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Lucas Alves Vianna

Sazonalidade, epidemiologia molecular e virulência do Vírus Sincicial Respiratório (VSR): uma perspectiva dentro do Programa Brasileiro de Vigilância da Influenza

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia do Centro de Ciências da Saúde da Universidade Federal do Espírito Santo, como requisito parcial para obtenção do título de Doutor em Biotecnologia.

Orientador: Dr. Iuri Drumond Louro

Coorientadora: Dra. Paola Cristina Resende

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UNIVERSIDADE FEDERAL DO ESPÍRITO SANTO
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REGISTRO DE JULGAMENTO DA TESE DA CANDIDATA AO GRAU DE DOUTOR PELO PPG BIOTECNOLOGIA.

A Comissão Examinadora da Tese de Doutorado intitulada "Sazonalidade, epidemiologia molecular e virulência do Vírus Sincicial Respiratório (VSR): uma perspectiva dentro do Programa Brasileiro de Vigilância da Influenza" elaborada por **LUCAS ALVES VIANNA**, candidato ao Grau de Doutor em Biotecnologia, recomendou, após apresentação da Tese, realizada no dia 24 de maio de 2021, que a mesma seja (assinale um dos itens abaixo):

(**X**) Aprovada

( ) Reprovada

Os membros da Comissão deverão indicar a natureza de sua decisão através de sua assinaturana coluna apropriada que segue:

Aprovada	Reprovada
Iúri Drumond Louro	
Patrícia Machado Bueno	
FernandesLiliana Cruz Spano	
Rita Catarina Medeiros Sousa	
Eurico de Arruda Neto	

DEDICATÓRIA

Eu acredito que tudo que somos e fazemos é produto de uma mistura de comportamento definido geneticamente com o estímulo do ambiente e das pessoas que nos cercam. Nessa lógica, esta tese foi indiretamente elaborada por várias pessoas que, de alguma forma, influenciaram minha forma de ser e de pensar e, por isso, deixo aqui minha dedicação:

Ao meu pai, Ivam de Souza Vianna, e à minha mãe, Edna Eunice Alves Vianna, que a vida toda me deram nada mais do que amor, suporte e incentivo; procurando sempre me oferecer tudo que eles mesmos não puderam ter. Serei eternamente grato por toda abdicação e por acreditarem no meu potencial. Espero deixá-los orgulhosos;

Aos meus irmãos e conselheiros, Vitor Alves Vianna e Beatriz Alves Vianna, que sempre estiveram ao meu lado, me dando forças, me estimulando a ser, a cada dia, um ser humano melhor;

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Aos meus amigos do LACEN, por todo companheirismo e aprendizado;

Aos meus amigos da turma do curso de Ciências Biológicas, 2007/1, da Universidade Federal do Espírito Santo. Há 14 anos a vida me presenteava com amigos por quem eu só tenho admiração e amor.

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“Em algum lugar, alguma coisa incrível está esperando para ser descoberta”

(Carl Sagan)

RESUMO

VIANNA, L.A., RESENDE, P.C, LOURO, I.D. **Sazonalidade, epidemiologia molecular e virulência do Vírus Sincicial Respiratório (VSR): uma perspectiva dentro do Programa Brasileiro de Vigilância da Influenza** 2021. 94f. Tese (Doutorado em Biotecnologia) – Programa de Pós-Graduação em Biotecnologia, UFES, Espírito Santo. Brasil.

Infecções pelo Vírus Sincicial Respiratório (VSR) são a principal causa de morbidade e mortalidade pediátrica. A evolução complexa do VSR enseja a necessidade de vigilância global, o que pode auxiliar no entendimento de múltiplos aspectos virais. Este estudo visou a investigar vários aspectos do VSR dentro do Programa Brasileiro de Vigilância da Influenza, avaliando o papel da carga viral e da diversidade genética na gravidade da infecção e o impacto de fatores climáticos na sazonalidade viral. Nós investigamos a prevalência do VSR em crianças até 3 anos de idade com Síndrome Respiratória Aguda Grave (SRAG) no Espírito Santo (ES), Brasil, de 2016 a 2018. Empregamos a RT-qPCR para detectar o vírus e determinar a carga viral e, a partir disso, estudar sua associação com fatores clínicos e entender a sazonalidade do VSR em nível local. Com o sequenciamento do gene G e a reconstrução filogenética foi possível entender a diversidade genética no Espírito Santo. Dos 632 casos de SRAG, 56% foram causados pelo VSR, com ambos os subtipos (VSR-A e VSR-B) cocirculando em todos os anos. Uma relação inversa e discreta entre temperatura média e circulação viral foi observada. Não encontramos conexão entre carga viral e gravidade da doença, não obstante, crianças infectadas pelo VSR-A apresentaram maior mediana de Pontuação de Gravidade Clínica (PGC), permaneceram mais tempo hospitalizadas e necessitaram mais frequentemente de suporte ventilatório e cuidados intensivos do que aquelas infectadas pelo VSR-B. Acerca da diversidade genética, encontramos alguns grupos genéticos locais dentro dos genótipos VSR-A ON1 e VSR-B BA, com linhagens apresentando modificações na cadeia de aminoácidos do gene G. Estudos com VSR dentro do Programa Brasileiro de Vigilância da Influenza são importantes para avaliar a adequação e viabilidade de uma só rede para a vigilância de outros vírus respiratórios. O entendimento da sazonalidade, virulência e da diversidade genética pode garantir a eficácia de futuros fármacos antivirais e vacinas, bem como auxiliar na administração de estratégias profiláticas.

Palavras-chave: Infecção. Gravidade. Carga Viral. Filogenia. Sazonalidade. Mutação.

Seasonality, molecular epidemiology, and virulence of Respiratory Syncytial Virus (RSV): a perspective into the Brazilian Influenza Surveillance Program

ABSTRACT

VIANNA, L.A., RESENDE, P.C, LOURO, I.D. **Seasonality, molecular epidemiology, and virulence of Respiratory Syncytial Virus (RSV): a perspective into the Brazilian Influenza Surveillance Program, 2021**. 94p. Thesis (Doctoral in Biotechnology) - Postgraduation Biotechnological Program, UFES, Espirito Santo. Brazil.

Respiratory Syncytial Virus (RSV) is the main cause of pediatric morbidity and mortality. The complex evolution of RSV creates a need for worldwide surveillance, which may assist in the understanding of multiple viral aspects. This study aimed to investigate RSV features under the Brazilian Influenza Surveillance Program, evaluating the role of viral load and genetic diversity in disease severity and the influence of climatic factors in viral seasonality. We have investigated the prevalence of RSV in children up to 3 years old with severe acute respiratory infection (SARI) in the Espirito Santo State (ES), Brazil, from 2016 to 2018. RT-qPCR allowed for viral detection and viral load quantification, to evaluate association with clinical features and mapping of local viral seasonality. Gene G sequencing and phylogenetic reconstruction demonstrated local genetic diversity. Of 632 evaluated cases, 56% were caused by RSV, with both subtypes A and B co-circulating throughout the years. A discrete inverse association between average temperature and viral circulation was observed. No correlation between viral load and disease severity was observed, but children infected with RSV-A presented higher clinical severity score (CSS) median, stayed longer in the hospital, required intensive care and ventilatory support more frequently than those infected by RSV-B. Regarding RSV diversity, some local genetic groups were observed in the main genotypes circulation RSV-A ON1 and RSV-B BA, with strains showing modifications in the G gene amino acid chain. Local RSV studies using the Brazilian Influenza Surveillance Program are relevant to assess the suitability and viability of a single network for the surveillance of other respiratory viruses. Understanding seasonality, virulence and genetic diversity can support the suitability of future antiviral drugs and vaccines and assist in the administration of prophylactic strategies.

Key words: Infection. Severity. Viral load. Phylogeny. Seasonality. Mutation.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

Ct	Limiar do ciclo (do inglês cycle threshold)
DNA	Ácido Desoxirribonucleico (do inglês Deoxyribonucleic acid)
DNAdf	DNA dupla fita
Et al.	e colegas (do latim *et alii*)
IFN	Interferon
ITRI	Infecção no trato respiratório inferior
kb	Kilobases
LACEN/ES	Laboratório Central do Espírito Santo
nm	Nanômetros
OMS	Organização Mundial da Saúde
pb	Pares de bases
PGC	Pontuação de Gravidade Clínica
RHV	Região Hiper Variável
RNA	Ácido Ribonucleico
RNP	RNAse P
RT-qPCR	Reação em Cadeia da Polimerase em tempo real precedida de retrotranscrição
SRAG	Síndrome Respiratória Aguda Grave
VSR	Vírus Sincicial Respiratório
µL	Microlitro

SUMÁRIO

1. INTRODUÇÃO

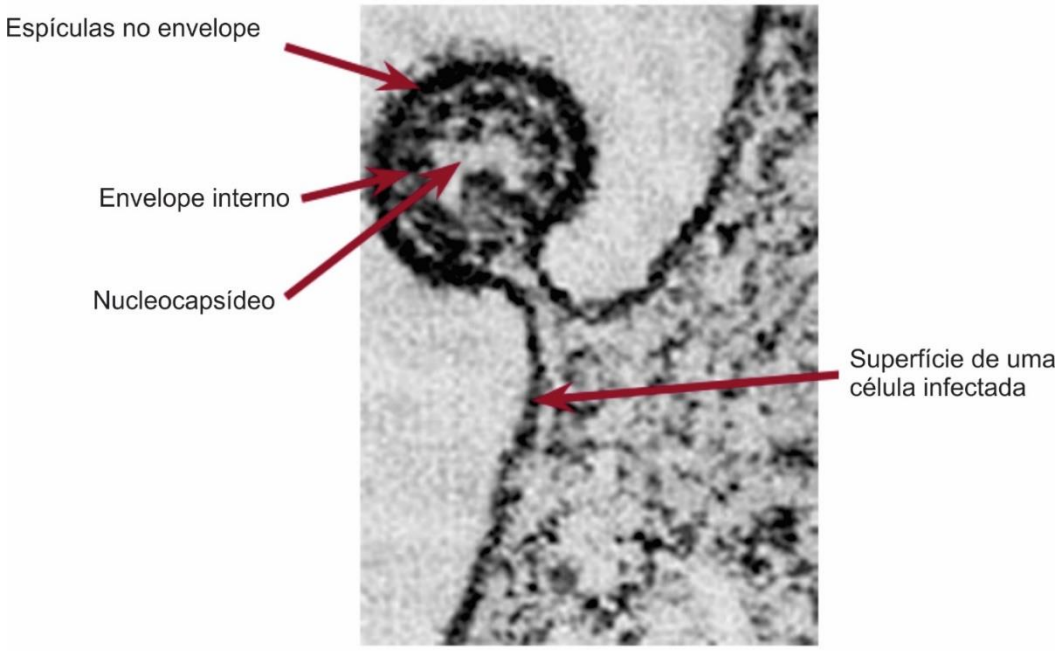
1.1 HISTÓRICO, CLASSIFICAÇÃO E ESTRUTURA VIRAL

O Vírus Sincicial Respiratório humano (hVSR; ou apenas VSR) foi isolado pela primeira vez em 1956, no estado de Maryland, nos Estados Unidos, a partir de amostras de *swab* de orofaringe coletadas de chimpanzés com sintomas respiratórios (MORRIS; BLOUNT; SAVAGE, 1956). Cerca de dois anos depois, o VSR foi caracterizado, também, como responsável por infecções graves de trato respiratório inferior em bebês (CHANOCK; ROIZMAN; MYERS, 1957). Sabe-se, hoje, que o VSR lidera as causas de infecções no trato respiratório inferior (ITRI) em crianças menores de 5 anos, causando principalmente bronquiolite e pneumonia (SHI et al., 2017).

Estudos filogenéticos apontam que o VSR pertence ao gênero *Orthopneumovirus*, classificado na família *Pneumoviridae* e ordem Mononegavirales (GRIFFITHS; DREWS; MARCHANT, 2017). Embora um único sorotipo seja identificado, as diversidades genética e antigênica do táxon são suficientes para sua classificação em dois subgrupos: VSR-A e VSR-B (MUFSON et al., 1985).

O VSR apresenta em sua estrutura um envelope lipídico e mede aproximadamente 150 nm, possuindo um formato geralmente esférico (GOWER et al., 2005) (Figura 1).

Figura 1 - Fotomicrografia eletrônica do VSR mostrando um vírion de VSR brotando através da membrana plasmática de uma célula infectada.

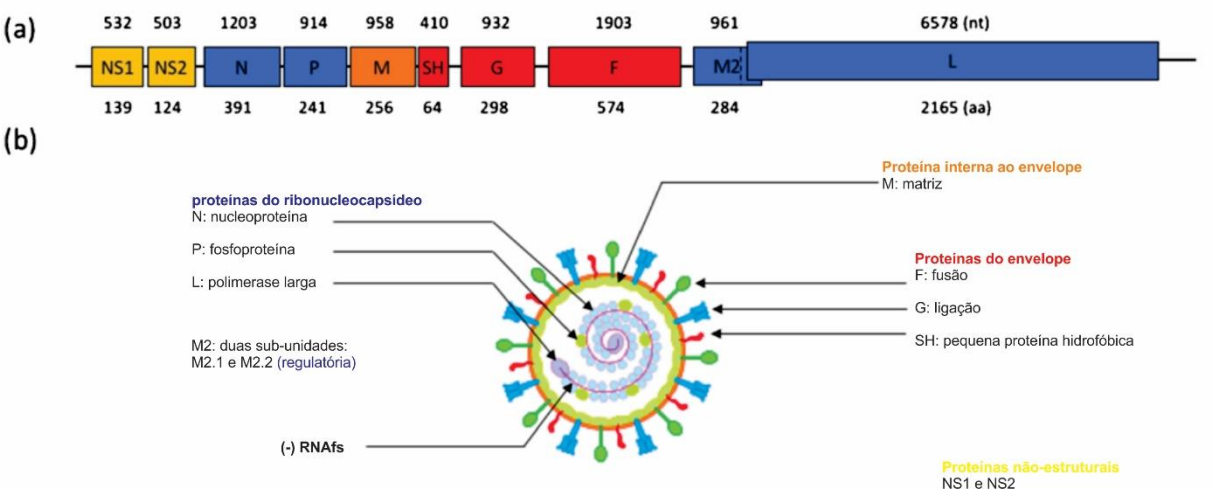


Fonte: COLLINS; GRAHAM, 2008, adaptado.

O VSR apresenta um genoma não-segmentado de RNA fita simples e polaridade negativa, o qual compreende 15,2 kb, e abriga 10 genes que codificam 11 proteínas: 2 não-estruturais e 9 estruturais (COLLINS; FEARNS; GRAHAM, 2013) (Figura 2). Na superfície do vírus são encontradas três proteínas: G, F e SH (REY-JURADO; KALERGIS, 2017). A glicoproteína G tem como função a adsorção do vírus à célula, enquanto a proteína F é responsável pela fusão do envelope viral à membrana celular, o que pode ocasionar a formação de sincícios, motivo pelo qual estes microrganismos foram batizados de Vírus Sincicial Respiratório (TRIPP; JORQUERA, 2016). A proteína SH (pequena proteína hidrofóbica) possui um papel menos claro, mas parece estar associada ao aumento da capacidade de infecção (TRIPP; JORQUERA, 2016).

A estrutura viral interna ao envelope é composta pela proteína M (matriz) (KINGSBURY, 1991). A proteína longa (L) é a maior subunidade da polimerase viral, na qual é encontrado o sítio catalítico. A fosfoproteína (P) é um cofator essencial à síntese do RNA e acredita-se que se associe com nucleoproteínas (N) e L livres para mantê-las na forma solúvel para montagem e interação com nucleocapsídeos (DUPUY et al., 1999). M2-1 são proteínas associadas ao nucleocapsídeo viral e são fatores de transcrição (LI et al., 2014; NAIR et al., 2010). A proteína regulatória M2-2 é responsável pela síntese do RNA durante a montagem do vírion (COLLINS, 1991). Por fim, NS1 e NS2 são as proteínas não-estruturais cuja função parece estar relacionada à inibição da liberação de interferon (IFN) em células infectadas (BARIK, 2013; CHIN et al., 2016).

Figura 2 - Esquema do genoma e proteoma do VSR; (a) Mapa do genoma de RNA de sentido negativo genoma em que nt e aa indicam, respectivamente, o comprimento dos nucleotídeos e aminoácidos. (b) Mapeamento das 11 proteínas no vírion VSR e suas classes correspondentes.



Fonte: TALEB et al., 2018, adaptado.

As glicoproteínas G e F, por sua posição no envelope viral, podem induzir significativamente a resposta imune (Wagner et al., 1989). A proteína G é uma glicoproteína de membrana tipo-II que apresenta de 289 a 299 aminoácidos. Em seu ectodomínio são encontradas duas regiões hipervariáveis: RHV-1 e RHV-2, separadas por um domínio altamente conservado de 13 aminoácidos. Há vasta literatura que indica que mudanças na composição da sequência de aminoácidos nessas regiões podem alterar as propriedades antigênicas do vírus (ANDERSON et al., 1985; JOHNSON et al., 1987; PALOMO; CANE; MELERO, 2000; PERET et al., 2000; VENTER et al., 2001). Há cerca de somente 53% de homologia entre as glicoproteínas G dos dois subgrupos e, por isso, a variabilidade antigênica entre eles é determinada pelas variações nessa glicoproteína (JOHNSON et al., 1987). Embora a proteína G seja alvo de anticorpos neutralizantes, sua alta variabilidade antigênica dificulta a criação de vacinas que tenham como alvo essa proteína (TALEB et al., 2018). Por outro lado, a proteína F é altamente conservada e possui mais epítopos alvos de anticorpos neutralizantes do que a proteína G. Além disso, considerando sua função de fusão (*i.e.* entrada) nas células, a proteína F tem sido o principal alvo de desenvolvimento de vacinas (BEELER; VAN WYKE COELINGH, 1989; MAGRO et al., 2012; NGWUTA et al., 2015).

De todo o genoma do VSR, a RHV-2 apresenta a maior diversidade genética, fazendo com que seja a região mais frequentemente utilizada em análises de filogenia molecular (ANDERSON et al., 1985; JOHNSON et al., 1987; MUFSON et al., 1985).

Por meio de análises de variabilidade genética da região RHV-2, vários genótipos são identificados dentro dos subgrupos VSR-A e VSR-B (MELERO; MAS; MCLELLAN, 2017). Atualmente são listados pelo menos 15 genótipos para o VSR-A (GA1-GA7, SAA1 e SAA2, NA1-NA4, CB-A, ON1) (BAEK et al., 2012; CUI et al., 2013a; ESHAGHI et al., 2012; PERET et al., 1998, 2000; PRETORIUS et al., 2013; SHOBUGAWA et al., 2009; TRENTO et al., 2015; VENTER et al., 2001) e 28 genótipos para VSR-B (GB1-GB4, SAB1-SAB4, URU1 e URU2, BA1-BA14, BA-C, THB, CB-B e CB1) (ARNOTT et al., 2011; AUKSORNKITTI et al., 2014; BLANC et al., 2005; CUI et al., 2013a, 2013b; DAPAT et al., 2010; GIMFERRER et al., 2016; KHOR et al., 2013; PERET et al., 2000; TRENTO et al., 2006; VENTER et al., 2001).

