva, and 80.3% of the children were classified as secretors. Twenty-one FUT2 gene SNPs were identified by Sanger sequencing, and two new mutations were observed. The F167L RV1 was significantly more detected (86.4%) in Le (a+b+) secretors compared to non-secretors. The study highlights the association between RV1 shedding and HBGAs as a marker for evaluating vaccine efficiency.
Techniques	Life sciences techniques, Genomic analysis [DNA sequencing];
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Rotavirus G1P[8] vaccine shedding and HBGA host genetic susceptibility in a birth community-cohort, Rio de Janeiro, Brazil, 2014-2018.

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Abstract

Recent studies have investigated whether the human histo-blood group antigen (HBGAs) profile could affect the effectiveness of the oral rotavirus vaccines, suggesting secretor positive individuals develop a more robust response. We investigated the G1P[8] vaccine (RV1) shedding in association with the host susceptibility profile in children from a birth community-cohort in Rio de Janeiro, Brazil, from 2014 to 2018. A total of 132 children were followed-up between 0 to 11-month-old, stool samples were collected before/after the 1st/2nd RV1 vaccination doses and saliva samples were collected during the study. Rotavirus shedding was screened by RT-qPCR and G/P genotypes determined by multiplex RT-PCR and/or Sanger nucleotide sequencing. The sequencing indicated an F167L amino acid change in the VP8* P[8] gene in 20.5% of children in the RV1 shedding follow-up, and mutant viral subpopulations were quantified by pyrosequencing. The HBGA/secretor status was determined by enzyme immunoassay in saliva, and 80.3% of the children were classified as secretors. Twenty-one *FUT2* gene SNPs were identified by Sanger sequencing, and two new mutations were observed. The F167L RV1 was significantly more detected (86.4%) in Le (a+b+) secretors compared to non-secretors. The study highlights the association between RV1 shedding and HBGAs as a marker for evaluating vaccine efficiency.

Introduction

After implementation of the rotavirus vaccines, Rotarix[®] (RV1, GlaxoSmithKline Biologicals, Rixensart, Belgium) and RotaTeq[®] (RV5, Merck Inc., USA), in over 100 countries worldwide¹, the burden of severe group A rotaviruses (RVA) diarrhea has decreased substantially, with reductions in hospitalizations and deaths in many countries, including Brazil²⁻⁶. However, RVA still are one of major causes of severe viral diarrhea in infants and young children <5-years-old worldwide^{6,7}.

Due to the zoonotic potential, variability of the RVA strains and host genetic factors, the surveillance of circulating RVA strains is necessary for evaluating and monitoring the effectiveness of the implemented immunization programs, mainly in low-income countries⁸. Currently, 36 G- and 51 P- genotypes have been described⁹ and globally, six G/P combinations are the most prevalent in humans: G1P[8], G2P[4], G3P[8], G9P[8], G4P[8] and G12P[8]¹⁰.

Studies involving the histo-blood group antigens (HBGA) have shown the importance of the VP8* domain (subunit of the VP4 protein of RVA) in the early stages of the pathogenesis of RVA infection¹¹⁻¹³. HBGAs are complex glycans (type 1 H, Le^a, Le^b) present on cell surfaces and in biological fluids, such as blood, breast milk, saliva, and intestinal mucosa¹⁴. These glycans are catalyzed by glycosyltransferases, through sequential addition of monosaccharides to an initial precursor; these transferases are encoded by the *ABO*, *FUT2* (Secretor) and *FUT3* (Lewis) genes¹⁵. Secretor status is a host susceptibility factor in several infectious diseases, such as viral gastroenteritis by RVA and noroviruses, and gastritis-ulcers by *H. pylori*¹⁶.

The HBGAs are highly polymorphic and differ across populations worldwide^{15,17}. The epistatic interaction between *FUT2* (*Se*) and *FUT3* (*Le*) genes determines the Lewis phenotype¹⁸ and, principally, missense mutations in these genes interfere with the level of expression and activity of the $\alpha(1,2)$ -fucosyltransferase and $\alpha(1,3/1,4)$ -fucosyltransferase enzymes, determining 'non-secretor' and 'Lewis negative' Le (a-b-) phenotypes, respectively¹⁹⁻²¹. The HBGA genes' mutations distributed worldwide as single nucleotide polymorphisms (SNPs) work as identity markers favoring certain conditions of susceptibility or resistance to infections or disorders.

In most studies on interaction between HBGAs and RVAs, the P[4] and P[8] VP8* genotypes preferentially infect the secretor and/or (Le^b) individuals²²; while non-secretor individuals (Le^a) were less susceptible to these genotypes²³⁻²⁵. Recent studies have investigated

whether the HBGA profile could contribute to the effectiveness of oral RVA vaccines²⁶⁻³¹, suggesting secretor positive individuals develop a more robust response²⁶⁻²⁸.

This prospective study aimed to follow newborns up to < 1-year of age in a low-income community-cohort in Manguinhos, Rio de Janeiro, Brazil, vaccinated with RV1 between November 2014 and November 2018, in order to assess the G1P[8] vaccine shedding in association to HBGA profile.

Results

Sampling of the children from Manguinhos community, Rio de Janeiro The 132 children were followed for 16,212 child-days, ranging from 7-285 days, with 79% monitored up to at least 90 days. A total of 569 stool samples were collected and a median of five samples were obtained per child (no less than two samples/per child) and 132 saliva samples were obtained (1 sample/per child). Gender distribution was 50.8% (67) male and 49.2% (65) female.

Rotavirus A and G1P[8] vaccine shedding A total of 19.2% (109/569) of stool specimens were positive for RVA by one-step reverse transcription-quantitative polymerase chain reaction (RT-qPCR), corresponding to 62.1% (82/132) of all children enrolled in this prospective study. By age range analysis, the highest RVA detection rate (39.3%, 92/234) was observed in children aged between 2 and 5 months (vaccination period) (Table 1). RVA acute diarrheic episodes (ADE) and non-ADE cases corresponded to 26.8% (11/41) and 18.5% (98/528) of stool samples respectively, and the G1P[8] was the most prevalent genotype (88.1%, 96/109). The G12P[8] (1.8%, 2/109), G3P[8] (0.9%, 1/109), and G3P[9] (0.9%, 1/109) genotypes were also detected. Four samples (3.7%) were G- not typed (G[NT]P[8]) and five samples (4.6%) were G- and P-not typed (G[NT]P[NT]).

Through nucleotide analysis of the VP8* gene by sanger sequencing, the G1P[8] strains were characterized as vaccine shedding in 88.5% of stool samples (85/96), with a range of

99.6-100% and 99.5-100% for nucleotide (nt) and amino acid (aa) similarity with RV1, respectively. The G1P[8] vaccine strain was detected before the 1st dose (3 non-vaccinated cases) and from day 1 to day 58 (median of 7 days) post-vaccination (1st dose: 59 stool samples; 2nd dose: 23 stool samples), with the Ct value (RT-qPCR) varying from 19.6 to 40 (median Ct value 35.6, range from 1.6×10^{-1} to 2.4×10^5 genome copies/mL).

In relation to the other non-G1P[8] genotypes detected in this cohort in acute diarrheic episodes (ADE), the G3P[9] genotype was observed 11 days after the 1st dose of RV1 (RT-qPCR Ct value of 19) and G3P[8] was detected in one 6 month-old child (RT-qPCR Ct value of 32.5). The G12P[8] genotype was detected in one 4-month-old child (7 days after the RV1 2nd dose, RT-qPCR Ct value of 39.1) and one 10-month-old child (RT-qPCR Ct value of 36.4).

Detection of VP8* P[8] gene mutation The F167L G1P[8] VP8* gene mutation in the vaccine strain was detected in 20.5% (27/132) of children monitored. RVA shedding of the F167L G1P[8] vaccine strain was present in 23 children after the 1st dose, 2 children after the 2nd dose, 1 child before the 1st dose and persisting after the 1st dose, and 1 child before the 1st vaccine dose (see [Supplementary Table S1 online](#)). The F167L G1P[8] vaccine strain was detected from day 5 to day 31 (median of 8 days, except for 2 cases detected before the 1st dose), with a median RT-qPCR Ct value of 32.9. The vaccine shedding period (days post-vaccination) for children releasing RV1 particles either with or without the F167L mutation was similar (median of 7 and 8 days).

Nucleotide analysis of the VP8* RVA vaccine shedding by sanger sequencing showed a mutation at nt 499T>C (1st position of the codon, T/CTT) in 26 samples (30.6%, 26/85), besides the mutation at nt 501T>A (3rd position of the codon, T/CTA/T) in 2 samples (2.4%, 2/85), both positions resulting in an altered aa in position 167 in the VP4 protein (phenylalanine - Phe, substituted by leucine - Leu) ([Table 2](#)). Results obtained from pyrosequencing showed an allelic quantification of C/T for the 1st position and T/A for the 3rd position of the T/CTA/T codon,

demonstrating a mixture of virus subpopulations containing Phe (TTT) and Leu (CTT, TTA or CTA) aa in RV1 shedding. The stool samples varied from 1 to 95.5% (TTT) and 4.5% to 99% (CTT) in the 1st position (see [Supplementary Fig S1 and TableS1 online](#)), 14 to 4% (CTA) and 86 to 96% (CTT), and 68 to 89% (TTA) and 32 to 11% (TTT) in the 3rd position. Besides that, two other aa substitutions were detected in the F167L RV1 shedding (in two stool samples): one (Y80H) in a conserved region and the other (N87S) in a hypervariable region (antigenic site 8-4). Furthermore, one silent mutation was detected in the nt position 219A>G (aa 73T), in other sample.

HBGA phenotyping and FUT2 genotyping Regarding the secretor status, 80.3% (106/132) of the children were classified as secretors and 15.9% (21/132) were non-secretors. Secretor status definition for 3.8% (5/132) of children was not possible. The Lewis phenotypes detected were: 59.8% (79/132) Le (a+b+), 15.9% (21/132) Le (a-b-), 13.6% (18/132) Le (a-b+), and 10.6% (14/132) Le (a+b-).

FUT2 genotyping was performed for 78 children with Le (a+b+) secretor and 21 with non-secretor status. Twenty-one SNPs were determined in 82.1% (64/78) of the Le (a+b+) secretor children and 17.9% (14/78) of them did not present any SNP. Twenty-eight genotypes were identified in Le (a+b+) secretors children (see [Supplementary Table S2 online](#)) and the most frequent genotype was *Se*^{171A>G, 216C>T, 357T>C, 428G>A, 739G>A, 960A>G, 1009A>G, 1011T>C} (35.9%, 28/78). The rs281377 SNP (357C>T) was detected in high frequency (80.7%, 63/78) and two new mutations were detected in the Le (a+b+) secretor phenotype (107T>A and 257C>T). Twelve SNPs were detected at a low frequency: rs1800021 (40A>G), rs138507381 (212T>C), rs200157007 (302C>T), rs28362836 (315C>A), rs1800026 (375A>G), rs1800027 (480C>T), rs1800025 (481G>A), rs148371614 (544G>A), rs142741127 (771G>A), rs141630650 (855A>C), rs916106939 (880T>C), and rs144809245 (969C>T). The rs1047781 SNP (385A>T) was not detected in any children phenotyped as Le (a+b+) in this cohort. Twelve (57.1%, 12/21)

children classified as non-secretor presented the following homozygous genotype: *se*^{171A>G}, 216C>T, 357T>C, 428G>A, 739G>A, 960A>G, 1009A>G, 1011T>C (see [Supplementary Table S3 online](#)). In addition, one non-secretor child presented a new mutation (257C>A) and the SNPs rs28362836 (315C>T), rs138954645 (542C>T), rs148371614 (544G>A) and rs142741127 (771G>A) were identified at a low frequency in three children, and were considered heterozygous at these positions (see [Supplementary Table S3 online](#)). One non-secretor child did not present any SNP in the *FUT2* gene.

Six genotypes in the *FUT2* gene were identified in 69.6% (16/23) of the Le (a+b+) secretor children with F167L RV1 shedding, and rs281377 (357C>T) was present in 100% (16/16) of them.

RV1 shedding and host susceptibility In relation to G1P[8] vaccine shedding and the host genetic susceptibility profile of the cohort, the F167L mutation (*VP8** gene) was more commonly detected in children with secretor status ($p = 0.0433$), and a Le (a+b+) phenotype ($p = 0.0354$) (Table 3 and 4).

Other RVA genotypes isolated in ADE in this cohort were detected in the secretor children, these being G12P[8] in Le (a-b+) and Le (a+b+) and G3P[8] in Le (a+b+). The G3P[9] RVA was isolated from a child both Le (a-b-) and positive for fucose detection. A P[8] genotype, untypable for G[NT], was detected in three Le (a+b+) secretor children and in one secretor Le (a-b+) child. Untypable (G[NT]P[NT]) RVA cases were detected in Le (a+b+) secretors (four children) and in one non-secretor Le (a+b-) child.

Discussion

This prospective study focused mainly on assessing the RV1 shedding in association to the HBGA profile in a birth community-cohort in Rio de Janeiro, Brazil. Through monitoring the G1P[8] vaccine shedding, it was possible to detect a mutation in the F167L *VP8** P[8] gene from stool samples of children from prior to the 1st dose and during the RV1 immunization

period. The evaluation of these children's host susceptibility profile showed that secretor as well as Lewis b positive children were significantly more likely to shed RV1 vaccine with occurrence of the F167L VP8* mutation.

Via monitoring of the stool specimens, 88.5% of the G1P[8] RVA detected were identified as RV1, mainly after the 1st dose (96.5%), at 2 months of age, similar to previously documented in clinical trials³²⁻³⁴. The G1P[8] genotype was detected in eight non-vaccinated children (before the 1st dose) and due to the low viral load, only three cases could be sequenced and determined as vaccine strain (RV1). The horizontal transmission of vaccine viruses has been previously demonstrated between vaccinated and unvaccinated infants or other close contacts^{33,34}. RV1 shedding was reported in two diarrhea cases after the 1st dose, with the F167L mutation being detected in one of them. Cases of diarrhea in vaccinated children have been previously reported due to nutritional impairment. However, it is noteworthy that in this study we observed a low diarrhea incidence, mainly in the first 4 months of age, which could be related to RV1 protection and the effect of exclusive breastfeeding (and transferred maternal antibodies) in these first months of life.

In the few diarrhea cases detected in this study, a rare G3P[9] was identified in one 2-month-old child after the 1st dose, with the VP8* P[9] gene exhibiting 97% nucleotide similarity to an *AU-1-like* Brazilian strain (KJ820906) collected from an inpatient 2-year-old, indicating a possible feline/canine-to-human interspecies transmission³⁵. The effectiveness of the RVA vaccines against genogroups 1 and 2 (*Wa-like* and *DS-1-like* strains, respectively) has been proven, but still remains unclear against the genogroup 3 (*AU-1-like*)^{36,37}. As to this rare genotype detected in Manguinhos, further studies are being conducted to determine the complete genomic constellation.

Through our molecular characterization of the RV1 shedding, it was possible to observe a high frequency of the mutation in the 167 position of the VP8* P[8] gene. In our

study, only the VP8* domain was analyzed because it is directly related to interactions with HBGAs. In the previous studies with the G1P1A[8] rotavirus vaccine candidate 89-12, the precursor to RV1, Ward et al.³⁸ identified five aa changes in the VP4 gene (G51D and L167F in VP8* domain; D331Y, D385Y and N695I in VP5* domain) that occurred during vaccine attenuation. It was proposed that these alterations could reduce cross neutralizing antibody responses. Indeed, according to Gonzalbo-Rovira et al.³⁹, the 167 position is placed at the bottom of the sugar-binding pocket, and Phe residue contained in RV1 could influence the interaction between the vaccine strain and the sugar of the cellular receptor, due to hydrophobic residue decreasing the binding affinity. 32.9% of the RV1 shedding samples detected in this birth-cohort presented the mutation in the 167 position, with a mixture of virus subpopulations of both amino acids, Leu (CTT, TTA, CTA) and Phe (TTT), in different percentages. We could hypothesize that this suggest an initial process of reversion of attenuation or a selective pressure, through the one and/or two alteration(s) in this codon (positions nt 499, 501), favoring the highest amount of circulation of strains containing the Leu residue, which has a greater avidity for the HBGA's binding site in the secretor profile. Zeller et al.⁴⁰ also reported the mutation in position 167 in the 3rd position of the codon (nt 501), in one vaccine derived strain (BE00048) detected in Belgium, in 2009.

Positive secretor status was the most prevalent (80.3%) in this community-cohort, and the Le (a+b+) profile (59.8%) frequency was similar to that observed in younger children from the Amazon (58.8%) as presented in our previous study⁴¹. The rs1047781 (385A>T) SNP, responsible for the weak genotype in the Asian population, was not detected in Le (a+b+) children in the Manginhos community, Rio de Janeiro state. The rs281377 (357C>T), a synonymous mutation, was the most frequent SNP detected in this Le (a+b+) secretor profile, and this SNP has been detected in the Brazilian population^{41,42}. According to Ferrer-Admetlla et

al.¹⁷, the rs281377 (357C>T) SNP has been detected in a worldwide distribution profile in the natural population, and the *se*^{357/385} haplotype is frequently detected in the Asian population.

We could explain the high frequency of the Le (a+b+) phenotype detected in children in Rio de Janeiro, Brazil under an evolutionary view that, at some point in the molecular clock, the *se*^{357/385} haplotype had diverged and separated from the rs1047781 (385A>T) SNP.

However, epistatic interactions between rs281377 (357C>T) and other SNPs could affect the expression of the *FUT2* gene. The failure in detecting the rs1047781 (385A>T) SNP, which has been attributed as responsible for the weak secretor phenotype detected in Le (a+b+), in the children in the Manguinhos community in the state of Rio de Janeiro could be explained by the presence of new SNPs in conjunction with rs281377 (357C>T). Further studies encompassing larger populations, including Le (a-b+) secretors will be needed to evaluate whether particular SNPs in *FUT2* gene can explain the phenotypes Le (a+b+) vs Le (a-b+) (or similar).

