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Aspectos imunológicos dos casos de Influenza A no Rio Grande do Sul e análise molecular das cepas circulantes

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Lista de abreviaturas utilizadas

ARDS: Acute respiratory distress syndrome
ANF: Aspirado nasofaríngeo
$CD4^+$: Linfócitos T CD4 positivos
$CD8^+$: Linfócitos T CD8 positivos
CT: Valor de ciclo limiar
DCs: Células dendríticas
HA: Hemaglutinina
HPAIV: Vírus influenza aviário altamente patogênico
IAV: Vírus influenza A
IL: Interleucina
INF: Interferon
IP10: IFN-γ-induced protein
IRA: Infecção respiratória aguda
M1: Proteína de matriz 1
M2: Proteína de matriz 2
MCP-1: Monocyte chemoattractant protein
MHC: Complexo de histocompatibilidade
MIP1-α: Macrophage inflammatory protein
MS: Ministério da Saúde
N: nucleoproteína
NA: Neuraminidase
NEP: Proteína de exportação nuclear
NP: Nucleoproteína
NS1: Proteína não-estrutural 1
OMS: Organização Mundial da Saúde
ORF: Fase aberta de leitura
PA: Polimerase ácida
PB1: Polimerase básica 1
PB2: Polimerase básica 2
RNA: Ácido ribonucleico
RNP: Ribonucleoproteína
RT-PCR: Transcrição reversa seguida por Reação em Cadeia da Polimerase
SA: Ácido siálico
SARS: Severe acute respiratory syndrome
SRAG: Síndrome respiratória aguda grave
TH1: Linfócitos T classe 1
TNF: Fator de necrose tumoral
TNFR: Receptor do fator de necrose tumoral
VIOP: Influenza de origem suína
vRNP: Ribonucleoproteínas virais

Resumo da Tese

Introdução

Um novo virus influenza A H1N1, de origem suína, causou a primeira pandemia do século XXI, a qual foi considerada de virulência moderada. No Brasil, as regiões Sudeste e Sul foram as mais afetadas e apresentaram as maiores taxas de mortalidade. Após a pandemia, um sistema de vigilância epidemiológica mundial foi instalado para caracterizar sequências genômicas do influenza A e buscar marcadores de virulência.

Objetivos

Analisar os fatores clínicos, o perfil de citocinas e mutações virais que estão envolvidos na infecção do vírus Influenza A no Rio Grande do Sul.

Material e Métodos

Foi realizada RT-qPCR para diagnóstico e carga viral de amostras de aspirado nasofaríngе de pacientes com Síndrome Respiratória Aguda e dosagens citocinas/quimiocinas a partir do soro. O genoma completo de 56 isolados virais foram sequenciados para análises filogenéticas e identificação de mutações nas proteínas virais.

Resultados

A carga viral no período pandêmico de 2009 foi significativamente maior para o vírus A(H1N1)pdm09 que amostras de vírus sazonal e essa relação inverteu no ano de 2011. No entanto, nos dois períodos, pandêmico e pós pandêmico estudados, o maior número de casos fatais foi causado pelo vírus influenza A(H1N1)pdm09. Houve um perfil de resposta imune inata/ inflamatória e adaptativa associado aos casos fatais, perfil este representado

pelas citocinas/quimiocinas IL-8, IL-4, MIP1-**α**, IL-15 e TNF-α. Houve constante evolução viral a partir do período pandêmico, principalmente nos anos de 2012 e 2013. Nenhuma mutação de resistência ao oseltamivir, (H275Y) foi encontrada.

Conclusões

Um perfil de elevação na expressão de citocinas/quimiocinas teve relação direta com a gravidade da infecção: IL-8, IL-4, MIP1-**α**, IL-15 e TNF-α. A análise das 56 sequências genômicas virais completas, revelou um maior acúmulo de mutações nas proteínas HA, NA e PA, as quais estão relacionadas com a antigenicidade e com a capacidade replicativa do vírus. Tais mutações poderiam determinar a adaptação do vírus ao hospedeiro, sua virulência e a eficiência da vacina anti-influenza. Este estudo contribui para o conhecimento e a vigilância do influenza A no Rio Grande do Sul.

1. Introdução

A infecção respiratória aguda (IRA) é um dos maiores desafios para a saúde pública uma vez que atinge 113 milhões de pessoas ao redor do mundo sendo a causa de 3,5 milhões mortes, anualmente (Murray and Lopez, 2013). O amplo acometimento da população ocorre devido à facilidade de disseminação dos agentes infecciosos causadores da IRA ocasionando elevada morbidade e mortalidade, especialmente entre pacientes pediátricos (menores de 6 anos de idade) e idosos (maiores de 65 anos de idade) (Kesson, 2007; Murray and Lopez, 2013). Entre 50 a 90% dos casos de IRA são causados por vírus, incluindo o vírus respiratório sincicial, vírus influenza A e B, vírus parainfluenza 1, 2 e 3, rinovírus e o adenovírus (Thomazelli e cols., 2007; Seo e cols., 2014). Recentemente, novos métodos de triagem molecular baseados na amplificação dos ácidos nucléicos dos vírus e na análise das sequências genômicas permitiram a identificação de vírus respiratórios outrora desconhecidos, como por exemplo alguns coronavírus (Konig e cols., 2010), influenza A H5N1 e influenza A (H1N1)pdm09, metapneumovírus humano (hMPV) e bocavírus humano (HBoV) (Kahn, 2006; Williams e cols., 2006; Yang e cols., 2009). O quadro clínico de pacientes com IRA viral é inespecífico e inclui sintomas que variam desde um simples resfriado com congestão nasal e rinorréia, faringite e crupe, até quadros mais severos representados por bronquiolite e pneumonia (Kesson, 2007). Complicações nos quadros sintomáticos podem levar ao agravamento da disfunção respiratória e, consequentemente, à morte.

O vírus influenza A, agente causador da enfermidade popularmente

conhecida como gripe, pertence à família *Orthomyxoviridae*, a qual compreende seis gêneros: i) Influenzavirus A, ii) Influenzavirus B, iii) Influenzavirus C, iv) Isavirus, v) Quaranjavirus e vi) Thogotovirus (ICTV, 2014). Os ortomixovírus são vírus de RNA envelopados, com genoma segmentado constituído por RNA fita-simples senso-negativo (Lamb e Krug, 2007).

O vírus influenza A é classificado em diferentes subtipos com base nas especificidades antigênicas determinadas pelas glicoproteínas de membrana viral, a Hemaglutinina (HA) e a Neuraminidase (NA). Já foram caracterizados e documentados 18 tipos de HA e 11 tipos de NA, sendo as aves aquáticas o principal reservatório desses subtipos; apenas três tipos de HA (H1, H2 e H3) e dois de NA (N1 e N2) afetam a espécie humanos (Hilleman, 2002; Hsieh e cols., 2006). Os tipos A e B contêm oito segmentos genômicos distintos, em que cada um codifica pelo menos uma proteína (Palese and Shaw, 2007; König e cols., 2010). Estudos recentes sobre o vírus influenza A revelaram que mecanismos moleculares tais como *splicing* alternativo e diferentes fases de leitura (ORF) podem levar à produção de um número variado de proteínas virais, de modo a facilitar a infecção e replicação viral na célula hospedeira (Vasin e cols., 2014). No total são 10 proteínas bem descritas e outras 8 proteínas recentemente descobertas que desempenham importante papel na formação do cenário de virulência do vírus, incluindo a adaptação da infecção e transmissão em novas espécies de hospedeiro, a capacidade de modular a resposta imune do hospedeiro, e a capacidade de replicar de forma eficiente baixas temperaturas (Chen e cols., 2001; Arias e cols., 2009; Wise e cols., 2011).