1.2 EPIDEMIOLOGIA E SAZONALIDADE

Infecções no trato respiratório inferior (ITRI) estão entre as principais causas de morbidade e mortalidade em crianças de até 5 anos no mundo (LIU et al., 2016), estando o VSR na liderança dos patógenos virais identificados nestes pacientes (MEISSNER, 2016) (Figura 3). Nair e colaboradores (2010) estimaram que, em 2005, cerca de 33,8 milhões crianças pequenas apresentaram ITRI devido à infecção por VSR e, destas, 10% necessitaram de admissão hospitalar (NAIR et al., 2010). Os dados ainda apontaram que até 199.000 crianças podem ter ido a óbito em função do VSR. Mais recentemente, em uma revisão sistemática da literatura, foi estimado que em todo o mundo, em 2015, cerca de 33,1 milhões de crianças menores do que 5 anos apresentaram ITRI devido à infecção por VSR, resultando em aproximadamente 3,2 milhões de internações hospitalares e quase 60 mil óbitos (SHI et al., 2017). Quando considerada a mortalidade geral pelo VSR (combinado de casos hospitalizados e na comunidade), é possível estimar algo em torno de 118 mil óbitos de crianças menores do que 5 anos (SHI et al., 2017).

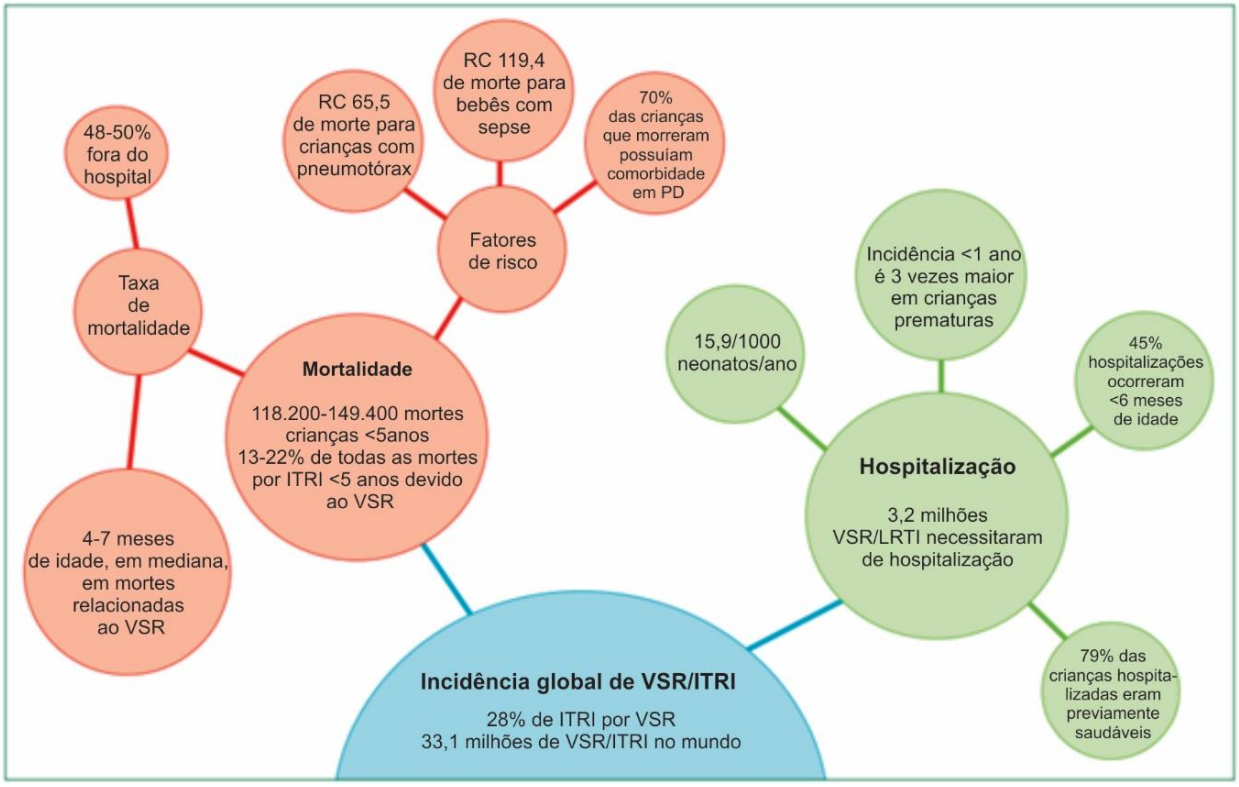
Embora as crianças sejam o grupo etário mais frequentemente acometido, o VSR também pode causar quadros clínicos graves em idosos (FALSEY; WALSH, 2005).

Evidências apontam que, nos Estados Unidos, a mortalidade em pessoas com mais de 65 anos seja de 7,2 por 100.000 pessoas-ano (THOMPSON et al., 2003), e que 8% dos pacientes hospitalizados com ITRI vão a óbito (FALSEY; WALSH, 2005).

Recém-nascidos prematuros e crianças com cardiopatia, pneumopatia ou imunodeficiência compõem o grupo com maior risco de acometimento grave (PERET et al., 2000; ZLATEVA et al., 2005).

No Brasil, segundo os boletins de Influenza e outros Vírus Respiratórios, o VSR apresenta um papel importante nos casos de Síndrome Respiratória Aguda Grave (SRAG), com prevalência variando de 9,75% até 17,3% entre 2016 e 2019 (MINISTÉRIO DA SAUDE, 2021). Em 2019, 5% dos óbitos em casos de SRAG foram causados pelo VSR MINISTÉRIO DA SAUDE, 2021).

Figura 3 – Carga global de VSR em crianças com menos de 5 anos de idade. A incidência é mostrada mundialmente para crianças com menos de 5 anos de idade, salvo indicação em contrário. A taxa de admissão hospitalar é de 15,9 admissões hospitalares por 1000 neonatos por ano, em países em desenvolvimento. A taxa de internação hospitalar de VSR ALRI de 15,9 entre neonatos é relatada por 1000 indivíduos, por ano, nos países em desenvolvimento. RC = razão de chances. ITRI = infecção do trato respiratório inferior. VSR = vírus sincicial respiratório. PD = país de alta renda.



Fonte: MAZUR et al., 2018, adaptado.

A circulação do VSR é cosmopolita e estima-se que 60% das crianças sejam infectadas por esse vírus durante o primeiro ano de vida (GLEZEN, 1986), com 100% delas sendo acometidas até o segundo ano de vida (SORCE, 2009).

Linhagens de ambos os subgrupos (VSR-A e VSR-B) cocirculam anualmente, porém é comum um subgrupo predominar sobre o outro (GILCA et al., 2006; IMAZ et al., 2000). Além disso, vários genótipos estão presentes simultaneamente em um mesmo local, independentemente da estação do ano, embora a composição das linhagens circulantes possa diferir mesmo em regiões geográficas contíguas (FLETCHER et al., 1997; PERET et al., 2000).

Novos genótipos de VSR-A (ON1) e VSR-B (*cluster* BA) emergiram nas últimas duas décadas, tendo sido identificados pela primeira vez nas cidades de Ontário, no Canadá (ESHAGHI et al., 2012), e em Buenos Aires, na Argentina (TRENTO et al., 2006), respectivamente. Um fator em comum a esses genótipos são duplicações nucleotídicas na RHV-2. ON1 possui 72 nucleotídeos duplicados, sendo um genótipo que rapidamente se disseminou para outros continentes como África, Ásia, Europa e demais países das Américas (CUI et al., 2013b; PIERANGELI et al., 2014; VALLEY-OMAR et al., 2013). Da mesma forma, o *cluster* de genótipos BA do VSR-B possui 60 nucleotídeos duplicados na região RHV-2, após o nucleotídeo 792 do gene da glicoproteína G (TRENTO, 2003), sendo um grupo de genótipos que se tornou prevalente globalmente, substituindo os demais genótipos (TRENTO et al., 2010). Acredita-se que o sucesso evolutivo desses genótipos esteja ligado ao escape da resposta imunológica do hospedeiro, visto que essas duplicações podem ter alterado a antigenicidade da proteína G, favorecendo o vírus com essas modificações (THONGPAN et al., 2017).

1.3 TRANSMISSÃO, PATOGENESE E IMPLICAÇÕES CLINICAS

O VSR possui tropismo tanto por tecidos oculares quanto pelos respiratórios, sendo possível causar infecção no trato respiratório superior a partir do duto nasolacrimal (BELSER; ROTA; TUMPEY, 2013). A transmissão ocorre via aerossol de gotículas de

secreção de nasofaringe por indivíduos infectados, as quais penetram nas membranas mucosas dos olhos, nariz e boca, por meio de contato próximo ou autoinoculação após contato com superfícies contaminadas (HALL, 1981).

A infecção é, normalmente, restrita às células superficiais do epitélio respiratório (JOHNSON et al., 2007; WELLIVER et al., 2007). As células ciliadas dos pequenos bronquíolos e os pneumócitos do tipo 1 nos alvéolos são os principais alvos de infecção nas vias aéreas inferiores (JOHNSON et al., 2007). A infecção e o dano tecidual tendem a ser irregulares em vez de difusos. Existem sinais abundantes de obstrução das vias aéreas devido à descamação de células epiteliais, secreção de muco e células imunes acumuladas. Os sincícios às vezes são observados no epitélio brônquico, mas não são comuns (COLLINS; GRAHAM, 2008).

A infecção resulta em replicação do vírus na nasofaringe, com período de incubação que varia de 4 a 6 dias, podendo se disseminar para as vias aéreas inferiores após alguns dias (COLLINS; CROWE, 2007; HALL, 2001; MCNAMARA; SMYTH, 2002; MEISSNER, 2016).

Os achados patológicos incluem necrose das células epiteliais, proliferação ocasional do epitélio bronquiolar, filtrados de monócitos, células T centralizadas nas arteríolas bronquiolares e pulmonares e neutrófilos entre estruturas vasculares e pequenas vias aéreas (COLLINS; GRAHAM, 2008).

Tosse, rinorreia e febre baixa são sintomas comuns (COLLINS; GRAHAM, 2008), sendo que a febre acomete cerca de 50% dos bebês (MEISSNER, 2016) e raramente é alta (BORCHERS et al., 2013). Sinais clínicos de bronquiolite incluem aumento da resistência das vias aéreas, aprisionamento de ar e respiração ofegante, sendo alguns desses sinais comuns até mesmo em bebês com quadros clínicos brandos (COLLINS; GRAHAM, 2008). A hipóxia, frequentemente detectada em bebês acometidos por VSR, é sinal de pneumonia (COLLINS; GRAHAM, 2008). Apneia pode ser observada em crianças pequenas e em bebês prematuros. Esses sinais clínicos são acompanhados de arquejos e/ou finos estertores inspiratórios na ausculta (BORCHERS et al., 2013).

O diagnóstico da bronquiolite aguda é, geralmente, clínico e baseia-se na presença de sintomas e sinais respiratórios típicos, os quais podem estar acompanhados de letargia, recusa alimentar e irritabilidade (BORCHERS et al., 2013).

Cinquenta por cento ou mais dos bebês hospitalizados por infecção respiratória das vias inferiores devido ao VSR apresentam episódios posteriores de hiperventilação (*i.e* respiração ofegante), o que pode persistir até a idade de 11 anos ou mais (HALL, 2001; SIMOES et al., 2007)

A resposta imune induzida pelo VSR pode ser tanto protetiva quanto patogênica, e parece haver diferenças entre uma primeira infecção em um bebê soronegativo e reinfecções em crianças mais velhas ou adultos (MEISSNER, 2016).

Reinfecções por VSR podem ocorrer várias vezes ao longo da vida, mesmo que haja produção de anticorpos e resposta T-celular após uma infecção primária e ainda que na ausência de mudanças antigênicas nas glicoproteínas de superfície do VSR. Como este vírus escapa ou inibe as defesas do hospedeiro ainda não está totalmente elucidado (COLLINS; MELERO, 2011).

As teorias mais sólidas a respeito da patogênese da bronquiolite por VSR indicam que há tanto resposta imune exagerada quanto dano celular direto devido à replicação viral (COLLINS; MELERO, 2011). No entanto, necropsias de bebês que vieram a óbito devido à infecção por VSR apontam a presença de macrófagos, neutrófilos e uma ausência relativa de células T citotóxicas, juntamente com baixas concentrações de citocinas derivadas de linfócitos T clássicos (liberadas por células T CD4+ e CD8+). Esse cenário não é compatível com uma resposta inflamatória patológica (WELLIVER et al., 2007). Na verdade, a presença de antígenos virais abundantes sugere replicação ativa do vírus e citotoxicidade direta (WELLIVER et al., 2007).

O espectro de gravidade causado pela infecção por VSR varia desde um quadro clínico brando e autolimitado até casos de SRAG (BORCHERS et al., 2013). As causas exatas dessas diferenças ainda não são completamente entendidas, porém, a literatura é assente em indicar que neonatos prematuros, crianças menores de 6 meses de idade e aquelas com cardiopatias, pneumopatias, problemas neuromusculares e imunocomprometidas compõem o grupo de risco da evolução grave causada pelo VSR, com maiores taxas de morbidade e mortalidade (HALL;

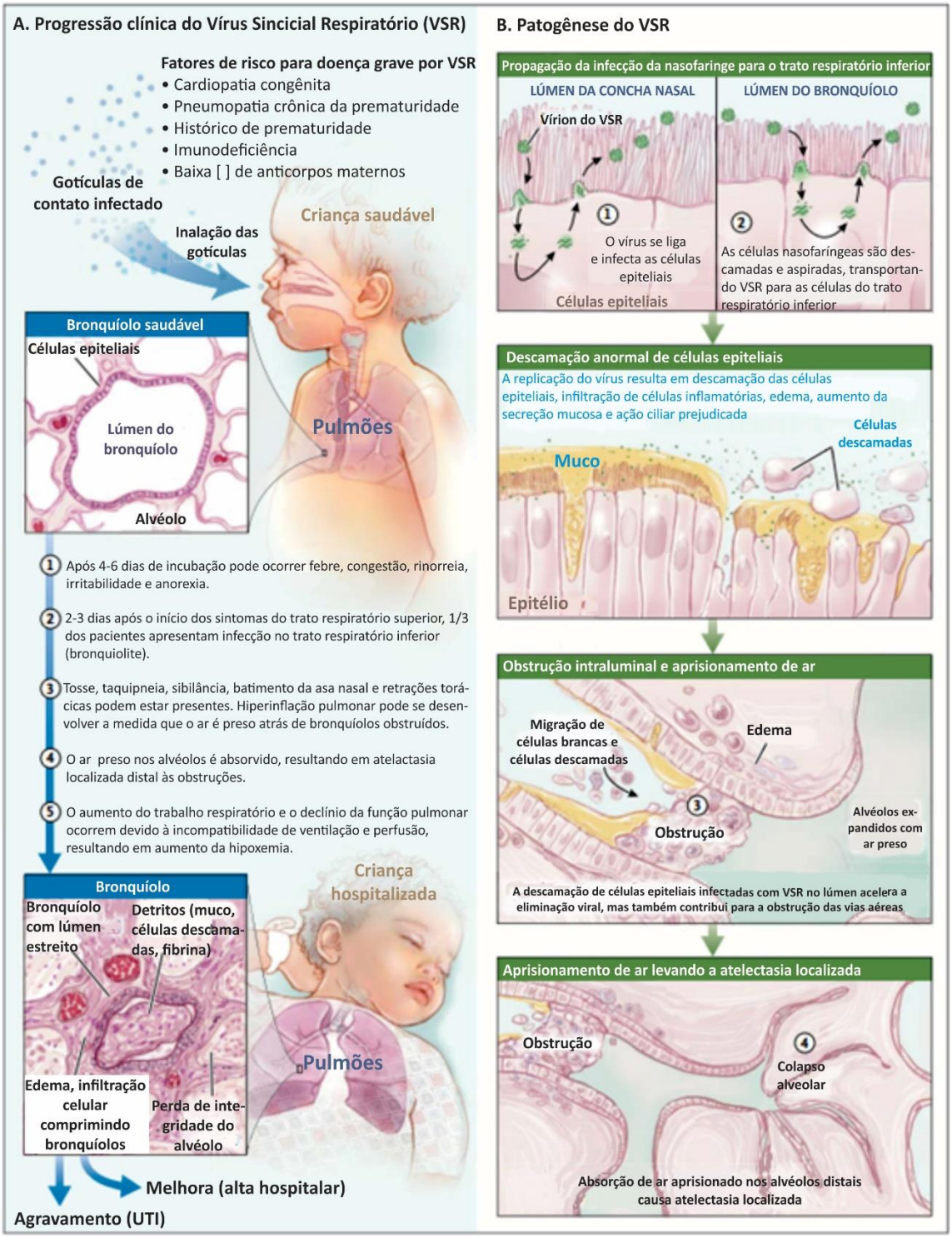
SIMÕES; ANDERSON, 2013; SOMMER, 2011). Apesar disso, grande parte das hospitalizações por VSR ocorrem em bebês previamente saudáveis e sem nenhum fator de risco (ALVAREZ et al., 2013; MEISSNER, 2016). A suscetibilidade genética do hospedeiro, a presença de coinfecções com outros patógenos, o genótipo e a carga viral possivelmente estão associados à gravidade da infecção (BORCHERS et al., 2013; HERVÁS et al., 2012; MURRAY et al., 2014), e acredita-se ainda que condições ambientais possam interferir no curso das infecções por VSR (COLLINS; GRAHAM, 2008).

A associação entre gravidade e os diferentes subgrupos (VSR-A ou VSR-B) é controversa. Embora alguns trabalhos indiquem uma maior virulência do VSR-A em relação ao VSR-B (MCCONNOCHIE et al., 1990; WALSH et al., 1997), outros não encontraram diferenças significativas entre eles (GRIFFITHS; DREWS; MARCHANT, 2017) ou até mesmo identificaram uma maior gravidade em pacientes infectados pelo VSR-B (HORNSLETH et al., 1998). Os fatores determinantes à gravidade da infecção pelo VSR podem estar mais ligados aos genótipos do que aos subgrupos (FLETCHER et al., 1997; GRIFFITHS; DREWS; MARCHANT, 2017; MARTINELLO et al., 2002; STRUCK et al., 2004). A predominância do subgrupo B do VSR em crianças menores de 1 ano, como demonstrado por uma meta-análise sistemática (ELAWAR et al., 2017), é intrigante e não está bem estabelecida.

Enquanto a relação entre gravidade de infecção pelo VSR e os diferentes genótipos permanece incerta, outras evidências indicam que o curso da infecção pelo VSR pode estar relacionado à carga viral (BAGGA et al., 2013; BUCKINGHAM; BUSH; DEVINCENZO, 2000; DEVINCENZO, 2004; DEVINCENZO et al., 2010; EL SALEEBY et al., 2011).

Reinfecções pelo VSR são observadas em 30% a 75% das crianças menores do que 2 anos de idade que tiveram a primo-infecção durante seus primeiros 12 meses de vida e, normalmente, ocorrem na sazonalidade seguinte (GLEZEN, 1986; HENDERSON et al., 1979). Mesmo em reinfecções, há a presença de sintomas em crianças pequenas, não obstante, a gravidade da doença é atenuada em exposições subsequentes, com menos crianças sendo acometidas por bronquiolite (ou outros sintomas de infecção de trato respiratório inferior), febre ou infecções de ouvido médio (HENDERSON et al., 1979).