Our study identified SNPs in the *FUT2* gene in children from the Manguinhos community, in Rio de Janeiro as rs1800021 (40A>G), rs48703160 (171A>G), rs681343 (216C>T), rs281377 (357T>C), rs601338 (428G>A), rs602662 (739G>A) and rs485186 (960A>G), also reported by Vicentini et al.⁴² in children from a Quilombola community (black population, slave descendants) in Espírito Santo (borders with the state of Rio de Janeiro, both states in Southeastern Brazil).

The Brazilian population has a high ethnical diversity and through this study in the Manguinhos children community-cohort, it was possible identify two new mutations: 107T>A and 257C>T/A and other SNPs, at a low frequency, not yet reported in Brazil: rs138507381 (212T>C), rs200157007 (302C>T), rs28362836 (315C>A/T), rs1800026 (375A>G), rs1800025 (481G>A), rs138954645 (542C>T), rs148371614 (544G>A), rs142741127 (771G>A), rs141630650 (855A>C), rs916106939 (880T>C), and rs144809245 (969C>T).

Le (a+b+) phenotype is very common in infants and children under 2 years-old, usually being a transient status in most children⁴³. In the infancy period, the Lewis phenotypes have not yet fully matured⁴⁴, due to the activity of the fucosyltransferases (*FUT2* and *FUT3* genes) not yet reaching the normal levels (enzyme activity of *Le* > *Se*)¹⁸.

In relation to the non-secretor phenotype, a unique child (0Le (a-b-)) did not present any SNP in the *FUT2* gene, i.e., not showing the nonfunctional allele *se*⁴²⁸ (*rs601338*)¹⁹. This child shed the RVA vaccine strain virus in both doses (6 days post-1st dose; 7 days post-2nd dose); however, only in the 2nd dose was the F167L mutation detected, with mixed virus subpopulations of 63.5% Phe/36.5% Leu. Possibly, other genetic factors could be related to this profile and vaccinal response.

The P[9] RVA genotype interacts with A-type HBGA antigen⁴⁵, and the ABO enzyme assay was performed in a saliva sample from the unique child that was infected with this genotype (G3P[9]) in this cohort, presenting positivity for type A antigen (ALe (a-b-) secretor) (data not shown), thus in line with the proposed susceptibility of A to genotype P[9].

The Manguinhos cohort showed a high prevalence of secretors, being a common profile in Latin America^{28-29,41-42,46}. In this study, we were able to investigate RV1 shedding and a common mutation was identified in many vaccine samples. This mutational event suggests that the vaccine is replicating and therefore a more robust immune response may be produced in these infants. In summary, our study demonstrated that Le (a+b+) secretors can influence vaccine replication.

This study highlights the detection of the new mutations in the *FUT2* gene detected in the community of Manguinhos, Rio de Janeiro, Brazil and the association between vaccine strain shedding and HBGAs as a marker for evaluating vaccine efficiency. The results in this study indicate the importance of the molecular monitoring of the RV1 vaccine virus stool shedding, mainly if the F167L VP8* mutation is frequent in other populations, and how the

host susceptibility profile can influence this viral selective pressure, besides the questions about circulation and fluctuations of the emergent RVA genotypes and ensuring vaccine efficacy in a post-RVA vaccine era.

Material and methods

Study design, clinical specimens and Ethics Statement The population of this study consisted of the 132 newborn infants residing in the Manguinhos community, Rio de Janeiro, Brazil, and who were attended between November 2014 and November 2018, in routine pediatric visits at the Germano Sinval Faria Health Center (GSFHC), National School of Public Health, Fiocruz. Manguinhos is a low-income community within the Metropolitan region of the Rio de Janeiro city, Brazil, and has one of the lowest Human Development Indices (HDI) of the municipality⁴⁷.

The stool and saliva samples were obtained from a prospective cohort study that aims to perform acute gastroenteritis surveillance in Manguinhos, whose project was approved by the Ethics Committee of Evandro Chagas National Institute of Infectious Diseases (CEP 688.566/14).

The mothers that formally agreed to take part in the research study, were instructed to take emitted stool of the infants, at least every 7-15 days, during the RVA immunization period (pre and post-RV1 vaccination/1st and 2nd doses) or when the child had a diarrheic episode (passive diarrhea surveillance). Diarrhea was defined by the presence of ≥ 3 liquid or semi-liquid evacuations in a 24 h period, while an asymptomatic case was of no episodes of diarrhea for at least 1 week before the collection date.

Between October 2016 and November 2018, saliva samples were collected unpaired with stool samples from infants from 15 days to 3-year-old, with a median of 1-year and 4 months of age, using sterile cotton-swabs (CHEMBIO®, Medford, NY, USA). All clinical specimens were sent to the Regional Rotavirus Reference Laboratory/ Laboratory of

Comparative and Environmental Virology (RRRL-LCEV) and kept at -20°C, until the moment of processing and further analysis.

RNA extraction from fecal specimens and viral RVA detection Viral RNA was extracted from clarified stool specimens (10-20% w/v) using an automatic RNA extraction procedure according to the manufacturer's instructions (QIAcube[®] Automated System and QIAamp[®] Viral RNA Mini kit; Qiagen, CA, USA). RVA detection was performed using RT-qPCR on an Applied Biosystems[®] 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as previously described⁴⁸. The RVA RT-qPCR result was considered positive if the cycle threshold (Ct) value was ≤ 40 .

G- and P- RVA genotyping Semi-nested multiplex reverse transcription-polymerase chain reaction (RT-PCR) was performed for G- and P- RVA genotyping using SuperScript[®] III One-Step RT-PCR System with Platinum[®] Taq DNA Polymerase High Fidelity (Invitrogen) and 9con1L/VP7deg (VP7) and 4con3/4con2 (VP8*) primers to generate 904-base-pair (bp) and 876-bp fragments, respectively. These amplicons were used as a template in a second round of amplification with genotype-specific primers as described on WHO/IVB/08.17⁴⁹.

VP8* RVA molecular characterization Products of one-step reverse transcription-polymerase chain reaction (RT-PCR) using 4con3/4con2 (876-pb) were purified using Wizard[®] SV Gel and a PCR Clean-Up System kit (Promega, Madison, USA) following the manufacturer's instructions. The purified amplicons of the VP8* gene of RVA were analyzed by Sanger sequencing using a BigDye[®] Terminator v3.1 Cycle Sequencing Kit and the ABI Prism 3730 or 3500 Genetic Analyser[®] (Applied Biosystems, Foster City, CA, USA). Chromatograms were analyzed and nt sequences (consensus) were edited using the BioEdit 7.2.1 Sequence Alignment Editor⁵⁰ and nt similarity was assessed using the Basic Local Alignment Search Tool

(<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). RVA genotypes were assigned using the RotaC^{2.0}

automated genotyping tool for Group A rotaviruses (<https://rotac.regatools.be/>)⁵¹. Deduced aa sequences of VP8* (G1P[8]) were aligned and compared with the RV1 (JX943612) and G1P[8] *Wa-like* (JX406750) strains, and nt and aa similarities values between RV1 and G1P[8] strains were verified using the BioEdit 7.2.1 Sequence Alignment Editor⁵⁰. The representative gene sequences of VP8* RVA obtained in the current study were submitted to GenBank under the accession numbers MN366044-MN366074.

Pyrosequencing of RV1 VP8* P[8] gene For mutation analysis in RV1 shedding, a pyrosequencing assay was performed using PyroMark Q96 ID (QIAGEN Valencia, CA, USA). The following primers were designed to amplify a 203-bp fragment of the VP8* P[8] subdomain containing the target (cytosine or thymine/ nt 499, and adenine or thymine/ nt 501): forward (VP8*RV1F) 5'-AGCAATTTAATGTGAGTAACGA-3' (nt 346-368) and reverse (VP8*RV1R) 5'-BIOTIN-AAATTTGCAGTACTTGAAGTCA-3' (nt 548-525) using PyroMark[®] Assay Design 2.0 software (QIAGEN Valencia, CA, USA). The forward (VP8*RV1-S1) (5'-TGATACCAGACTTGTAGGA-3'; nt 447-465) was designed for the pyrosequencing assay. The biotinylated fragment (203-pb) was amplified using SuperScript[®] III One-Step RT-PCR System with Platinum[®] Taq DNA Polymerase (Invitrogen). Initially, the samples were denatured with primers at 97°C/7 min. Next, the master mix was added in each tube, and incubated under the following thermocycling conditions: cDNA synthesis and pre-denaturation at 50°C/30 min, denaturation at 94°C/10 min, then 40 cycles of 94°C/15 s, 53°C/30 s and 68°C/1 min, followed by a final elongation step (68°C/5 min). Each sample and controls (positive and negative) were tested in duplicate and the quality of the RT-PCR products were checked by agarose gel electrophoresis. Biotinylated amplicons were hybridized to streptavidin-coated beads and purified using the PyroMark Q96 Vacuum Prep Workstation (Qiagen, Valencia, CA, USA)

according to the manufacturer's instructions. Pyrosequencing reactions were performed using the PyroMark Gold Q96 SQA Reagents in the PyroMark Q96 ID (QIAGEN) following the manufacturer's instructions, and the analysis of the peaks was performed using PyroMark ID software. The allelic quantification results (%) were calculated by the duplicate media. The primers designed for this analysis and thermocycling conditions were previously tested and validated. As a positive control, a fragment of the VP8* P[8] region (encoding aa 7-257) of the RV1 stool shedding was isolated. The fragment without the mutation (nt 499/ T) was ligated into the pCR[®] 4-TOPO[®] vector using the TOPO TA Cloning[®] Kit for Sequencing (Invitrogen, Life Technologies, UK) and then transformed into competent *Escherichia coli* Top 10 (Invitrogen, Life Technologies, UK) following the classical methodologies previously described⁵².

HBGA phenotyping and secretor status in saliva An enzyme immunoassay (EIA) was performed to detect AB0 (H) histo-blood groups and Lewis phenotypes, and the secretor status was evaluated in Le (a-b-) saliva samples by *Ulex europaeus* lectin EIA as described previously^{41, 53}.

DNA extraction from saliva and FUT2 genotyping Genomic DNA recovered from epithelial cells was extracted from each saliva sample collected from children phenotyped as Le (a+b+) secretor and non-secretor Le (a+b-) or Le (a-b-) and were used in the touchdown PCR for the genotyping of SNPs in the *FUT2* gene, by Sanger sequencing as previously described^{41, 54}.

Statistical analysis Statistical analysis was performed using GraphPad Prism 8 v.8.2.0 (GraphPad Software, San Diego, CA, USA). Detection of the G1P[8] RVA genotype (RV1 stool shedding) in association with the host susceptibility profile was investigated, when appropriate, through the Chi-square or Fisher's exact test, with a significance level of 5.0%.

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Additional Information

Competing Interests The authors declare no competing interests.

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Author Contributions

C.P.C, M.P.M., J.P.G.L. and M.T.B.M., proposed the study and designed the experiments.

D.C.C. and P.B., designed the clinical study, collected the samples and followed-up on the children.

C.P.C, A.J.V., R.M.S.A., F.C.A.M. and J.J.F.B, performed laboratory experiments and analysis.

C.P.C, M.P.M., J.P.G.L. and M.T.B.M., interpreted the data and wrote the manuscript.

J.N. and L.S., discussed the results and critically reviewed the final version.

RV1 immunization period (age group)	n children (follow-up)	RVA detection rate (%)	RVA genotypes (ADE or non-ADE cases)	Secretor profile in RVA cases
Before the 1 st dose (<2 months)	127	11/304 (3.6)	5 G1P[8] and 3 G1P[8] RV1* (8 non-ADE)	8 <i>Se</i>
			G[NT]P[NT] (3 non-ADE)	3 <i>Se</i>
1 st and 2 nd doses (2-5 months)	125	92/234 (39.3)	78 G1P[8] RV1* and 6 G1P[8] (84 non-ADE) ^a	55 <i>Se</i> ^b and 12 <i>se</i> ^c
			G1P[8] RV1* (3 ADE)	2 <i>Se</i> and 1 <i>se</i>
			G12P[8] (1 ADE)	1 <i>Se</i>
			G3P[9] (1 ADE)	1 <i>Se</i>
			G[NT]P[NT] (2 non-ADE)	1 <i>Se</i> and 1 <i>se</i>
			G[NT]P[8] (1 non-ADE)	1 <i>se</i>
After the 2 nd dose (6-11 months)	24	6/31 (19.4)	G1P[8] RV1* (1 ADE) and G3P[8] (1 ADE)	1 <i>Se</i> (same child)
			G12P[8] (1 ADE)	1 <i>Se</i>
			G[NT]P8 (3 ADE)	3 <i>Se</i>
Total	132	109/569 (19.2)		

Table 1: Stool samples collected from the 132 infants/children in acute diarrheic episodes (ADE) or non-ADE, rotavirus A (RVA) detection rate and G- and P-genotyping (NT = not typed) in the different age groups. Distribution between RVA genotypes detected and the Secretor (*Se*: secretor, *se*: non-secretor) profile corresponding to 82 children followed. The birth community-cohort study was conducted between November 2014 and November 2018, in Rio de Janeiro, Brazil. a. In three children (5 G1P[8] cases), the secretor profile was considered inconclusive. b. eight children with 2 RVA cases, c. four children with 2 RVA cases. *In this cases, the G1P[8] was characterized as RV1 by Sanger sequencing (VP8* gene).

Rotavirus A P[8] strains (GenBank accession numbers)	amino acid position 167			
	Codon			
	1 st (nt 499)	2 nd (nt 500)	3 rd (nt 501)	aa
<i>Wa-like</i> (JX406750)	T	T	G	Leu
Prototype P1A[8] unpassaged 89-12	T	T	G	Leu
RV1-RIX 4414, passage 89-12 (JX943612)	T	T	T	Phe
RV1 shedding (this study)	T	T	T	Phe
	C	T	T	Leu
	T/C	T	A/T	Leu

Table 2: Nucleotide (nt) substitutions and corresponding 167 amino acid substitution (aa, Leu: leucine or Phe: phenylalanine) in the VP8* gene in different rotavirus A (RVA) strains: G1P[8] *Wa-like*, vaccine prototype, RV1 vaccine and RV1 shedding detected in the Manguinhos community-cohort, Rio de Janeiro, Brazil, from November 2014 to November 2018.

Secretor/ Lewis profile	G1P[8] vaccine shedding ^a		F167L G1P[8] vaccine shedding ^b		<i>p</i> value ^{c,d}
	n = 40	(%)	n = 21	(%)	
Secretor	30	(75)	21	(100)	0.0433
Non-secretor	9	(22.5)	0	(0)	
Indetermined	1	(2.5)	0	(0)	
Le(a+b-)	6	(15)	0	(0)	0.0354
Le(a-b+)	5	(12.5)	2	(9.5)	
Le(a+b+)	23	(57.5)	19	(90.5)	
Le(a-b-)	6	(15)	0	(0)	

Table 3: Association between Secretor and Lewis profile and G1P[8] RV1 shedding (n = 61 children), with and without a F167L mutation, in the birth community-cohort, Rio de Janeiro, Brazil, from 2014 to 2018. a. The immunization schedule of the children with G1P[8] shedding: 28 after 1st dose, 12 after 2nd dose and 1 non-vaccinated child. b. All children were vaccinated with the 1st dose, except one (non-vaccinated). c. Twelve children (with 2 RVA positive results each) were removed of the statistic analysis. d. *P* values were determined by Chi-square test.

Code Child	Immunization schedule	Days post-vaccine	RVA RT-qPCR Ct value	VP8* gene (Sanger sequencing)
004	3	NA	38,3	F167L RV1
033	1	8	38,5	F167L RV1
037	1	8	39,5	RV1
043	1	5	34,1	F167L RV1
053	1	5	39,7	RV1
057	1	8	34,9	F167L RV1
071	2	5	33,7	RV1
072	2	5	30,3	RV1
090	1	10	33,1	F167L RV1
094	1	9	31,6	F167L RV1
096	1	6	36,6	RV1
097	1	8	37,2	RV1
099	1	16	38,1	RV1
101	1	8	34,1	F167L RV1
113	1	7	28,4	F167L RV1
122	1	7	35,8	RV1
	2	?	37,5	F167L RV1
142	1	17	36,7	F167L RV1
144	1	12	26,3	F167L RV1
160	1	12	33,9	F167L RV1
164	2	7	36,7	RV1
173	2	9	35,4	RV1
204	1	8	38,0	RV1
211	1	8	32,8	F167L RV1
225	1	7	23,3	F167L RV1
226	2	8	37,7	RV1
233	1	8	37,2	RV1
234	1	5	24,5	F167L RV1
	2	9	35,4	RV1
235	1	8	21,9	F167L RV1
236	1	8	19,8	F167L RV1
240	1	9	39,6	F167L RV1
244	1	8	19,6	F167L RV1
245	1	6	21,6	RV1
249	2	5	34,5	RV1
250	1	6	22,6	RV1
253	1	22	30,0	F167L RV1
259	1	6	26,5	F167L RV1
	2	7	30,7	RV1
261	1	7	22,7	RV1
	2	24	36,1	RV1
264	1	5	27,1	RV1
265	2	58	38,6	RV1
266	2	10	33,7	RV1
270	3	NA	21,0	F167L RV1
	1	31	32,9	F167L RV1
275	1	6	20,3	RV1
281	1	6	19,9	F167L RV1
282	1	3	32,0	RV1
284	1	4	26,6	RV1
292	1	4	22,9	RV1
293	1	5	35,9	RV1

Table 4: Detection of the vaccine shedding, with or without a F167L mutation, in children phenotyped as Le (a+b+) secretors (n=47) in the birth community-cohort, Rio de Janeiro, Brazil, from 2014 to 2018. G1P[8] RVA samples were firstly genotyped by G/P multiplex RT-PCR. The VP8* gene was analysed by sanger nucleotide sequencing and the results showed to be G1P[8] vaccine strain

(RV1). Information about these derived vaccine samples are summarized: the immunization schedule (1: 1st dose, 2: 2nd dose, 3: non-vaccinated), number of days of vaccine shedding (NA: not applicable, ? : no information) and rotavirus (RVA) RT-qPCR Ct value.