Uma pandemia é definida como uma epidemia que ocorre em nível mundial, a qual cruza as fronteiras internacionais e afeta um grande número de pessoas. Este evento envolve o surgimento de um novo vírus influenza, o qual deve ser capaz de causar enfermidade e disseminar-se entre os hospedeiros humanos (Poland e cols., 2007). Mais especificamente, o surgimento de um novo vírus influenza representa o surgimento de proteínas virais inéditas, principalmente a hemaglutinina e neuraminidase para o qual a população não apresenta resposta imunológica ou apresenta de maneira pouco reativa. Este novo vírus pode surgir como consequência de um rearranjo de dois ou mais vírus de origem distintas, principalmente entre vírus humanos sazonais e um ou mais vírus de origem animal. A história registra três episódios pandêmicos de influenza A no século XX: a Influenza espanhola H1N1 (“Gripe Espanhola”) entre 1918 e 1920; a Influenza asiática H2N2 entre 1957 e 1960; e a Influenza de Hong Kong H3N2 de 1968 a 1972 (Figura 1). Ressalta-se ainda a transmissão direta do vírus influenza aviária de alta patogenicidade A (H5N1) ao homem, gerando surtos de elevada

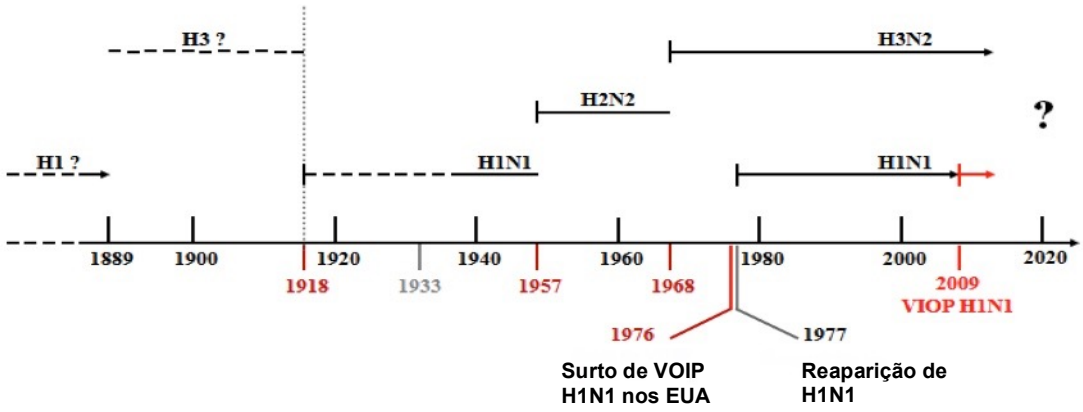

Figura 1. **Cronologia da circulação dos vírus influenza A em humanos**. Desde o século XIX houve cinco ocorrências de modificações antigênicas maiores (*antigenic shift*), com aparecimento de cepas distintas daquelas circulantes, culminando em pandemias (1889–1891, 1918–1920, 1957–1958, 1968–

1969, e 2009–2010). Os vírus H1N1, descendentes da Influenza Espanhola de 1918, foram introduzidos na população de suínos em algum momento após 1918. Três subtipos de hemaglutinina (H1, H2 e H3) e 2 de neuraminidase (N1 e N2) têm sido isolados de humanos desde 1933 (primeiro isolado viral). Em 1976 foi registrado um surto de Influenza de origem suína (VIOP), e em 1977 reapareceu a cepa H1N1 humana. As linhas descontínuas indicam evidência sorológica sem registro de isolados virais. As linhas contínuas indicam circulação sazonal de cada cepa (as quais continuamente sofreram modificações antigências menores (*drift*) durante os períodos interpandêmicos e as interrogações significam que a data exata da circulação e/ou origem do subtipo não está definida (Modificada deMedina, 2010).

letalidade na Ásia (Lai e cols, 2016).

A Gripe Espanhola foi uma pandemia responsável por mais de 50 milhões de mortes em todo o mundo entre 1918–1920, sendo uma das mais letais de toda história médica contemporânea. Depois do reaparecimento do subtipo H1N1 em 1977, provavelmente reintroduzida por um erro de manejo laboratorial, esta cepa co-circulou na população humana juntamente com o subtipo H3N2, a qual teve origem no ano 1968 (Medina, 2010); ambas constituem subtipos causadores de epidemias sazonais.

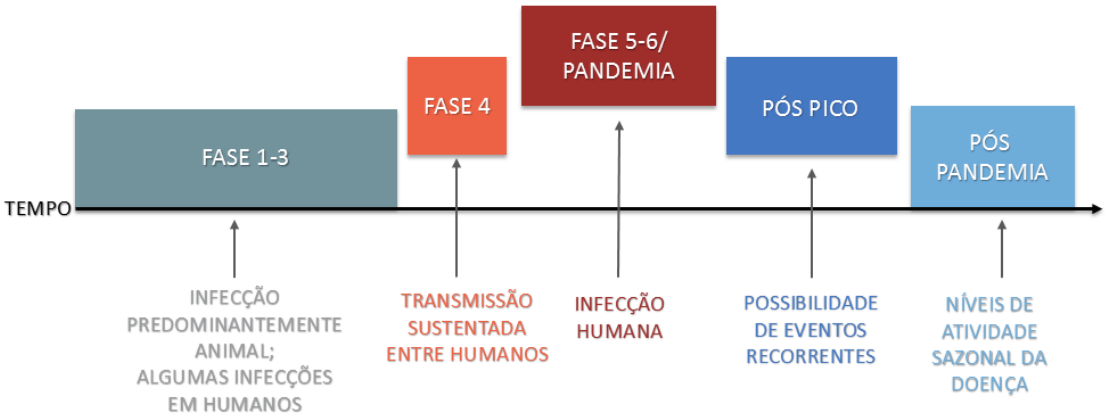

Figura 2. Fases de uma pandemia segundo a OMS. (Modificada de http://www.who.int/csr/disease/swineflu/phase/en/)

O primeiro evento pandêmico do século XXI foi causado por um novo vírus H1N1, A(H1N1)pdm09, que surgiu no México entre fevereiro e março de 2009 e foi substituindo a cepa sazonal H1N1, co-circulando na atualidade com o subtipo H3N2 (Fineberg, 2014). A cepa pandêmica tem origem suína e é um exemplo de recombinação tripla, evento que deu origem a segmentos genômicos totalmente distintos dos originais (Garten e cols., 2009; Wang and Palese, 2009). Foi estimado que durante o primeiro ano de circulação da cepa A(H1N1)pdm09 houve de 151.700 a 575.400 casos ao redor do mundo e que mais de 18 mil pessoas morreram em mais de 200 países, segundo dados da Organização Mundial da Saúde (OMS) (WHO, 2013). Uma característica desta pandemia foi a maior prevalência em pessoas com menos de 65 anos (80%), embora este último grupo seja usualmente o principal alvo das epidemias sazonais de influenza (Kesson, 2007). A Figura 2 resume as fases da pandemia de 2009, segundo a OMS. Na fase 1, os vírus Influenza circulam somente em animais, na fase 2 o risco de transmissão entre humanos é elevada devido a circulação do vírus em animais domésticos e é na próxima etapa, a fase 3, que ocorre transmissão eventual entre humanos. Na fase 4, ocorre a transmissão sustentada entre humanos mas ainda em nível local, sendo a próxima etapa a fase 5, onde dois ou mais países de uma mesma região da OMS é afetada pelo mesmo Influenza isolado na fase 4. Na próxima etapa, adicionalmente aos critérios definidos na fase 5, o mesmo vírus causa transmissão sustentada em grupos de pessoas em pelo menos uma segunda região definida pela OMS. A fase pós pico pandêmico é definida como a etapa onde uma vigilância eficaz foi aplicada para conter a doença, porém podem ocorrer ondas de infecção onde

a atividade viral volta a ser elevada. Finalmente, a fase pós pandêmica é caracterizada por níveis de atividade semelhantes à Influenza sazonal, na maioria dos países afetados, devido à vigilância adequada.

1.1. Aspectos clínicos e epidemiológicos da doença

Durante uma epidemia sazonal de Influenza, cerca de 5 a 15% da população é infectada, resultando em aproximadamente 3 a 5 milhões de casos graves por ano e de 250 a 500 mil mortes no mundo, principalmente entre idosos e portadores de doenças crônicas (Saúde, 2015). A Influenza ou gripe se apresenta com um amplo espectro clínico, desde infecção assintomática até quadros letais. A Síndrome Respiratória Aguda Grave (SRAG), é caracterizada pelos sintomas: febre, tosse e dispneia. Em sua forma clínica clássica a síndrome gripal (SG) caracteriza-se por sintomas de início súbito, com febre, calafrios, prostração, cefaleia, mal-estar, mialgia, tosse, congestão nasal e dor de garganta. Ocasionalmente, a Influenza pode causar broncoespasmo, bronquite ou pneumonia. As infecções bacterianas (sinusite, otite, pneumonia) são as complicações mais comuns em crianças e em idosos. A evolução é, em geral, benigna, com desaparecimento dos sintomas em 7 dias, embora a tosse, o mal-estar e a prostração possam permanecer por algumas semanas. Sinais de agravamento, como mialgia intensa, piora de sintomas gastrointestinais, dispneia e persistência ou aumento da febre por mais de 3 a 5 dias pode indicar pneumonite primária pelo vírus da Influenza ou secundária à infecção bacteriana ou, raramente, miocardite, pericardite, mielite transversa e encefalite. A vigilância da influenza no Brasil é composta pela vigilância sentinela de Síndrome Gripal e

de Síndrome Respiratória Aguda Grave e pela vigilância universal de SRAG. A vigilância sentinela conta com uma rede de unidades distribuídas em todas as regiões geográficas do país e tem como objetivo principal identificar os vírus respiratórios circulantes para subsidiar, com os isolamentos virais, a composição da vacina contra gripe, além de permitir o monitoramento da demanda de atendimento por essa doença. A vigilância da SRAG monitora os casos hospitalizados e óbitos, além de casos de SRAG em gestantes e surtos ocasionais, com o objetivo de identificar o comportamento da influenza no país para orientar na tomada de decisões em situações que requeiram novos posicionamentos do Ministério da Saúde e Secretarias de Saúde Estaduais/Municipais.