Figura 4 - Patogênese da bronquiolite pelo VSR. A infecção é adquirida pela inoculação da mucosa nasal ou conjuntival com secreções contaminadas ou por inalação de grandes gotículas respiratórias (>5 μm de diâmetro) contendo vírus, dentro de um raio de 2 metros de um paciente infeccioso. Após um período de incubação de 4 a 6 dias, a replicação viral no epitélio nasal resulta em congestão, rinorreia, irritabilidade e falta de apetite. Febre ocorre em aproximadamente 50% das crianças infectadas. Uma vez no trato respiratório inferior, o



vírus infecta as células epiteliais ciliadas da mucosa dos bronquíolos e pneumócitos nos alvéolos. Duas glicoproteínas de superfície do VSR, F e G, medeiam a ligação viral ao

glicocálice da célula alvo. A adsorção viral inicia uma mudança conformacional na proteína F para uma estrutura pós-fusão, que facilita a fusão do envelope viral à membrana plasmática da célula hospedeira, resultando na entrada viral na célula. A replicação viral inicia um influxo de células natural killer, CD4+ auxiliares, linfócitos T CD8+ citotóxicos e granulócitos ativados. Infiltração celular do tecido peribronquiolar, edema, aumento da secreção mucosa, descamação de células epiteliais infectadas e batimento ciliar prejudicado causam vários graus de obstrução intraluminal. Durante a inspiração, é gerada uma pressão intrapleural. A pressão positiva de expiração estreita ainda mais as vias aéreas, produzindo maior obstrução, o que causa sibilância. As respostas imunes inatas e adaptativas estão envolvidas na eliminação viral, e a maioria das crianças hospitalizadas recebe alta após 2 a 3 dias. A regeneração do epitélio bronquiolar começa dentro de 3 a 4 dias após a resolução dos sintomas. ICU denota unidade de terapia intensiva.

Fonte: MEISSNER, 2016, adaptado.

1.4 TRATAMENTO, PROFILAXIA E VACINAS

Não há disponível atualmente uma terapêutica específica que amenize o curso da doença e que abrevie a resolução dos sintomas em pacientes infectados pelo VSR (KFOURI et al., 2017). Portanto, o tratamento costuma ser de suporte (RALSTON et al., 2013). A ribavirina é o único antiviral disponível para tratamento do VSR. No entanto, seu uso não é recomendado devido às dificuldades de administração, alto custo e risco para os cuidadores (FRIEDMAN; RIEDER; WALTON, 2014; TURNER et al., 2014).

Na década de 1960, a administração de uma vacina inativada em formalina contra VSR apresentou resultados inesperados. Pacientes vacinados, quando infectados naturalmente pelo VSR, apresentaram agravamento dos sintomas, e dois óbitos foram associados à consequência dessa vacina (OLMSTED et al., 1986). Entretanto, ao longo dos anos diversos grupos vêm trabalhando em busca de uma vacina eficaz para redução desse problema de saúde pública. Atualmente, várias vacinas candidatas estão sendo desenvolvidas, muitas em fases avançadas de teste e com resultados promissores (GLENN et al., 2016; KFOURI RA et al., 2017; MCLELLAN; RAY; PEEPLES, 2013; REZAEE et al., 2017).

Por fim, a imunização passiva pelo Palivizumab é o único método farmacológico profilático aprovado para uso no Brasil (GESKEY; THOMAS; BRUMMEL, 2007). Tratam-se de anticorpos monoclonais (mAb) IgG-1 humanizados direcionados a um epítopo no sítio antigênico A da glicoproteína F e que é capaz de neutralizá-la, inibindo a fusão do VSR com o epitélio ciliado do trato respiratório (WANG; BAYLISS; MEADS,

2011). A administração deve ser realizada em 5 doses, sendo a primeira um mês antes do início da sazonalidade e as demais a cada mês a partir da primeira dose (GESKEY; THOMAS; BRUMMEL, 2007). Por se tratar de um medicamento oneroso, o Palivizumab é recomendado apenas para grupos específicos, como bebês prematuros e portadores de pneumopatias ou cardiopatias (GESKEY; THOMAS; BRUMMEL, 2007). A administração do Palivizumab em pacientes desses grupos provou reduzir a taxa de hospitalização, o tempo de internação e a morbidade (WANG; BAYLISS; MEADS, 2011). O uso do Palivizumab nos demais grupos não é recomendado devido à baixa relação custo/benefício (WANG; BAYLISS; MEADS, 2011).

Reflexo da preocupação global em relação ao VSR é o recente Projeto Piloto da Organização Mundial de Saúde (OMS), que tem como objetivo primário testar a viabilidade e adequação da Rede Mundial de Vigilância e Resposta à Gripe à vigilância concorrente do VSR (OMS, 2017). Esse piloto é resultado de uma crescente preocupação global com o impacto do VSR na saúde pública, e conta com a participação de 10 países, dentre os quais destaca-se o Brasil. São alguns dos objetivos do projeto:

- Identificar a sazonalidade em diferentes países e regiões geográficas;
- Determinar a idade e os grupos de risco para quadros graves de VSR;
- Testar as definições de caso mais eficazes para diferentes grupos de risco;
- Padronizar os procedimentos laboratoriais para a detecção do VSR e para a garantia da qualidade dos exames;
- Auxiliar, com evidências científicas, a tomada de decisões políticas nacionais e mundiais.

Não obstante a miríade de pesquisas relacionadas as mais diversas facetas do VSR, são inúmeras as lacunas no conhecimento que precisam ser preenchidas. O entendimento dos padrões de circulação local é imperativo na adoção de políticas de saúde pública eficazes, sobretudo no que tange a administração oportuna do Palivizumab. De maneira semelhante à vigilância da Influenza é necessário acompanhar em tempo real a diversidade genética do VSR, de forma a garantir a adequação de futuras vacinas e fármacos antivirais às linhagens circulantes. Por fim, a investigação de quais fatores estão relacionados à gravidade clínica é essencial na estimativa e redução da morbidade e mortalidade causado pela infecção.

Destarte, neste projeto, investigamos a prevalência da infecção pelo VSR-A e VSR-B em crianças de até 3 anos de idade com SRAG no Espírito Santo (ES), entre 2016 e 2018, confrontando os diferentes genótipos ou grupos genéticos circulantes com fatores como diferenças na carga viral e nos sintomas, gravidade e desfecho clínico (óbito *vs* cura). A criação de uma Pontuação de Gravidade Clínica (PGC) permitiu analisar a diferença de gravidade entre subtipos e diferentes cargas virais. As amostras utilizadas neste estudo foram oriundas do Laboratório Central de Saúde Pública do Espírito Santo (LACEN/ES), com representatividade de 60 dos 70 municípios do Estado.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a prevalência do Vírus Sincicial Respiratório em crianças de até 3 anos de idade com SRAG, no Espírito Santo, entre 2016-2018, e testar a relação de fatores climáticos na sua circulação e da carga viral e da diversidade genética na gravidade clínica, no contexto do Programa Brasileiro de Vigilância da Influenza.

2.2 OBJETIVOS ESPECÍFICOS

- Inferir o padrão de sazonalidade do VSR no ES, entre 2016 e 2018, e analisá-lo em conjunto com dados climáticos;
- Identificar a prevalência de infecções pelo VSR entre crianças de até 3 anos de idade com SRAG no Espírito Santo, a fim de aferir a importância desse agravo na saúde pública infantil capixaba;
- Investigar a prevalência dos subtipos VSR-A e VSR-B, bem como caracterizar geneticamente os casos positivos;
- Determinar se a carga viral e a diversidade genética estão correlacionadas à gravidade da infecção;
- Analisar as relações filogenéticas dos genótipos circulantes de VSR-A e VSR-B no ES com cepas de referência para cada genótipo já identificado e avaliar a prevalência de cada um dentro de contextos epidemiológicos;
- Investigar a presença de alterações na cadeia de aminoácidos da glicoproteína G.

3. MATERIAIS E MÉTODOS

3.1 ASPECTOS ÉTICOS E LEGAIS

Este projeto obteve aprovação do Comitê de Ética em Pesquisa com Seres Humanos do Centro de Ciências em Saúde da Universidade Federal do Espírito Santo – CEP/CCS/UFES; n° 018577/2018, CAAE: 84633518.1.0000.5060, bem como da Comissão para Análise de Pesquisas no Âmbito da Rede Estadual de Saúde do Núcleo Especial de Desenvolvimento de Recursos Humanos – NUEDRH/SESA/ES (Anexo A), este último exigido por se tratar de um estudo envolvendo amostras e dados clínicos da Secretaria de Estado de Saúde do Espírito Santo (SESA/ES). A necessidade de consentimento dos pais ou responsáveis foi dispensada pelo comitê de ética.

3.2 DELINEAMENTO AMOSTRAL

No presente estudo foram utilizadas amostras de secreções respiratórias (*swabs* de oronasofaringe, lavados brônquicos e aspirados traqueais e bronco-alveolares) coletadas de crianças de até 36 meses de idade entre 07 de março de 2016 a 14 de dezembro de 2018, totalizando 34 meses de amostragem. Ao todo, foram coletadas amostras de 632 pacientes, residentes de 60 municípios do Espírito Santo. Todos os casos eram de pacientes notificados com SRAG pela SESA/ES e referenciados ao LACEN/ES para o diagnóstico dos vírus Influenza e VSR. As amostras foram gentilmente cedidas para este projeto após as devidas aprovações.

3.3 EXTRAÇÃO DOS ÁCIDOS NUCLEICOS

Os ácidos nucleicos foram extraídos e purificados utilizando-se 200 µl das amostras respiratórias com o kit comercial PureLink™ Viral RNA/DNA Mini Kit (Invitrogen®, Thermo Fisher Scientific©) – conforme manual do fabricante e, posteriormente, eluídos em 60 µl água livre de nucleases e armazenados a -70°C.

3.4 IDENTIFICAÇÃO POR RT-QPCR

A detecção do RNA viral do vírus Influenza e do VSR foi realizada por TaqMan® RT-qPCR em termociclador 7500 Real-Time PCR System (Applied Biosystems®). O diagnóstico da Influenza seguiu o protocolo de triagem para a detecção dos casos de Influenza A e B, e subtipagem, nos casos positivos de Influenza A, dos subtipos H1N1 e H3, conforme preconizado pelo Ministério da Saúde (CDC, 2009). A detecção dos casos positivos de VSR foram feitas segundo protocolo descrito por FRY et al., 2010. O RNA do gene da RNAse P também foi retrotranscrito e amplificado para cada amostra, de forma a permitir a realização do controle interno dos aspectos relacionados à qualidade das amostras, do protocolo de extração de RNA, da retrotranscrição e da qPCR. O quadro 1 mostra os *primers* e sondas que foram utilizados na detecção dos vírus respiratórios supracitados. As reações de RT-qPCR foram realizadas com o kit GoTaq® Probe 1-Step RT-qPCR System (Promega®) e seguiram o seguinte programa: 45°C por 25 minutos, 95°C por 2 minutos e 45 ciclos de 95°C por 15 segundos e 55°C por 30 segundos.

Quadro 1 - Sequências dos *primers* e sondas utilizados nos testes de identificação dos vírus da Influenza A e B e VSR.

Vírus	Sequência	Referência
Flu A	F: GAC CRA TCC TGT CAC CTC TGA C	CDC, 2009
	R: AGG GCA TTY YGG ACA AAK CGT CTA	
	S: TGC AGT CCT CGC TCA CTG GGC ACG	
Flu B	F: TCC TCA ACT CAC TCT TCG AGC G	CDC, 2009
	R: CGG TGC TCT TGA CCA CCA AAT TGG	
	S: CCA ATT CGA GCA GCT GAA ACT GCG GTG	
VSR	F: GGC AAA TAT GGA AAC ATA CGT GAA	Fry et al., 2010
	R: TCT TTT TCT AGG ACA TTG RAY TGA ACA G	
	S: CTG TGT ATG TGG AGC CTT CGT GAA GCT	

3.5 CÁLCULO DA CARGA VIRAL E ANÁLISE DA GRAVIDADE

Para a inferência da quantidade de cópias de vírus por célula (carga viral) nas amostras clínicas, estas foram submetidas a um novo protocolo de RT-qPCR. Nessa etapa, sequências sintéticas específicas de VSR e do gene β-globina foram utilizadas para a construção de curvas padrão. Cada ponto da curva foi obtido aplicando-se diluições seriadas e de quantidade de cópias conhecida dos fragmentos de VSR e do gene da β-globina. As amostras biológicas foram então amplificadas em conjunto com as diluições padrão e o *Ct* (Cycle Threshold) das amostras foi comparado com o *Ct* dos pontos da curva padrão.

Para a obtenção das soluções seriadas, utilizamos plasmídeos recombinados de *Escherichia coli* com um inserto sintético de 150 pares de bases, cuja sequência inclui a região de hibridização dos *primers* e sonda de VSR utilizados na etapa de detecção do vírus. Os plasmídeos foram ressuspendidos a 100 ng de DNA/µl, diluídos e estocados a 10^8 cópias DNA/µl a -70°C. Para fins deste cálculo, seguimos a seguinte equação (WHELAN; RUSSELL; WHELAN, 2003):

$$\text{n°de cópias/µl} = \frac{\text{g/µl de DNA}}{\text{Tamanho do Fragmento em pb} \times 660} \times 6{,}022\times10^{23}$$

A construção da curva padrão com um fragmento sintético do gene da β-globina foi realizada com o fim de normalizar as possíveis diferenças de carga viral causadas por fatores como qualidade da coleta, do transporte e armazenamento dos espécimes e dos diferentes prazos de coleta desde a data do início dos sintomas. O referido gene possui duas cópias em cada célula, encontradas no braço curto dos cromossomos 11, e assim foi possível calcular o número de células que uma determinada amostra possui a partir da comparação dos *Ct* das amostras com os pontos da curva padrão da β-globina. De posse da média do número de cópias do VSR em uma determinada amostra, dividimos esse valor pelo número de células obtido pela análise da curva padrão do gene da β-globina, encontrando a média do número de cópias por célula.

As curvas padrão de ambos os marcadores foram construídas com diluições seriadas de 10^6 a 10^1 cópias DNA/µl, e o perfil de termociclagem e os oligonucleotídeos utilizados foram os mesmos da etapa de triagem dos casos positivos de VSR.

Para avaliar a gravidade dos casos de SRAG, uma Pontuação de Gravidade Clínica (PGC) – adaptada de Martinello et al. (2002) – foi utilizada. Trata-se de uma escala de 6 pontos (0 até 5) em que 0 indica a condição clínica mais branda e 5 a mais grave. Admissão em UTI, duração da hospitalização ≥5 dias, saturação de oxigênio ≤95% e uso de métodos não invasivos de oxigenoterapia contabilizaram 1 ponto cada. Dois pontos foram adicionados aos casos que necessitaram de ventilação mecânica.

3.6 SUBTIPAGEM DOS CASOS POSITIVOS DE VSR

A identificação dos subtipos VSR-A e VSR-B nos casos positivos também foi realizada por TaqMan® RT-qPCR em termociclador 7500 Real-Time PCR System (Applied Biosystems®). Para tanto, utilizamos *primers* e sondas específicos para cada subtipo (DE-PARIS et al., 2012; HU et al., 2003), empregando o seguinte programa de termociclagem: 45°C por 25 minutos, 95°C por 2 minutos e 45 ciclos de 95°C por 15 segundos e 55°C por 30 segundos.

3.7 AMPLIFICAÇÃO E SEQUENCIAMENTO PARCIAL DO GENE G

Para a obtenção de fragmentos de 711 pb para VSR-A e 726 pb para VSR-B do gene G, realizamos reações de RT-PCR convencional utilizando a enzima QIAGEN OneStep RT-PCR Kit (Qiagen) empregando *primers* descritos previamente (ZLATEVA et al., 2005). A transcrição reversa foi realizada a 55°C por 30 minutos, e a amplificação dos produtos a 94°C por 30 segundos, 60°C por 1 minuto, 72°C por 1 minuto totalizando 40 ciclos e uma extensão final de 72°C por 10 minutos. Para visualização dos produtos, 5 µl do DNA amplificado foram misturados a 1 µl de GelRed® e 2 µl BlueJuice™ 10 X e submetidos a eletroforese em gel de agarose a 1%. O DNA foi purificado com o kit ExoSap-IT (Affymetrix, Inc., USA) e empregado na reação de sequenciamento utilizando o kit Bigdye terminator (Applied Biosystems, Foster City, CA, USA) utilizando *primers* a 3,2µmolar. A leitura dos fragmentos obtidos pelo sequenciamento foi realizada pelo ABI 3130XL Genetic Analyzer (Applied Biosystems).

3.8 ANÁLISES FILOGENÉTICAS

As sequências consenso foram geradas a partir da montagem dos eletroferogramas obtidos frente a uma sequência de referência, utilizando o software Sequencher 5.1 (Gene Codes Corporation, Ann Arbor, MI, USA). A nomenclatura utilizada neste estudo para cada amostra obtida foi “subtipo de VSR/País_UF/número da amostra no laboratório de origem/ano”.

As sequências produzidas neste trabalho foram depositadas na plataforma GenBank, sob o número de acesso MW026969 – MW027004 e MW030961-MW030981, e na plataforma GISAID, sob o número de acesso EPI_ISL_549271 – EPI_ISL_549327.

3.9 CARACTERIZAÇÃO DOS GENÓTIPOS DE VSR BASEADO NA 2ª Região Hiper Variável (RHV-2)

Para a classificação dos genótipos das nossas amostras, um conjunto de dados contendo sequências referência dos genótipos já descritos na literatura para ambos os subtipos VSR-A e VSR-B foram recuperados do Genbank (ver S2 Table). A maior parte dos genótipos descritos basearam-se apenas nas divergências encontradas na RHV-2, e a descrição de novos genótipos leva em consideração sequências que se agrupam com um suporte acima de 0,7 (70%) e com uma distância máxima de pares de bases (*pairwise distance/p-distance*) de 0,07 entre este grupo e o grupo filogeneticamente mais próximo a ele (ARNOTT et al., 2011; BLANC et al., 2005; VENTER et al., 2001). O conjunto de dados de sequências de genótipos referência e as sequências obtidas neste estudo foram alinhadas utilizando o MUSCLE, ferramenta integrada ao MEGA 6.0. Após o alinhamento, edições manuais foram necessárias para refinar o alinhamento e remover as bases extras a RHV-2. Assim, utilizamos sequências de 336 pb para VSR-A e 318 pb para VSR-B. A relação filogenética entre as sequências foi inferida por Máxima Verossimilhança (MV) com *bootstrap* de 1000 replicatas. O melhor modelo de substituição de nucleotídeos foi escolhido pelo programa JModel Test.

3.10 RECONSTRUÇÃO FILOGENÉTICA DO GENE G

Para a reconstrução filogenética, utilizamos um fragmento do gene G de 711 pb para VSR-A e 726 pb para VSR-B. Para verificar sequências similares às sequências do Espírito Santo, utilizamos a ferramenta BLAST (Basic Local Alignment Search Tool), disponível em https://blast.ncbi.nlm.nih.gov/Blast.cgi. As sequências obtidas foram classificadas de acordo com o país e ano de coleta. Sequências com similaridade maior do que 99,5% foram removidas do alinhamento com o a ferramenta CD-Hit70, disponível on-line em http://weizhongli-lab.org/cd-hit/servers.php. Foram mantidas somente algumas sequências por grupo, representativas de diferentes países e anos de coleta. As sequências selecionadas foram alinhadas juntamente com as geradas neste trabalho para serem incluídas nas análises filogenéticas. A inferência das relações filogenéticas entre as sequências geradas e as disponíveis em bancos de dados de DNA foi realizada pelo método de Máxima Verossimilhança (*Maximum Likelihood* – *ML*) no software Mega 6.0.