Supplementary information:

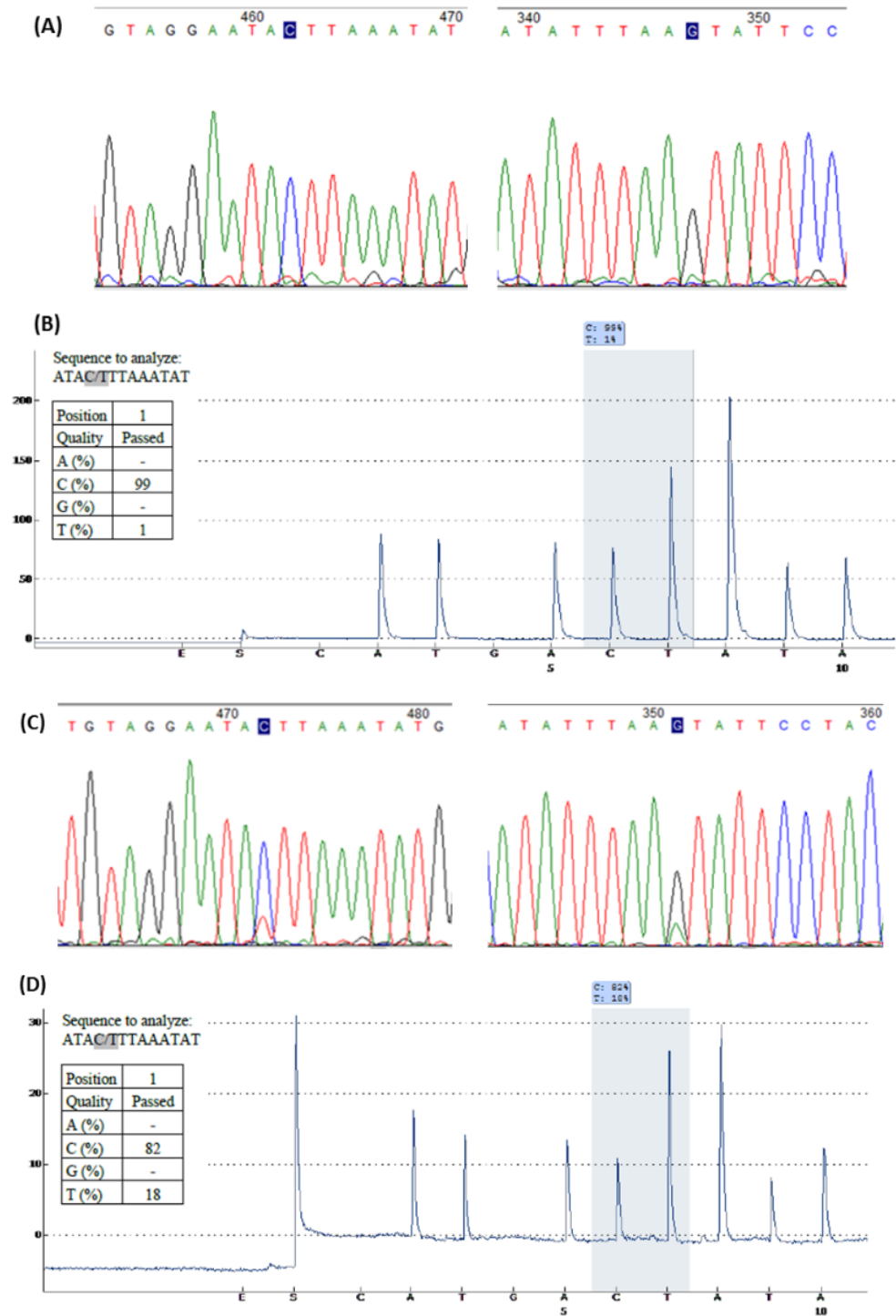


Figure S1: Chromatograms (Forward and Reverse, Sanger Nucleotide Sequencing, A and C) and Pyrograms (Pyrosequencing Method, B and D). Pyrosequencing showed a sensitivity of 1% in a

mixture sample (F167L) in RV1 shedding (nt 499, 1% TTT, Phenylalanine and 99% CTT, Leucine).

Child code	Secretor status	Lewis status	Immunization schedule	Days post-vaccination	RVA RT-qPCR Ct value	Sanger Sequencing	Pyrosequencing	
						peak nt 499	% TTT (Phe)	% CTT (Leu)
004	Se	Le (a+b+)	3	NA	38.3	T>C	73.5	26.5
101	Se	Le (a+b+)	1	8	34.1	C>T	13	87
033	Se	Le (a+b+)	1	8	38.5	C>T	17	83
043	Se	Le (a+b+)	1	5	34.1	C>T	15.5	84.5
057	Se	Le (a+b+)	1	8	34.9	C>T	9	91
079	Se	Le (a-b+)	1	8	31	T>C	63.5	36.5
113	Se	Le (a+b+)	1	7	28.4	C>T	29	71
090	Se	Le (a+b+)	1	10	33.1	T>C	57	43
103	se	Le (a-b-)	2	7	36.3	T>C	63.5	36.5
094	Se	Le (a+b+)	1	9	31.6	C	2.5	97.5
144	Se	Le (a+b+)	1	12	26.6	C	2	98
122	Se	Le (a+b+)	2	?	37.5	C	1	99
142	Se	Le (a+b+)	1	17	36.7	C	11.5	88.5
158	Se	Le (a-b+)	1	7	33.3	C>T	10	90
211	Se	Le (a+b+)	1	8	32.8	C	1.5	98.5
160	Se	Le (a+b+)	1	12	34	T>C	60.5	39.5
236	Se	Le (a+b+)	1	8	19.8	T>C	55.5	44.5
225	Se	Le (a+b+)	1	7	39.6	C	32.5	67.5
234	Se	Le (a+b+)	1	5	24.5	T>C	64.5	35.5
235	Se	Le (a+b+)	1	8	22	C	9	91
244	Se	Le (a+b+)	1	8	19.6	C	20	80
253	Se	Le (a+b+)	1	22	30	C	7.5	92.5
259	Se	Le (a+b+)	1	6	26.5	C>T	42.5	57.5
276	Se	Le (a-b+)	1	7	20.7	C	15.5	84.5
281	Se	Le (a+b+)	1	6	19.9	C	3	97
270	Se	Le (a+b+)	3	NA	21	C	9.5	90.5
			1	31	33	C	7.5	92.5
240*	Se	Le (a+b+)	1	9	39.6	C	95.5	4.5

Table S1: Secretor profile (Se: secretor, se: non-secretor) in children (n = 27) who presented F167L RV1 shedding, and information about the immunization schedule (1: 1st dose, 2: 2nd dose, 3: non-vaccinated), number of days of vaccine shedding (NA: not applicable, ? : no information) and rotavirus (RVA) RT-qPCR Ct value. Comparison between results in Sanger Nucleotide Sequencing and Pyrosequencing (Phe: phenylalanine, Leu: leucine) methods in these derived vaccine samples. a. This unique child showed different results in the nucleotide analysis, probably due to these samples having been obtained from the distinctive viral extraction protocols.

<i>FUT2</i> genotyping/ Le (a+b+) secretor phenotype	allele	n (%)
<i>Se</i> ^{315C>A}	heterozygous	1 (1.3)
<i>Se</i> ^{357T>C}	heterozygous	2 (2.6)
<i>Se</i> ^{357T>C}	homozygous	3 (3.8)
<i>Se</i> ^{40A>G, 357T>C}	homozygous	1 (1.3)
<i>Se</i> ^{(40A>G), 357T>C}	homozygous	1 (1.3)
<i>Se</i> ^{40A>G, 357T>C}	heterozygous	2 (2.6)
<i>Se</i> ^{357T>C, 480C>T}	heterozygous	1 (1.3)
<i>Se</i> ^{357T>C, 481G>A}	homozygous	1 (1.3)
<i>Se</i> ^{357T>C, 481G>A}	heterozygous	1 (1.3)
<i>Se</i> ^{357T>C, 428G>A, 480C>T}	heterozygous	1 (1.3)
<i>Se</i> ^{212T>C, 357T>C, 969C>T}	homozygous	1 (1.3)
<i>Se</i> ^{357T>C, 428G>A, 739G>A, (960A>G), (1009A>G), (1011T>C)}	heterozygous	1 (1.3)
<i>Se</i> ^{216 C>T, 357T>C, 428G>A, 739G>A, 960A>G, 1009A>G, 1011T>C}	heterozygous	1 (1.3)
<i>Se</i> ^{107T>A, 171A>G, 216C>T, 357T>C, 428G>A, 739G>A, 855A>C, 960A>G, 1009A>G, 1011T>C}	heterozygous	1 (1.3)
<i>Se</i> ^{171A>G, 216C>T, (357T>C), (428G>A), 739G>A, 960A>G, 1009A>G, 1011T>C}	homozygous	1 (1.3)
<i>Se</i> ^{171A>G, 216 C>T, 357T>C, 428G>A, 739G>A, 960A>G, 1009A>G, 1011T>C}	heterozygous	28 (35.9)
<i>Se</i> ^{171A>G, (216 C>T), (357T>C), 428G>A, (739G>A), 960A>G, 1009A>G, 1011T>C}	heterozygous	1 (1.3)
<i>Se</i> ^{171A>G, 216 C>T, 357T>C, 428G>A, 739G>A, 1009A>G, 1011T>C}	heterozygous	1 (1.3)
<i>Se</i> ^{171A>G, 216 C>T, (357T>C), 375A>G, 428G>A, 739G>A, 960A>G, 1009A>G, 1011T>C}	heterozygous	1 (1.3)
<i>Se</i> ^{171A>G, 216C>T, 357T>C, 428G>A, 480C>T, 739G>A, 960A>G, 1009A>G, 1011T>C}	heterozygous	2 (2.6)
<i>Se</i> ^{171A>G, 216C>T, (357T>C), 428G>A, 739G>A, 880T>C, 960A>G, 1009A>G, 1011T>C}	heterozygous	1 (1.3)
<i>Se</i> ^{171A>G, 216C>T, (357T>C), 428G>A, 739G>A, 960A>G, 1009A>G, 1011T>C}	heterozygous	2 (2.6)
<i>Se</i> ^{40A>G, 171A>G, 216C>T, (357T>C), 428G>A, 544G>A; 739G>A, 771G>A, 960A>G, 1009A>G, 1011T>C}	heterozygous	1 (1.3)
<i>Se</i> ^{40A>G, 171A>G, 216C>T, 302C>T, 357T>C, 428G>A, 480C>T, 739G>A, 960A>G, 1009A>G, 1011T>C}	heterozygous	1 (1.3)
<i>Se</i> ^{40A>G, 171A>G, 216C>T, (357T>C), 428G>A, 739G>A, 960A>G, 1009A>G, 1011T>C}	heterozygous	4 (5.1)
<i>Se</i> ^{171A>G, 216C>T, (357 T>C), 428G>A, 481G>A, 739G>A, 960A>G, 1009A>G, 1011T>C}	heterozygous	1 (1.3)
<i>Se</i> ^{40A>G, 171A>G, 216C>T, 257C>T, (357T>C), 428G>A, 739G>A, 960A>G, 1009A>G, 1011T>C}	heterozygous	1 (1.3)
<i>Se</i> ^{40A>G, 107T>A, 171A>G, 216C>T, 257C>T, (357T>C), 428G>A, 739G>A, 960A>G, 1009A>G, 1011T>C}	heterozygous	1 (1.3)
without SNPs	-	14 (17.9)

Table S2: *FUT2* genotypes in secretors Le (a+b+) children (n = 78 saliva samples), in the birth community-cohort, Rio de Janeiro, Brazil, 2014-2018. Bolded bases are new single nucleotide polymorphisms (SNPs). Bases in parentheses suggest homozygous or heterozygous in relation the genotyping defined in allele column.

<i>FUT2</i> genotyping (non-secretor children)	allele	n (%)
<i>se</i> 171A>G, 216C>T, 357T>C, 428G>A, 739G>A, 960A>G	homozygous	2 (9.5)
<i>se</i> 171A>G, 216C>T, 357T>C, 428G>A, (544G>A), 739G>A, (771G>A), 960A>G	homozygous	1 (4.8)
<i>se</i> 171A>G, 216C>T, 357T>C, 428G>A, 739G>A, 960A>G, 1009A>G, 1011T>C	homozygous	14 (58.3)
<i>se</i> 171A>G, 216C>T, (315C>T), 357T>C, 428G>A, 739 G>A, 960A>G, 1009A>G, 1011T>C	homozygous	1 (4.8)
<i>se</i> 171A>G, 216C>T, (257 C>A), 357T>C, 428G>A, 739 G>A, 960A>G, 1009A>G, 1011T>C	homozygous	1 (4.8)
<i>se</i> 171A>G, 216C>T, 357T>C, 428G>A, (542C>T), 739G>A, 960A>G, 1009A>G, 1011T>C	homozygous	1 (4.8)
without SNPs	-	1 (4.8)

Table S3: *FUT2* genotypes in non-secretor (Le (a+b-) or Le (a-b-)) children (n = 21 saliva samples), in the birth community-cohort, Rio de Janeiro, Brazil, 2014-2018. The bolded base is a new single nucleotide polymorphism (SNP). Bases in parentheses suggest heterozygous.

Norovirus infection and HBGA host genetic susceptibility in a birth community-cohort, in Manguinhos, Rio de Janeiro, Brazil.

Este estudo está relacionado aos objetivos 1, 2 e 4.

Este manuscrito está em fase final de elaboração e será submetido à: Infection, Genetics and Evolution

Resumo: Neste estudo foi avaliada a frequência, incidência e diversidade genética dos norovírus em crianças vacinadas com a RV1 e a relação com o perfil de susceptibilidade (HBGAs) do hospedeiro, em uma coorte infantil na comunidade de Manguinhos, durante o período de novembro de 2014 a novembro de 2018. Os resultados revelaram que os norovírus infectaram 21,2% das crianças, com uma taxa de incidência de 5,8 infecções em 100 criança-meses. A razão de detecção dos norovírus foi de 5,6%, sendo em 17,1% dos casos diarrêicos e em 4,7% dos assintomáticos. A análise molecular da região B/C dos norovírus identificou seis diferentes genótipos (GII.4 Sydney 2012[P31], GII.4 Sydney 2012[P16], GII.4 Sydney 2012[P4 New Orleans 2009], GII.2[P16], GII.6[P7] e GI.3[P13]), sendo o GII.4 Sydney 2012 detectado em 50% das amostras caracterizadas, circulando associado a três diferentes polimerases durante o período do estudo. Em relação à susceptibilidade do hospedeiro, embora a maioria das noroviroses tenha acometido crianças com perfil secretor, a associação entre norovírus e os HBGAs não pode ser estabelecida. O gene *FUT3* das crianças de fenótipo Lewis negativo foi avaliado, e sete novas mutações foram detectadas.

Norovirus infection and HBGA host genetic susceptibility in a birth community-cohort, Rio de Janeiro, Brazil.

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Highlights

- Norovirus incidence and host susceptibility in a birth community-cohort in Brazil.
- Genetic diversity of norovirus observed in the Manguinhos cohort.
- GII.4 Sydney circulated associated with three different polymerase types.
- New *FUT3* gene mutations in Le (a-b-) individuals not yet detected in Brazil.
- *FUT3* genotyping suggests proximity between Africa and Rio de Janeiro population.

Abstract

Norovirus has emerged as an important viral agent of acute pediatric gastroenteritis, with a growing genetic diversity reported in the last decades. Histo-blood group antigens (HBGAs) present on the surface of enterocytes can be recognized as receptors for norovirus infection differing among populations, and the binding specificity among genotypes/genogroups could affect the epidemiology and evolution of these viruses. This study aims to determine the frequency, incidence and genetic diversity of noroviruses in a cohort of children vaccinated against rotavirus, correlating with the host HBGA (Secretor/Lewis) genetic susceptibility profile. Norovirus genogroups I and II (GI/GII) were screened by RT-qPCR in 569 stool samples from 132 children followed-up from birth to 11 months of age during 2014-2018 period. Norovirus was identified in 21.2% of children enrolled in this study, with a norovirus detection rate of 5.6% (32/569), in 17.1% and 4.7% of acute diarrheic episodes (ADE) and non-ADE, respectively. The norovirus incidence was of 5.8 infections per 100 child-months. The B/C region partial nucleotide sequencing characterized six different norovirus genotypes, with GII.4 Sydney 2012 being detected in 50% of them, circulating associated with three different polymerase genotypes (GII.P31, GII.P16 and GII.P4 New Orleans 2009). *FUT3* genotyping was performed by Sanger sequencing in DNA recovery from epithelial cells from saliva samples, and seven new mutations were detected in this population. Significant association between norovirus symptomatic infection and secretor profile could be inferred.

Key words: norovirus - GII.4 Sydney - Susceptibility - Secretor - Lewis - birth community-cohort

1. Introduction

Noroviruses have emerged as a leading cause of viral of acute pediatric gastroenteritis worldwide, especially after the rotavirus A (RVA) vaccines implementation (Koo et al., 2013), as well as the widespread use of sensitive molecular methods to detect norovirus in global surveillance scale (Glass et al., 2000; van Beek et al., 2018). Nowadays, noroviruses are associated with 18% of all cases in <5-year-old, and an estimated 212,000 deaths annually (Ahmed et al., 2014; Pires et al., 2015).

Noroviruses are genetically diverse positive-stranded RNA viruses that belong to the genus *Norovirus* within the *Caliciviridae* family (Green, 2013). The genogroups I and II (GI and GII) are the most frequently related with human diseases; and the accumulation of point mutations and recombination drives the evolution of norovirus genotypes (Kroneman et al., 2013). The binary typing system involving both polymerase and capsid sequences (RdRp = P type and VP1 = capsid genotype) (Kroneman et al., 2013) has been used for molecular characterization and currently 41 P-types and 31 capsid genotypes have been described (Vinjé, 2015; Chhabrat et al., 2019).

Noroviruses belonging to GII.4 genotype have been the most prevalent viruses circulating in the last two decades in epidemic and sporadic acute gastroenteritis cases in Brazil (Fioretti et al., 2014; Fumian et al., 2016; Costa et al., 2017) and worldwide (Siebenga et al., 2009; Hoa Tran et al., 2013; Vinjé, 2015; Cannon et al., 2017). On average, every 2 to 7 years new GII.4 variants arise, and since 2012 GII.4 Sydney has been the dominant variant (Siebenga et al., 2007; Kroneman et al., 2013), although other emergent genotypes (GII.17 and GII.2) have emerged locally, especially in Asian countries in the last years (Tohma et al., 2017; Sang & Yang, 2018). Currently there is no licensed norovirus vaccine, albeit some candidates are under development. Among them, the phase IIb trials of the bivalent GI.1/GII.4 (nonreplicating virus-like particle/VLP) prototype are ongoing (Mattison et al., 2018).