Durante a pandemia de 2009, a região Sul do Brasil foi a mais afetada, com 18.349 casos confirmados até a semana epidemiológica (SE) 47 de 2009. As taxas de incidência (66,2/100.000 habitantes) e de mortalidade (2,32/100.000 habitantes) também foram mais elevadas no Sul do que em outras regiões do Brasil (Gorini da Veiga e cols., 2012a). No Rio Grande do Sul (RS) foram confirmados 3.585 casos de Influenza A(H1N1)pdm09, dos quais 298 foram a óbito. Já no ano de 2010 não foram confirmados casos de Gripe A no RS e em agosto deste mesmo ano foi declarada a fase pós-pandêmica da Influenza A(H1N1)pdm09 pela Organização Mundial da Saúde (OMS); porém em 2011 voltaram a ser confirmados 103 casos de infecções por A(H1N1)pdm09, dos quais 14 foram a óbito (Gregianini e cols., 2011; Martins e cols., 2012).

Dos 812 casos de Síndrome Respiratória Aguda Grave (SRAG) causadas pelo vírus Influenza A no RS em 2012, 527(65%) foram causados por

influenza A(H1N1)pdm09, levando 68 indivíduos ao óbito, enquanto o vírus sazonal H3N2 levou 9 indivíduos a óbito, apesar dos 285 casos confirmados; nesse mesmo ano os estados vizinhos de Santa Catarina e Paraná reportaram 752 casos com 73 óbitos e 1.097 casos com 36 óbitos, respectivamente (Martinse cols., 2012). Em 2013 o RS confirmou 731 casos de SRAG por influenza A, sendo 277 casos de influenza A H3N2 e 454 casos de influenza (H1N1)pdm09, e um total de 71 óbitos. No ano de 2014 esses números caíram para 177 casos de SRAG causados por Influenza A e, desses, 25 foram a óbito. Já no ano de 2015, segundo dados da Vigilância Epidemiológica do Ministério da Saúde (MS), o Rio Grande do Sul registrou 7 óbitos por influenza A (5 óbitos por H3N2 e 2 por influenza A não subtipado), mas nenhum caso ou óbito por influenza (H1N1)pdm09 foi confirmado nesse ano (Saúde, 2015). A tabela 1 resume os dados epidemiológicos do período do estudo, juntamente com os dados atualizados do ano de 2016.

Tabela 1. Número de casos de SRAG e casosde influenza no RS, 2009-2016.

Ano	SRAG		A H1N1pdm09		A H3N2		A (?)		Influenza B		Referência
	Casos	Mortes	Casos	Mortes	Casos	Mortes	Casos	Mortes	Casos	Mortes	
2009	8338	494	3585	298	N.I.	N.I.	172	N.I.	N.I.	N.I.	Martins et al., 2011
2010	716	60	0	0	N.I.	N.I.	0	0	25	0	Martins et al., 2011
2011	1501	125	103	14	N.I.	N.I.	N.I.	N.I.	N.I.	N.I.	Martins et al., 2011
2012	3951	214	527	68	285	9	0	0	49	0	CEVS, 2013a
2013	3176	326	454	58	277	13	0	0	131	2	CEVS, 2013b
2014	1956	177	29	12	142	13	1	0	17	0	CEVS, 2014
2015*	2332	211	0	0	47	5	13	2	27	2	CEVS, 2015
2016**	4399	469	1096	177	2	0	118	14	0	0	CEVS, 2016

*Várias amostras não foram subtipadas.

** Até 8/11/16, 178 casos ainda não haviam sido analisados.

N.I.: não informado.

Apesar de a cepa pandêmica (H1N1)pdm09 ser antigenicamente similar à cepa H1N1 espanhola (1918), a magnitude das manifestações clínicas no evento pandêmico de 2009 foi muito menor que aquela apresentada pela

Gripe Espanhola, com um índice de mortalidade 100 vezes menor que a pandemia de 1918 (Watanabe and Kawaoka, 2011). Sobre esse fato, alguns estudos têm demonstrado a ocorrência de proteção cruzada, ou seja, produção de anticorpos protetores por exposição prévia a uma cepa distinta da atual, mas antigenicamente relacionada, em pessoas com mais de 60 anos (Medina e cols., 2010; Xu e cols., 2010). Adicionalmente, a leve enfermidade associada à cepa pandêmica de 2009 é consistente com a ausência de marcadores de virulência, os quais foram identificados previamente em cepas humanas e aviárias (Hatta e cols., 2001; Medina and Garcia-Sastre, 2011).

É sabido que em suínos, que também constituem um importante reservatório dos vírus influenza, ocorre o fenômeno de recombinação entre as mais distintas cepas, e um vírus já recombinado, como o (H1N1)pdm09, pode vir a sofrer rearranjos dos seus segmentos novamente e dar origem a novos marcadores de virulência, como por exemplo o surgimento de uma HA com maior afinidade às células do trato respiratório inferior, induzindo assim pneumonia viral (Vincent e cols., 2008). O desafio dos novos estudos é investigar quais fatores contribuem para a patogenicidade viral, a qual pode ser incrementada com a exposição das cepas circulantes ao sistema imunológico do hospedeiro, à co-circulação com outras cepas como a sazonal humana H3N2 e, por fim, com a exposição dos vírus às drogas antivirais. Não obstante, durante os surtos ou pandemias o diagnóstico rápido e preciso se torna fundamental para a prevenção da disseminação da doença e para estudos epidemiológicos (Nishiura e cols., 2009; Zhou and Wentworth, 2012)

1.2. Etiologia

O vírus Influenza A pertence à família *Orthomyxoviridae e* possui genoma segmentado composto por 8 fitas simples de RNA com polaridade negativa envoltos por uma nucleoproteína (NP), que se unem ao complexo de transcrição/replicação viral formado pela polimerase básica 1 (PB1), pela polimerase básica 2 (PB2) e pela polimerase ácida (PA). Este conjunto de RNA viral e proteínas se denomina complexo ribonucleoprotéico (RNP) (Figura 3a). No caso do vírus Influenza A, cada segmento codifica uma ou duas proteínas funcionalmente importantes.

A partícula viral do influenza A é pleomórfica (forma esférica ou oval), mede 80–120nm de diâmetro e está envolta por um envelope de bicamada lipídica, do qual emergem duas glicoproteínas de superfície − hemaglutinina (HA) e neuraminidase (NA), codificadas pelos segmentos 4 e 6 do RNA genômico, respectivamente. A HA atua na adesão e fusão do vírus à célula hospedeira, enquanto a NA evita a agregação das partículas virais e auxilia na liberação das novas partículas de dentro da célula infectada, após a replicação do vírus (Figura 3b); assim, ambas são determinantes para a infectividade, além de serem os principais antígenos contra os quais a resposta humoral do hospedeiro é direcionada. O segmento M do genoma viral codifica as proteínas da matriz, M1 e M2. A M1 localiza-se logo abaixo do envelope viral e participa da montagem do vírus na célula hospedeira; a M2 é uma proteína tetramérica que forma um canal iônico entre o interior do vírus e o meio, permitindo mudanças de pH durante a síntese da HA e o desencapamento viral. O segmento 8 codifica duas proteínas não-estruturais:

a NS1, que antagoniza a ação supressora do interferon (INF), e a NEP, proteína de exportação nuclear (Hilleman, 2002; Hsiehe cols., 2006). Adicionalmente, estudos recentes têm identificado novas proteínas como a PB1-N40 (função desconhecida), PA-X94 a qual é supressora da expressão gênica celular as quais são codificadas por PB1 e PA, respectivamente (Hayashi e cols., 2015). Outras duas formas de PA foram identificadas e nomeadas PA-N155 e PA-N182, com papel no ciclo replicativo do vírus. Alguns vírus expressam a proteína pró-apoptótica PB1-F2, a qual é codificada por uma segunda ORF no segmento PB1 (Vasin e cols., 2014).

O genoma do vírus pandêmico (H1N1)pdm09 tem aproximadamente 13.580 nucleotídeos, sendo que o tamanho dos segmentos variam de 890 a 2.341 nucleotídeos. Além da região codificadora, cada segmento contém regiões não-codificadoras, que na extremidade 5’ variam de 20 a 58 nucleotídeos, sendo 13 deles conservados em todos os segmentos, e na extremidade 3’ variam de 19 a 45 nucleotídeos, sendo 12 deles conservados (Hoper e cols., 2011).