3.11 ANÁLISES ESTATÍSTICAS

As análises estatísticas foram realizadas no software SPSS 20.0 (SPSS, Inc., Chicago, IL) e R v.3.4.4. Os testes de qui-quadrado, teste exato de Fisher, Mann-Whitney, Kaplan-Meier e Kruskal Wallis foram usados quando apropriado. O modelo de regressão de Cox foi usado para avaliar se a carga viral teve um efeito estatisticamente significativo no tempo de permanência na UTI, e os resíduos de Schoenfeld foram usados para verificar a suposição de riscos proporcionais. Para testar a associação entre os dados climáticos e a circulação do VSR, foi realizado o teste de correlação de *Spearman*. Um valor de *p* inferior a 0,05 foi considerado estatisticamente significativo.

4. RESULTADOS E DISCUSSÃO

O item “Resultados e discussão” será apresentado no formato de artigos.

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Carta de aceite: Anexo B.

Full Title: Seasonality, molecular epidemiology, and virulence of Respiratory Syncytial Virus (RSV): a perspective into the Brazilian Influenza Surveillance Program

Short Title: Landscape of Respiratory Syncytial Virus (RSV)

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Abstract

Background: Respiratory Syncytial Virus (RSV) is the main cause of pediatric morbidity and mortality. The complex evolution of RSV creates a need for worldwide surveillance, which may assist in the understanding of multiple viral aspects.

Objectives: This study aimed to investigate RSV features under the Brazilian Influenza Surveillance Program, evaluating the role of viral load and genetic diversity in disease severity and the influence of climatic factors in viral seasonality.

Methodology: We have investigated the prevalence of RSV in children up to 3 years of age with severe acute respiratory infection (SARI) in the state of Espirito Santo (ES), Brazil, from 2016 to 2018. RT-qPCR allowed for viral detection and viral load quantification, to evaluate association with clinical features and mapping of local viral seasonality. Gene G sequencing and phylogenetic reconstruction demonstrated local genetic diversity.

Results: Of 632 evaluated cases, 56% were caused by RSV, with both subtypes A and B co-circulating throughout the years. A discrete inverse association between average temperature and viral circulation was observed. No correlation between viral load and disease severity was observed, but children infected with RSV-A presented a higher clinical severity score (CSS), stayed longer in the hospital, and required intensive care, and ventilatory support more frequently than those infected by RSV-B. Regarding RSV diversity, some local genetic groups were observed within the main genotypes circulation RSV-A ON1 and RSV-B BA, with strains showing modifications in the G gene amino acid chain.

Conclusion: Local RSV studies using the Brazilian Influenza Surveillance Program are relevant as they can bring useful information to the global RSV surveillance. Understanding seasonality, virulence, and genetic diversity can aid in the development and suitability of antiviral drugs, vaccines, and assist in the administration of prophylactic strategies.

Introduction

Respiratory Syncytial Virus (RSV) is the most common pathogen associated with acute respiratory tract infections (ARTI), as well as the main cause of bronchiolitis and pneumonia in infants and small children [1]. RSV infection can cause a range of symptoms, varying from mild upper respiratory tract illness to severe lower respiratory tract infection [2]. The reason for different outcomes is still unclear, however, it can be related to the underlying conditions, genetic or acquired host factors, and/or viral characteristics [3,4]. Some studies have evaluated the association between viral load and disease severity with significant associations [4,5]. However, most of these studies did not use standardized methods of viral load measurement, therefore, this relationship must be more carefully evaluated. Understanding the role of the viral load in RSV infection may be a tool to establish its relationship with disease progression, severity, clinical outcome, and drug intervention timeframe [6].

RSV treatment is based only on supportive care and infection prevention is limited to passive immunoprophylaxis (Palivizumab) and case isolation [2]. No approved RSV vaccine is available, but promising candidates are currently in development and in advanced clinical trial phases [7]**.**

RSV strains can be classified into two serogroups: RSV-A and RSV-B [8]. The potential virulence attributed to a specific group remains controversial: some authors have pointed RSV-A [9,10] or RSV-B [11] as the most virulent subtype, while another study has not found significant differences between them [12]. Multiple genotypes were described for RSV-A and RSV-B, based on the gene G second hypervariable region (HVR-2) [13,14]. In the past two decades, important genetic changes occurred with the emergence of new RSV-A and RSV-B genotypes: RSV-A ON1 containing a duplication of 72 nucleotides, and RSV-B BA with a duplication of 60 nucleotides in the HVR-2 gene G [14,15]. These genotypes replaced previous

ones and have spread globally. Understanding their genetic diversity may reveal the virus's ability to cause re-infections throughout life, and help in the development of antiviral drugs, diagnostic assays, and vaccines [13].

In 2017, the World Health Organization (WHO) launched the Global Respiratory Syncytial Virus Surveillance Pilot to test the feasibility of using the Global Influenza Surveillance and Response System (GISRS) for RSV surveillance without adversely affecting influenza surveillance [16]. This pilot study results from the global concern about RSV's impact on public health. Brazil, one of four countries in the Americas included in the pilot, has a remarkable respiratory virus surveillance program, however, more data are required for a better understanding of factors such as RSV circulation, evolution, and pathogenicity. In this study, we used the Brazilian Influenza Surveillance Program to analyze the local prevalence of RSV in children with SARI and to evaluate which factors are potentially associated with disease severity. We also explored the viral seasonality and investigated the influence of climatic factors on circulation. Finally, we conducted a phylogenetic study to understand how the local genetic diversity of RSV behaves when compared to what is observed in the rest of the world.

Materials and methods

Population sampling, study period, and location

This study is a retrospective investigation of respiratory samples (nasopharyngeal secretions, tracheal and bronchoalveolar aspirates, and bronchoalveolar lavages) collected from the Brazilian Influenza Surveillance Program over 34 months. (March 7th, 2016, to December 14th, 2018). A total of 632 samples collected from pediatric patients (from 0 to 36 months old) classified as SARI, residents of 60 municipalities in the state of Espirito Santo (ES), were enrolled in this study. ES is located in southeastern Brazil (**S1 Fig.**) and has a territory of

46,074.447 km^2, with a population of approximately 4,1 million inhabitants [17]. These samples were screened by real-time RT-qPCR for RSV and Influenza A/B at the ES Central Public Health Laboratory (LACEN/ES), one of 26 Brazilian laboratories that integrate the Brazilian Ministry of Health Influenza Surveillance Program.

RSV and Influenza detection and subtyping

Nucleic acids were extracted from respiratory samples using the PureLink™ Viral RNA/DNA Mini Kit (Invitrogen®, Thermo Fisher Scientific©), according to the manufacturer's protocol. All samples were initially tested for Influenza A and B in a TaqMan® one-step real-time RT-PCR (RT-qPCR) assay using specific primers and probes for influenza (CDC, USA), according to the manufacturer's recommendations. Additionally, an RT-qPCR assay was performed to identify positive RSV cases using a GoTaq® Probe 1-Step RT-qPCR Kit (Promega, Madison, WI, EUA). RSV positive samples (*i.e.* those with cycle threshold [CT] ≤ 40) were subtyped using specific primers and probes for RSV-A and RSV-B N genes. In parallel, Ribonuclease P RNA (RNase P) was used as an internal control for each sample and, in all batches, RNA extraction negative control (MOCK) and a PCR negative control (NTC) were used. All primers and probes are described in the **S1 Table**.

Clinical and epidemiological data collection

Clinical and epidemiological data were retrieved mainly from the Brazilian Notifiable Diseases Information System (SINAN) database and, in some cases, – when the SINAN form was incomplete – patients' Medical Records were assessed to fill in missing information. The main information retrieved from SINAN were: 1) clinical outcome (recovery or death); 2) hospitalization length of stay; 3) need for oxygen administration and type (invasive or not

invasive); 4) intensive care unit (ICU) need and length of stay; 5) clinical characteristics (fever, cough, dyspnea, O_2 saturation, respiratory distress, comorbidities), and 6) epidemiological and demographical features (age, town or area of residence).

We have used the Brazilian Ministry of Health's definition of SARI, which is: hospitalized patients with fever and cough or sore throat, and presenting dyspnea or O_2 saturation <95%, or respiratory distress [18]. A Clinical Severity Score (CSS) was adapted from Martinello *et al*. [19]. A 6-point scale (0 to 5 spectrum) was used, where 0 indicated the mildest condition and 5 indicated the most severe. ICU admission, hospitalization length of stay ≥5 days, oxygen saturation ≤95%, and oxygen therapy noninvasive methods accounted for 1 point each. Two points were assigned for mechanical ventilation.

Viral load quantification

RSV viral load was determined by RT-qPCR using a protocol adapted from Álvarez-Argüelles *et al*. [20], including a synthetic *β-globin* dsDNA as a template. To quantify the RSV copy number, expressed in copies per cell (c/c), we designed a dsDNA containing the annealing regions of RSV primers and probe, as well as the upstream and downstream regions (150 bp). This synthetic DNA was incorporated into a pMA-T plasmid, which was used in the RT-qPCR. Standard curves for absolute quantification of RSV and *β-globin* gene were generated by 10-fold serial dilutions (10^6-10^1 gene copies), in triplicate. The RSV primers, probe, and thermal cycling protocol adopted were the same used in the diagnostic phase. *β-globin* primers and probe are listed in the **S1 Table**. All amplification assays were carried out in an ABI 7500 equipment (Applied Biosystems, Foster City, CA, USA). The viral load status was compared with different clinical features and epidemiological data.

Climate data collection

Climate data (precipitation, temperature, and humidity) of five cities – representing the different geographic regions of the state – were collected daily and kindly provided by the Capixaba Institute of Research, Technical Assistance, and Rural Extension (INCAPER), Vitoria, Espirito Santo, Brazil. The weekly average was accessed by assembling daily data from all collection sites for each epidemiological week (EW). The definition of the RSV epidemic period was based on a previously described protocol [21], which considers RSV outbreak onset, peak, and end. Seasonality onset was defined as the first of 2 consecutive weeks when ≥10% of tested samples for respiratory pathogens were positive for RSV. Similarly, RSV season end was defined when the proportion of positive RSV tests fell below 10% for two consecutive weeks. Peak was determined as the week when the maximum number of RSV positive cases occurred [21].

Partial amplification and sequencing of glycoprotein gene

RSV-A and RSV-B positive samples were selected for sequencing based on the following criteria: a) cycle threshold (ct) value less than 30, due to the difficulty in sequencing samples with ct higher than this; b) representativeness by collection date; c) distinct clinical outcomes; and d) different viral load values.

The partial gene G amplification (about 730 bp) was performed at LVRS/IOC/FIOCRUZ, the National Influenza Center, by conventional RT-PCR, using the QIAGEN OneStep RT-PCR Kit (Qiagen) and a pair of primers (**S1 Table**) for each subtype. The reverse transcription was performed at 55°C for 30 minutes and the cDNA was amplified by PCR (40 cycles of 94°C/30 seconds, 60°C /1 minute, 72°C/1 minute and a final extension at 72°C/10 minutes). Amplification was confirmed in a 1% agarose gel. DNA was purified using an ExoSap-IT Kit

(Affymetrix, Inc., USA) and submitted for sequencing reaction using a BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and primers at 3.2 μmolar. The reads were obtained in the ABI 3130XL Genetic Analyzer (Applied Biosystems). Consensus sequences were built from electropherograms comparison with a reference sequence in the software Sequencher 5.1 (Gene Codes Corporation, Ann Arbor, MI, USA). The adopted nomenclature pattern hereon was "hRSV subtype/country/ES-sample number/year."

RSV genotyping and gene G phylogenetic reconstruction

RSV-A and RSV-B gene G DNA sequences (711 bp and 726 bp, respectively) were used to reconstruct phylogenetic relationships. Genotyping was based on gene G HVR-2, using RSV-A and RSV-B sequences (336 bp and 318 bp, respectively). Reference sequences of previously described genotypes are shown in the **S2 Table**. Additionally, to place our sequences in a global context we performed a BLAST search (Basic Local Alignment Search Tool), available at https://blast.ncbi.nlm.nih.gov/Blast.cgi. These sequences (**S3 Table**) were labeled with country of origin and collection year, and those with more than 99.5% genetic similarity using the CD-HIT tool (http://weizhongli-lab.org/cd-hit/servers.php) were removed from the final dataset. Alignments were conducted using Muscle algorithm, via MEGA 6.0 software [22], and, when necessary, they were adjusted manually. The phylogenetic trees were constructed using the Maximum Likelihood (ML) method, complete deletion for gap or missing data treatment, and 1000 replicates of bootstrap probabilities tools, and analyzed using the Mega 6.0 software. General Time Reversible + Gamma (GTR+G) was the nucleotide substitution model elected for all analyses on JModelTest software, except for RSV-A, where the Tamura-Nei + Gamma (TrN+G) substitution model nucleotide recommended for the analysis was used [23]. Mega 6.0 software was employed to calculate the average pairwise distance (p-distance) and to compare

the amino acid changes between Brazilian samples and the reference sequences of ON1 (JN257693) and BA (AY333364).

Statistical treatment

Statistical analyses were performed using SPSS 20.0 (SPSS, Inc., Chicago, IL) and R v.3.4.4 software. Chi-square, Fisher exact, Mann–Whitney, Kaplan-Meier, and Kruskal Wallis were used whenever appropriate. The Cox regression model was used to assess whether the viral load had a statistically significant effect on length of stay in ICU, and Schoenfeld Residuals were used to check the proportional hazards assumption. To test the association between climate data and RSV circulation, we performed the Spearman correlation test. A p-value of less than 0.05 was considered statistically significant.

Data availability

The sequences produced here were deposited on the GenBank platform, under the accession number MW026969–MW027004 and MW030961-MW030981, and in the GISAID platform, under the accession number EPI_ISL_549271–EPI_ISL_549327. Laboratory, epidemiological, clinical, and climatic data can be found in the **S4 Table**.

Ethics Statement

This project was approved by the Human Research Ethics Committee of the Health Sciences Center of the Federal University of Espirito Santo (UFES), under the number: 018577/2018; CAAE: 84633518.1.0000.5060. The need for consent from parents or guardians was waived by the ethics committee.

Results

RSV clinical and epidemiological data

A total of 632 respiratory samples collected from children under 3 years of age were tested by RT-qPCR for Influenza A, Influenza B, and RSV. RSV is the most prevalent pathogen found in these samples (56%; 352/632) (**Table 1**). From the RSV positive cases, 51% (180/352) were RSV-A, 42% (147/352) were RSV-B, and co-detections with both subtypes were found in 1.4% (5/352). Twenty samples could not be subtyped (5.7%). Influenza frequency was 7.4% (47/632), of which 74% (35/47) were Influenza A H1N1 pdm09, 15% (7/47) were H3N2, and 11% (5/47) were Influenza B. The median age was 4 months old (1-11.0 interquartile range; IQR). Of the positive cases, 99.7% (351/352) were classified as SARI and 14 deaths (4%) were reported.

Table 1. Number of tested samples, RSV positivity, subtype prevalence, and demographic data from each year and the whole study period.

	2016 n (%)	**2017 n (%)**	**2018 n (%)**	**2016-18 n (%)**
General data				
Sample n°	251/632 (40%)	135/632 (21%)	246/632 (39%)	632/632 (100%)
RSV +	155/251 (62%)	80/135 (59%)	117/246 (48%)	352/632 (56%)
RSV -	96/251 (38%)	55/135 (41%)	129/246 (52%)	280/632 (44%)
Flu +	27/251 (11%)	6/135 (4%)	14/246 (6%)	47/632 (7%)
RSV+ deaths	6/155 (4%)	5/80 (6%)	3/117 (3%)	14/352 (4%)
Subtyped samples	141/155 (91%)	78/80 (98%)	113/117 (97%)	332/352 (94%)
Subtypes				
RSV-A	58/141 (41%)	14/78 (18%)	108/113 (96%)	180/352 (51%)
RSV-B	80/141 (57%)	63/78 (81%)	4/113 (4%)	147/352 (42%)
RSV-A and RSV-B	3/141 (2%)	1/78 (1%)	1/113 (1%)	5/352 (1.4%)
Demographic data (RSV+)				
Median age (months)	4 (1-12.0)	4 (1-10.5)	3 (1-8.0)	4 (1-11.0)
Gender				

Male	72/155 (46%)	49/80 (61%)	61/117 (52%)	182/352 (52%)

Table 2 shows patients' clinical features of RSV+ and subtypes. The most frequent symptom reported was cough (93%, 318/341), followed by respiratory distress (88%, 269/307), and fever (86%, 288/336). Seventy-four percent (252/342) of the children needed oxygen therapy and 38% (95/252) of these required mechanical ventilation. The median hospitalization length of stay was 8 (6-14 IQR) days. Intensive care was needed for 61% (202/333) of patients and the median number of days in ICU was 6 (3-10 IQR). The Kaplan-Meier test was used as a survival analysis technique and revealed that patients' recovery took, on average, 8 days from the date of admission to the ICU (**S4 Fig and S5 Table**).

Table 2. Summary of clinical and epidemiological data by RSV+ and each subtype. Statistically significant values are highlighted in bold. Although the study included 352 patients with RSV, it is possible to observe that the denominators in the clinical profile differ from this number. This occurred because not all clinical data were recorded for all children.

	RSV+ n (%)	**RSV-A n (%)**	**RSV-B n (%)**	***p-value***
Demographic profile				
Sample number	352	180	147	
Age				
Median age: months (IQR[1])	4 (1-11)	4 (1-10.0)	4 (1-12.5)	0.78
Gender				
Male (%)	182/352 (52%)	92/180 (51%)	78/147 (53%)	0.725
Clinical profile				
Fever	288/336 (86%)	147/174 (84%)	124/139 (89%)	0.223
Cough	318/341 (93%)	162/176 (92%)	134/142 (94%)	0.418
Dyspnea	251/331 (76%)	135/172 (78%)	97/136 (71%)	0.148
O_2 saturation ≤95%	169/277 (61%)	101/150 (67%)	56/109 (51%)	**0.009**
Respiratory distress	269/307 (88%)	154/167 (92%)	96/120 (80%)	**0.002**
O_2 Therapy	252/342 (74%)	138/177 (78%)	98/143 (68%)	0.092
Invasive	95/252 (38%)	56/138 (41%)	33/98 (34%)	
Noninvasive	157/252 (62%)	82/138 (59%)	65/98 (66%)	
Intensive Care	202/333 (61%)	113/168 (67%)	78/142 (55%)	**0.03**
Median hospitalization days	8 (6-14)	9 (6-15)	8 (5-14.0)	0.15
Median days in Intensive Care	6 (3-10)	7 (4-11.0)	6 (3-9)	0.13
Deaths RSV+	14/352 (4%)	3/180 (2%)	8/147 (5%)	0.07

[1]IQR: interquartile range.

When compared to RSV-B, patients affected by RSV-A showed a higher frequency of respiratory distress (92% vs 80%, p=0.002), and more often manifested O_2 saturation ≤95% (67% vs 51%, p=0.009) and higher requirement for intensive care (67% vs 55%, p=0.03). Our data also indicate that patients affected by RSV-A stayed one day longer in the hospital and intensive care units than those affected by RSV-B, however, these data were not statistically significant. Lastly, the RSV-A viral load showed more than twice the number of virus copies per cell (median=57.41 copies/cell) than RSV-B (median=27.35 copies/cell). RSV-A CSS median was 4 and RSV-B's was 3, and children infected by RSV-A were most frequently classified in higher severity scores than those infected by RSV-B (**Table 3**).