Previous studies have shown that susceptibility to norovirus infections can differ among individuals (Lindesmith et al., 2003; Huang et al., 2005; Thorven et al., 2005). Norovirus strains recognize the histo-blood group antigens (HBGAs) present on the surface of enterocytes through the P2 subdomain of VP1 (Hutson et al., 2003). The HBGAs are polymorphic and vary between different populations (Marionneau et al., 2001) and the binding specificity of norovirus seems to differ among genogroups and genotypes (Huang et al., 2005), affecting norovirus molecular epidemiology and evolution worldwide (Nordgren & Svensson, 2019). The HBGAs are complex carbohydrates and their synthesis is directed by transferases encoded by the *ABO*, *FUT2 (Se)* and *FUT3 (Le)* genes (Marionneau et al., 2001). Single nucleotide polymorphisms (SNPs) can alter the expression of the $\alpha(1,2)$ -fucosyltransferase and $\alpha(1,3/1,4)$ -fucosyltransferase, originating non-secretors and Lewis negative individuals, respectively (Kelly et al., 1995; Le Pendu, 2004). Substitution of amino acids caused by specific mutations in the *FUT3* gene, such as 59T>G, 202T>C, 314C>T, 508G>A and 1067T>A, can lead to an inactivation or decreased activity of the Lewis enzyme (Koda et al., 1993; Elmgren et al., 1996), resulting in a Le (a-b-) phenotype. Previous studies have shown that non-secretors are more resistant to GII.4 infection (Thorven et al., 2005, Tan et al., 2008); however, many questions regarding norovirus susceptibility remain unanswered (Ramani et al., 2016; Cannon et al., 2019; Nordgren & Svensson, 2019). Considering this matter, vaccine trials and challenge studies should consider the individuals' secretor profile in order to understand the impact of genetic susceptibility on norovirus vaccine efficacy (Mattison et al., 2018).

Birth cohort studies are important to understand the immunity following natural infections (Lopman & Kang, 2014; Cannon et al., 2019), as well as to investigate the circulation of norovirus genotypes and shedding in healthy individuals, especially in developing countries (Lopman et al., 2015); and how the host genetic profile can affect the burden of disease, in order to achieve the best vaccination strategies.

In Brazil, some norovirus community-based studies have been reported ([Linhares et al., 1989](#); [Vicentini et al., 2013](#); [Siqueira et al., 2017](#)), although none of these have evaluated the incidence of norovirus infection in a birth community-cohort, the circulating genotypes and host genetic susceptibility profile ([Cannon et al., 2019](#)). We describe the results of a longitudinal study aimed to investigate the frequency/incidence of norovirus and its genetic diversity in a birth community-cohort of children vaccinated against RVA, and the relation of norovirus infection and the HBGA host genetic susceptibility profile.

2. Material and Methods

2.1. Study design and collection of the clinical samples

This study approved by the Ethics Committee of Evandro Chagas National Institute of Infectious Diseases/FIOCRUZ (CEP 688.566/14), and is part of a longitudinal study of acute gastroenteritis surveillance conducted in the Manguinhos community, neighboring the main *campus* of the Oswaldo Cruz Foundation, Rio de Janeiro city, Brazil.

One-hundred thirty-two children enrolled in a birth to 11-month-old community-cohort from November 2014 to November 2018 were followed up for 16,212 child-days, ranging from 7 to 285 days. Clinical samples were sent to the Regional Rotavirus Reference Laboratory/ Laboratory of Comparative and Environmental Virology (RRRL-LCEV) and were frozen at -20°C for further analysis. A median of five stool samples per child (ranging from 2 to 9 samples) totalizing 569 samples and 132 saliva samples (1 sample/child) were obtained. Previously, all stool specimens were screened for RVA, and saliva samples were used to investigate the HBGA secretor status and FUT2 genotyping ([Cantelli et al., 2019, unpublished results](#)). Diarrhea was characterized as \geq three liquid/semi liquid evacuations in a 24-h period, and asymptomatic cases were defined with no episodes of diarrhea for at least 1 week before the collection date. A new norovirus episode was defined when it occurred $>$ 4 weeks after the previous episode.

2.2. RNA extraction from fecal specimens and viral detection

Total nucleic acid was extracted from clarified stool specimens (20% w/v) using an automatic RNA extraction procedure according to the manufacturer's instructions (QIAcube[®] Automated System and QIAamp[®] Viral RNA Mini kit, Qiagen, CA, USA). RNA viral samples were screened for the presence of norovirus (GI/GII) using a multiplex one-step reverse transcription-quantitative polymerase chain reaction (RT-qPCR) on an Applied Biosystems[®] 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as previously described ([Kageyama et al., 2003](#); [Fumian et al., 2016](#)). The norovirus RT-qPCR result was considered positive if the cycle threshold (Ct) value was < 40 and presented a characteristic sigmoid curve. Besides that, samples which were positive in the first screening were confirmed after the second test, in triplicate.

2.3. Norovirus genotyping and phylogenetic analysis

RT-PCR was performed for norovirus genotyping using Qiagen One-step RT-PCR (Qiagen) and Mon431/G2SKR (GII) and Mon432/G1SKR (GI) primers as previously described ([Cannon et al., 2017](#)) to generate 570-base-pair (bp) and 579-bp fragments, respectively. The following thermocycling conditions were performed: DNA synthesis and pre-denaturation at 42°C/30 min, denaturation at 95°C/15 min, then 40 cycles of 95°C/1 min, 50°C/1 min and 72°C/1 min, followed by a final elongation step (72°C/10 min). When this first round did not yield any products, a second round of amplification (semi-nested PCR) was performed in a subset of samples using the primers COG2F/G2SKR (GII) or COG1F/G1SKR (GI) to generate 390-pb and 380-pb fragments, respectively ([Kojima et al., 2002](#); [Kageyama et al., 2003](#)).

To assess the major epitopes of the VP1 protein region in GII.4 norovirus strains, the P2 subdomain was amplified using EVP2F/EVP2R primers (674-bp fragment) as previously described ([Vega et al., 2014](#)). The amplicons were purified using Wizard[®] SV Gel and a PCR Clean-Up System kit (Promega, Madison, USA) or ExoSAP-IT[®] PCR Product Cleanup Reagent (Applied Biosystems, Foster

City, CA, USA), following the manufacturer's instructions. The purified products were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit and the ABI Prism 3730 or 3500 Genetic Analyzer® (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences (consensus) and electropherograms were analyzed using the BioEdit 7.2.1 Sequence Alignment Editor (Hall, 1999). Nucleotide similarity was assessed using the Basic Local Alignment Search Tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and norovirus genotypes were assigned using the Norovirus Automated Genotyping Tool (<https://www.rivm.nl/mpf/typingtool/norovirus/>) (Kroneman et al., 2011).

Phylogenetic analysis was performed using the maximum-likelihood method, with the K2+G+I and K2 models applied for analysis of portions of the regions encoding the polymerase (RdRp, region B) and VP1 (region C), respectively. Phylogenetic trees were constructed with 2,000 bootstrap replicates in MEGA v.7.0.26 (Kumar et al., 2016). Deduced amino acid sequences of the P2 subdomain (GII.4 strain) were compared with the Brazilian (KF434585) and American (KX907727, MK762561) strains circulating in different years, using the BioEdit 7.2.1 Sequence Alignment Editor (Hall, 1999). The sequences obtained in this study were submitted to GenBank under the accession numbers MH393567, MH393572-MH393573, MN428922-MN428933 and MN428935-MN428939.

2.4. HBGA phenotyping and the secretor status in saliva

The HBGA secretor status was previously evaluated for this cohort (Cantelli et al., 2019, unpublished results). An enzyme immunoassay (EIA) was performed to detect ABO histo-blood groups and Lewis phenotype, and the secretor status was tested in Le (a-b-) saliva samples by *Ulex europaeus* lectin EIA as previously described (Nordgren et al., 2014; Moraes et al., 2019).

2.5. Genomic DNA extraction from saliva and *FUT2* and *FUT3* genotyping

DNA total was extracted from each saliva specimen collected from children phenotyped as Le (a+b+) secretor and non-secretor, Le (a-b-) or Le (a+b-), aiming to confirm the phenotype *status*. In

our previous study (Cantelli et al., 2019, unpublished results), the touchdown PCR was performed to genotyping of SNPs in the *FUT2* gene by Sanger nucleotide sequencing as previously described (Nordgren et al., 2014; Moraes et al., 2019). In a subset of saliva samples (n = 21) phenotyped as Le (a-b-), touchdown PCR was performed to genotyping of SNPs in the *FUT3* gene as previously described (Nordgren et al., 2014; Olivares et al., 2019, unpublished results).

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism v.8.2.0 (GraphPad Software, San Diego, CA, USA). Frequencies of norovirus detection in different age groups and susceptibility profiles were investigated using the Chi-square or Fisher's exact test, with a significance level of 5.0%. The incidence rate for norovirus infection (child-time) was based on the total follow-up duration for each child (new norovirus episodes during follow-up divided by sum of the person-time-at-risk during the observation time).

3. Results

3.1. Frequency and incidence of norovirus in the Manguinhos cohort

In total, 569 samples were obtained, representing 41 from acute diarrheic episodes (ADE) and 528 from non-ADE. We detected norovirus in 32 of 569 samples (5.6%) and observed the highest detection rate (16.1%, 5/31) in children belonging the group between 6 and 11 months old ($p < 0.001$) (Table 1). Regarding the viral load, the median Ct value observed was 27.7 and 34.3 for ADE and non-ADE, detected in 7 of 41 (17.1%) and 25 of 528 (4.7%), respectively, that represented 21.2% (28/132) of children enrolled in this study. Considering all norovirus-positive samples, the Ct value ranged from 13.2 to 39.4 (median of 32.9). During the period of the study, no norovirus seasonality was observed and the incidence rate was 5.8 infections per 100 child-months (or 69 infections per 100 child-years), between 14 days and 10 months of age.

Norovirus GII and GI were detected in 30 (93.8%) and 2 (6.2%) samples, respectively. Of these, we detected GII norovirus in 7 ADE and 23 non-ADE cases, whilst GI was only detected in two

non-ADE cases (Table 2). It was observed that 26 of 28 children (92.9%) had one norovirus infection, and two children showed sequential infections. In one child, norovirus GII was detected at the age of 20 days and 5 months, both as non-ADE cases. A 1-month-old child who presented non-ADE case was observed to shed the norovirus GII.2[P16] genotype during an interval of 8 days and, after 30 days, norovirus GII persisted, being detected in an ADE case. And one other 6-month-old ADE child was observed to shed the norovirus GII.4 Sydney 2012[P4 New Orleans 2009] genotype during an interval of 10 days.

3.2. Molecular characterization of norovirus in the Manguinhos cohort

Norovirus was genotyped in 18 of 32 positive samples (56.3%) (Figure 1), with 13 samples being genotyped both the B (RdRp) and C (Capsid) regions. Six different genotypes were detected. The most frequent norovirus genotype detected was GII.6[P7] (n = 4), followed by GII.4 Sydney 2012[P31] (n = 2), GII.4 Sydney 2012[P16] (n = 2), GII.2[P16] (n = 2), GII.4 Sydney 2012[P4 New Orleans 2009] (n = 2) and GI.3[P13] (n = 1). In 15.6% of samples (n = 5) could only be amplified using the semi-nested PCR (C region), and norovirus was characterized as GII.4 Sydney 2012 (n = 3) and GII.2 (n = 2) strains.

The GII.4 Sydney 2012 genotype was detected circulating during the entire period of the study in association with three different polymerase types along the years, GII.P31 detected in 2015, GII.P16 in 2016 and GII.P4 New Orleans 2009 in 2017, and the genotype GII.2, harboring the polymerase type GII.P16, was identified circulating in 2017 (Table 2). We also characterized the P2 subdomain of six GII.4 Sydney 2012 strains, and the molecular analysis showed amino acid substitutions in the positions 297 and 372 (epitope A), 333 (epitope B), 340 (epitope C), and 357 (epitope G) (Table S1) between samples evaluated.

3. Norovirus infection and host susceptibility status

In order to associate norovirus infection and host susceptibility profile in this cohort, the HBGA phenotyping previously performed for this population was considered (Cantelli et al., 2019b, unpublished results). Regarding the secretor status, 80.3% (106) were secretors, 15.9% (21) non-secretors and 3.8% (5) inconclusive. Concerning the Lewis phenotype, 59.8% (79) of the children were Le (a+b+), 13.6% (18) Le (a-b+), 15.9% (21) Le (a-b-), and 10.6% (14) Le (a+b-).

The noroviruses were detected in our cohort study in 24 infants/children secretors (92.3%) and in only two non-secretors. All norovirus detected in ADE were reported from secretors (Le^b) ($p = 0.02$) (Table 3), and in asymptomatic norovirus cases (non-ADE), a significantly proportion was detected in Le (a+b+) secretors (80.9%). In this cohort, twenty-one children were phenotyped as Lewis negative, 9 being secretors and 9 non-secretors, and the norovirus was detected in two of them children, one secretor and one non-secretor.

3.4. FUT2 and FUT3 genotyping

The *FUT2* gene was previously sequenced in children phenotyped as secretor Le (a+b+) and non-secretor (Cantelli et al., 2019, unpublished results) and the distribution of the *FUT2* genotypes (SNPs identified in the Manguinhos population) in norovirus positive children is described in Table 4.

For 21 children phenotyped as Le (a-b-), *FUT3* genotyping was performed, and twenty-six SNPs were identified, of which the most frequent were rs28362459 (59T>G) and rs3745635 (508G>A) (66,7%), followed by rs812936 (202T>C), rs778986 (314C>T) and rs28381968 (808G>A) (23.8%), rs28362463 (484G>A, 19.0%), rs28362460 (61C>T), rs5655905 (732C>T) and rs3894326 (1067T>A) (14.3%) (Figure 2). Seven new mutations were identified: 13G>A (19%), 362T>C, 588G>A, 601A>G, 602A>G, 607G>A (4.8%) and, 1052A>G (9.5%) and 10 other SNPs were detected in low frequency: rs28362461 (72G>C), rs749920542 (393C>A), rs372597863 (606G>A), rs28362465 (612A>G),

rs148170391 (645T>C), rs765224654 (696G>A), and rs28381969 (974C>T) (4.8%), rs28362466 (667G>A), rs750177892 (691A>C) and, rs150418165 (968G>C) (9.5%).

4. Discussion

This study reports the frequency and incidence of norovirus infection as well as the circulating genotypes in infants/children from a community-cohort conducted in Manguinhos, Rio de Janeiro. The study previously assessed the G1P[8] rotavirus vaccine (RV1) shedding and the HBGA host genetic susceptibility profile in infants from birth to 11 months of age ([Cantelli et al., 2019b](#), [unpublished results](#)). During this cohort study noroviruses were more frequent than rotaviruses, and a high diversity of norovirus genotypes circulated among these infants/children, even in asymptomatic episodes.

Several studies have shown the importance of establishing the cut-off Ct values for norovirus diagnosis, relating to disease burden in children with acute gastroenteritis ([Phillips et al., 2009](#); [Trang et al., 2015](#)). In our study, most of the samples evaluated were in asymptomatic infants/children in the follow-up. Besides that, several studies with asymptomatic individuals described prolonged periods of norovirus shedding as a common and important characteristic for norovirus transmission efficiency ([Phillips et al., 2010](#); [Teunis et al., 2015](#)). As a criterion for evaluating the positivity for norovirus in this sampling, we verified that some norovirus positive samples could be genotyped with high RT-qPCR Ct values, one of them being with a Ct value of 39.2. For this reason, in this study, the Ct value < 40 was considered.

The frequency of norovirus in the Manguinhos cohort was 5.6%, with 17.1% detected in ADE and 4.7% in non-ADE cases. Norovirus prevalence in non-ADE cases was similar (5%, 95% CI: 4-8%) to that reported by [Nguyen et al. \(2017\)](#). A meta-analysis study recently describing the prevalence of asymptomatic norovirus infection worldwide found a higher detection rate (7%) compared to rate found in the present study ([Qi et al., 2018](#)). On the other hand, the detection rate in ADE cases in

Manguinhos community was similar to rates found by these recently published meta-analysis studies (17-18%) (Nguyen et al., 2017; Qi et al., 2018).

According to child age stratification, the highest norovirus detection rate in the cohort was after 6 months of age (16.1%), consistent with other birth studies worldwide, as in a MAL-ED study (Rouhani et al, 2016; Cannon et al., 2019), although in this age group (6-11 months old), a considerable number of children had discontinued the follow-up, and a low number of stool samples was provided.

In our study, we found an incidence of norovirus infection of 5.8 infections per 100 child-months between 0 and 11 months of age. This norovirus incidence rate was similar to the rate found in a study conducted in Chile, using samples from a birth-cohort collected between 2006 and 2008 from asymptomatic infections from children aged 0-5 months (5.7 cases per 100 child-months) (O'Ryan et al., 2009). Similar findings were also described in an Ecuador cohort in 2009 that showed a peak incidence of 6.3 cases per 100 child-months among 6 to 11 months of age (Lopman et al., 2015).

In relation to the norovirus infections detected in the study, the majority of the noroviruses were identified in non-ADE cases from 0 to 5 months of age (78%, 25/32). This can be explained by breastfeeding during the first months of life in the birth cohort, by which maternal antibodies and human milk oligosaccharides rich in α 1,2-linked fucosylated glycans (depending on the mother's secretor/lewis status) can act as first line of defense against severe norovirus disease (diarrhea) in breastfed infants (Jiang et al., 2004; Newburg et al., 2004; Lopman & Kang, 2014; Saito et al, 2014; Bode, 2015; Siqueira et al., 2017; Krammer & Bouckaert, 2018).