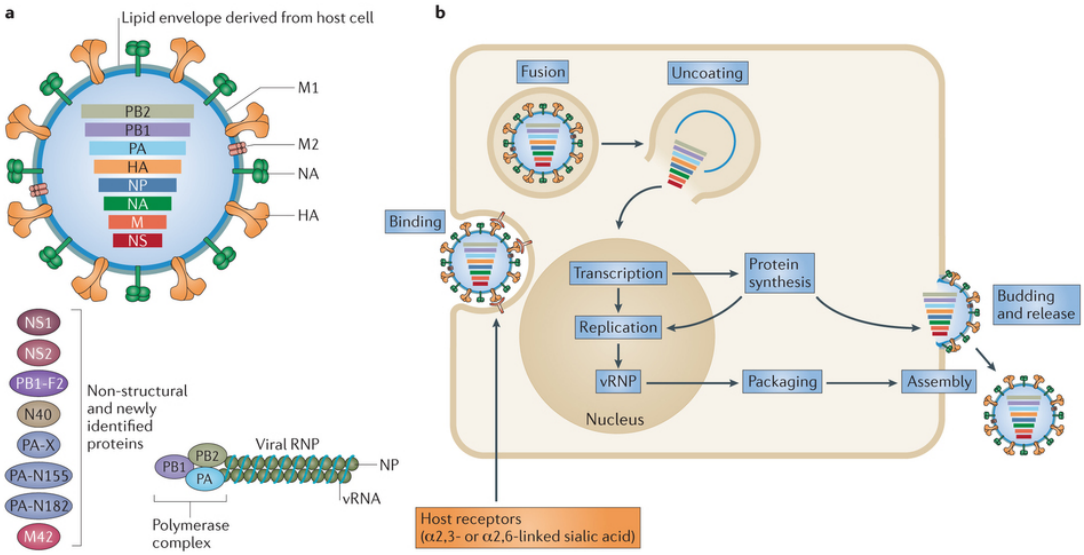

Figura 3. **Características da partícula viral do Influenza A e sua replicação**. **a)** A partícula viral do vírus Influenza A possui genoma com 8 segmentos (genes) de RNA de fita simples, de sentido negativo que juntos codificam as proteínas demonstradas no esquema. Estes segmentos genômicos se empacotam em complexos de ribonucleoproteínas virais (vRNP) juntamente com as polimerases PB1, PB2 e PA (codificadas por seus respectivos segmentos). Os segmentos não estruturais codificam a proteína de exportação nuclear NS2 e o antagonista da resposta viral do hospedeiro NS1; o segmento da matriz codifica a proteína M1, M2 proteína de canal iônico, e a M42, uma proteína relacionada à M2. O segmento HA codifica a glicoproteína de ligação ao receptor celular HA; e o segmento NA codifica a neuraminidase, a qual cliva moléculas de ácido siálico (SA) (Fineberg, 2014). Adicionalmente, as duas novas proteínas identificadas recentemente N40 (função desconhecida) e PA-X94, a qual é um repressor da expressão gênica celular, são codificadas por PB1 e PA, respectivamente. Outras 2 formas de PA foram recentemente identificadas e nomeadas PA-N155 e PA-N182, com papel no ciclo replicativo do vírus. Alguns vírus expressam a proteína pró-apoptótica PB1-F2, a qual é codificada por uma segunda ORF no segmento PB1. **b)** A infecção viral é iniciada pela ligação do vírus ao SA do receptor da célula hospedeira e sua entrada é mediada por endocitose. A clivagem da HA por proteases celulares é requerida para expor o peptídeo de HA, que é responsável pela fusão entre o envelope viral e a membrana endossomal a um pH baixo, processo que abre o canal de íon M2, acidifica o interior viral e libera os segmentos do genoma viral dentro do citoplasma. Estes segmentos são então translocados para o núcleo onde são transcritos e replicados pelo complexo polimerase RNA-dependente. As novas partículas virais são montadas na membrana celular e liberadas por brotamento para o fluído extracelular, processo mediado pela NA e sua atividade de neuraminidase, a

qual destrói SA das glicoproteínas celulares e virais que, caso contrário, reteriam o vírus na membrana celular. Para que a nova partícula viral seja infecciosa, a proteína HA deve ser cortada por proteases extracelulares similares à tripsina. Estas proteases estão localizadas somente nas mucosas intestinais e respiratórias, o que restringe a infecção pelo influenza A nestas áreas (Shi e cols., 2014).

1.2.1. Tropismo tecidual e patogênese

Em humanos, após a transmissão por via respiratória, o vírus influenza A liga-se e penetra nas células epiteliais da traqueia e dos brônquios. Ocorre então a replicação viral que resulta em destruição da célula hospedeira. A viremia raramente é bem documentada, porém nas secreções respiratórias o vírus é detectado por pelo menos 10 dias após a infecção, dependendo essencialmente da condição imunológica do paciente, com um pico durante as 48 horas seguidas ao contágio (Gorini da Veigae cols., 2012a). Tanto o processo infectivo/replicativo como o desenvolvimento da enfermidade dependem de fatores moleculares virais e do hospedeiro e da interação destes fatores (Figura 4).

A proteína HA do vírus Influenza A, além de modular a antigenicidade, também modula o tropismo tecidual e a patogênese viral através da sua ligação específica a um receptor celular. Por esse motivo, a maioria dos subtipos virais de influenza são hospedeiro-específicos, embora alguns subtipos possam ser capazes de circular em mais de uma espécie de hospedeiro, como por exemplo os subtipos H1N1 e H3N2, os quais são endêmicos em humanos, aves e suínos. O vírus entra na célula hospedeira através de uma interação específica entre a HA e moléculas de ácido siálico (SA) unidos em uma conformação α-2,6 ou α-2,3 às porções glicanas das

glicoproteínas de superfície celular. Classicamente, os subtipos de Influenza A humanos se unem ao receptor de conformação α-2,6 SA, os quais são abundantes nas células do epitélio nasofaríngeo e traqueal do trato respiratório superior; nas células epiteliais do trato respiratório inferior humano, por outro lado, predomina a forma α-2,3 SA. Os vírus de origem aviária, por outro lado, têm preferência pela forma α-2,3 SA do receptor; essa diferença de especificidade explica o fato de subtipos de influenza A aviário altamente patogênicos apresentarem baixa transmissibilidade entre humanos.

A adaptação de uma cepa a diferentes hospedeiros se origina por mutações que resultam em trocas de resíduos pontuais na HA, ou então por rearranjo dos segmentos de subtipos diferentes em uma mesma célula hospedeira; tal adaptação pode originar eventos zoonóticos (transmissão de um animal não-humano para humanos ou vice-versa) que, por sua vez, podem causar pandemias na população (Wilks e cols., 2012). Portanto, um novo subtipo viral pode surgir pela coinfecção de um hospedeiro intermediário, como é o caso dos suínos, os quais possuem ambos tipos de receptores (α-2,3 SA e α-2,6 SA) em uma mesma célula. Esse mecanismo pode explicar a tripla recombinação de segmentos genômicos virais que deu origem ao vírus pandêmico de 2009, o qual possui segmentos de cepas humanas (H1N1 de 1918 e H3N2 sazonal), e H1N1 de suínos. Apesar da especificidade da ligação da HA ao receptor celular, para cada subtipo específico parece haver um conjunto de diferentes resíduos que influenciam nesta ligação e, consequentemente, modula sua patogenicidade. Um exemplo é a mutação D222G, encontrada em isolados do vírus pandêmico

(H1N1)pdm09, a qual foi associada com doença grave e morte (Kilander e cols., 2010; Chan e cols., 2011). Essa cepa mostrou ter uma afinidade aumentada pelo receptor α-2,3 SA, sem alterar sua capacidade de se ligar ao receptor α-2,6 SA humano. Portanto, uma pequena alteração na especificidade de ligação ao receptor pode aumentar o potencial patogênico do vírus, permitindo a infecção de células do trato respiratório inferior, desta forma modulando a indução de pneumonia em casos severos da infecção, ou mesmo para que o vírus possa adaptar-se e adquirir a capacidade de cruzar a barreira interespécie aves-humanos ou ser transmitido de humano para humano. Portanto, várias substituições de aminoácidos estão sendo caracterizadas como marcadores de virulência pela capacidade de modificar diretamente as proteínas virais e assim causar efeitos graves ou fatais no hospedeiro.