Table 3. Clinical Severity Score (CSS): scores varied from 0 to 5. Higher values indicated more severe illness. Need for ICU, O_2≤95%, hospitalization length of stay >5 days, and requirement of O_2 therapy accounted for 1 point each. The need for mechanical ventilation accounted for 2 points. Patients infected with RSV-A were most commonly classified into the most severe scores.

Clinical Severity Score (CSS)						
CSS	**RSV-A n (%)**	**RSV-B n (%)**	***p-value***	**Viral load median (IQR)**	**n**	***p-value***
0	1 (1%)	10 (15%)	**0.003**	54.06 (6.12-603.61)	8	0.089
1	8 (8%)	8 (12%)		217.41 (96.38-370.56)	9	
2	19 (20%)	11 (17%)		41.18 (6.53-112.59)	16	
3	14 (15%)	15 (23%)		17.31 (6.33-125.40)	14	
4	26 (27%)	9 (14%)		12.05 (4.32-36.63)	9	
5	28 (29%)	13 (20%)		11.81 (1.14-54.24)	18	

Viral load

A total of 156 (44%) samples were submitted to the viral load analysis (**Table 4**). According to age, the median viral load was higher in children with 4 to 6 months of age (63.0 cop/cell, p=0.007). Regarding patients' clinical conditions, we found a lower viral load in patients with

fever (26.15 cop/cell) than in those without (111.29 cop/cell; p<0.001), and a higher viral load (70.24 cop/cell) in patients without the need for oxygen therapy (22.69 cop/cell; p=0.02). Deceased patients had a lower viral load (2.80 cop/cell; p=0.02) in comparison to the others (37.96 cop/cell). Although lacking statistical support (p=0.089), a noteworthy observation is the tendency towards a lower viral load in patients with elevated CSS. The viral load analysis was performed regardless of the time between symptoms onset and date of collection, which, in theory, could cause an analytical bias due to the natural decrease in viral load over the course of the disease. However, a segmented analysis (0-3; 4-7 and >7 days between symptom onset and sample collection) revealed very similar results. Furthermore, of the 156 samples used to measure viral titers, only 26 (16%) were collected 7 days after symptoms onset. Therefore, we opted to maintain full sampling for viral load analysis.

The Cox regression model showed that the viral load did not have a statistically significant effect on ICU length of stay (p=0.29; 95% CI: 0.99-1.00). Schoenfeld Residuals (**S5 Fig and S6 Table**) showed that the proportional hazards assumption was met (p=0.95).

Table 4. Comparison of viral load values between gender, age, outcome, and clinical condition. Statistically significant p-values are highlighted in bold.

Demographic data				
Parameter		**N**	**Median (IQR[1])**	***p-value***
Gender	Male	78	51.40 (8.13-265.31)	0.08
	Female	78	24.63 (4.46-88.29)	
Age (months)	0-3	86	51.40 (6.12-152.90)	**0.007**
	4-6	22	63.09 (32.12-211.67)	
	7-12	21	39.29 (2.32-236.91)	
	>12	26	7.77 (1.72-36.92)	
Outcome	Recovery	130	37.96 (6.72-122.71)	**0.02**
	Death	7	2.80 (0.04-21.49)	
Clinical data				
Fever	Yes	121	26.15 (4.33-104.46)	**<0.001**
	No	27	111.29 (51.80-408.21)	
Cough	Yes	144	41.53 (4.86-148.15)	0.59
	No	7	11.52 (7.98-106.29)	

Dyspnea	Yes	106	37.96 (3.91-154.88)	0.69
	No	40	42.05 (8.58-120.16)	
O_2 saturation ≤ 95%	Yes	71	26.41 (3.95-150.65)	0.40
	No	51	50.16 (8.36-196.81)	
Respiratory distress	Yes	115	39.29 (4.78-150.13)	0.27
	No	18	75.69 (12.66-214.26)	
Days of hospitalization	1-4	20	79.36 (11.10-245.08)	0.20
	5-8	49	39.45 (11.89-176.21)	
	>8	54	24.42 (4.08-78.04)	
Ventilatory support	*No*	48	70.24 (11.41-342.96)	**0.02**
	Yes (total)		22.69	
	Yes - noninvasive	65	26.41 (6.26-105.11)	0.35
	Yes - invasive	40	17.31 (3.95-68.70)	
Intensive Care	Yes	82	30.01 (4.41-113.44)	0.73
	No	67	39.29 (6.90-154.61)	
Days of Intensive Care	1-4	20	34.74 (3.60-226.28)	0.547
	5-8	16	16.27 (2.09-106.22)	
	>8	24	36.24 (9.10-106.65)	
Days of symptom until collect	0-3	51	36.63 (5.99-220.48)	0.19
	4-6	67	39.98 (7.65-135.24)	
	7-9	24	19.98 (0.53-77.39)	
	>9	12	10.45 (3.92-50.98)	
Subtype	RSV-A	64	57.41	**0.03**
	RSV-B	76	27.35	

[1] IQR: interquartile range.

Viral seasonality and climatic analysis

In 2016 and 2017, the RSV season started in the 12th EW (March, early fall season), peaked between the 16th–20th EW, and ended in the winter season, between the 31st–32nd EW (**Fig 1**; **S7 Table**). In 2018, the beginning of RSV seasonality was observed earlier, with the first cases occurring in the 3rd EW, (January, in the middle of summer). The peak took place in the 14th EW and the end occurred in the 27th EW. Thus, the RSV seasonal period in 2016, 2017, and 2018 lasted 20, 19, and 24 weeks, respectively.

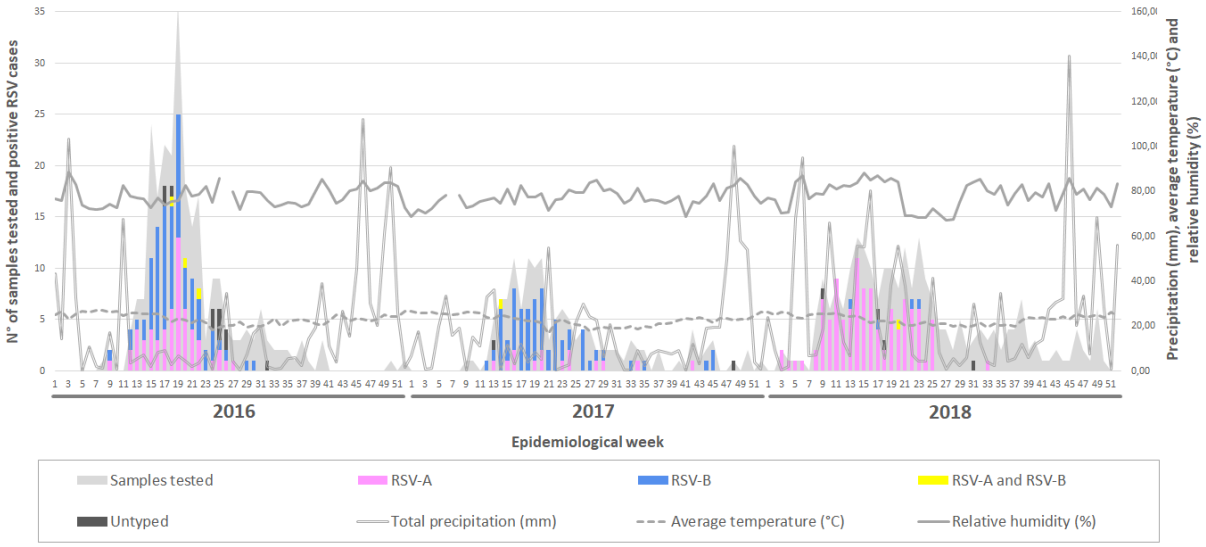

Fig 1. Circulation of RSV-A and RSV-B between 2016 and 2018 in Espirito Santo State. The X-axis shows the epidemiological weeks (EW) for each year. The primary Y-axis displays the number of positive cases for each of the subtypes and the secondary Y-axis shows the values of the climatic variables. The gray zone indicates the total number of samples tested in each EW.

Precipitation rate and relative humidity percentage have not been shown to influence the distribution of RSV cases by Spearman's correlation test (p=0.55 and 0.11, respectively). The mean temperature, however, showed a minor and inverse correlation with RSV infections (-0.16; p=0.05).

Although RSV-A and RSV-B co-circulated each year, it is noteworthy that the subtype distribution changed over the years. In 2016, RSV-B predominated (n=80; 58%) over RSV-A (n=58; 42%). In 2017 this difference increased, and RSV-B was responsible for 82% of the cases (n=63). Finally, in 2018, there was a shift in this pattern and almost all RSV cases were caused by RSV-A (n=108; 96%).

Phylogeny of RSV and genetic analysis

The phylogenetic reconstructions revealed that 36 RSV-A were classified as GA2.ON1 genotype and 21 RSV-B were classified as BA genotypes, based on the 2nd HVR (**S2 and S3**

Figs). Some local genetic groups of both genotypes and a slightly higher diversity among the RSV-A strains (p-distance=1.8%) were observed in comparison to RSV-B (p-distance=1.6%) (**Figs 2 and 3**).

RSV-A ES Brazilian strains, from 2016 to 2018, are clustered with strains that circulated in North America, South America, Asia, Africa, and Oceania, from 2011 to 2018. A Brazilian main local cluster BR.1 (L142S, L274P, Y304H, and T320A) circulated in ES state, from 2016 to 2018. Additionally, two new subclusters, BR.1.1 (E106G,) and BR.1.2 (N103T, S144I, E224V, S270P, and/or P298L) were detected co-circulating in the ES state in 2018. Amino acid substitutions, compared with the RSV-A GA2.ON1 reference strain (JN257693), can be observed in the **S8 Table**. The average CSS inside the BR.1 cluster was 2.84, while the average in the rest of the BR strains was 3.78, showing that the BR.1 cluster may be more associated with lower severity disease than the other strains. The viral load seemed to be higher on BR.1 strains when compared to other Brazilian strains.

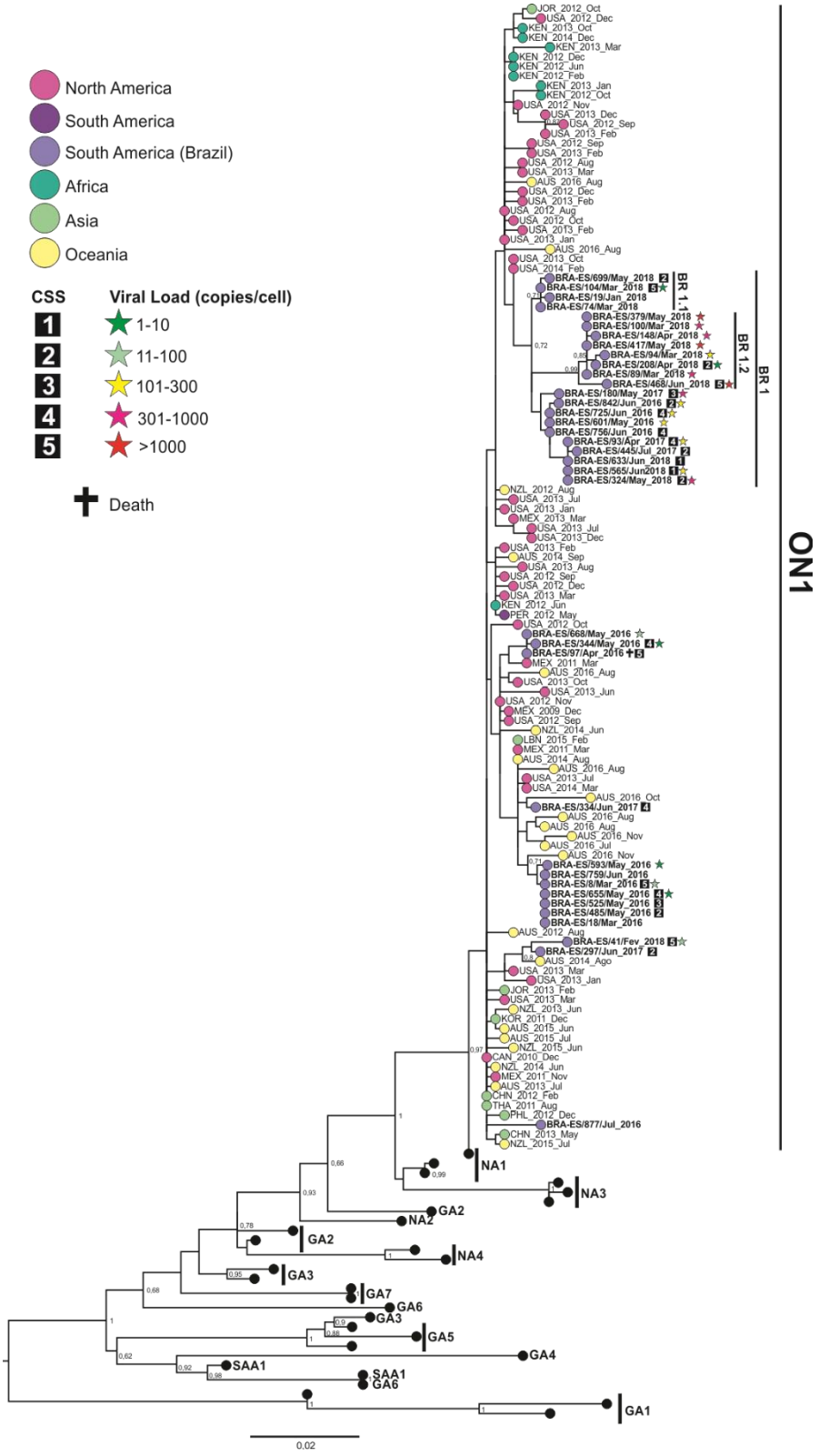

Fig 2. RSV-A phylogenetic tree. The tree was built using the maximum likelihood method on MEGA 6.0 software from a MUSCLE alignment of G gene sequences of 711 bp. Previously published sequences from known genotypes were retrieved from the NCBI database. Numbers from 1 to 5 within the squares indicate the patients' CSS. The cross indicates patients who died due to RSV infection. The stars indicate the viral load, categorized by color (in copies per cell).

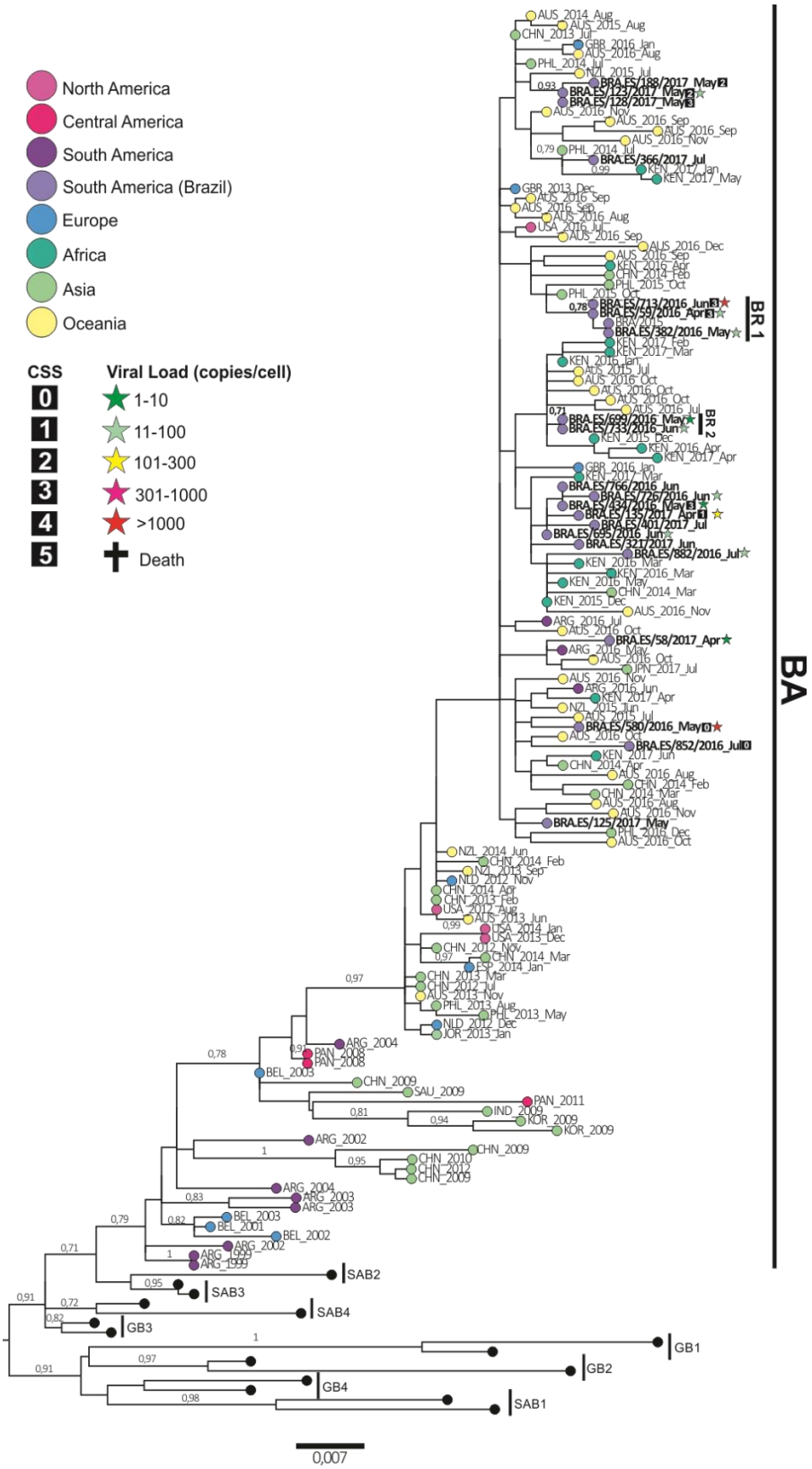

Fig 3. RSV-B phylogenetic tree. The tree was built using the maximum likelihood method on MEGA 6.0 software from a MUSCLE alignment of G gene sequences of 726 bp. Previously published sequences from known genotypes were retrieved from the NCBI database. Numbers from 1 to 5 within the squares indicate the patients' CSS. The cross indicates patients who died due to RSV infection. The stars indicate the viral load, categorized by color (in copies per cell).

RSV-B gene G phylogenetic reconstruction (**Fig 3**) revealed that Brazilian strains from 2016 to 2018 belonged to a cluster containing global strains circulating since 1999. ES Brazilian strains were distributed through this main cluster and presented punctual amino acid substitutions, some of them with a potential loss of O-glycosylation, such as T229N and/or S287F (strains from 2017). Inside the main cluster, some local subclusters were observed, such as the BR.1 (S101G loss glycosylation site, P217L, and T248A loss glycosylation site) and BR.2 (G136S and S269P), in samples from 2016, revealing a large diversity among RSV-B viruses circulating in the ES State during that year. Additionally, two strains from 2017 presented an insertion of tree nucleotides at codon 228. All these amino acid substitutions, compared with the RSV-B BA reference strain (AY333364), are described in the **S9 Table**. CSS and viral load data were unavailable for most of the RSV-B sequences, therefore, we could not compare those data with the genetic strains observed.