Most of the norovirus strains screened were classified as GII (93.8%) and five different genotypes were identified. Overall, the GII.4 Sydney 2012 variant was the most prevalent genotype (50%, 9/18) in the Manguinhos cohort, in agreement with several surveillance studies in Brazil and worldwide (Fioretti et al., 2014; Vinjé, 2015; Cannon et al., 2017; Costa et al., 2017). This variant

circulated harboring three different polymerase types: GII.P31, GII.P16 and GII.P4 New Orleans 2009 according to that reported in our previous study (Cantelli et al., 2019) during the same period (2015-2017), in children between 1 to 4 years of age in the Manguinhos community, besides USA outbreaks reported in a relatively close period (Cannon et al., 2017; Barclay et al., 2019). The analysis of the P2 subdomain of the GII.4 Sydney 2012 strains (GII.4[P31], GII.4[P16] and GII.4[P4]) showed amino acid substitutions with variability patterns similar to those previously described by Cannon et al. (2017) and Barclay et al. (2019) in their studies with this variant, in fact, the previously described G and H (NERK motif) epitopes (Tohma et al., 2019) were analyzed in this study. Tohma et al. (2019) suggest the 352, 357 and 378 residues (epitopes C and G) seem to play a major role in the antigenic topology and emergence of new GII.4 variants.

In the Manguinhos cohort, the emergent norovirus polymerase type GII.P16 was identified in association with two capsid genotypes, GII.4 Sydney 2012 and GII.2 in 2016 and 2017, respectively. These both strains have been described causing acute gastroenteritis outbreaks worldwide in these same years (Barreira et al., 2017; Li et al., 2017; Niendorf et al., 2017; Hata et al., 2018; Medici et al., 2018).

In 2017, we reported the emergent recombinant GII.2[P16] for the first time in Brazil (Cantelli et al., 2019) in 2 ADE children aged 22 and 48 months. In the present birth-cohort study, this recombinant was identified from an asymptomatic child 1-month-old, and molecular analysis showed a high similarity (> 99% nt identity) between this strain detected in Brazil with German strains (Niendorf et al., 2017).

Concerning the genetic susceptibility, studies in association with norovirus infection in asymptomatic individuals are rare (Nordgren & Svensson, 2019); the majority of norovirus asymptomatic infections were detected in Le (a+b+) secretor infants/children, associated with the most common capsid genotypes, GII.4 Sydney 2012 and GII.6 (Currier et al., 2015; Sharma et al.,

2019). However, one non-secretor child was infected with GII.4 Sydney 2012 (in non-ADE case), similar to that reported by some studies (Frenck et al., 2012; Nordgren et al., 2013). Besides that, significant correlation between norovirus symptomatic infection and secretor *status* (Le^b) was observed.

The high percentage of children characterized as secretors found in our cohort, in agreement with other studies conducted in Latin America (Vicentini et al., 2013; Lopman et al., 2015; Bucardo et al., 2018; Moraes et al., 2019), coupled with a limited number of samples representative of each genotype, hamper the assessment of any association between host susceptibility vs norovirus genotypes, and could be cited as a limitation of our study. Secretor individuals with a Le (a+b+) phenotype can be susceptible to different norovirus genotypes, both secretor-dependent or -independent, probably due to a lower expression of secretor glycans on gut epithelial surfaces (Nordgren & Svensson, 2019).

According to our results, low detection of norovirus in Le (a-b-) children could suggest this profile has 'some degree' of resistance to norovirus, mainly when associated to the synergistic effect of the non-secretor *status* (approximately 50% of the Le (a-b-) children were characterized as non-secretors). According to studies conducted in Burkina Faso (Nordgren et al., 2013) and Nicaragua (Bucardo et al., 2009), Lewis negative secretor individuals were susceptible to norovirus, indicating the importance of the *FUT2* gene as a mediator of susceptibility (Bucardo et al., 2009; Kindberg & Svensson, 2009; Nordgren & Svensson, 2019).

In our study we sequenced the *FUT3* gene to evaluate the occurrence of the main SNPs in children with Le (a-b-) *status* in Manguinhos cohort. The main mutations rs28362459 (59T>G), rs812936 (202T>C), rs778986 (314C>T), rs3745635 (508G>A), and rs3894326 (1067T>A) leads to reduction or inactivation of the $\alpha(1,3/1,4)$ -fucosyltransferase. These mutations appear to be distributed worldwide (Koda et al., 1993; Pang et al., 1998), and were also identified in our study and

in other previously conducted in Brazil (Corvelo et al., 2013; Olivares et al., 2019, unpublished results). Pang et al. (1998) reported alleles nonfunctional $le^{484,667}$ and $le^{484,667,808}$ in *FUT3* gene, in South African individuals (including the black Xhosa-Africans). Few studies have estimated the distribution of the SNPs *FUT3* gene in African Americans (Cakir et al., 2002), and in Manguinhos cohort was possible to identify the occurrence of the mutations rs28362463 (484G>A), rs28362466 (667G>A), and rs28381968 (808G>A) in our population, suggesting a genetic proximity between Africa and Rio de Janeiro population.

5. Conclusion

Our study highlights the norovirus incidence/frequency, circulating genotypes and host susceptibility profile results in the Manguinhos cohort, in Rio de Janeiro, consistent with other studies, and describes for the first time the evaluation of norovirus incidence and host susceptibility profile of children from a birth-community cohort in Brazil. This study emphasizes the norovirus diversity circulating during the rotavirus vaccination period, mainly in asymptomatic episodes, demonstrating the emergency of these viruses and the importance of surveillance studies in communities after the introduction of rotavirus vaccines and in this norovirus pre-vaccination era. These results will be able to contribute to understanding of these questions that have an impact on public health.

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Table 1: Norovirus detection from stool samples collected from the infants/children with acute diarrheic episodes (ADE) or not (non-ADE) according to age group.

Age group	Number of followed-up children	Clinical status Norovirus positive		Clinical status Norovirus negative		Number of positive samples/Number of stool samples investigated (%)	<i>p</i> value ^a
		ADE	non-ADE	ADE	non-ADE		
		< 2 months	127	0	14		
2 - < 6 months	125	2	11	14	207	13/234 (5.6)	
6 - 11 months	24	5	0	15	11	5/31 (16.1)	
Total	132	7	25	34	503	32/569 (5.6)	

^a*P* value was determined by Fisher's exact test.

Table 2. Norovirus (genogroups/genotypes) detection in 28 children from a birth community-cohort, Manguinhos, Rio de Janeiro city, Brazil, 2014-2018*. Information about norovirus RT-qPCR Ct value, clinical symptoms (acute diarrheic episode/ADE or non-ADE) and age are described.

Year	Norovirus	Code child	Ct value	ADE	non-ADE	Age
2015	GII.4 Sydney 2012[P31]	029, 009	23.9, 15.8	-	2	2m 8d, 5m 10d
	GII.6[P7]	014, 016	16.4, 20.5	-	2	2m 8d, 4m 20d
	GII.4 Sydney 2012	095	39.2	-	1	14d
	GI	001	39.4	-	1	4m
	GII	017	35.7	-	1	1m 15d
	GII	224	37.6	-	1	1m
	GII	071	38.8	-	1	4m 26d
	GII	096	33.6	-	1	1m 18d
2016	GII.4 Sydney 2012[P16]	169, 222	17.8, 33.2	2	-	9m, 10m
	GI.3[P13]	160	34.3	-	1	4m 13d
	GII.4 Sydney 2012	219	32.4	1	-	10m
	GII	144	36.4	-	1	5m 27d
	GII	212	38.2	1	-	5m 16d
	GII	225	31.7	-	2	20d, 5m
	GII	226	32.7	-	1	2m 17d
	GII.6[P7]	250, 289	29.7, 19.3	-	2	1m 1d, 5m 3d
2017	GII.4 Sydney 2012[P4 New Orleans 2009]	265 ^a	13.2	1	-	6m 2d
	GII.2[P16]	275 ^b	16.2	-	1	1m 3d
	GII	275 ^b	27.7	1+	-	2m 14d
	GII.4 Sydney 2012	291	32.7	-	1	1m 16d
	GII.2	274, 282	35.3, 34.4	-	2	27d, 28d
	GII	234	38.8	-	1	14d
	GII	293	38.9	-	1	1m

Notes:

*At the end of 2014 and at the beginning of 2018, norovirus strains were not detected in children between 0-11 months of age. m= months, d= days;

^aThis child was observed to shed the GII.P4 New Orleans 2009-GII.4 Sydney 2012 genotype after an interval of 10 days.

^bThis child was observed to shed the GII.P16-GII.2 norovirus genotype after an interval of 8 days (Ct value 16.7), and approximately after 30 days, the GII was detected. *In this sample a G1P[8] rotavirus vaccine shedding was codetected.

Table S1: Amino acid sequences of the P2 subdomain of the VP1 capsid protein of GII.4 Sydney 2009 with different polymerase genotypes (GII.P31, GII.P16 and GII.P4 New Orleans 2009). # Sites within HBGA binding site and +NERK motif.

GII.4 Sydney 2012		Deduced amino acid sequences of the structural P2 subdomain of the capsid protein (VP1)																												
		Epitope A						Epitope B		Epitope C			Epitope D			Epitope E			Epitope G				Epitope H							
Year	B region	2	2	2	2	2	3	3	3	3	3	3	3	3	3	3	3	3	3	4	4	4	3	3	3	3	3	3	3	3
		9	9	9	9	9	6	7	7	3	8	4	7	7	9	9	9	9	9	0	1	1	5	5	5	5	5	6	0	1
		4	5	6	7	8	8	2	3	3	2	0	6	8	3	4	5	6	7	7	2	3	2	5	6	7	9	4	9	0
		#																												
2015	[P31]	T	G	S	H	N	E	N	H	V	K	T	E	N	S	T	T	H	R	S	N	T	Y	S	A	E	A	R	N	N
2016	[P16]	T	G	S	R	N	E	D	H	M	K	T	E	N	S	T	T	H	R	S	N	T	Y	S	A	D	A	R	N	N
2017	[P4]	T	G	S	R	N	E	D	H	M	K	A	E	N	S	T	T	H	R	S	N	T	Y	S	A	D	A	R	N	N

Table 3: Distribution the Secretor/Lewis children (n = 26) with norovirus in acute diarrheic episode (ADE) and non-ADE in a birth community-cohort, Manginhos, Rio de Janeiro city, Brazil, between November 2014 and November 2018.

Secretor/Lewis profile	Norovirus ADE		Norovirus non-ADE		p value ^a
	n = 5	(%)	n = 21	(%)	
Secretor	5	(100)	19	(90.5)	0.47
Non-secretor	0	(0)	2	(9.5)	
Le(a+b-)	0	(0)	1	(4.8)	0.02
Le(a-b+)	3	(60)	1	(4.8)	
Le(a+b+)	2	(40)	17	(80.9)	
Le (a-b-)	0	(0)	2	(9.5)	

^ap values were determined by Chi-square test.

Table 4: Norovirus (capsid genotypes) and host susceptibility (Secretor/Lewis) status and FUT2 genotyping in children from a birth community-cohort, Manguinhos, Rio de Janeiro city, Brazil.

Norovirus genotypes	n children	Secretor status	Lewis status	FUT2 genotyping
GII.4 Sydney 2012	3	<i>Se</i>	Le (a+b+)	<i>Se</i> 171A>G, 216 C>T, 357T>C, 428G>A, 739G>A, 960A>G, 1009A>G, 1011T>C
	1	<i>se</i>	Le (a+b-)	<i>se</i> 171A>G, 216 C>T, 357T>C, 428G>A, 544G>A, 739G>A, 771G>A, 960A>G, 1009A>G, 1011T>C
	3	<i>Se</i>	2 Le (a-b+), 1 Le (a-b-)	NI
	1	<i>Se</i>	Le (a+b+)	without SNP
GII.6	3	<i>Se</i>	2 Le (a+b+), 1 Le (a-b+)	<i>Se</i> 171A>G, 216 C>T, 357T>C, 428G>A, 739G>A, 960A>G, 1009A>G, 1011T>C
GII.2	3	<i>Se</i>	Le (a+b+)	<i>Se</i> 171A>G, 216 C>T, 357T>C, 428G>A, 739G>A, 960A>G, 1009A>G, 1011T>C
GI.3	1	<i>Se</i>	Le (a+b+)	<i>Se</i> 171A>G, 216 C>T, 357T>C, 428G>A, 739G>A, 960A>G, 1009A>G, 1011T>C

Notes:

Se = heterozygous secretor, *se* = homozygous non-secretor, NI = no information; SNP = single nucleotide polymorphism.

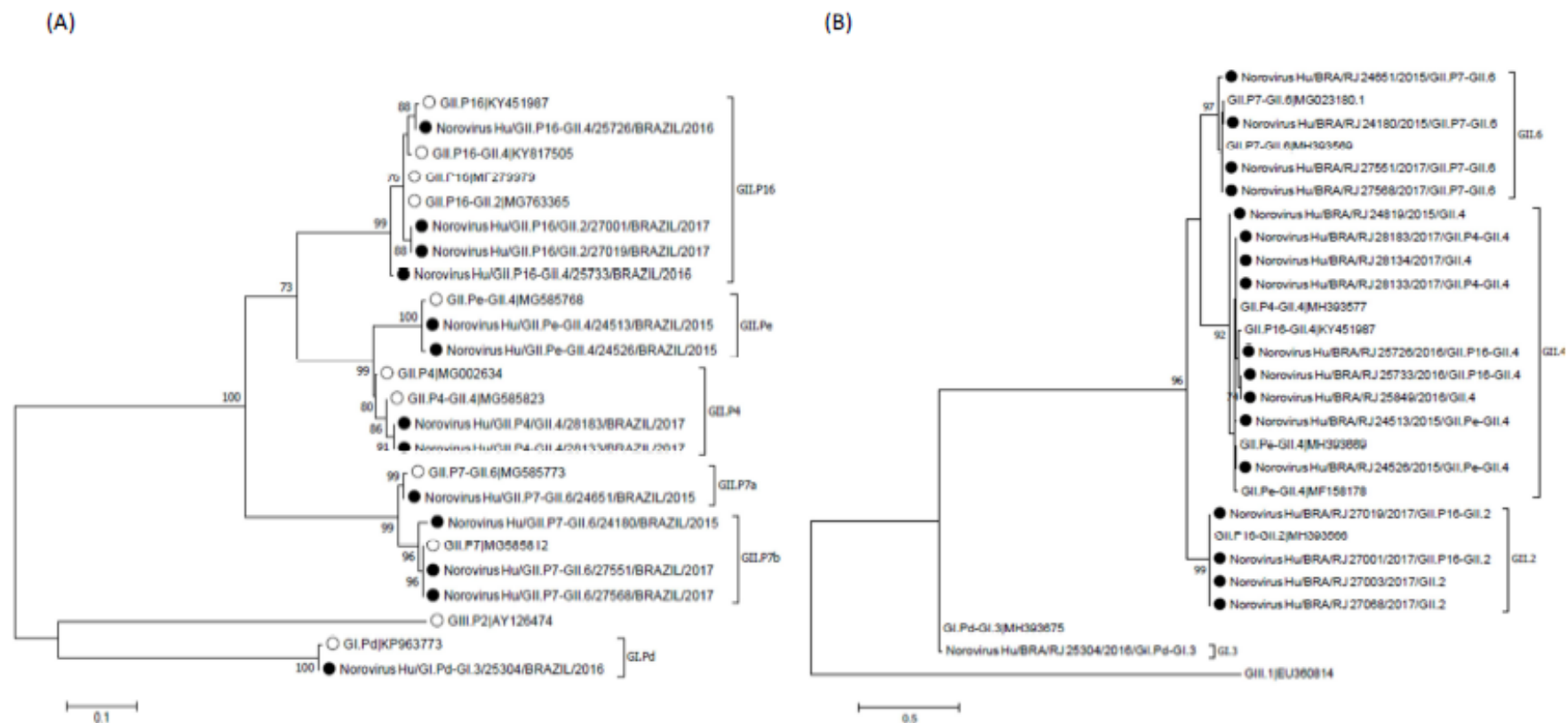


Figure 1: Phylogenetic analysis of GI/GII noroviruses based on partial nucleotide sequences of the polymerase and capsid regions from infants and children from a birth community-cohort, from 2014 to 2018, in Manguinhos, Rio de Janeiro city, Brazil. (A) Phylogenetic tree of a 199-bp portion of the B region (polymerase), and (B) Phylogenetic tree of a 215-bp portion of the gene encoding the C region (VP1). Nucleotide sequences were analyzed using the maximum-likelihood method with the K2+G+I and K2 nucleotide substitution models, respectively, and bootstrap values >70% are shown at the nodes of the tree as percentages based on 2,000 replicates. The strains reported in this study are indicated by filled black circles. The reference strains of norovirus genotypes are shown with their respective GenBank accession numbers.