Além da HA, outras proteínas virais podem modular e conferir ao vírus vantagem replicativa em humanos e levar a um aumento da virulência. Estudos têm documentado que mutações no complexo da polimerase viral, como T271A em PB2 (Bussey e cols., 2010) e T97I em PA (Song e cols., 2009) aumentam a síntese de RNA viral em cepas do influenza A H5N1, um subtipo aviário altamente patogênico. A lisina (627K) da proteína PB2, por sua vez, é um determinante de infecção em humanos (Subbarao e cols., 1993). Mais recentemente, uma substituição de Aspartato para uma Asparagina (N) na posição 701 da PB2 também foi associada à adaptação de vírus aviário ao crescimento em células de mamíferos (Gabriel e cols., 2005; de Jong e cols., 2006). Polimorfismos em NS1 podem causar elevação da sua capacidade de inibir IFN e, consequentemente, diminuição da resposta

protetora do hospedeiro.

A evolução genética do vírus influenza A em humanos destaca-se por sua imprevisibilidade devido à sua capacidade de adquirir mutações através do tempo, as quais podem alterar constantemente a patogenicidade independente dos fatores do hospedeiro. Por essa razão torna-se essencial o monitoramento dos polimorfismos no genoma viral por metodologias avançadas de sequenciamento, como por exemplo o sequenciamento de alta performance (*deep sequencing*), a fim de definir novos fatores de virulência e sua relação com a magnitude da enfermidade, além da resposta ao tratamento antiviral e à vacina.

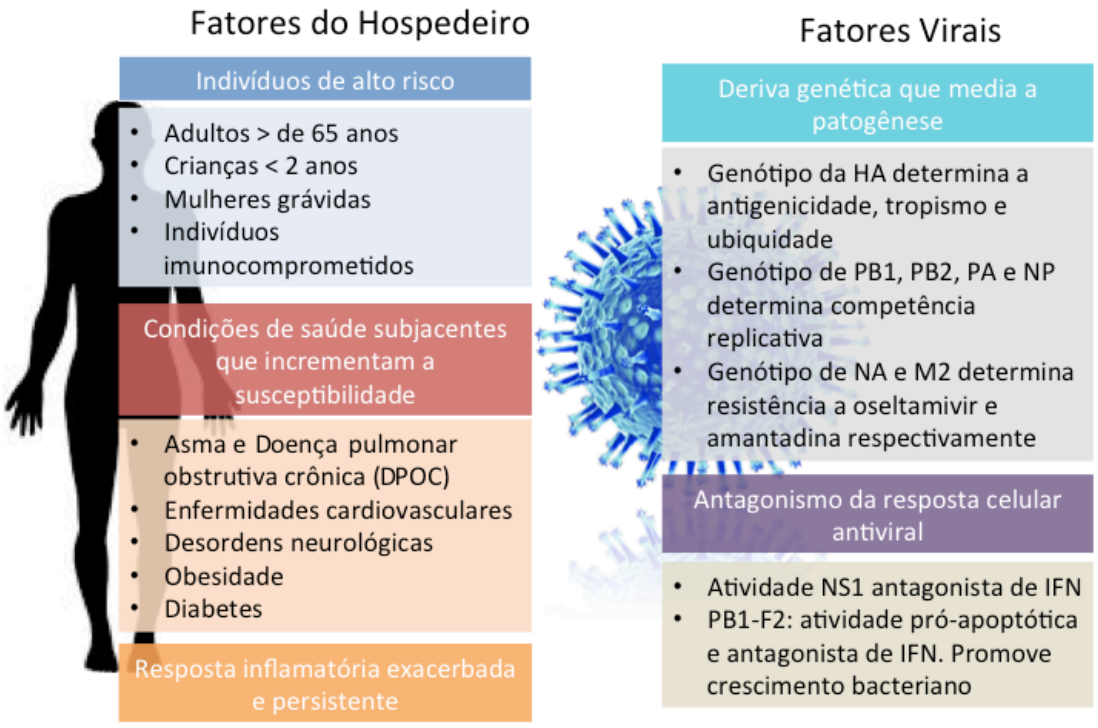

Figura 4. **Fatores do hospedeiro e fatores virais que influenciam na severidade da doença**.

1.2.2. Mudanças Antigênicas do Vírus Influenza A e Resposta Imunológica do Hospedeiro

O acúmulo de mutações nos sítios antigênicos da HA diminuem ou inibem a ligação de anticorpos neutralizantes, permitindo assim o surgimento de novas cepas virais que se disseminam na população, uma vez que tais variantes podem escapar da imunidade desenvolvida por infecção ou vacinação prévias. Este acúmulo de mutações pontuais é um mecanismo de variabilidade genética do vírus influenza conhecido como variação antigênica menor (*antigenic drift*) e é a explicação molecular para as epidemias sazonais de gripe, uma vez que, para o vírus mutante, a resposta imunológica é ineficaz ou incompleta. Em uma variação antigênica menor, são produzidos mutantes antigênicos como resultado da modificação da sequência de aminoácidos das glicoproteínas do envelope vira HA e NA, e do padrão de glicosilação de ambas. Esses vírus mutantes são selecionados como vírus predominantes na medida que se diferenciam do vírus antecessor, o qual é suprimido por anticorpos específicos que surgem na população como resposta à infecção. Este ciclo se repete continuamente.

Quando surge um vírus de tipo A com HA e/ou NA diferentes daquelas presentes nos vírus circulantes na população, temos a chamada variação antigênica maior (*antigenic shift*). As variações maiores surgem provavelmente em consequência da recombinação genética entre segmentos de diferentes tipos virais (humano e/ou animal) que infectam uma mesma célula (Frank, 2002). Como os vírus influenza de vários subtipos circulam em diversas espécies animais, especialmente em aves migratórias, o risco de

transmissão inter-espécies e a adaptação ao homem é real e contínuo.

Outro segmento imunogênico importante é a proteína viral neuraminidase (NA), a qual, ao sofrer mutação pontual, pode levar à resistência do vírus às drogas antivirais que têm como alvo a NA – os chamados inibidores de neuraminidase. Um exemplo é a resistência ao oseltamivir (Tamiflu) através do surgimento de substituições de aminoácidos, como H227Y a qual anula a união do composto ativo do fármaco à NA (Gubareva e cols., 2010).

Embora a infecção por Influenza seja frequente, um padrão para a resposta inflamatória ou a regulação da resposta imune e a patogênese do efeito citopático em humanos ainda não está completamente esclarecida. A maioria das evidências são oriundas de estudos com modelos animais e a patofisiologia dos modelos utilizados podem variar substancialmente em humanos.

1.3. Imunidade e o vírus influenza A

A proteção contra o vírus da gripe depende da presença de anticorpos neutralizantes no hospedeiro, enquanto a eliminação da infecção é mediada por imunidade celular citotóxica (resposta TH1). Uma resposta TH1 ótima consiste em secreção específica de IFN-γ contra os vírus por células $CD4^+$ e células citotóxicas $CD8^+$, as quais lisam as células infectadas. Já as células dendríticas (DCs) representam o principal grupo de células apresentadoras de antígenos para as células T e, assim, iniciam a resposta imune primária. Antes de migrarem aos gânglios linfáticos, as DCs sofrem maturação e

regulam positivamente o MHC de classe II e as moléculas co-estimulatórias da liberação de citocinas pró-inflamatórias e das quimiocinas, as quais auxiliam na sua função de estimular as células T, levando assim à iniciação de respostas imunes adaptativas específicas contra o patógeno. Antes de contribuir para a resposta imune de memória, as DCs contribuem para a resposta imune inata, secretando IFN-α/β, uma potente citocina antiviral (Ramos and Fernandez-Sesma, 2015).

A proteína NS1 do influenza A inibe a produção de IFN pelas células infectadas (Garcia-Sastre, 2002), incluindo DCs humanas, modulando tanto a resposta inata como a adaptativa (Fernandez-Sesma e cols., 2006). Ao inibir a produção de INF em DCs humanas, esta proteína permite que o vírus complete seu ciclo replicativo; consequentemente, a infecção se estabelece. Adicionalmente, a proteína viral PB1-F2 é um importante fator de virulência, pois pode ativar a apoptose das células infectadas principalmente representadas pelas células do sistema imune, mais especificamente os macrófagos. Esse efeito apoptótico em macrófagos desregula a resposta imune inata e também adaptativa, causando uma maior incidência de infecções secundárias e pneumonia (Chene cols., 2001).