Discussion

In this paper, we investigated RSV features using the Brazilian Influenza Surveillance Program and addressed some RSV issues listed in the WHO global RSV surveillance pilot objectives [16], such as the RSV burden in hospitalized children and mapping of local seasonality. Additionally, we described the molecular characteristics of gene G which revealed RSV-A and RSV-B local clusters co-circulating in Brazil.

RSV is prevalent in Brazilian children with SARI

RSV prevalence in different Brazilian regions is highly diverse, ranging from 7.7% to 77.6% [24–26]. In the ES state, from 2016 to 2018, the prevalence in hospitalized children up to 3 years of age was 56%. These differences are probably related to the use of diverse methods of

RSV detection (*e.g.* RT-PCR or immunofluorescence) or patient inclusion criteria (*e.g.* age, symptoms, period of the year). During the 1997-98 season, Checon *et al.* found a prevalence of 28% in the capital of ES State [26]. This lower prevalence in comparison to our study can be attributed to the less sensitive method used by the authors (immunofluorescence) and a broader target population age (children ≤ 5 years old).

In our study, the median age of four months in hospitalized children with RSV confirms the higher prevalence in children younger than one year of age [2], which justifies why RSV vaccine candidates are aiming to protect, primarily, infants and young children [7]. Although the median hospitalization length of stay found here is similar to some other studies [27,28], notably, most of them report a shorter duration [1,4,25]. One hypothesis that could explain this finding is the fact that all children included in our study were diagnosed with SARI, which makes our study group a cohort with severe RSV infection. Another hypothesis is linked to the possibility that most of the children in the study had an infection in the lower respiratory tract. Aerosol transmission increases the chances of inhaling viral particles in the lower airways, while larger droplets are retained in the upper airways [29]. Naturally, aerosol infections tend to trigger a more severe course of infection [30].

The subtype but not the viral load appears to be associated with disease severity

RSV infection can cause a range of clinical outcomes [2], but factors attributed to a worse outcome remain unclear [3,4]. Several studies have shown that the male gender is a risk factor for RSV infection [2], while others have not observed such a connection [31]. Although not statistically significant, we observed that male children were slightly more affected than female,

which could support the hypothesis that male children are at higher risk. Nevertheless, the CSS median was three for both genders.

Although some authors have found no correlation between subtypes and disease severity [32,33], many others indicate RSV-A as the most virulent subtype [9,10,12,34,35]. We have found that children infected with RSV-A revealed a higher clinical score index (CSS median=4) – therefore, a more severe disease – when compared to those infected with RSV-B (CSS median=3). Children infected by RSV-A required O_2 therapy more often than those infected by RSV-B and, of all children who needed O_2 therapy, those affected by subgroup A needed mechanical ventilation more frequently. Although these data did not have statistical support, other studies found the same connection [9,10]. Our data also show that children infected by subgroup A required ICU more often (p=0.03) and remained hospitalized and in ICU a day longer, on average, when compared to those infected by RSV-B, which is in agreement with previous studies [35,36]. Notwithstanding, we highlight that only one genotype was found for each subtype (ON1 and BA), thus, those differences in severity could be a consequence of differences in the genotype's virulence, rather than in the subtype's.

The correlation between disease severity and viral load remains controversial. While several authors have shown that the severity of the infection follows the viral load [37,4,5,38], others have not [7,12,33]. Some studies found an association between viral load and symptom frequency, but not severity itself [39,40]. Viral load measurement methods are widely variable between studies: some authors use plaque assay [4] or semi-quantitative analyses, such as ct [5,7,32], others use quantitative methods [38–41]. Moreover, most studies that use quantitative methods do not normalize the measurements. Respiratory samples are naturally heterogeneous and the collection technique can influence viral genome concentration [38].

In this study, we used a standardized method for measuring the viral load. Interestingly, we found a lower viral load in patients with fever (p=0.00), with the need for ventilatory support

(p=0.02), and in those who died (p=0.02). Our data conflict with previous studies that demonstrated a positive association between viral load and the presence of cough, fever [39], and the need for intubation [37]. However, two recent studies reported a higher viral load in less severe RSV disease [42,43]. Piedra *et al.* observed a positive correlation between viral load and mucosal concentration of proinflammatory cytokines that may suggest that high RSV loads can protect from disease progression due to the promotion of an early robust innate immune response [42,43]. Conflicting results between studies could be attributed to the different methods used to calculate viral load, various study designs, and indicators of disease severity.

The seasonal period of RSV may fluctuate and its circulation is slightly associated with temperature

In temperate countries, RSV peak activity occurs in the winter and several studies have shown the connection between cold temperatures and viral circulation [44]. In contrast, in tropical countries, there is a wide range of variability in the timing and duration of epidemics and the correlation between climatic factors and viral activity is controversial [21,45]. Although in the Southern Hemisphere the RSV wave usually starts between March and June and decreases between August and October [21], in Brazil, a continental country with five geographic regions, a wide variation in the seasonality is seen, such as those observed in the northeastern [46] and southern [47] regions.

Here, we showed that RSV's activity was very similar between the 2016 and 2017 seasons, with the circulation onset occurring in March (EW 12) and ending in July/August (EW 31-32), during the winter season. These data are in accord with the Brazilian Society of Pediatrics, which recommends the administration of Palivizumab from February to July [48]. Nonetheless, in 2018, we observed an early occurrence of the seasonality onset by nine weeks, with the

beginning of circulation occurring in January (summer season) and the end taking place in the Fall instead of Winter.

In the southeastern region, it was observed that the RSV peak usually happens in early April [49]. Our data shows that, in 2016, the RSV peak occurred in May, suggesting subtle differences even inside the same geographical region. In 2018, there was an extension of RSV's seasonality duration by 4.5 weeks when compared to the average in 2016-2017. Those observations are especially worrisome since major variations could make a preventive measure harder to implement. Understanding local epidemics is important in managing the time of prophylaxis, supporting vaccine development, and following morbidity and mortality caused by RSV infection [44]. Thus, establishing RSV surveillance in real-time may allow for the identification of patterns and possible variations in prophylaxis time. RSV seasonality usually lasts five to six months [21]. In our study, the longest seasonal period occurred in 2018 (6 months), followed by 2016 (5 months) and 2017 (4.75 months). Interestingly, the prevalence of RSV-A was high in 2018 (96%), medium in 2016 (41%), and low in 2017 (18%). These data reinforce the theory that RSV-A may lengthen the seasonality [50].

Climatic factors, such as humidity, rainfall, and temperature have been assumed to impact RSV seasonality [44,51]. However, this association remains controversial. An inverted correlation between RSV circulation, temperature, and humidity was observed in a Brazilian study, carried out in the state of São Paulo [52]. In this study, a minor correlation was found between temperature decrease and case number increase. However, no correlation was found concerning humidity or precipitation.

ON1 and BA were the only genotypes detected

All RSV-A isolates were ON1 genotype and all RSV-B were BA, which confirms the fast-global dissemination of RSV with nucleotide duplication. These findings are consistent with recently published reports performed in other countries, such as the Philippines [53], Kenya [54], Italy [55], USA, and Puerto Rico [56].

Overall p-distance during the study period in RSV-A was 1.8%. A recent study observed an overall p-distance of 1.4% within ON1 [13]. A noteworthy observation is the fact that in 2017 we found the lowest prevalence of RSV-A in ES (18%), and yet, still, the highest genetic diversity. Phylogeny showed that 2017 strains were distributed in almost all genetic clusters, which showed high diversity that year. RSV-A phylogenetic analysis revealed ongoing genetic changes, with BR.1 grouping the most recent strains, suggesting that BR.1 strains may be under positive selective pressure. Changes in the circulation of RSV strains have been considered a mechanism for evading immune response generated by previous strains, which possibly allows for re-infections to occur [57].

As demonstrated, in 2018 RSV-B was responsible for only 4% of cases. Therefore, the phylogenetic analysis did not include any RSV-B samples from that year. Older strains, from 2009 to 2014, are positioned at the base of the BA cluster, however, sample strains collected between 2015 and 2018 did not form genetic groups related to the year of collection. This observation may suggest an absence of positive pressure.

Although we found clusters composed exclusively of ES samples, it is necessary to expand the sequencing of RSV samples globally to verify if there is, in fact, the formation of local genetic groups or if the observation is caused by a sample bias.

Previous studies showed that a large part of the genetic variability between RSV strains comes from changes in the O-glycosylation profile and that this may be associated with an evolutionary mechanism of immune response evasion [58]. Here, we investigated and listed strain amino acid substitutions and also those shared within and between clusters. However, we

did not carry out an in-depth analysis to understand the role of these mutations, as our objective was purely observational. Among the mutations found, one of the most interesting was the insertion of three nucleotides at codon 228 in RSV-B. Further studies are essential to understand virus evolution and pathogenicity mutation consequences.

Limitations of this study include the fact that the majority of patients had an acute infection, thus, the prevalence found refers only to SARI, and the absence of a mild infection group prevents further analysis of severity influencing factors. Furthermore, clinical data were taken from notification forms, which often contain inconsistencies and missing data. Despite those caveats, we believe the data provide valuable epidemiological, genetic, and clinical information on RSV.

Conclusion

In this study, we observed a high prevalence of RSV in children under three years of age even when using the Brazilian Influenza Surveillance Program. This result is important because it shows that the establishment of global RSV surveillance within the Influenza surveillance system allows for the detection of a large number of cases. Our data suggest that RSV-A is, in fact, more virulent than RSV-B. Notably, no correlation between viral load and disease severity was observed. The observation of a marked early onset of the seasonal period is worrisome since this can make it difficult to administer prophylactic measures at the right time, however, it is necessary to expand the historical series of seasonality in the state of Espirito Santo. The average temperature was the only climatic factor to show interference with the viral circulation. Our data show the annual co-circulation of RSV-A and RSV-B, however, with considerable fluctuations in the prevalence of subtypes. ON1 and BA were the only genotypes found in the studied period, which corroborates a series of recent studies. The establishment of a global and

standardized real-time RSV surveillance may allow for the collection of data that will help to understand the complex mechanisms of viral evolution and will facilitate the development of future vaccines and antiviral drugs.

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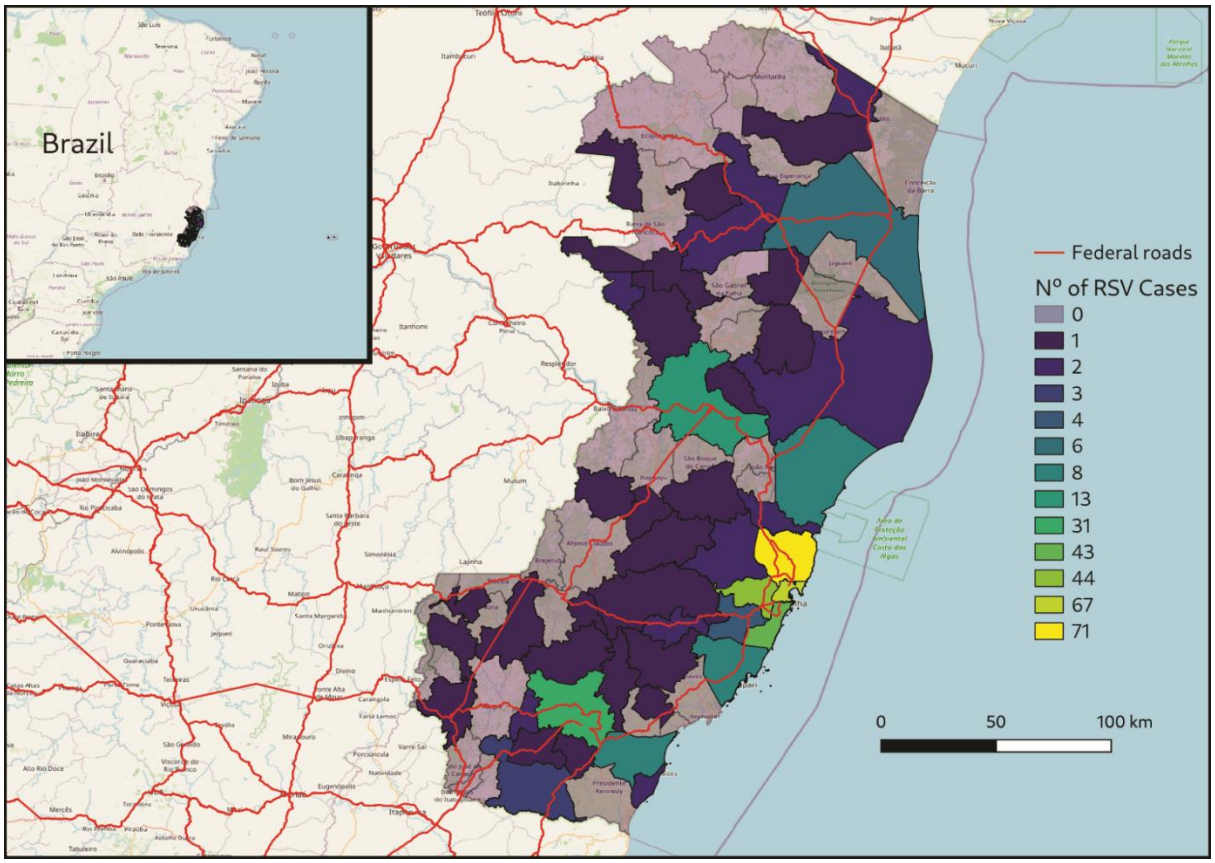
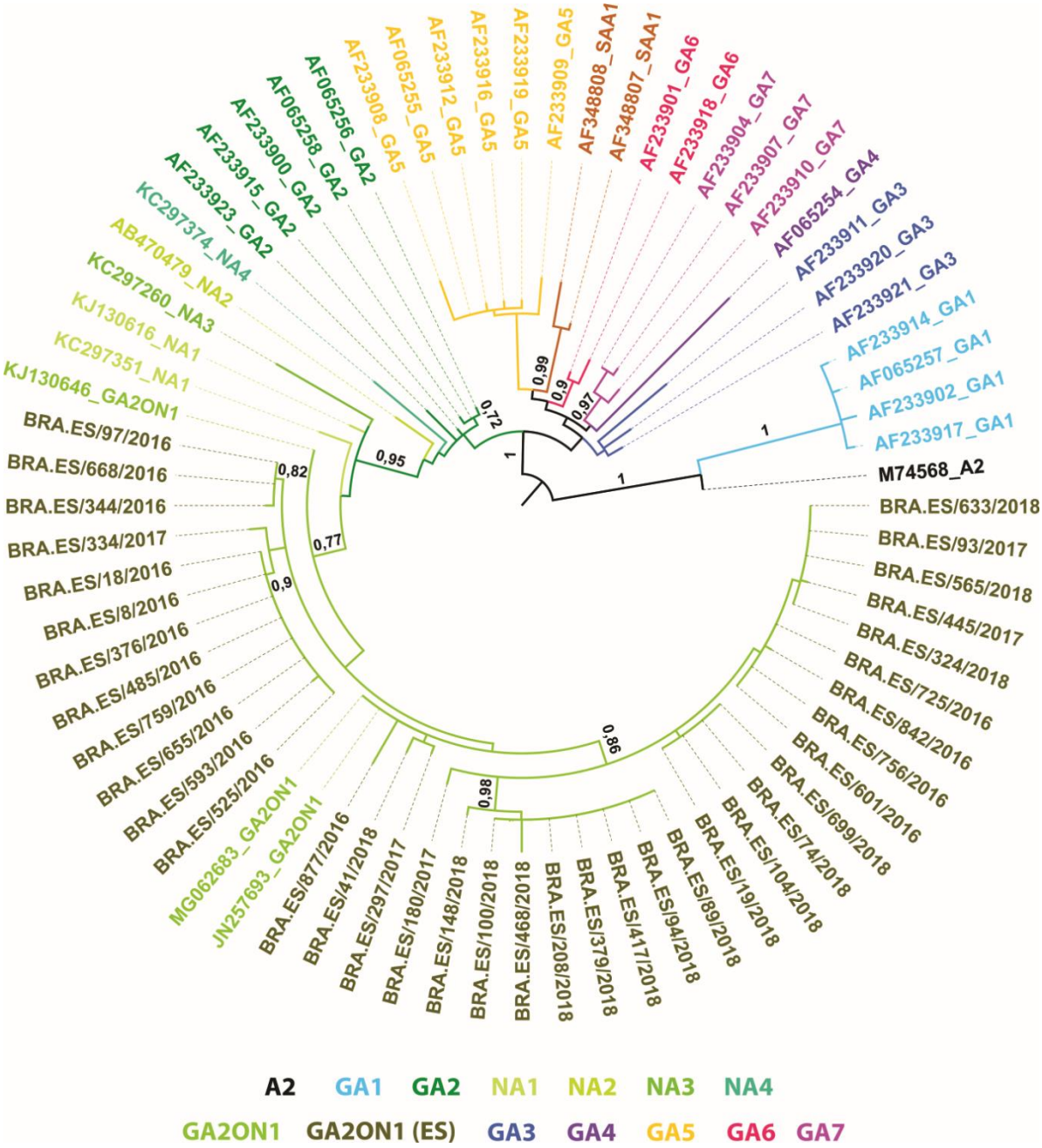
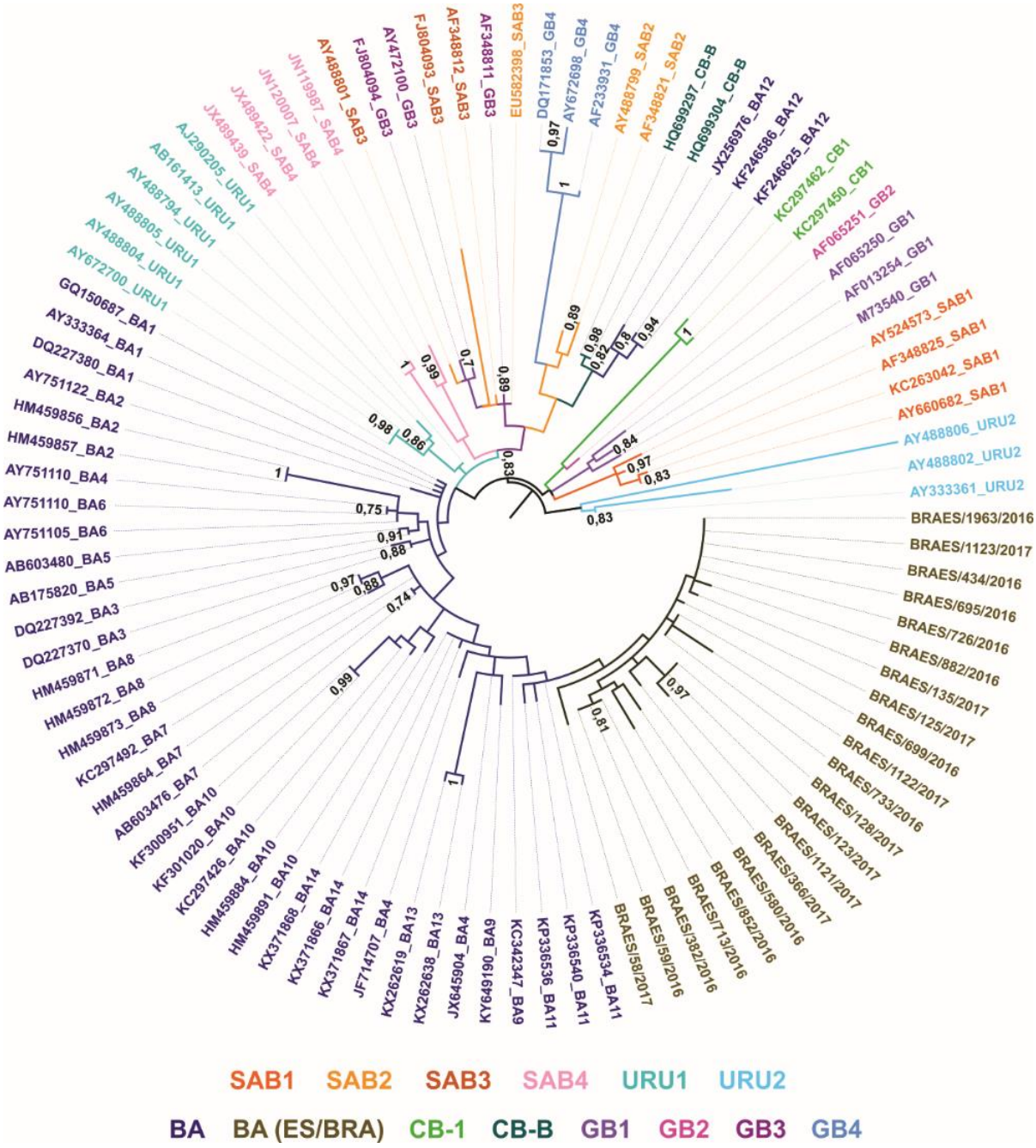
Supporting information