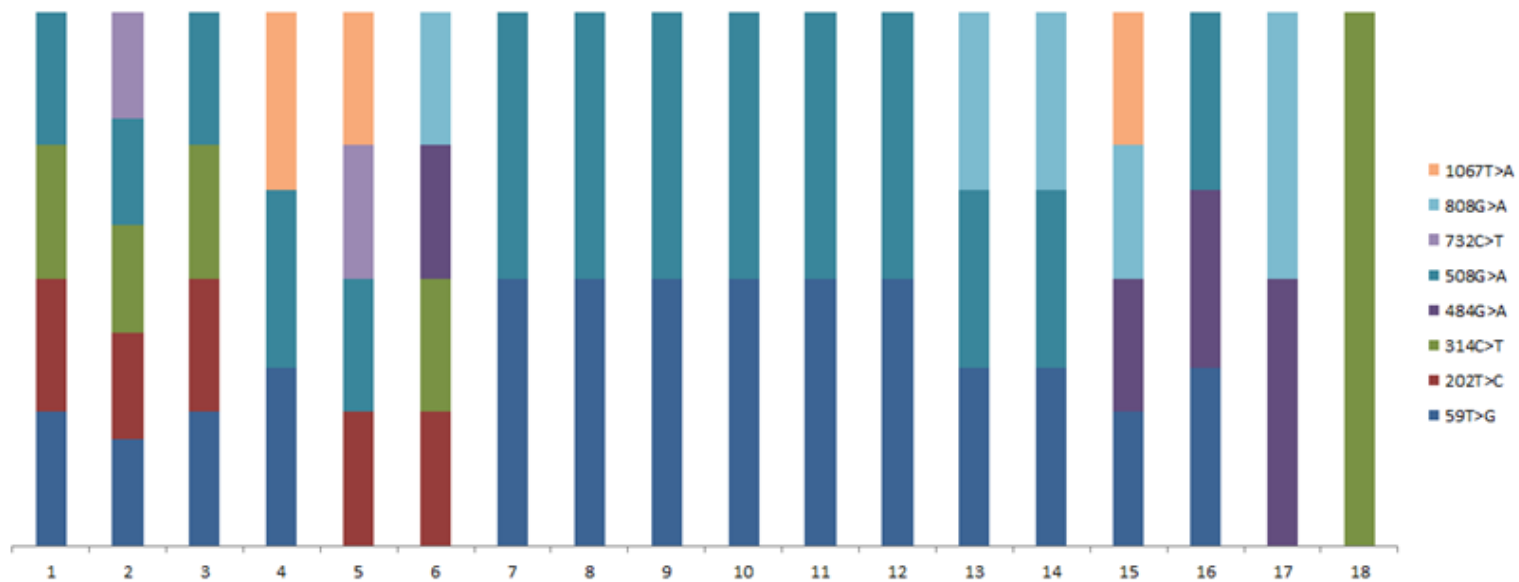


Figure 2: Distribution of eight SNPs in *FUT3* gene more frequent in the children (n = 18) from birth community-cohort phenotyped as Le (a-b-). Norovirus was detected in three children (4, 7 and 10) in non-acute diarrheic episodes (non-ADE), from 1 month to 2 months and 8 days of age. The study was conducted between November 2014 and November 2018, in Manguinhos, Rio de Janeiro city, Brazil.

High genetic diversity of noroviruses in children from a community-based study in Rio de Janeiro, Brazil, 2014-2018.

Este estudo está relacionado ao objetivo 5.

Publicado: Archives of Virology

Resumo: Neste estudo foi realizada a detecção e caracterização dos genótipos de norovírus que circularam entre as crianças da comunidade de Manguinhos, Rio de Janeiro, na faixa etária de 1 a 4 anos, entre o período de novembro de 2014 a abril de 2018. Os resultados demonstraram uma alta frequência (47,5%) de norovirose nesta população (em 46,7% dos casos diarreicos e 50% dos casos assintomáticos), além de uma alta diversidade de genótipos circulantes (GII.4 Sydney 2012[P31], GII.4 Sydney 2012[P16], GII.4 Sydney 2012[P4 New Orleans 2009], GII.2[P16], GII.17[P17], GII.7[P7], GII.6[P7], GI.7[P7], GI.3[P13] e GI.1[P1]), sendo detectado pela primeira vez o recombinante GII.2[P16] no Brasil.



High genetic diversity of noroviruses in children from a community-based study in Rio de Janeiro, Brazil, 2014-2018

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Abstract

We report on the occurrence and diversity of noroviruses in children (younger than 5 years old of age) from a low-income urban area in Rio de Janeiro, Brazil. Sixty-one stool specimens collected from children between 1 and 4 years old with acute diarrhoeic episodes (ADE) and non-ADE were investigated. RT-qPCR and sequencing of PCR products after conventional RT-PCR analysis were performed. Noroviruses were detected in 29 (47.5%) samples: 21 (46.7%) from cases with ADE and 8 (50%) from non-ADE cases. Molecular characterization showed 10 different genotypes circulating in this community between November 2014 and April 2018.

Acute gastroenteritis (AGE) is the second leading cause of morbidity and mortality in children under 5 years old worldwide [1]. Rotavirus A (RVA) and norovirus are the most important viral pathogens in AGE [2]. After the introduction of RVA vaccines, noroviruses replaced RVA as the main cause of AGE affecting children [3], and GII.4 has been

the predominant genotype for over 2 decades [4–6]. However, uncommon genotypes such as GII.17 and GII.2 have recently emerged and caused outbreaks in many countries worldwide [6, 7]. Currently, there are several norovirus vaccine candidates. It is important to understand the burden of norovirus-associated AGE as well as the genetic diversity of noroviruses prior to the introduction of an effective vaccine [8, 9]. In this study, we investigated the occurrence and genetic diversity of norovirus in stool samples from children with acute diarrhoeic episodes (ADE) and non-ADE from a low-income urban area, Manguinhos community, which is part of the metropolitan region of Rio de Janeiro, Brazil.

Stool specimens were obtained from children aged 1–4 years with diarrhoea (≥ 3 liquid or semi-liquid evacuations in a 24-h period) or who were asymptomatic (with no episodes of diarrhoea for at least 1 week before collection date) and were undergoing routine pediatric examinations at the Germano Sinval Faria Health Center (GSFHC), National School of Public Health, Oswaldo Cruz Foundation, between November 2014 and April 2018. Forty-nine children were enrolled in this study, and 61 stool samples were obtained. Ten children returned to the health unit during the study period, and a stool sample was collected each time. Thus, 2 or 3 stool samples were obtained from some of these children regardless of whether they had ADE. Samples were collected from children whose parents had formally agreed to take part in the research study, and none of the children

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had blood relatives (siblings). A new norovirus episode was defined when it occurred more than 2 weeks after the previous episode. Viral RNA was extracted from stool suspensions (10% w/v) using an automatic RNA extraction procedure according to the manufacturer's instructions (QIAcube® Automated System and QIAamp® Viral RNA Mini kit; QIAGEN, CA, USA). Norovirus screening was performed using reverse transcription quantitative polymerase chain reaction (RT-qPCR) on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using primers, probes and conditions described previously by Kageyama et al. [10].

For norovirus genotyping, RT-PCR was performed using the primers Mon431/G2SKR (GII) and Mon432/G1SKR (GI) to generate 570-base-pair (bp) and 579-bp fragments, respectively [11]. The resulting amplicons were purified using Wizard® SV Gel and a PCR Clean-Up System kit (Promega, Madison, USA) following the manufacturer's instructions, and they were analysed by Sanger sequencing using a BigDye® Terminator v3.1 Cycle Sequencing Kit and an ABI Prism 3500 Genetic Analyser® (Applied Biosystems, Foster City, CA, USA). Consensus sequences were obtained using the BioEdit 7.2.1 Sequence Alignment Editor [12]. Genotypes were assigned using the Norovirus Automated Genotyping Tool (<https://www.rivm.nl/mpf/typingtool/norovirus/>) [13], and nucleotide similarity was assessed using the Basic Local Alignment Search Tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences obtained in the current study were deposited in the GenBank database under the accession numbers MH393565-MH393566, MH393568-MH393571, MH393574-MH393580, and MH393582-MH393588. Phylogenetic analysis were performed using the maximum-likelihood method, with the K2+G+I model applied for analysis of portions of the regions encoding the polymerase (RdRp/region B) and major capsid protein (VP1/region C). Phylogenetic trees were constructed with 2,000 bootstrap replicates in MEGA v.7.0.26 [14].

Twenty-nine (47.5%) out of the 61 stool specimens collected were positive for norovirus, representing 53.1% (26/49) of the children who were examined at the GSFHC (19 out of 35 with ADE, 6 out of 9 without ADE and 1 out of 5 who provided samples during both ADE and non-ADE). Norovirus was detected in 46.7% (21/45) and 50% (8/16) of ADE and non-ADE cases, respectively. The B-C typing region of 21 (72.4%) out of 29 norovirus-positive samples was sequenced in order to determine the norovirus genotype (Fig. 1). Ten different genotypes were detected, and the most frequent were GII.P4-GII.4 (4/21), GII.P7-GII.6 (4/21), and GI.7-GI.7 (4/21), followed by GII.P16-GII.2 (2/21), GII.P17-GII.17 (2/21), GII.P16-GII.4 Sydney (1/21), GII.Pe-GII.4 Sydney (1/21), GII.P7-GII.7 (1/20), GI.Pd-GI.3 (1/21),

and GI.P1-GI.1 (1/21) (Table 1). Of the positive samples, 21 (72.4%) were identified as GII, seven (24.1%) as GI, and one (3.5%) as a GI/GII mixed infection (GI.P7-GI.7 and GII.P17-GII.17). Norovirus GII was observed in 14 ADE and seven non-ADE cases, while GI was present in seven ADE cases. Twenty-four (92.3%) out of 26 positive children had one norovirus episode, and 7.7% (2/26) had two. Two children, aged 15 and 20 months (both non-ADE), were positive for norovirus GII in the first episode (both untypable), and in the second episode, one 19-month-old child with ADE shed norovirus GII.P4-GII.4 and the other, a 27-month-old child (non-ADE), shed norovirus GII.P7-GII.6. One 12-month-old child who presented with ADE was observed to shed norovirus GI.P7-GI.7 genotype after an interval of 7 days. Analysis according to age group showed that the highest norovirus detection rate was observed in children aged 12-23 months (48.7%, 19/39).

The frequent detection and genetic diversity of noroviruses in non-ADE children observed in this community may be a consequence of frequent exposure to these viruses. This can result in asymptomatic episodes due to some degree of acquired mucosal immunity by children who are constantly challenged by noroviruses [3, 15]. Individuals living in this community have limited access to public services, especially sanitation; as such, it is considered a precarious area of the city in which to live. Presumably, such an environment, together with the environmental stability of noroviruses and the low infective dose, would drive viral transmission, causing norovirus-associated ADE in susceptible children [16, 17]. Post-symptomatic norovirus shedding can be detected after resolution of symptoms for several weeks or months [18, 19], and several studies focusing on non-ADE children have demonstrated that asymptomatic excretion of norovirus in stool samples is common, particularly in low income/hygiene settings and does not necessarily reflect a pre- or post-symptomatic event [16, 18, 20]. Norovirus detection rates ranging between 0% and 49% have been reported worldwide [19, 21, 22]. In developing countries, studies have addressed the prevalence of noroviruses in hospitalized children, an approach that causes the prevalence of viral infection to be underestimated, because it does not include genotypes circulating in asymptomatic individuals, particularly those living in communities [3, 23, 24].

The highest norovirus rate detected in children aged 12-23 months (48.7%, 19/39) was consistent with other studies of outpatient children from developing countries [25, 26]. Here, noroviruses were detected during all seasons, similar to observations in Cochabamba, Bolivia [24], and in rural communities in the Vhembe district of South Africa [26]. Xavier et al. [27] studied ADE in a community of the city of Salvador in northeastern Brazil, in the pre-RVA vaccination era and found 9.0% human caliciviruses in children up to 3

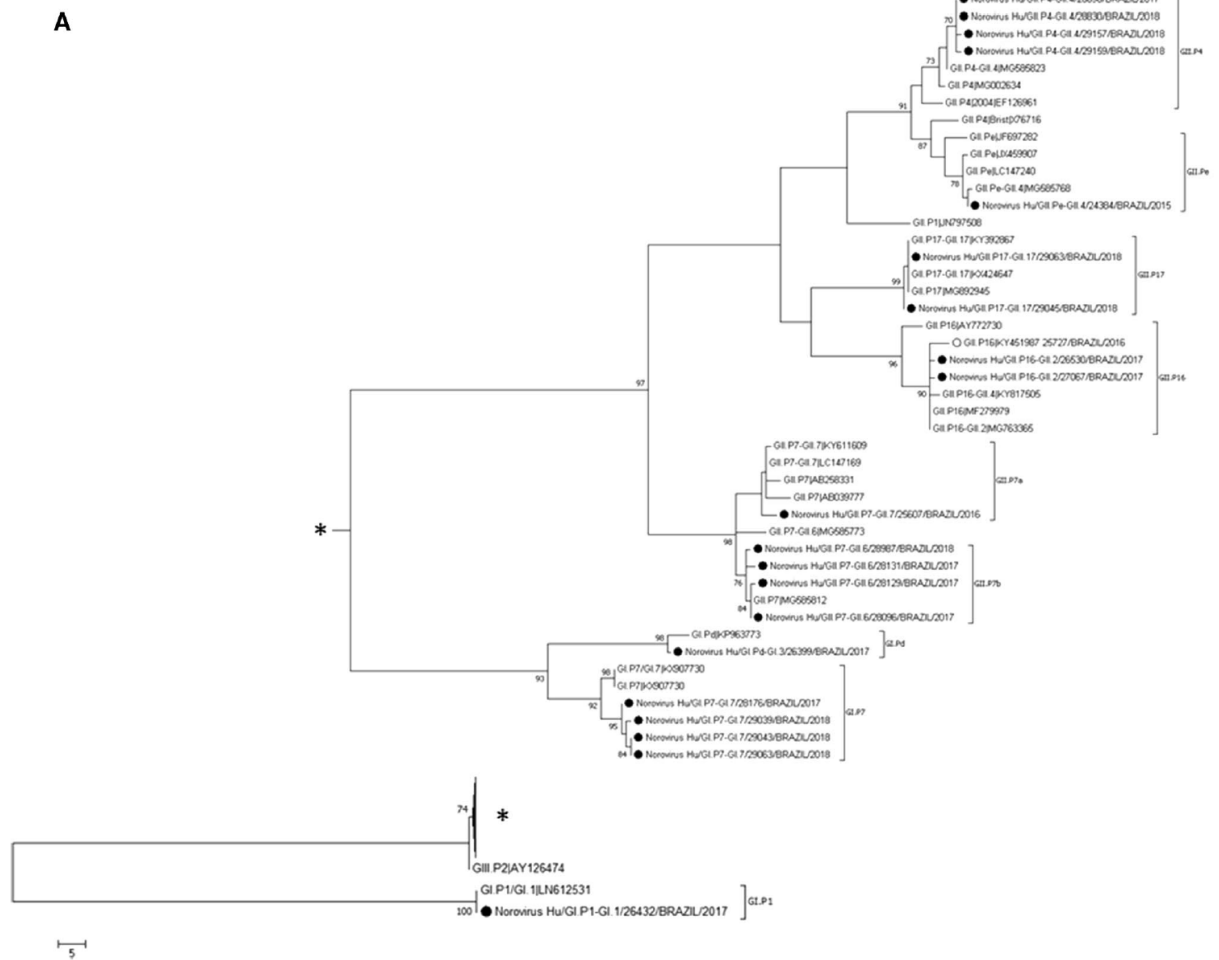


Fig. 1 Phylogenetic analysis of GI/GII noroviruses based on partial nucleotide sequences of the polymerase and capsid regions from children exhibiting acute diarrhoeic episodes (ADE) or those without ADE in the Manguinhos community, Rio de Janeiro, Brazil, from 2015 to 2018. (A) Phylogenetic tree of a 179-bp portion of the polymerase gene (RdRp/region B). (B) Phylogenetic tree of a 215-bp portion of the gene encoding the major capsid protein (VP1/region C). The names of the reference strains of norovirus genotypes are

with their respective GenBank accession numbers. Nucleotide sequences were analysed using the maximum-likelihood method with the K2+G+I nucleotide substitution model, and bootstrap values > 70% are shown at the nodes of the tree as percentages based on 2,000 replicates. The norovirus strains reported in this study are indicated by filled black circles. The strain GII.P16-GII.4 reported by Barreira et al. [34] (KY451987) is indicated by an empty circle

years old. The MAL-ED group detected norovirus in 23.5% of ADE (range, 7.1–32.8%) and 19.0% of non-ADE (range, 2.2–30.4%) in children up to 2 years old between November 2009 and February 2012 [28].

Norovirus GII was the most prevalent genogroup (73.3%), with circulation of 7 genotypes, which was consistent with studies in Brazil [23] and worldwide [29], showing the co-circulation of different GII genotypes in Manguinhos between 2014 and 2018. The predominant genotype in non-ADE children was GII.P7-GII.6 (3 cases), while GII.

P4-GII.4 was predominant in those with ADE (2 cases). According to partial RdRp analysis (Fig. 1A), GII.P7 formed two different clusters (GII.P7a and GII.P7b), suggesting some degree of variability in this genotype. The GII.P7-GII.7 strain was detected in a child with ADE in May 2016, while GII.P7-GII.6 viruses were detected in September 2017 in three non-ADE children and one ADE child in February 2018 at different sites in Manguinhos.