Os casos leves da infecção pelo influenza são limitados à replicação viral no trato respiratório superior, e os sintomas nesses casos são devido à indução de inflamação logo após o estabelecimento da infecção viral neste local. As complicações da infecção pelo vírus Influenza A são mais frequentes em pessoas que apresentam comorbidades, tais como doença pulmonar crônica ou cardíaca, asma, imunossupressão e diabetes mellitus

(Figura 4). Isso inicia-se quando o vírus alcança o epitélio do trato respiratório inferior, onde um quadro severo de injúria ao epitélio alveolar pode resultar em disfunção respiratória ou síndrome respiratória aguda grave (SRAG) (Short e cols., 2014). Grande parte da patologia pulmonar durante esta síndrome é sabido estar associada à liberação de citocinas e quimiocinas; os altos níveis de inflamação evidenciam que o dano tecidual é causado por uma resposta imune inflamatória exacerbada (Bruder e cols., 2006; Shorte cols., 2014). Estudos demonstram que em casos graves de influenza altamente patogênico (HPAIV-*High Pathogenic Avian Influenza Virus*) em humanos, altos níveis de IP10, MCP-1, IL-8, IL-6, IL-10 e diversos outros fatores pró-inflamatórios estão envolvidos na patogênese dos casos de influenza (Beigel e cols., 2005; de Jonge cols., 2006). Segundo a revisão publicada por Ramos e Fernandez-Sesma (2015), recentes estudos têm proposto terapia anti-inflamatória para atenuar o dano tecidual. Além disso, segundo esses mesmos autores, existem diferenças na patogenicidade entre os subtipos do vírus influenza A. No caso dos vírus influenza sazonais, as complicações são na maioria das vezes associadas com infecção bacteriana secundária. Já a maioria dos casos de pneumonia primária viral severa tem sido associada ao influenza pandêmico, como o influenza A(H1N1)pdm09 ou H1N1 de 1918 (Ramos and Fernandez-Sesma, 2015).

Durante a pandemia de 2009, muitos casos graves foram associados à presença de comorbidades pré-existentes, contudo a alta severidade em adultos saudáveis ainda carece de associações com fatores de risco. No Brasil, a maior incidência de casos de influenza e a maior morbi-mortalidade esta concentrada na região sul e sudeste (Gorini da Veigae cols., 2012a;

Baccin e cols., 2013). Entender os fatores do hospedeiro e os fatores virais que levam à maior incidência de casos graves nestas regiões é de suma importância para direcionar as ações de vigilância epidemiológica, monitoramento viral, e manejo clínico do paciente.

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3. Justificativa

Compreender o papel do vírus da gripe A na saúde pública em uma área geográfica específica tem sido o foco de muitos estudos epidemiológicos e moleculares, em particular a análise genômica viral, que permite a caracterização de fatores de virulência e as relações filogenéticas entre cepas circulantes locais, bem como entre estas cepas e aquelas oriundas de outras regiões do mundo. A falta de dados moleculares das cepas de influenza A de amostras clínicas colhidas no Brasil, especificamente no estado do Rio Grande do Sul, um dos estados mais afetados pela gripe, com elevados índices de mortalidade, justifica a necessidade e a importância da realização do presente estudo.

4. Objetivos

Analisar os aspectos epidemiológicos, o perfil de citocinas e as mutações genômicas virais que estão envolvidos na infecção do vírus Influenza A em pacientes com Síndrome Respiratória e Síndrome Respiratória Aguda Grave, e investigar fatores que podem predispor à gravidade da doença.

Objetivos específicos

a) Determinar as características epidemiológicas e clínicas dos indivíduos diagnosticados com infecção pelo vírus Influenza A, no período de 2009 a 2015.

b) Avaliar a resposta imune inflamatória, bem como marcadores da resposta imune adaptativa, desenvolvida após a infecção em uma coorte de pacientes de 2009, os primeiros a entrarem em contato com o novo vírus pandêmico, no Rio Grande do Sul.

c) Analisar a diversidade genômica das cepas isoladas e compará-las, na busca de alterações gênicas que possam estar envolvidas na severidade da doença.

5. Metodologia

Amostra

Foram analisadas amostras de aspirado nasofaríngeo de indivíduos com Síndrome Gripal (SG) ou Síndrome Respiratória Aguda Grave (SRAG) notificadas no Rio Grande do Sul e encaminhadas ao LACEN-RS nos anos de 2009 a 2015. As amostras foram colhidas no período de 3 a 7 dias após o aparecimento dos primeiros sintomas. Para as amostras de soro colhidas durante o período pandêmico de 2009, com diagnóstico positivo para Influenza A, foi analisada a resposta imunológica, ou seja, citocinas e quimiocinas envolvidas na resposta imune inata e adaptativa. A partir do ano de 2011, as amostras que foram incluídas no estudo foram somente de pacientes hospitalizados com SRAG. Os resultados preliminares com amostras de 2009 e 2011 já foram publicados (Gorini da Veiga e cols., 2012b; Baccine cols., 2013). Para cada amostra foi preenchida a *Ficha de Investigação Influenza Humana* (Ministério da Saúde, 2010), que serve de base para análise dos dados epidemiológicos (CEP-UFCSPA, Par. nº 873/09 de 18/06/2009).

Foi estimado um tamanho amostral de 120 amostras de aspirado nasofaríngeo com diagnóstico positivo para influenza A, colhidas entre 2009 e 2015., correspondendo a um total de 2,5% das amostras que foram positivas para influenza neste período. Estas amostras foram selecionadas aleatoriamente (adultos e crianças) durante o período proposto. Este tamanho amostral foi baseado na frequência do marcador de gravidade da doença, substituição nucleotídica D259G,

(Ferreira e cols., 2011), juntamente com a taxa de mortalidade relacionada à infecção pelo Influenza A (no ano de 2009: 2.1/10000 habitantes) para um nível de significância de 0.05% e um grau de confiança de 0.20 (beta). A figura 5 demonstra geograficamente a proveniência das amostras deste estudo, após a obtenção das sequências genômicas. Seguida à realização das análises laboratoriais previstas neste estudo, as amostras biológicas foram descartadas.

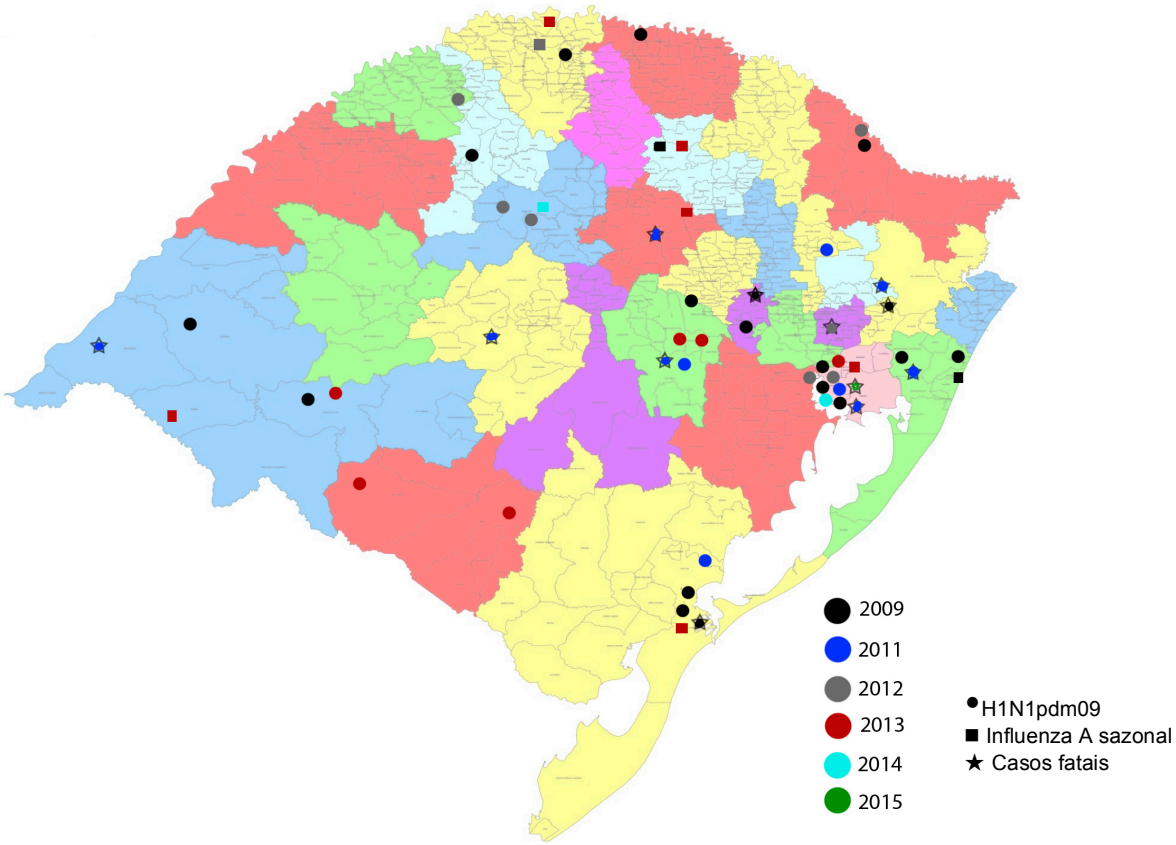

Figura 5. Locais de proveniência das amostras de aspirado de nasofaringe, segundo as regiões do RS e de acordo com os anos de colheita.