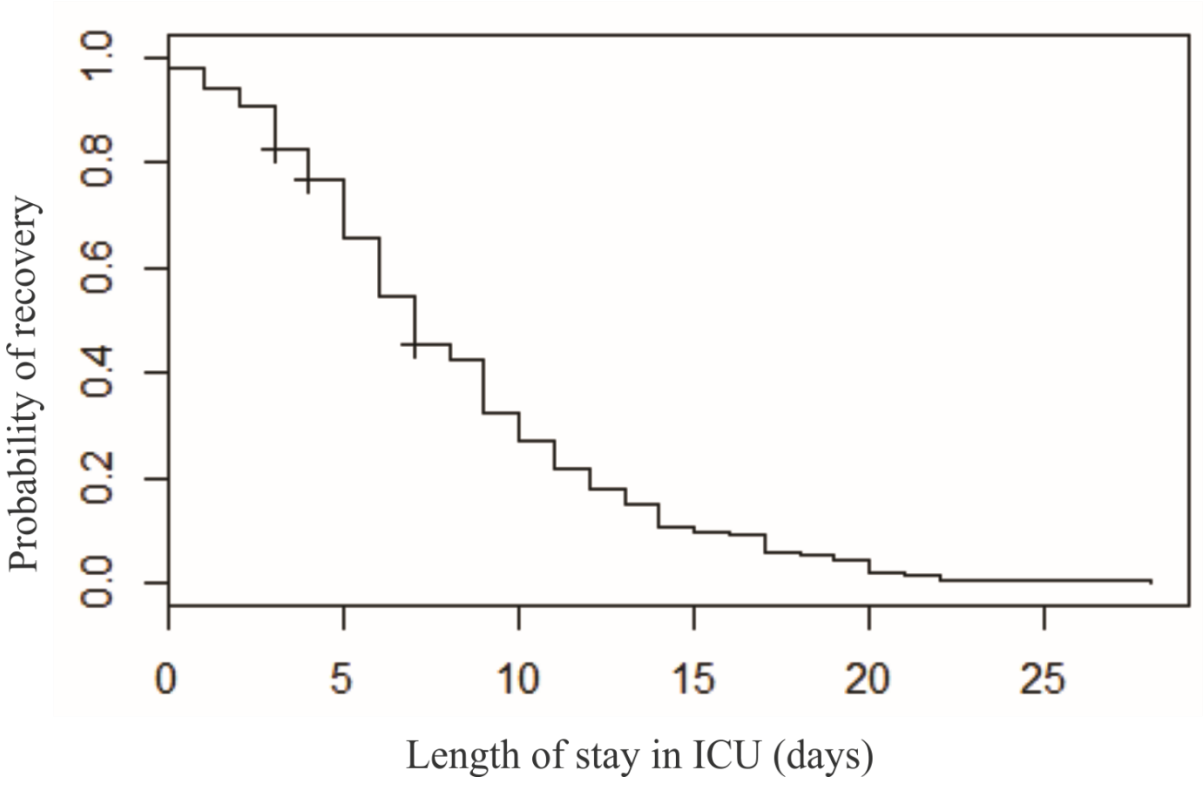
S1 Fig. Map of the Espirito Santo State (Brazil) and its federal highways. The state is divided into 78 municipalities, of which 60 were represented by children with SARI and 46 with children with confirmed RSV infection. The colors of the municipalities represent the number of positive RSV cases.



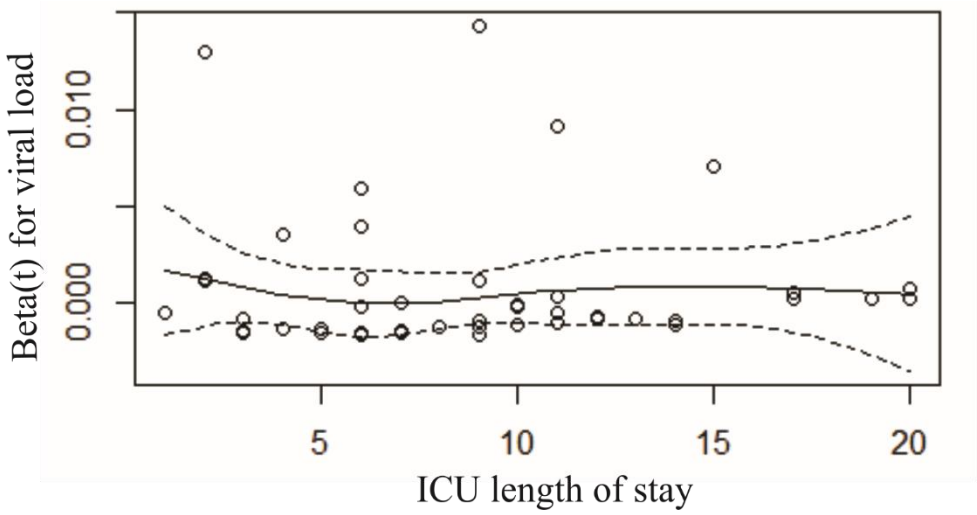
S2 Fig. RSV-A phylogenetic tree based on 336 bp of the HVR-2 of G gene. The tree was built using the maximum likelihood method on MEGA 6.0 software from a MUSCLE alignment, with some manual editions. Reference sequences from each described genotype were downloaded from the NCBI GenBank and used in the phylogenetic reconstruction. The genotypes were classified by colors and all ES strains were grouped within the ON1 genotype.



S3 Fig. RSV-A phylogenetic tree based on 318 bp of the HVR-2 of G gene. The tree was built using the maximum likelihood method on MEGA 6.0 software from a MUSCLE alignment, with some manual editions. Reference sequences from each described genotype were downloaded from the NCBI GenBank and used in the phylogenetic reconstruction. The genotypes were classified by colors and all ES strains were grouped within the BA genotype.



S4 Fig. Survival curve in relation to ICU length of stay estimated by the Kaplan-Meier test. Given the small number of deaths, it was necessary to modify the analysis to assess the likelihood of cure.



S5 Fig. Graph of Schoenfeld residues: there was no marked trend, indicating that the premises for the application of the Cox model were met.

S1 Table. Primers, probes, and DNA fragments used in the study. "F", "R", and "P", represent the sequence of the forward and reverse primers, and the probe, respectively. A synthetic DNA fragment from RSV was included in a pMA-t vector.

Target	Sequence (3'-5')	Reference
Influenza A[1]	F: GAC CRA TCC TGT CAC CTC TGA C R: AGG GCA TTY YGG ACA AAK CGT CTA P: TGC AGT CCT CGC TCA CTG GGC ACG	CDC, 2009
Influenza B[1]	F: TCC TCA AYT CAC TCT TCG AGC G R: CGG TGC TCT TGA CCA AAT TGG P: CCA ATT CGA GCA GCT GAA ACT GCG GTG	CDC, 2009
RNase P[1]	F: AGATTTGGACCTGCGAGCG R: GAGCGGCTGTCTCCACAAGT P: TTCTGACCTGAAGGCTCTGCGCG	CDC, 2009
RSV screening[1]	F: GGC AAA TAT GGA AAC ATA CGT GAA R: TCT TTT TCT AGG ACA TTG RAY TGA ACA G P: CTG TGT ATG TGG AGC CTT CGT GAA GCT	Fry *et al*., 2010
β-globin[1]	F: GAGCCATCTATTGCTTACATTTGCTTCTGA R: CCTGCCCAGGGCCTCACCACCAACTTCATC P: CACGTTCACCTTGCCCCACAGG	Huang *et al*., 1989
RSV-A[2]	F: GCTCTTAGCAAAGTCAAGTTGAATGA R: AACATGCCACATAACTTATTGAT P: ACACTCAACAAAGATCAACTTCTGTCATCCAGC	Hu *et al*. 2003 de Paris *et al*., 2012
RSV-B[2]	F: GATGGCTCTTAGCAAAGTCAAGTTAA R: TGTCAATATTATCTCCTGTACTACGTTGAA P: TGATACATTAAATAAGGATCAGCTGCTGTCATCCA	Hu *et al*. 2003
RSV sequencing[3]	F: AGAGACCCAAAAACACYAGCCAA R: ACAGGGAACGAAGTTGAACACTTCA	Zlateva, *et al*., 2005
RSV fragment sequence	GAGCCATCTATTGCTTACATTTGCTTCTGACACAACTGTGTTCACTAGCA ACCTCAAACAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGCC GTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAG GCCCTGGGCAGG	-
β-globin synthetic fragment	GAGCCATCTATTGCTTACATTTGCTTCTGACACAACTGTGTTCACTAGCA ACCTCAAACAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGCC GTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAG GCCCTGGGCAGG	-

PCR conditions:

[1] 50°C/30 minutes, 95°C/2 minutes, 45 cycles of 95°C/15 seconds, and 55°C/30 seconds.

[2] 45°C/25 minutes, 95°C/2 minutes, 45 cycles of 95°C/15 seconds, and 55°C/30 seconds.

[3] 55°C/30 minutes, 40 cycles of 94°C/30 seconds, 60°C/1 minute, 72°C/1 minute, and a final extension at 72°C/10 minutes.

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S2 Table. List of the sequences used to build the phylogeny based on HVR-2 of gene G for both subtypes RSA-A and RSV-B.

Subtype	Accession n°	Genotype	Origin
RSV-A	M74568	A2	ND
	AF233902	GA1	USA
	AF233914	GA1	USA
	AF233917	GA1	USA
	AF233923	GA2	USA
	AF233900	GA2	USA
	AF233915	GA2	USA
	KJ130646	ON1	CHN
	JN257693	ON1	CAN
	MG062683	ON1	MEX
	AF233911	GA3	CAN
	AF233920	GA3	USA
	AF233921	GA3	USA
	AF065254	GA4	USA
	AF233912	GA5	USA
	AF233916	GA5	USA
	AF233919	GA5	USA
	AF233908	GA5	CAN
	AF233909	GA5	USA
	AF233901	GA6	USA
	AF233918	GA6	USA
	AF233904	GA7	CAN
	AF233907	GA7	CAN
	AF233910	GA7	USA
	KC297351	NA1	CHN
	KJ130616	NA1	CHN
	AB470479	NA2	JPN
	KC297260	NA3	CHN
	KC297374	NA4	CHN
	AF348808	SAA1	ZAF
	AF348807	SAA1	ZAF
	AF065255	GA5	USA
	AF065256	GA2	USA
	AF065257	GA1	USA
	AF065258	GA2	USA

RSV-B	M17213	GB1	ND
	AF065250	GB1	USA
	AF013254	GB1	ND
	M73540	GB1	USA
	AF065251	GB2	USA
	AF348811	GB4	ZAF
	DQ171853	GB4	NZL
	AY672698	GB4	ARG
	AF233931	GB4	USA
	AF348825	SAB1	ZAF
	AY660682	SAB1	KEN
	KC263042	SAB1	KEN
	AF348821	SAB2	ZAF
	AY488799	SAB2	URU
	JX489439	SAB4	BRA
	JX489422	SAB4	BRA
	JN120007	SAB4	KHM
	JN119987	SAB4	KHM
	AY488804	URU1	URU
	AY488805	URU1	URU
	AY488794	URU1	URU
	AB161413	URU1	JPN
	AJ290205	URU1	GBR
	AY333361	URU2	ARG
	AY488802	URU2	URU
	AY488806	URU2	URU
	KC297462	CB1	CHN
	KC297450	CB1	CHN
	AY333364	BA1	ARG
	DQ227380	BA1	ARG
	GQ150687	BA1	ESP
	HM459857	BA2	JPN
	HM459856	BA2	JPN
	AY751122	BA2	BEL
	DQ227370	BA2	ARG
	DQ227392	BA3	ARG
	AB175820	BA5	JPN
	AB603480	BA5	JPN
	AY751105	BA6	BEL
	AY751110	BA6	BEL
	HM459864	BA7	JPN
	KC297492	BA7	CHN
	AB603476	BA7	JPN
	HM459873	BA8	JPN
	HM459872	BA8	JPN
	HM459871	BA8	JPN
	KC342347	BA9	THA
	KY649190	BA9	IND
	HM459891	BA10	JPN
	HM459884	BA10	JPN
	KC297426	BA10	CHN
	KF301020	BA10	PAN

ND: not determined.

S3 Table. List of the sequences used to build the phylogeny based on gene G for both subtypes RSV-A and RSV-B. The collection date of some sequences was unavailable.

Subtype	Accession n°	Genotype	Collection date	Origin
RSV-A	KP792358	NA1	2007	ESP
	Z33494	GA5	1990	URU
	Z33455	GA7	1992	ESP
	Z33427	GA1	1990	URU
	Z33426	GA3	1990	URU
	Z33422	GA2	1989	URU
	Z33417	GA7	1992	ESP
	Z33416	GA3	1990	ESP
	Z33414	GA3	1993	ESP
	M11486	GA1	1985	USA
	KP258696	SAA	1986	USA
	KF300972	NA1	2010	PAN
	KP792359	NA1	2008	ESP
	KC297381	NA4	2007	CHN
	KC297324	NA4	2011	CHN
	KC297277	NA3	2011	CHN
	KC297292	NA3	2011	CHN
	AY114150	GA5	2003	SGP
	DQ985132	SAA	1997	BEL
	JQ901453	NA2	2002	NLD
	AF065254	GA4	1998	ND[1]
	AF065407	GA1	1998	USA
	AF065255	GA5	1998	ND[1]
	AY114149	GA2	2002	SGP
	DQ985132	GA6	1986	USA
	AF065256	GA2	1998	USA
	KC297260	NA3	2011	CHN
	DQ985131	GA6	1988	BEL
	KM042392	ON1	2013 Feb	USA
	MH760648	ON1	2015 Jul	AUS
	MH760647	ON1	2015 Jun	AUS
	MH760646	ON1	2014 Ago	AUS
	MH760643	ON1	2014 Sep	AUS
	MH760640	ON1	2014 Aug	AUS
	MH760632	ON1	2016 Aug	AUS
	MH760629	ON1	2016 Aug	AUS

	MH760628	ON1	2016 Aug	AUS
	MH760625	ON1	2016 Aug	AUS
	MH760624	ON1	2016 Aug	AUS
	MH760623	ON1	2016 Aug	AUS
	MH760617	ON1	2016 Oct	AUS
	MH760611	ON1	2016 Nov	AUS
	MH760610	ON1	2016 Nov	AUS
	MH760607	ON1	2013 Jul	AUS
	MH760602	ON1	2012 Aug	AUS
	MH760590	ON1	2016 Jul	AUS
	MH182025	ON1	2014 Dec	KEN
	MH181993	ON1	2013 Oct	KEN
	MH181990	ON1	2013 Mar	KEN
	MH181984	ON1	2013 Jan	KEN
	MH181975	ON1	2012 Dec	KEN
	MH181935	ON1	2012 Oct	KEN
	MH181924	ON1	2012 Jun	KEN
	MH181923	ON1	2012 Jun	KEN
	MH181908	ON1	2012	KEN
	MG793382	ON1	2015	LBN
	MG062688	ON1	2011	MEX
	MG062687	ON1	2013	MEX
	MG062685	ON1	2011	MEX
	MG062683	ON1	2009	MEX
	MF614947	ON1	2013	CHN
	KY865205	ON1	2011	MEX
	KY654512	ON1	2012	PHL
	KX894805	ON1	2013	USA
	KX894803	ON1	2013	USA
	KX765970	ON1	2014	NZL
	KX765939	ON1	2013	NZL
	KX765932	ON1	2015	NZL
	KX765926	ON1	2012	NZL
	KX765925	ON1	2015	NZL
	KX765915	ON1	2014	NZL
	KX655644	ON1	2012	JOR
	KX655626	ON1	2013	JOR
	KU950677	ON1	2012	USA
	KU950673	ON1	2012	USA
	KU950628	ON1	2013	USA
	KU950627	ON1	2013	USA

	KU950615	ON1	2012	USA
	KU950610	ON1	2013	USA
	KU950592	ON1	2012	USA
	KU950590	ON1	2013	USA
	KU950583	ON1	2013	USA
	KU950567	ON1	2012	USA
	KU950550	ON1	2012	USA
	KU950544	ON1	2013	USA
	KU950531	ON1	2013	USA
	KU950523	ON1	2014	USA
	KU950521	ON1	2012	USA
	KU950520	ON1	2013	USA
	KU950513	ON1	2012	USA
	KU950502	ON1	2012	USA
	KU950493	ON1	2012	USA
	KU950492	ON1	2012	USA
	KU950486	ON1	2012	USA
	KU950472	ON1	2013	USA
	KU839637	ON1	2014	USA
	KM042390	ON1	2013	USA
	KJ672475	ON1	2013 Jan	USA
	KJ672471	ON1	2013_Mar	USA
	KJ672469	ON1	2013 Feb	USA
	KJ672465	ON1	2012 Dec	USA
	KJ672441	ON1	2013 Mar	USA
	KJ672433	ON1	2013 Feb	USA
	KJ672432	ON1	2013 Mar	USA
	KJ672429	ON1	2013 Feb	USA
	KJ672428	ON1	2013 Jan	USA
	KJ627264	ON1	2012 May	PER
	KC559440	ON1	2012 Feb	CHN
	KC342413	ON1	2011 Aug	THA
	JX627336	ON1	2011 Dec	KOR
	JN257693	ON1	2010 Dec	CAN
RSV-B	AF065250	GB1	1990-91	USA
	M73542	GB1	1985	USA
	AF065251	GB2	1992-93	USA
	KC297470	GB2	2011	CHN
	AF065252	GB3	1992-93	USA
	AF065253	GB3	1992-93	USA
	AY333361	GB4	1990	MON