Norovirus GII.4 was detected in association with multiple polymerase genotypes, including GII.P4, GII.Pe and

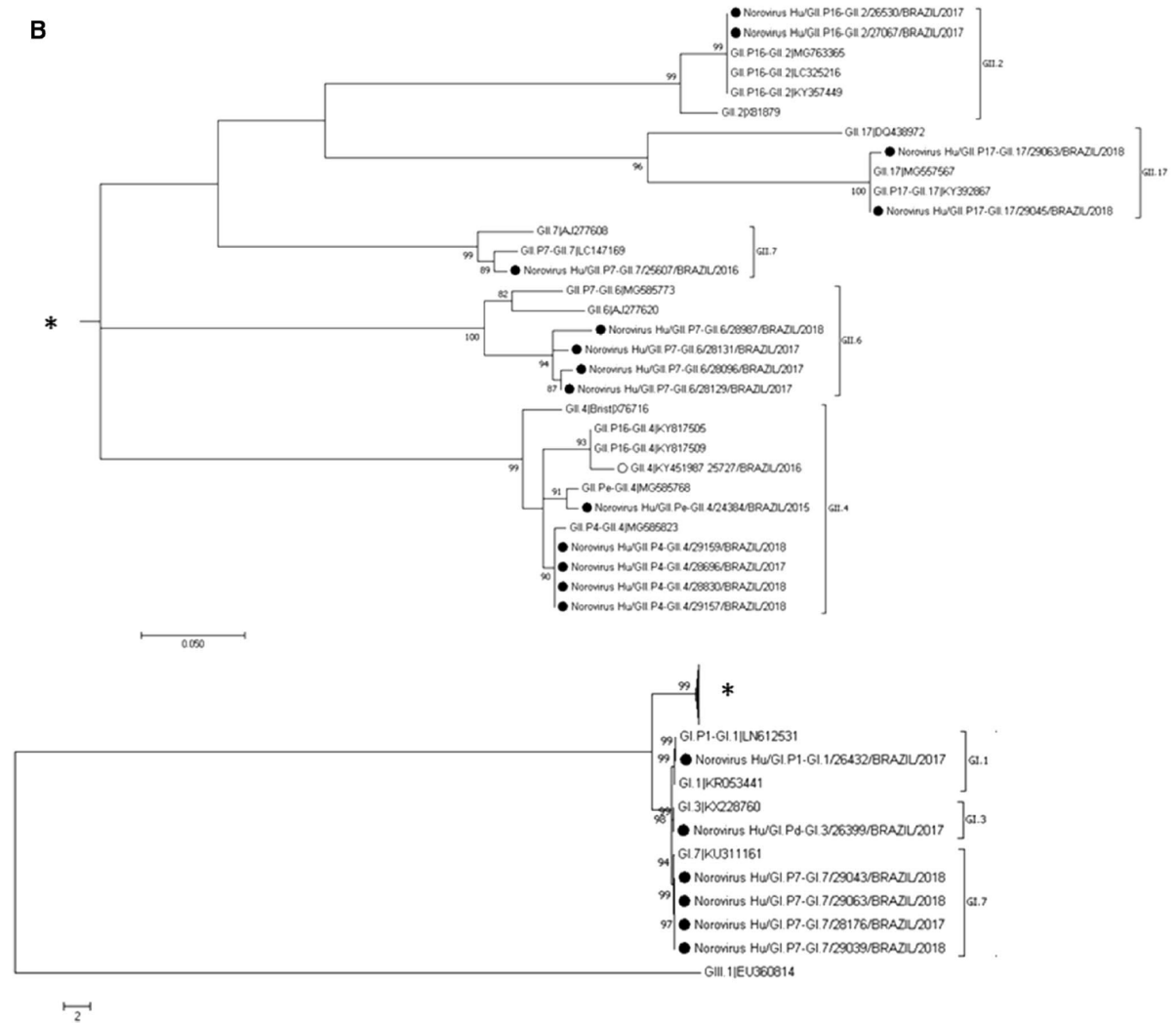


Fig. 1 (continued)

GII.P16. Furthermore, the GII.P16 genotype was detected in association with two GII variants, GII.4 and GII.2, both of which emerged as a major cause of ADE outbreaks in different countries during 2016-2017 [30–34]. The GII.P16-GII.2 viruses exhibited significant similarity (99% identical nucleotides) to strains described in 2016 in Germany [30] and subsequently detected in Italy, China and Japan [31–33]. This study shows original data on detection of recombinant GII.P16-GII.2 in Brazil. The finding of the GII.P17-GII.17 genotype in both ADE and non-ADE

cases suggests that this genotype had continued to circulate in the Brazilian population since 2015, when it was first described [35].

In conclusion, this study highlights the diversity of noroviruses co-circulating in the low-income community of Manguinhos, Rio de Janeiro, Brazil. These results contribute to our understanding of norovirus strain diversity and emphasize the importance of surveillance studies in communities both in the norovirus pre-vaccination period and as a follow-up in the post-RVA vaccination era.

Table 1 Norovirus genotypes of 21 strains detected in children with acute diarrhoeic episodes (ADE) or non-ADE in the Manguinhos community, Rio de Janeiro, Brazil, from 2015 to 2018

Year	Norovirus genotype	Number of cases (%)	ADE	Age (months)	Non-ADE	Age (months)
2015	GII.Pe-GII.4	1 (4.8)	1	13	-	-
2016	GII.P16-GII.4	1 (4.8)	-	-	1	17
	GII.P7-GII.7	1 (4.8)	1	16	-	-
2017	GII.P16-GII.2	2 (9.5)	2	22, 48	-	-
	GI.Pd-GI.3	1 (4.8)	1	19	-	-
	GII.P7-GII.6	3 (14.2)	-	-	3	23, 27, 29
	GI.P7-GI.7	1 (4.8)	1	23	-	-
	GII.P4-GII.4	1 (4.8)	1	29	-	-
	GI.P1-GI.1	1 (4.8)	1	21	-	-
2018	GII.P7-GII.6	1 (4.8)	1	12	-	-
	GII.P4-GII.4	3 (14.2)	2	19, 30	1	26
	GI.P7-GI.7	3 (14.2)	2	12, 13	1	30
	GII.P17-GII.17	2 (9.5)	1	14	1	30

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the Ethics Committee of Fiocruz (CEP 311/06; CEP 688.566/14).

Informed consent Informed consent was obtained from the parent or guardian of each child included in this study.

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4 DISCUSSÃO

Com o objetivo de associar o perfil de susceptibilidade genético (HBGAs) frente às infecções para os RVA e norovírus, este estudo avaliou o perfil de susceptibilidade de 132 crianças acompanhadas em uma coorte infantil, desde o nascimento até os 11 meses de idade. A estratégia de se estudar uma coorte de crianças que recebam a vacina RV1 monitorando a eficácia desta vacina, além das infecções naturais ocorridas no período de estudo também objetivou contribuir para o entendimento de como o perfil do hospedeiro pode influenciar a epidemiologia e evolução destes vírus.

Os resultados obtidos neste estudo demonstraram uma alta frequência do perfil secretor (80,3%), destacando-se o fenótipo Le (a+b+) (59,8%). A alta prevalência de indivíduos secretores em países da América Latina é relativamente comum, confirmando os dados apresentados em estudos conduzidos em outros países como Chile, Equador e Nicarágua (O' Ryan et al, 2009, Lopman et al, 2015, Bucardo et al, 2018), assim como em outras regiões do Brasil, como a região Amazônica, estado de Roraima (Moraes et al, 2019) e Espírito Santo, esta última de uma comunidade quilombola, composta de descendentes de escravos (Vicentini et al, 2013).

O perfil Le (a+b+) secretor é um fenótipo relativamente comum em crianças < 2 anos de idade, podendo ser considerado um *status* transiente nesta faixa etária (Cooling, 2015). Postula-se que o fenótipo de Lewis ainda não tenha atingido sua maturidade, devido às fucosiltransferases (FUT2/FUT3) ainda não alcançarem níveis normais neste grupo etário (Henry et al, 1995). Em populações asiáticas, este fenótipo é atribuído ao SNP rs1047781 (385A>T), sendo designado 'fraco secretor' (*weak*, Se^w) (Kelly et al, 1995), sugestionando a este perfil um 'certo' grau de resistência à infecção grave pelos RVA, em comparação ao *status* Le (a-b+) secretor (Yang et al, 2017). Na coorte aqui avaliada, em 17,9% das crianças fenotipadas como Le (a+b+) secretor, nenhum SNP no gene *FUT2* foi detectado, podendo tratar-se de um *status* Lewis transiente.

A caracterização nucleotídica realizada no gene *FUT2* em crianças com o perfil Le (a+b+) secretor da coorte de Manguinhos demonstrou que o SNP

rs1047781 (385A>T) não estava presente na população do Rio de Janeiro, sendo similar ao descrito no estudo conduzido por Moraes e colaboradores (2019) na população de Roraima. As crianças da coorte de Manguinhos com este perfil apresentaram uma alta frequência do SNP rs281377 (357C>T). De acordo com Ferrer-Admetlla e colaboradores (2001) este SNP está distribuído naturalmente no mundo, sendo o haplótipo *se*^{357/385} detectado em alta frequência na população asiática (Pang et al, 2001, Park et al, 2005, Guo et al, 2017). Possivelmente, através da ótica evolucionária, o haplótipo *se*^{357/385} poderia ter divergido e segregado o SNP rs1047781 (385A>T) na população brasileira. Interações epistáticas do SNP rs281377 (357C>T) com outros, poderia afetar a expressão do gene *FUT2*, explicando este fenótipo Le (a+b+) secretor em nossa população. Contudo, estudos envolvendo um número amostral mais robusto, assim como a avaliação completa do perfil Le (a-b+) secretor, são fundamentais para a obtenção de valores estatisticamente significativos e, que possam comprovar esta hipótese.

Em relação ao *status* não secretor (*se*), somente uma das crianças classificada neste fenótipo (1/21) não apresentou o SNP rs601338 (428G>A). Este SNP é responsável pela não funcionalidade da $\alpha(1,2)$ -fucosiltransferase (enzima *Se*) (Kelly et al, 1995), e este resultado sugere que outros fatores genéticos poderiam estar relacionados a este perfil sem atribuição ao SNP rs 601338 428G>A (Daniels, 2013). Dentre eles, mutações no gene *FUT1* (antígeno H) (Cooling, 2015, Michalewska et al, 2018) poderiam explicar este fenótipo na população.

Poucos estudos descrevem a caracterização dos genes *FUT2/FUT3* na população brasileira (de Mattos et al, 2002, Corvelo et al, 2013, Vicentini et al, 2013, Moraes et al, 2019, Olivares et al, 2019, resultados não publicados). As genotipagens aqui realizadas demonstram a diversidade de genótipos circulantes na população do Rio de Janeiro, inclusive destacando novas mutações ainda não descritas na literatura, indicando que novos estudos são necessários para uma melhor compreensão da importância epidemiológica destes genes nas infecções por RVA e norovírus.

Em relação ao monitoramento epidemiológico dos RVA circulantes, os resultados indicaram que durante o período estudado, o vírus vacinal (G1P[8], RV1) foi o genótipo predominantemente caracterizado (sequenciamento nucleotídico) nas amostras investigadas. Outros genótipos de RVA foram detectados em quatro

crianças: G3P[8], G12P[8] (2 crianças) e G3P[9]; e algumas amostras não puderam ser genotipadas (G[X]P8, G[X]P[X]). Assim, a detecção de 3,9% das estirpes que consideramos 'não vacinais', indicaram uma baixa prevalência de RVA na coorte de Manguinhos.

O alto percentual do vírus vacinal excretado nesta coorte possibilitou realizarmos o monitoramento desta estirpe. Visando associar este genótipo ao perfil de susceptibilidade, elegeu-se caracterizar o gene VP8*/VP4, visto este subdomínio participar da interação com os HBGAs. Os resultados da análise nucleotídica demonstraram uma alta frequência da mutação F167L nas amostras derivadas de vacinas. Estudos recentes utilizando cristalografia (Gozalbo-Rovira et al, 2019, Sun et al, 2019) demonstraram que a posição 167 está localizada em uma região que interage com os açúcares dos HBGAs. Ward e colaboradores (2006) demonstraram que a estirpe vacinal, passou a expressar o aminoácido fenilalanina durante o processo de atenuação, conferido a esta posição caráter hidrofóbico. Desta forma, esta natureza pode indicar uma diminuição da afinidade na interação RV1-HBGA, quando comparada à leucina identificada na linhagem selvagem (*Wa-like*). Os resultados obtidos pela técnica de pirosequenciamento convergiram com a análise por Sanger e, através da quantificação, confirmou-se a existência de subpopulações contendo ambos os aminoácidos nas amostras vacinais excretadas.

A ocorrência da mutação sugere que a vacina está replicando de forma eficaz no trato intestinal, e uma maior interação RV1-HBGA poderia ocorrer através da subpopulação viral contendo o aminoácido leucina. Este dado indica, inclusive, a importância de realização do monitoramento das estirpes vacinais excretadas, para verificação desta ou de outras mutações que poderiam indicar reversão da atenuação, podendo ou não afetar a resposta vacinal.

Um alto percentual (86,4%) de crianças com perfil Le (a+b+) secretor apresentando a mutação F167L foi identificado, e neste contexto, possivelmente o *status* do hospedeiro contribuiu na dinâmica da interação/replicação do vírus vacinal. De acordo com o observado neste estudo, o perfil HBGA poderia ser utilizado como um importante marcador na avaliação da eficácia da vacina, e isto explicaria, inclusive, o baixo percentual de RVA detectado nesta coorte.

Durante o estudo, foi possível verificar uma maior detecção de norovírus em relação aos RVA, principalmente em casos assintomáticos, entre 0-5 meses de

idade, enfatizando a importância destes vírus nesta faixa etária. Certamente, uma explicação para as infecções assintomáticas se deve à ação dos anticorpos e HBGAs maternos durante a fase de amamentação, agindo como primeira linha de defesa em relação à gravidade destas infecções (Jiang et al, 2004, Newburg et al, 2004, Lopman & Kang, 2014, Saito et al, 2014, Bode, 2015, Siqueira et al, 2017, Krammer & Bouckaert, 2018).

Nas infecções assintomáticas pelo norovírus pode ser verificada a excreção destes vírus por prolongados períodos, facilitando o sucesso de sua transmissão (Phillips et al, 2010, Teunis et al, 2015). Neste âmbito, este estudo de coorte permitiu verificar a frequência dos casos assintomáticos e avaliar a epidemiologia dos genótipos circulantes, comparando com estudos realizados anteriormente em outros países (Lopman & Kang, 2014, Lopman et al, 2014). Por outro lado, são raros os estudos de susceptibilidade em indivíduos com infecção assintomática por norovírus (Nordgren & Svensson, 2019).

As frequências de detecção dos norovírus em amostras fecais desta coorte, dentre os casos diarrêicos (17,1%) e assintomáticos (4,7%) foram similares aos descritos em recentes estudos de meta-análise global (Nguyen et al, 2017, Qi et al, 2018). Além disto, também não foi observado um padrão de sazonalidade durante o período de avaliação. Os norovírus foram detectados em 21,2% das crianças, com incidência de 5,8 infecções em 100 criança-meses, no grupo etário de 0-11 meses. Este resultado de incidência foi similar aos descritos nos estudos de coorte realizados no Chile, na faixa etária entre 0-5 meses (O' Ryan et al, 2009) e no Equador entre 6-11 meses de idade (Lopman et al, 2015).

Na coorte de Manguinhos (0-11 meses) foi observada a circulação de seis diferentes genótipos (GII.4 Sydney 2012[P31], GII.4 Sydney 2012[P16], GII.4 Sydney 2012[P4 New Orleans 2009], GII.2[P16], GII.6[P7] e GI.3[P13]), e dentre as amostras caracterizadas, a variante GII.4 Sydney 2012 foi a que apresentou maior ocorrência durante o período analisado, confirmando a prevalência global deste genótipo (Fioretti et al, 2014, Vinjé, 2015, Cannon et al, 2017, Costa et al, 2017). Inclusive, pode ser verificado que esta variante circulou associada a três diferentes genótipos da polimerase ([P31], [P16] e [P4 New Orleans 2009]), corroborando aos dados epidemiológicos dos surtos descritos em período similar nos EUA (Cannon et al, 2017, Barclay et al, 2019).

Em relação ao perfil de susceptibilidade do hospedeiro, estudos de associações com infecções assintomáticas pelo norovírus são raros (Nordgren & Svensson, 2019); em Manguinhos, a maioria das infecções assintomáticas pelo norovírus foram detectadas em crianças Le (a+b+) secretoras, associadas com os genótipos do capsídeo mais comuns, GII.4 Sydney 2012 e GII.6 (Currier et al., 2015; Sharma et al., 2019); significativa correlação entre infecção sintomática pelos norovírus e o *status* secretor (Le^b) foi observada ($p = 0,02$).

A diversidade de genótipos de norovírus identificada circulando entre as crianças da coorte (0-11 meses), impulsionou a investigação para uma faixa etária ampliada, de 1 a 4 anos, em crianças também residentes na comunidade de Manguinhos, durante o mesmo período de avaliação (2014-2018). Assim, 61 amostras de fezes obtidas de 49 crianças foram analisadas e os norovírus foram detectados em 47,5% (29/61) das amostras; sendo 21 (46,7%) dos casos diarreicos e 8 (50%) dos casos assintomáticos, e a faixa etária de 12-23 meses foi a que apresentou a maior taxa de detecção (19/39, 48,7%). Os resultados de caracterização demonstraram uma ampliação da diversidade genética, sendo detectados mais quatro outros genótipos (GI.7[P7], GI.1[P1], GII.7[P7] e GII.17[P17]), ainda não identificados nas crianças da coorte.

Neste estudo o recombinante GII.2[P16] foi detectado circulando nesta comunidade em 2017, em ambas as faixas etárias (0-11 meses e de 1-4 anos), sendo esta a primeira descrição no Brasil (Cantelli et al, 2019). Nossas amostras apresentaram >99% de similaridade nucleotídica com as estirpes recombinantes descritas pela primeira vez por Niendorf et al (2017), circulando na Alemanha. Além disso, o norovírus recombinante GII.4 Sydney 2012[P16], descrito pela primeira vez circulando no Brasil em 2016 (Barreira et al, 2017), foi observado circulando na comunidade de Manguinhos no mesmo ano. Alguns autores postulam que o recombinante GII.2[P16] poderia ser mais virulento, podendo ocupar a posição dos genótipos GII.4 Sydney 2012 e GII.17, em relação à prevalência destes nos últimos surtos; contudo, a vigilância necessita ser contínua para uma avaliação mais acurada do papel deste novo emergente (Fu et al, 2017, Niendorf et al, 2017, Li et al, 2018). A circulação dos dois genótipos recombinantes sugere a importância da polimerase P[16] para o *fitness* viral e sucesso destes emergentes, responsáveis pelos surtos de gastroenterite detectados no período de 2016-2017 em diversos

países e continentes (Barreira et al, 2017, Cannon et al, 2017, Niendorf et al, 2017, Hata et al, 2018, Li et al, 2018, Medici et al, 2018).

Os resultados deste estudo contribuem para uma melhor compreensão das infecções pelos RVA e norovírus em comunidades; particularmente considerando o *status* vacinal das crianças em relação à vacina RV1. Além disso, a análise dos HBGAs contribui para uma melhor compreensão da susceptibilidade às infecções por RVA e norovírus, demonstrando que os estudos em comunidade tornam-se relevantes, sendo contemporâneos e complementares aos dados obtidos na vigilância epidemiológica realizada nacional e mundialmente.