Extração de ácidos nucléicos

O RNA viral das amostras de aspirado nasofaríngeo dos pacientes foi extraído com o *QIAamp Viral RNA Mini kit* (Qiagen), de acordo com as

instruções do fabricante.

qRT-PCR

Para a identificação do vírus A(H1N1)pdm09 nas amostras, será utilizado o protocolo padrão do *Center for Disease Control and Prevention* (CDC, EUA), baseado em um conjunto de 4 pares de *primers* e sondas TaqMan® específicos: "InfA" identifica a presença de influenza A; "swInfA" identifica vírus influenza A de origem suína; "swH1" detecta especificamente o subtipo A(H1N1)pdm09; RP amplifica uma região do gene da RNase P de humanos e serve para controle interno da reação (WHO, 2009b).

Para as reações de qRT-PCR foi empregado o *SuperScriptIII Platinum One-Step Quantitative kit* e *Influenza A (H1N1) Primer and Probe Set* (Invitrogen) (WHO, 2009b), cada reação contendo 0,5 µl de SSIII/Platinum *Taq*, 0,5µl de *primer/probe*, 12,5µl 2X Master Mix, 0,05µl de ROX e 5µl de amostra de RNA, em volume total de 25µl. Foi utilizado o termociclador 7500 da Applied Biosystems® nas seguintes condições: 50^{o}C por 30 min e 95^{o}C por 2 min; 45 ciclos de 95^{o}C por 15s e 55^{o}C por 35s.

Quantificação da Carga Viral

Foi realizada a análise da carga viral relativa das amostras através do método $2^{-\Delta CT}$ (Livak & Schmittgen, 2001), com base nos resultados de qRT-PCR, considerando o valor de *Curve Threshold* (*C*T) de cada reação (de cada conjunto de *primers* e sonda. A fim de excluir qualquer possibilidade de variação de valores de *C*T decorrentes das diferenças nas quantidades iniciais de amostra empregadas nas reações, os

valores de *C*T das reações de cada amostra com os *primers* e sonda específicos para influenza A (infA, swInfA e swH1) foram normalizados com base no valor de *C*T da reação com os *primers* e sonda da RP para a respectiva amostra. Desta forma, a RNase P serve como padrão de referência para o cálculo de carga viral relativa.

Sequenciamento de Ácidos Nucleicos

Para obter o genoma completo do vírus das amostras clínicas originais, os RNAv foram sequenciados *de novo* diretamente do aspirado nasofaríngeo através de sequenciamento em massa (*next generation deep-sequencing,* plataforma Illumina® através do Programa de Sequenciamento Viral do J. Craig Venter Institute (JCVI), EUA.

Ensaios de quantificação da resposta imune: perfil de citocinas/quimiocinas

Quarenta e cinco amostras de soro, as quais foram enviadas para o Laboratório Central até a semana epidemiológica 28 de 2009, foram analisadas quanto a concentração de 18 citocinas/quimiocinas, incluindo concentração de GM-CSF, IFN2-α, IFN-γ, IL-10, IL-12 (p40), IL-13, IL-15, β IL1-, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IP-10, MCP-1, MIP1-α, TNF- α. Estes ensaios são multiplex e utilizam microesferas marcadas com fluorocromos e atuam tanto como o identificador, como superfície sólida. As reações são analisadas no equipamento Luminex 200™ baseado em citometria de fluxo, o qual fornece quantificação simultânea em pg/mL. O painel de citocinas/quimiocinas foi selecionado para o estudo com base em outras pesquisas anteriores descritos em uma recente revisão (Okomo-Adhiambo e cols., 2015). Como grupo

controle, soros de 31 indivíduos que não apresentaram sintomas de gripe, foram analisados para o mesmo painel e a mediana dos valores serviram para normalizar as dosagens dos soros dos pacientes.

Análise Estatística

As correlações entre as concentrações do painel de ciotocinas/quimiocinas para os quatro diferentes grupos: controles, pacientes ambulatoriais, pacientes hospitalizados e falecidos graves, foram comparados através do método paramétrico de ClusKal Wallis, inferido no programa SPSS, versão 17. Para analisar perfis entre os grupos, e para a construção de gráficos *dotplot* foi utilizado o programa "*R"*. O teste não-paramétrico de Mann-Whitney U-test foi utilizado para comparar amostras independentes. Para todas as análises estatísticas o valor de $p < 0,05$ foi considerado como indicativo de significância.

6. Artigo científico redigido em inglês, a ser submetido ao *Journal of Virology (JVI)*

Cytokines profile in patients infected with influenza A virus and full-genome sequences of strains that circulate in Southern Brazil

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Abstract

Influenza A virus infects the upper respiratory tract of humans, causing acute respiratory illness; some cases may progress to severe lower respiratory-tract complications and death. Pathogenesis of severe cases is associated with a complex interaction between the host and the virus, including, among others, cytokine/chemokine responses and virulence factors. This study analyzed the profile of serum expression of cytokines/chemokines of 45 patients infected during the 2009 pandemic according to severity, and the mutations spectrum of 56 full genome influenza A virus along the 2009–2015 seasons. Results suggest that there is a profile of inflammatory responses associated to diseases severity, represented by IL-8, IL-4, IL15 and TNF-α. The amino acid substitution analysis reflect a constant evolution of all segments, mainly those coding for the HA, NA and PA proteins. No resistance mutations in NA were found and there was no association between any specific genotype and death. Nevertheless, mutations observed only in some fatality cases might be associated with disease outcome, such as HA-D239G, which was found in two of nine fatality cases. Specific mutations that characterized the different seasons were observed, as well as an accumulation of mutations mainly in the 2013 strains. Results point to the importance of evaluating severity markers in order to better conduct treatment of patients, as well as disease control and prevention.

Keywords: Influenza A virus, cytokines/chemokines, viral mutations, molecular epidemiology.

Introduction

Seven years after the last influenza pandemic, we are still trying to determine the key factors that influence the transmissibility and pathogenesis of the 2009 pandemic influenza A virus (IAV) – (H1N1)pdm09 – in order to develop more accessible diagnostic tests, investigate more effective vaccines, and other treatment interventions based on a better understanding of the dissemination and evolution of influenza virus.

IAV infection usually lasts around one week and is characterized by a sudden onset of high fever, chills, cough, nasal obstruction, headache, sore throat and rhinitis. Most influenza cases are limited to viral replication in the upper respiratory tract, and in such cases symptoms arise from the induction of inflammation after the onset of local viral infection [1]. Complications from IAV infection are usually more common in people who have comorbidities, such as chronic heart or lung disease, asthma, immunosuppression and diabetes mellitus. Risk factors such as age – less than 2 years old and over 65 years old – and clinical conditions such as pregnancy and obesity have also been related to the severity of influenza [2].

IAV may disrupt the microbiome in the upper respiratory tract, promoting the proliferation of pathogenic bacteria. Complications begin when the infection reaches the epithelium of the lower respiratory tract, causing severe injury to the alveolar epithelium that eventually result in acute respiratory distress syndrome (ARDS), which may be fatal.

Infected epithelial cells produce cytokines that attract white blood cells, neutrophils and macrophages and activate adjacent endothelial cells, causing cell injury and triggering apoptosis [3]. Most of the pulmonary disorder is associated with the release of high levels of cytokines and other pro-inflammatory mediators [4]. The CD8+ T lymphocytes (CTLs) specific against influenza play a key role in the elimination of host cells infected with influenza virus in the lung through two well-defined effector activities: antigen-specific cytotoxicity and cytokines/chemokines. Traditionally, IFN-γ, TNF-α and IL2 are the most prominent effector cytokines produced by CTLs.

IFN-γ is a potent antiviral agent; it increases the cytotoxicity of other cells in the immune system, promotes activation of dendritic cells (DCs) and helps B cells in promoting antibody isotype switching. TNF-α is mainly a pro-inflammatory cytokine; it induces the nonspecific death of infected cells and regulates the function of other immune cells via TNFRIL-10 is typically produced by regulatory CD4+ T cells and/or CD4+ T helper cells; it is usually recognized as an immune anti-inflammatory regulating cytokine, which serves as a regulator of the ongoing inflammation [5].