	M73543	GB4	1989	ND[1]
	AY660682	SAB1	2003	KEN
	JF704213	SAB1	1998	ZAF
	AY327815	SAB2	2000	QAT
	DQ171866	SAB3	2001	NZL
	JF704216	SAB3	1998	ZAF
	DQ270231	SAB4	ND[1]	CHN
	KC297430	SAB4	2009	CHN
	AY333362	BA	1999	ARG
	AY333364	BA	1995-2001	ARG
	AY751108	BA	2001	BEL
	AY751110	BA	2003	BEL
	AY751117	BA	2002	BEL
	DQ227373	BA	2002	ARG
	DQ227381	BA	2002	ARG
	DQ227396	BA	2004	ARG
	DQ227389	BA	2003	ARG
	DQ227393	BA	2003	ARG
	KC297435	BA	2009	CHN
	KC297477	BA	2012	CHN
	KC297490	BA	2009	CHN
	KC297456	BA	2010	CHN
	DQ227395	BA	2004	ARG
	DQ985142	BA	2003	BEL
	KF300952	BA	2008	PAN
	KX371868	BA	2008	PAN
	KC297492	BA	2009	CHN
	KC297476	BA	1905	CHN
	KC710985	BA	2012	NLD
	KT781377	BA	2014	CHN
	KX765912	BA	2014	NZL
	KM586838	BA	2013	CHN
	KU950682	BA	2012	USA
	KX765959	BA	2013	NZL
	KM586835	BA	2012	CHN
	LC311394	BA	2013	PHI
	MH760724	BA	2013	AUS
	KM586840	BA	2013	CHN
	MH760666	BA	2013	AUS
	KT781376	BA	2014	CHN
	MF443156	BA	2014	ESP

	KC710996	BA	2012	NLD
	KX655648	BA	2013	JOR
	KT781363	BA	2014	CHN
	LC311396	BA	2013	PHI
	KU839625	BA	2014	USA
	KU950607	BA	2013	USA
	KY828376	BA	2015	BRA
	LC311365	BA	2015	PHI
	KX775817	BA	2015	KEN
	MH742858	BA	2016	KEN
	MH742865	BA	2016	KEN
	KT781370	BA	2014	CHN
	MH742866	BA	2016	KEN
	MH742869	BA	2017	KEN
	KM517573	BA	2013	CHN
	LC384997	BA	2014	PHI
	MH760725	BA	2014	AUS
	MH760729	BA	2015	AUS
	LC384999	BA	2014	PHI
	MH760672	BA	2016	AUS
	KY249660	BA	2013	GBR
	KY674984	BA	1905	USA
	LC311369	BA	2015	PHI
	MH760695	BA	2016	AUS
	MH760721	BA	2016	AUS
	MH760702	BA	2016	AUS
	MG839547	BA	2016	ARG
	KX765906	BA	2015	NZL
	MG773268	BA	2016	ARG
	MH760679	BA	2016	AUS
	MH742890	BA	2017	KEN
	MH742820	BA	2017	KEN
	MH760731	BA	2015	AUS
	KX775816	BA	2016	KEN
	MH742838	BA	2017	KEN
	MH760668	BA	2016	AUS
	MH760728	BA	2015	AUS
	MH760694	BA	2016	AUS
	KX775814	BA	2015	KEN
	MH742825	BA	2016	KEN
	MH742840	BA	2017	KEN

	MH742826	BA	2017	KEN
	MH760720	BA	2016	AUS
	KY249682	BA	2016	GBR
	KX765935	BA	2015	NZL
	KT781362	BA	2014	CHN
	KT781373	BA	2014	CHN
	MH760685	BA	2016	AUS
	KT781404	BA	2014	CHN
	MH760683	BA	2016	AUS
	MH760708	BA	2016	AUS
	BRA.ES/5	BA	7	BRA
	MG773266	BA	2016	ARG
	MH760687	BA	2016	AUS
	LC324678	BA	1905	JPN
	LC385006	BA	2016	PHI
	MH760671	BA	2016	AUS
	MH760722	BA	2016	AUS
	MH760673	BA	2016	AUS
	MH760680	BA	2016	AUS
	MH760714	BA	2016	AUS
	MH760675	BA	2016	AUS
	MH760710	BA	2016	AUS
	MH760723	BA	2016	AUS
	MH742920	BA	2016	KEN
	KT781360	BA	2014	CHN
	BRA.ES/5	BA	16	0/2
	KY249683	BA	2016	GBR
	MH760703	BA	2016	AUS
	MH742896	BA	2017	KEN
	MH742907	BA	2017	KEN
	MH760701	BA	2016	AUS
	MH760670	BA	2016	AUS
	MH760689	BA	2016	AUS
	JF714708	BA	2009	SAU
	KF246586	BA	2009	IND
	HQ699300	BA	2009	KOR
	HQ699304	BA	2009	KOR
	KF300951	BA	2011	PAN

S4 Table. General table that provides all epidemiological, clinical, and climatic data of the study.

S5 Table. Cox (Proportional Hazards) Regression: Given that the p-value is >0.05, it can be inferred that the viral load has no significant effect on ICU length of stay.

Average				Median			
Estimate	Standard error	Interval		Estimate	Standard error	Interval	
		LI	LS			LI	LS
8.18	0.45	7.30	9.05	7.00	0.56	5.91	8.09

S6 Table. Proportional hazards assumption test: the premises for the application of the Cox model were met.

Parameter	P	Q	p-value
Viral load	-0,009	0,003	0,95

S7 Table. Duration and climatic characteristics of RSV seasonality in the years studied.

Year	Onset			Peak			End			RSV+	Duration		Average precipitation (mm)	Average temperature (°C)	Average humidity (%)
	EW	Month	Season	EW	Month	Season	EW	Month	Season		Weeks	Months			
2016	12	March	Fall	19	May	Fall	32	August	Winter	150	20	5	7.78	22.23	77.83
2017	12	March	Fall	16 20	April May	Fall	31	July August	Winter	72	19	4.75	15.30	21.32	78.72
2018	3	January	Summer	14	April	Fall	27	July	Fall	115	24	6	29.24	23.13	78.36

S8 Table. List of amino acid changes in RSV-A. Residues in blue and red show potential losses and gains of O-glycosylation sites, respectively.

S9 Table. List of amino acid changes in RSV-B. Residues in blue and red show potential losses and gains of O-glycosylation sites, respectively.

5. CONCLUSÕES

O VSR lidera as causas de bronquiolite e pneumonia em crianças no mundo todo, contribuindo significativamente para morbidade e mortalidade em populações pediátricas. Além disso, não é infrequente estar associado a doenças respiratórias graves em idosos e pacientes com comorbidades. Neste trabalho pudemos avançar no conhecimento científico, gerando e interpretando dados inéditos no Espírito Santo. A maioria dos trabalhos científicos com VSR na região sudeste foram realizados em São Paulo e Rio de Janeiro, sendo as observações extrapoladas para toda a região. Um estudo local é importante para avaliar a viabilidade dessas extrapolações, sobretudo ao considerar que há variabilidade genética do VSR até mesmo entre territórios adjacentes. Aqui, fomos capazes de observar que o VSR é altamente prevalente em crianças de até 3 anos de idade com quadro de SRAG, o que reforça a necessidade do foco de novas vacinas nesse grupo etário. Além disso, nosso estudo mostrou pela primeira vez o perfil da circulação sazonal do VSR no território capixaba. Essa informação é especialmente importante pois pode ser incluída nas políticas de profilaxia com o Palivizumab. Preocupante foi a observação de uma mudança importante no início da sazonalidade em 2018, pois variações como essas podem prejudicar a eficácia deste anticorpo monoclonal. Por isso, acreditamos serem necessários novos estudos que contemplem um período amostral mais robusto, de forma a verificar o histórico sazonal do VSR no Espírito Santo. Nossos dados ainda confirmam a circulação exclusiva dos genótipos ON1 (VSR-A) e do grupo de genótipos BA (VSR-B). Curiosamente, pacientes com maiores cargas virais não apresentaram quadros clínicos mais graves do que aqueles com cargas virais menores, no entanto, pacientes infectados pelo VSR-A apresentaram maior gravidade clínica do que aqueles infectados por VSR-B. Nosso estudo é um dos poucos estudos com VSR a normalizar os dados de quantificação viral. Por fim, observamos a formação de *clusters* genéticos na construção filogenética, os quais devem ser investigados de maneira mais aprofundada. O projeto piloto de vigilância global do VSR no contexto da vigilância da Influenza, implementado pela OMS em 2017, deve auxiliar no entendimento dos padrões sazonais, além de permitir uma observação em tempo real da diversidade genética do vírus, facilitando a implementação de vacinas, drogas antivirais, e a adoção de políticas de saúde pública eficazes e contundentes.

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APÊNDICES

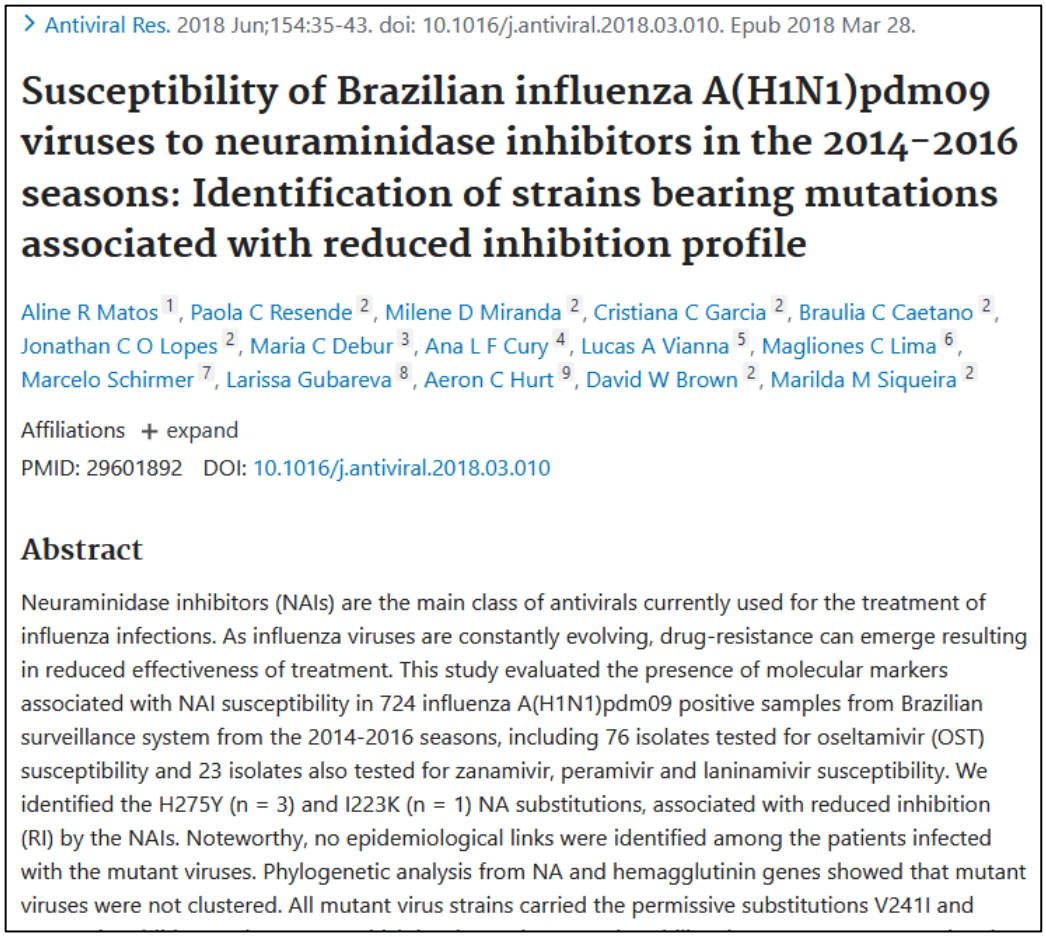
Outras publicações durante o doutorado:

1. Artigo publicado na revista **Antiviral Research**

   Fator de impacto: 4.9

   Ano: 2018

   Colaboração: diagnóstico por RT-qPCR das amostras oriundas do Espírito Santo, seleção e envio das amostras à Fiocruz para testes complementares (*e.g*. isolamento viral, testes de resistência a antivirais e sequenciamento genômico) e revisão do manuscrito.



› Antiviral Res. 2018 Jun;154:35-43. doi: 10.1016/j.antiviral.2018.03.010. Epub 2018 Mar 28.

Susceptibility of Brazilian influenza A(H1N1)pdm09 viruses to neuraminidase inhibitors in the 2014–2016 seasons: Identification of strains bearing mutations associated with reduced inhibition profile

Aline R Matos [1], Paola C Resende [2], Milene D Miranda [2], Cristiana C Garcia [2], Braulia C Caetano [2], Jonathan C O Lopes [2], Maria C Debur [3], Ana L F Cury [4], Lucas A Vianna [5], Magliones C Lima [6], Marcelo Schirmer [7], Larissa Gubareva [8], Aeron C Hurt [9], David W Brown [2], Marilda M Siqueira [2]

Affiliations + expand

PMID: 29601892 DOI: 10.1016/j.antiviral.2018.03.010

Abstract

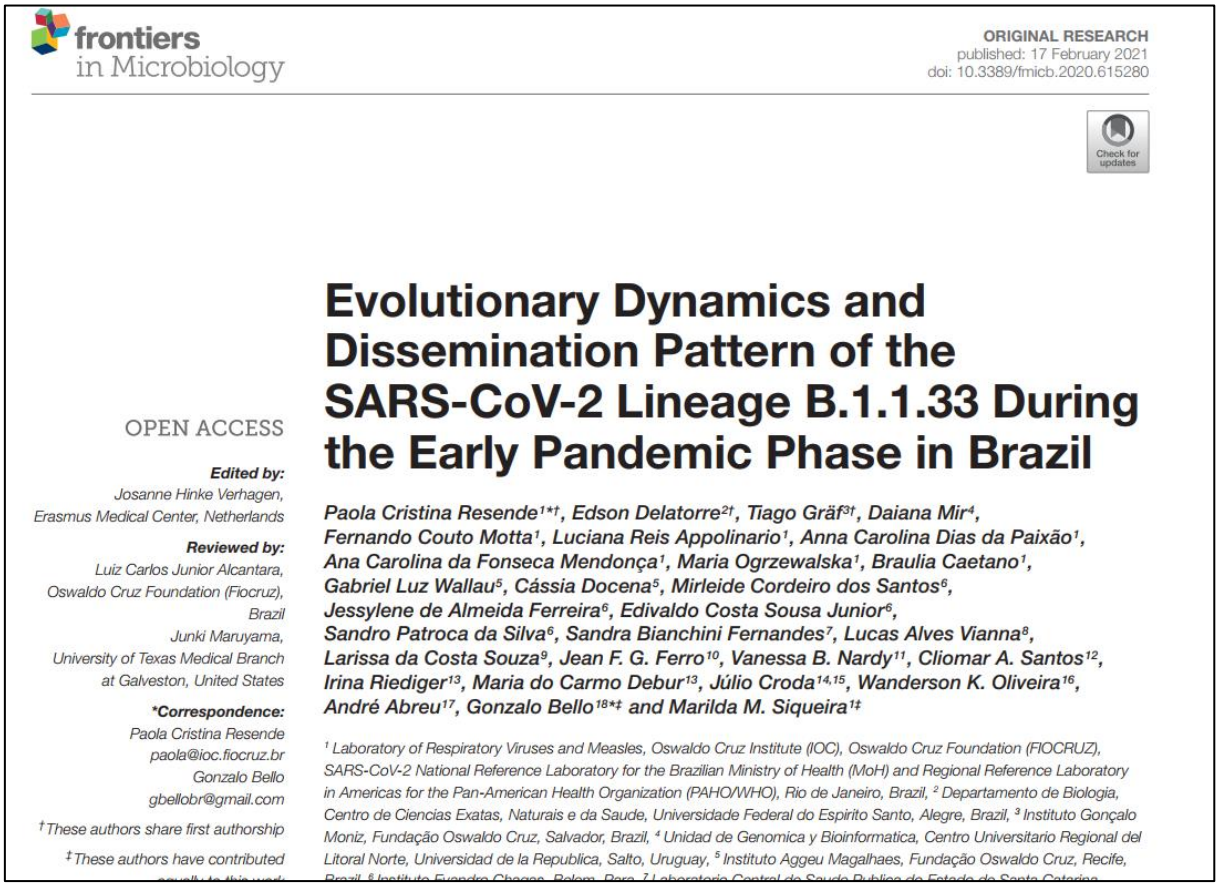
Neuraminidase inhibitors (NAIs) are the main class of antivirals currently used for the treatment of influenza infections. As influenza viruses are constantly evolving, drug-resistance can emerge resulting in reduced effectiveness of treatment. This study evaluated the presence of molecular markers associated with NAI susceptibility in 724 influenza A(H1N1)pdm09 positive samples from Brazilian surveillance system from the 2014-2016 seasons, including 76 isolates tested for oseltamivir (OST) susceptibility and 23 isolates also tested for zanamivir, peramivir and laninamivir susceptibility. We identified the H275Y (n = 3) and I223K (n = 1) NA substitutions, associated with reduced inhibition (RI) by the NAIs. Noteworthy, no epidemiological links were identified among the patients infected with the mutant viruses. Phylogenetic analysis from NA and hemagglutinin genes showed that mutant viruses were not clustered. All mutant virus strains carried the permissive substitutions V241I and

2. Artigo publicado na revista **Frontiers in Microbiology**

Fator de impacto: 4.1

Ano: 2021

Colaboração: diagnóstico por RT-qPCR das amostras oriundas do Espírito Santo, seleção e envio das amostras à Fiocruz e revisão do manuscrito.



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Evolutionary Dynamics and Dissemination Pattern of the SARS-CoV-2 Lineage B.1.1.33 During the Early Pandemic Phase in Brazil

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ANEXO A - Aprovação da Secretaria de Estado de Saúde do Espírito Santo para a execução deste projeto de pesquisa

GOVERNO DO ESTADO DO ESPÍRITO SANTO
SECRETARIA DE ESTADO DA SAÚDE

DECLARAÇÃO DE ANUÊNCIA PARA REALIZAÇÃO DE PROJETO DE PESQUISA

Gilsa Rodrigues, Gerente de Vigilância em Saúde da Secretaria de Estado de Saúde do Espírito Santo (GEVS/SESA), declara que está de acordo com o desenvolvimento do projeto de pesquisa em parceria com o Laboratório Central de Saúde Pública – LACEN – intitulado: **"Prevalência de genótipos do Vírus Sincicial Respiratório (RSV) e desfecho clínico de crianças com Síndrome Respiratória Aguda Grave (SRAG) no Espírito Santo (2016-2018)",** coordenado pelo Pesquisador Dr. Iuri Drumond Louro, professor na Universidade Federal do Espírito Santo. A participação do LACEN dar-se-á por meio do fornecimento de amostras biológicas referenciadas ao diagnóstico molecular da Influenza, bem como pelo acesso aos dados clínicos e epidemiológicos dos pacientes, conforme critérios definidos pela Gerência de Vigilância em Saúde e LACEN. A liberação das amostras e dos dados clínicos será realizada após a apresentação da carta de aprovação do Comitê de Ética e autorização do Grupo Técnico para Análise de Pesquisa no Âmbito da SESA/ES.

Vitória, 11 de abril de 2018

Gilsa A. Pimenta Rodrigues
Gerente de Vigilância em Saúde
SESA/GEVS
Nº func.: 1.527.207

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ANEXO B – Notificação formal do aceite do manuscrito intitulado “Seasonality, molecular epidemiology, and virulence of Respiratory Syncytial Virus (RSV): a perspective into the Brazilian Influenza Surveillance Program” na revista PLoS ONE.

Notification of Formal Acceptance for PONE-D-20-30854R3 - [EMID:80ad49a04ee8ccae] Caixa de entrada ×

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PONE-D-20-30854R3
Seasonality, molecular epidemiology, and virulence of Respiratory Syncytial Virus (RSV): a perspective into the Brazilian Influenza Surveillance Program

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