5 CONCLUSÕES

- O estudo do perfil de susceptibilidade (HBGA) das crianças da coorte de Manguinhos identificou uma alta frequência do perfil Le (a+b+) secretor (59,8%);
- As análises nucleotídicas do gene *FUT2* (*Se*) e *FUT3* (*Le*) demonstraram uma diversidade de genótipos circulando nesta população e a identificação de novas mutações ainda não descritas;
- O SNP rs281377 (357C>T) no gene *FUT2* foi detectado em uma alta frequência (80,7%) em indivíduos Le (a+b+) secretores, podendo ser, em termos evolutivos, uma importante mutação, que associada à outras mutações, seja responsável por este fenótipo em nossa população;
- Os resultados demonstraram que o genótipo vacinal G1P[8] (RV1) foi o que circulou predominantemente (78%) nas amostras RVA positivas investigadas na coorte;
- A correlação do perfil HBGA com a detecção da mutação F167L na proteína VP8* do RV1 demonstrou que o perfil Le (a+b+) favoreceu a replicação do vírus vacinal nas crianças da coorte, sugerindo que o perfil HBGA pode ser utilizado como um marcador para avaliação da eficácia vacinal;
- O pirosequenciamento demonstrou ser uma ferramenta importante na análise de subpopulações de aminoácidos (Phe/Leu) presentes nas amostras mutantes derivadas da vacina (RV1);
- Na coorte infantil, a incidência de norovírus foi de 5,8 infecções em 100 criança-meses, com detecção em 17,1% das amostras diarreicas e 4,7% das assintomáticas;
- A caracterização molecular dos norovírus revelou uma grande diversidade de genótipos circulantes na população infantil de Manguinhos, incluindo o recombinante GII.2[P16] detectado neste estudo pela primeira vez no Brasil;

- Significante associação entre infecção sintomática pelos norovírus e o *status* secretor (Le^b) foi observada.
- Avaliando as taxas de detecção entre os grupos etários em ambas as populações estudadas (0-11 meses e de 1-4 anos), verificou-se que na faixa etária de 6 meses aos 23 meses foi a de maior ocorrência de casos de norovirose;
- Na comunidade de Manguinhos não foi observado um padrão de sazonalidade dos norovírus em ambas as populações (coorte de 0-11 meses e 1-4 anos) no período estudado (2014 a 2018);

6 PERSPECTIVAS

Considerando os resultados obtidos neste trabalho e visando contribuir para os avanços na compreensão das infecções por RVA (estirpes selvagem e vacinal), norovírus e outros vírus gastroentéricos emergentes, podemos elencar algumas perspectivas de estudos:

- a) Caracterizar o gene *FUT2* dos indivíduos com o perfil Le (a-b+) secretor, e avaliar a frequência do SNP rs281377 (357C>T) em relação ao perfil Le (a+b+);
- b) Caracterizar o gene *FUT1* (*H*) da criança 0Le (a-b-) não secretora, que não apresentou o SNP rs601338 (428G>A) no gene *FUT2*, para avaliação de possíveis SNPs que possam correlacionar o fenótipo não secretor;
- c) Realizar análises nucleotídicas nos demais segmentos genômicos dos vírus vacinais excretados, buscando-se verificar a existência de outras mutações em regiões importantes como a região hipervariável do gene VP7 (capsídeo externo) e do gene que codifica a enterotoxina viral (NSP4), importantes na patogênese dos RVA;
- d) Investigar a ocorrência de possíveis rearranjos genéticos (*reassortments*) na estirpe vacinal excretada;
- e) Investigar, retrospectivamente, a ocorrência de outros vírus gastroentéricos emergentes (adenovírus, bocavírus e sapovírus) em amostras de fezes obtidas das crianças da coorte;
- f) Associar o perfil de susceptibilidade das crianças com outros vírus gastroentéricos detectados nas crianças da coorte;
- g) Ampliar estudos de coorte em comunidades, com um amostral representativo, avaliando os aspectos descritos neste estudo, como a diversidade viral genética,

dinâmica das infecções pelos RVA e norovírus e associações com o perfil HBGA.

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8 APÊNDICE – PARECER COMITÊ DE ÉTICA



Ministério da Saúde
Fundação Oswaldo Cruz
COMITÊ DE ÉTICA EM PESQUISA-CEP/FIOCRUZ

Rio de Janeiro, 15 de maio de 2006.

PARECER

Título do Projeto: "Detecção e Identificação de Vírus Responsáveis pela Etiologia da Gastroenterite Aguda"
Protocolo CEP: 311/06
Pesquisador Responsável: José Paulo Gagliardi Leite
Instituição: IOC
Deliberação: APROVADO

O estudo tem como objetivos detectar rotavírus A e C, astrovírus, calicivírus humanos e adenovírus entéricos, em amostras fecais de gastroenterite agudas, além de implementar metodologias para a detecção de outros vírus, associados a estas infecções, com caracterização molecular e análises genealógicas, na busca de variações geográficas e/ou temporais.

Segundo a Organização Panamericana de Saúde as gastroenterites agudas são, após as infecções respiratórias agudas, constituem o mais importante agravo à saúde em crianças menores de cinco anos. Associados ao quadro de desnutrição, estas são responsáveis por mais de 3,3 milhões de mortes no mundo, sendo que um milhão destas ocorre na América Latina. O trabalho pretende identificar outros vírus gastroentéricos causadores de doenças em nosso meio, objetivando a criação de um Programa de Vigilância Epidemiológica e Laboratorial de detecção e caracterização molecular (identificação) de vírus, no sentido de auxiliar a avaliação do impacto na introdução da vacina em nosso país.

Trata-se de um projeto integrado, envolvendo atividades de Graduação e Pós-Graduação.

Serão utilizadas fezes obtidas de amostras encaminhadas para diagnóstico e identificação viral que estão armazenadas no laboratório, onde a pesquisa será executada, ou enviadas pelos LACENs, ou unidades hospitalares.

O projeto não oferece nenhum risco físico aos participantes com referência à obtenção do material biológico para estudo.

O TCLE está claro, com as informações necessárias.

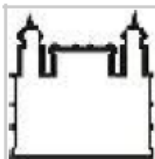
A equipe envolvida no estudo é qualificada para tal e o projeto será desenvolvido no Laboratório de Referência para Rotavirose, do Depto. de Imunologia, do Instituto Oswaldo Cruz.

Após análise das respostas às pendências emitidas no parecer datado de 28/04/2006 por este colegiado, tendo por referência as normas e diretrizes da Resolução 196/96, foi decidido pela APROVAÇÃO do referido protocolo.

Informamos, outrossim, que deverão ser apresentados relatórios parciais/anuais e relatório final do projeto de pesquisa.

Além disso, qualquer modificação ou emenda ao protocolo original deverá ser submetida para apreciação do CEP/Fiocruz.

Alda Maria Da-Cruz
Vice-Coordenadora do Comitê de Ética em Pesquisa
Em Seres Humanos da Fundação Oswaldo Cruz



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PARECER CONSUBSTANCIADO DO CEP

Elaborado pela Instituição Coparticipante

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: EPIDEMIOLOGIA DO ROTAVÍRUS EM RECÉM NATOS E LACTENTES DE UMA COORTE INFANTIL EM MANGUINHOS - DO PERÍODO PRÉ-VACINAL A 2 ANOS DE IDADE

Pesquisador: Patricia Brasil

Área Temática:

Versão: 2

CAAE: 31505914.8.3001.5240

Instituição Proponente: Instituto de Pesquisa Clínica Evandro Chagas - IPEC / FIOCRUZ

Patrocinador Principal: Instituto de Pesquisa Clínica Evandro Chagas - IPEC / FIOCRUZ

DADOS DO PARECER

Número do Parecer: 1.288.236

Apresentação do Projeto:

Este parecer refere-se à apreciação de EMENDA ao projeto "EPIDEMIOLOGIA DO ROTAVÍRUS EM RECÉM NATOS E LACTENTES DE UMA COORTE INFANTIL EM MANGUINHOS - DO PERÍODO PRÉ-VACINAL A 2 ANOS DE IDADE" pelo CEP/ENSP, pois tem a ENSP como Instituição Coparticipante. A pesquisadora responsável é Patricia Brasil e a instituição proponente é o IPEC/FIOCRUZ.

O projeto foi aprovado pelo CEP/IPEC conforme Parecer Consubstanciado número 904.309, de 09/12/2014. A apresentação do projeto foi descrita no Parecer Consubstanciado do CEP/IPEC/FIOCRUZ de número 688.566, emitido em 16/06/2014.

A Emenda ora em análise, foi apreciada pelo CEP/IPEC e recebeu o Parecer Consubstanciado de aprovação número 1.133.331 em 01/07/2015.

A justificativa da emenda resume-se a "Inclusão de coleta de saliva nos participantes do projeto para pesquisa de perfil genético com relação aos antígenos Lewis, H e perfil secretor do grupo sanguíneo ABO".

As doenças diarreicas agudas (DDA) representam uma importante causa de mortalidade infantil em países em desenvolvimento. Os rotavírus são reconhecidos como a causa isolada mais importante

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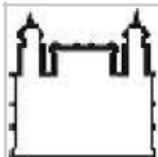
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de gastroenterite grave infantil no mundo, sendo o rotavírus A o responsável por mais de 600.000 mortes por ano. Em 2009, a OMS recomendou a inclusão da vacina contra o rotavírus em programas de imunização de todos os países, com ênfase na sua introdução em países onde a morte por diarreia é responsável por mais de 10% da mortalidade de crianças menores de cinco anos. Ainda é incerto se o uso disseminado da vacina irá causar pressão seletiva nos rotavírus humanos, disparando alterações genéticas e antigênicas que diminuirão a sua efetividade. A diversidade de sorotipos/genótipos detectada em rastreamentos em todo o mundo é observada principalmente nos países com grande desigualdade econômica. Isto, associado à possibilidade da emergência de genótipos incomuns, e a disseminação destes que podem tornar-se epidemiologicamente importantes, justifica a vigilância contínua de rotavírus em lactentes com e sem DDA.

Desenho do estudo: Coorte prospectiva.

População do estudo: Recém nascidos e lactentes da Coorte do Estudo de Incidência de Dengue em Manguinhos

Locais de estudo: o estudo será conduzido nos Serviços de Saúde da Família do Territórios Integrados de Atenção à Saúde (TEIAS) de Manguinhos, da Fiocruz do Rio de Janeiro (Centro de Saúde Manguinhos) e Clínica de Saúde da Família Victor Valla.

Seguimento: Os recém-natos e lactentes da coorte serão acompanhados nas consultas de rotina do projeto e durante os episódios de DDA.

Na primeira consulta ambulatorial, idealmente com 30 dias de vida, a saliva será coletada a partir de swab oral.

Amostras de fezes serão coletadas nos recém nascidos e lactentes da seguinte forma:

- Até 2 meses de vida com intervalos quinzenais.
- Após a 1ª dose da vacina contra rotavírus.
- Após a 2ª dose da vacina contra rotavírus.
- Durante episódios de doença diarreica aguda.

- Vigilância de diarreia: serão realizados telefonemas semanais e visitas domiciliares quinzenais para busca ativa de DDA, entre os participantes do estudo.

Coleta, transporte e conservação de amostras fecais:

- Coletores fecais plásticos sem conservantes serão utilizados para a coleta das amostras de fezes.

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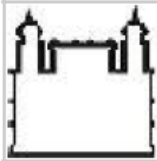
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As amostras serão retiradas com espátulas de madeira diretamente das fraldas descartáveis utilizadas pelas crianças após evacuação espontânea durante as consultas no CSEGSF/ENSP ou na Clínica de Saúde da Família Victor Valla. No caso de recém natos, a equipe de assistência irá buscar as fezes no domicílio do paciente.

- As amostras de saliva serão conservadas à temperatura de 4°C em refrigerador específico para amostras de material biológico e encaminhadas ao Laboratório de Virologia Comparada e Ambiental do Instituto Oswaldo Cruz.

- As amostras de fezes serão conservadas à temperatura de 4°C em refrigerador específico para amostras de material biológico e encaminhadas ao Laboratório de Virologia Comparada e Ambiental do Instituto Oswaldo Cruz.

Critério de Inclusão:

Eligibilidade: serão elegíveis todos os recém natos ou lactentes em seguimento da Coorte, ou em acompanhamento pelo Programa de Saúde da Família de Manguinhos (TEIAS).

Inclusão: Recém nato ou lactente até 2 anos de idade, morador de Manguinhos, cujos pais ou responsáveis tenha assinado o termo de consentimento livre e esclarecido (TCLE).

Critério de Exclusão:

Serão excluídas as crianças cujos pais retirarem o termo de consentimento, ou que desistam do acompanhamento pela equipe da Coorte.

Metodologia de Análise:

Ensaio laboratoriais para amostras de saliva:

a) Para detecção dos antígenos secretados na amostra oral, detecção imunoenzimática (ELISA) será utilizada, com anticorpos de detecção -A, -B (ABO) e -Lea e -Leb (Lewis)(Nordgren J et al, 2014; Bucardo F et al, 2013);

b) Após a detecção por ELISA amostras com perfil de secreção negativas, serão testadas para detecção do gene Fuc1-2-Gal utilizando ensaio imunoenzimático (ELISA) de lectina UEA-I (Nordgren J et al, 2014);

c) Para amostras com fenótipo positivo secretor duvidoso nos ELISA e provenientes de crianças infectadas por Rotavírus, será realizada a extração do DNA genômico utilizando kits comerciais e empregada as técnicas de PCR e sequenciamento nucleotídeo, utilizando oligonucleotídeos

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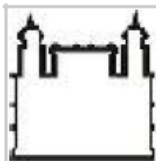
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específicos para o genes FUT2 e 3.

Ensaio laboratoriais para amostras de fezes:

- a) Ensaio imunoenzimático para detecção de rotavírus da espécie A (RVA);
- b) Detecção de rotavírus por eletroforese em gel de poliacrilamida em suspensões fecais;
- c) Detecção de RVA e norovírus (Nv) através da transcrição reversa associada à reação em cadeia da polimerase qualitativa e quantitativa (RT-PCR e RT-qPCR).
- d) Genotipagem dos RVA através de Multiplex Nested PCR.
- e) Caracterização molecular de RVA e genotipagem Nv através do sequenciamento genômico;
- f) Pesquisa de protozoários e bactérias em suspensões fecais dos recém natos e lactentes com DDA.

Objetivo da Pesquisa:

Objetivo Primário:

- Detecção de genótipos do Rotavírus diferentes dos presentes na vacina disponível no Programa Nacional de Imunização.

Objetivo Secundário:

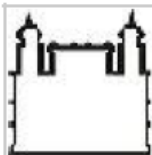
- Detectar os genótipos de rotavírus presentes nas fezes de recém natos e lactentes da coorte antes e após a primeira dose da vacina, e após a segunda dose da vacina até os 2 anos de idade;
- Avaliar se os recém natos/lactentes já entraram em contato com o rotavírus antes da primeira dose da vacina;
- Verificar se a primeira dose da vacina aumentaria a efetividade desta, conferindo maior proteção contra a infecção pelo rotavírus;-Detectar o agente etiológico da DDA em episódios de gastroenterite no paciente acometido e em seus familiares e, porventura de algum animal doméstico;
- Descrever o perfil sócio-econômico e ambiental dos recém natos e lactentes da coorte com DDA;
- Caracterizar o perfil genético de recém nascidos e lactentes da coorte, com relação aos antígenos de Lewis, H e perfil secretor do grupo sanguíneo ABO, a partir de amostras de saliva;
- Relacionar o perfil genético obtido com possível suscetibilidade à infecção pelos rotavírus.

Avaliação dos Riscos e Benefícios:

Riscos:

Segundo a pesquisadora, "Não há riscos relacionados com a participação da criança, já que a coleta da saliva será realizada com swab confeccionado de material macio e sem conservantes,

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diretamente da boca do bebê, e as fezes serão coletadas diretamente da fralda. Caso haja constrangimento em realizar a coleta de fezes na sala de espera do ambulatório, esta poderá ser coletada no banheiro do posto ou no consultório médico, durante o atendimento da criança".

Benefícios:

Ainda segundo a pesquisadora, "O paciente estará colaborando com um estudo científico que ajudará no entendimento da infecção pelo Rotavírus na população infantil até 2 anos de idade. A criança receberá atendimento pelos médicos da Pesquisa sempre que tiver doença diarreica aguda. As informações obtidas através dessa pesquisa serão confidenciais e sigilosas. Essas informações não serão divulgadas de forma a possibilitar a identificação e estarão protegidas por códigos e senhas".

Comentários e Considerações sobre a Pesquisa:

A emenda apresentada possui os elementos necessários à apreciação ética.

Considerações sobre os Termos de apresentação obrigatória:

Foi elaborado e apresentado novo TCLE que contempla a coleta de saliva para a pesquisa de perfil genético com relação aos antígenos Lewis, H e perfil secretor do grupo sanguíneo ABO.

Recomendações:

Não há.

Conclusões ou Pendências e Lista de Inadequações:

Não há.

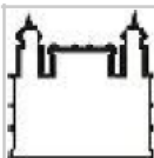
Considerações Finais a critério do CEP:

Em atendimento ao subitem II.19 da Resolução CNS nº 466/2012, cabe ao pesquisador responsável pelo presente estudo elaborar e apresentar relatório final "[...] após o encerramento da pesquisa, totalizando seus resultados", em forma de "notificação". O modelo de relatório de CEP/ENSP se encontra disponível em www.ensp.fiocruz.br/etica.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações	PB_INFORMAÇÕES_BASICAS_472842	04/06/2015		Aceito

Endereço: Rua Leopoldo Bulhões, 1480 - Térreo
Bairro: Manguinhos CEP: 21.041-210
UF: RJ Município: RIO DE JANEIRO
Telefone: (21)2598-2863 Fax: (21)2598-2863 E-mail: cep@ensp.fiocruz.br



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Continuação do Parecer: 1.288.236

Básicas do Projeto	E2.pdf	11:42:28		Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_472842 E2.pdf	02/03/2015 07:13:23		Aceito
Outros	AdendoProjetoRotavirusAlvaroCarinaMarciaZeFinal.pdf	02/03/2015 07:09:58		Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_377346 E1.pdf	07/11/2014 13:41:40		Aceito
Outros	termo anuência ENSP.jpg	15/09/2014 21:05:55		Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_377346 E1.pdf	15/08/2014 17:44:19		Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_335199.pdf	24/05/2014 19:00:41		Aceito
Projeto Detalhado / Brochura Investigador	Projeto rotavirus versão maio 2014.pdf	24/05/2014 18:36:38		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE CEP ENSP - projeto Rotavirus revisado abril 2014.pdf	24/05/2014 18:30:05		Aceito
Folha de Rosto	folha de rosto CEP assinada.pdf	24/05/2014 18:17:46		Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

RIO DE JANEIRO, 20 de Outubro de 2015

Assinado por:

Mônica Carvalho de Mesquita Werner Wermelinger
(Coordenador)

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