In a study by Gao et al., 2009, high levels of seven proteins were found in the lungs of fatal H1N1 influenza cases in 2009: IL1RA, IL6, TNF-α, IL8, MCP1, MIP1-β and IP10 [6]. Another similar study by To et al., 2009, found high plasma levels of G-CSF, TNF-α, IL1-α, IL6, IL10, IL15, IP10 and MCP1 in patients who developed acute respiratory distress syndrome (ARDS) [7]. High levels of IP10, MCP1 and MIP1-β were found in a study that evaluated groups

of hospitalized patients and in severe cases of IAV infection. Also, high levels of IL8, IL9, IL17, IL6, TNF- α, IL15, and IL12p7 were found in patients that were hospitalized, and IL6, IL12, and IL15 were considered markers of severe disease [8].

The pathogenesis of IAV infection is not restricted to host factors, since virulence factors have been also associated with changes in the viral genome. Such changes occur primarily in the genes of the immunogenic viral envelope glycoproteins, HA and NA, responsible for the annual appearance of epidemics and influenza outbreaks; nevertheless, changes in other viral proteins also play a role in pathogenesis. In the case of influenza A (H1N1)pdm09 virus, mutations as HA-D239G and PB2-K340N seem to have an increased occurrence in severe cases [9]. Furthermore, the NA-H275Y mutation is associated with resistance to the antiviral agent amantadine [10].

Genomic comparison with strains that are well established is important to characterize the genetic background of isolates. The seasonality of outbreaks and diversity in relation to disease severity may be associated with viral variants [11]. Therefore, understanding local viral diversity and reconstituting its genetic evolution may be useful for therapeutic approaches and for the development of vaccines. In this study, we examined the profile of the immune response through the levels of cytokines/chemokines in different groups of patients infected with IAV (H1N1)pdm09 in the State of Rio Grande do Sul, Brazil, where there is a high incidence of infections by this virus compared to other regions in Brazil. Additionally, 56 whole genomes of viruses circulating from 2009 to 2015, including (H1N1)pdm09 and H3N2 were assessed for their evolution and mutations related to virulence.

Methods

Study Subjects, Clinical Data, and Biological Samples

We retrospectively studied a group of 45 patients with laboratory-confirmed infection by IAV (H1N1)pdm09, among July and September 2009. Another 56 samples of nasopharyngeal aspirates collected between 2009 and 2015 were included in this study; the patients included in this study from 2011 onwards were only those who met the hospitalization criteria, or severe acute respiratory syndrome (SARS), and who were also infected by influenza A (H1N1)pdm09. For each patient, a clinical form was filled out by the attending physician/nurse, at time of collection [12]. The following data were obtained: demographic characteristics, date of notification, date of onset of symptoms, acute respiratory infection symptoms, comorbidities, pregnancy status and X-ray results (when available). All samples and forms were sent to the State Central Laboratory (LACEN-RS) for analysis.

This study has been approved by the Research Board and Ethics Committees of Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), and HNSC (Ethics Statements n. 1774/12 and n. 14199, respectively). The Committees waived the need for written informed consent from the donors because samples were routinely received for laboratory analyses of influenza in the State, which has become compulsory in Brazil and all information obtained is for epidemiological surveillance and research purposes. Experiments were

performed in compliance with relevant laws and in accordance with the ethical standard of the Declaration of Helsinki.

Identification and Quantitation of Influenza A Virus

In order to identify the virus, all samples were analyzed by reverse transcription followed by real time polymerase chain reactions (RT-qPCR) at the State Central Laboratory (LACEN-RS) using the SuperScript-III Platinum One-Step Quantitative kit and the Influenza A (H1N1)pdm09 Primer and Probe Set (Invitrogen-Life Technologies, Carlsbad, CA) as described elsewhere [12]. RNA extracted from 101 nasopharyngeal aspirate samples using the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) was used in RT-qPCR. Briefly, reactions were performed using 0.5ml of SSIII/Platinum Taq Mix, 1 mM of each primer, 250nM of probe, 12.5µl 2X Master Mix, 5µl of RNA sample and water, to a final volume of 25µl. All reactions were performed in 7500 Real Time PCR System (Applied Biosystems-Life Technologies, Carlsbad, CA). The following reaction conditions were applied: 50˚C for 30 min; 95˚C for 2 min; 45 cycles at 95˚C for 15s and 55˚C for 35s.

Multi-segment RT-PCR and Sequencing

Fifty six samples with high viral loads (twenty from the pandemic period and 36 from the post-pandemic period) were used for sequencing. RNA was extracted as mentioned above and used as template in 50 µl multi-segment RT-PCR, using Superscript III high-fidelity RT-PCR kit with influenza-specific universal primers complementary to the conserved 12-13 nucleotides at the end of all 8 genomic segments. Primer sequences and final concentrations in the reaction were as follows: Opti1-F1-5' GTTACGCGCCAGCAAAAGCAGG (0.1µM); Opti1-F2-5' GTTACGCGCCAGCGAAAGCAGG (0.1µM); Opti1-R1- 5' GTTACGCGCCAGTAGAAACAAGG (0.2µM). Amplicons were purified with 0.45x volume AMPure XP beads (Beckman Coulter). Next, samples were sent to the Mount Sinai Hospital Sequencing Core for whole genome deep sequencing.

Sequence Alignment and Concatenation

Sequences of IAV strains from Latin America were retrieved from the Influenza Research Database (http://www.fludb.org/). The genomic segments and genes were aligned using software MAFFT [13]. The calculation procedure consists of three stages: (i) all-to-all comparison, (ii) progressive alignment and (iii) iterative refinement. Aligned segment sequences were concatenated using Phyutility software. Amino acid substitutions discussed in this analysis were identified using MEGA 4 package [14]. Additionally, all sequences were analyzed in http://flusurver.bii.a- star.edu.sg/help/faq.html, using the sequences of archetype California/07/2009 as reference.

Phylogenetic Analysis

Phylogenetic analysis was conducted on all strains with full-length nucleotide sequences available for all 8 segments. The study dataset was compared with other sequences of South America obtained in an influenza database (Fludb.org). The phylogenetic tree with all 8 concatenated segments were reconstructed using the maximum-likelihood method implemented in the PhyML program (v3.0 aLRT) [15]. The GTR (general time reversible) substitution model was selected assuming an estimated proportion of invariant sites and four gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data. The reliability of internal branches was assessed using the aLRT test (SH-Like). The phylogenetic trees of each influenza protein where constructed using MEGA 4 package [14].

Structural Modeling of HA and Map of Mutations

A modeling test was performed to show de amino acid changes in the HA and NA molecular structures using a homology method tool in http://swissmodel.expasy.org/interactive using the crystal structure of A/California/07/2009 hemagglutinin as template. Molecular graphics and analyses were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311) [16].

Assays of Serum Concentrations of Cytokines/Chemokines

Forty five serum samples, which were sent to the State Central Laboratory from epidemiologic week 28/2009, together with nasopharyngeal aspirate to perform the influenza subtyping, where assayed for 18 cytokines/chemokines concentration including GM-CSF, IFN2-α, IFN-γ, IL-10, IL12(p40), IL-13, IL-15, IL1- β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IP-10, MCP-1, MIP1-α, TNF-α. These assays use a bead-based multiplex immunology assay-xMAP® microspheres, which are fluorescently dyed 6.45 μm magnetic microspheres that act as both the identifier and the solid surface to build the assay. The reactions are analyzed in Luminex® Analyzer: Luminex 200™ system: flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics and high-speed digital signal processors. Fluorescence flow-cytometry of the beads provides simultaneous quantification in pg/mL of a panel of cytokines/chemokines. These panel was selected for the study based on other previous researches described in a recent review [4]. Normal plasma of 31 individuals where measured to whole panel and served as control group to normalize the data of patients with the median of controls.

Statistical Analysis

The serum concentrations of cytokines/chemokines for all patients were normalized with controls median. Next, all ratio values were transformed into log10. Correlations between these concentrations number of three different groups: outpatients, severe hospitalized and deceased patients, were analyzed using a parametric method, Clustal Wallis 2-sided tests and applied Dunn post-hoc test (correction of Bonferroni). To analyze clusters between groups, we

used Pearson distances with Ward hierarchical clustering, with column and row wise, inferred in R program. Correlations between serum cytokines/chemokines concentration and clinical variables were analyzed using the non-parametric Fisher exact test. Statistical analysis was performed using SPSS software, version 17.0. To construct the dotplot to each parameter we used the R program. The non-parametric test of Mann-Whitney U-test was used to compare independent samples. For all statistic analyses p-value of<0.05 was considered to indicate significance.

Results and Discussion

Clinical Background of Patients: from cytokines/chemokines assays

A total of 45 serum samples of patients diagnosed with influenza A (41 pandemic strains and four H3N2) and 56 complete sequences of Influenza A (H1N1)pdm09 virus (2009 – 2015) were studied. For the 45 patients whose serum were analyzed, the age mean was 34.42 (±17S.D.) years and underlying medical conditions were present in 50% of cases. Gender distribution was not significantly different among groups (male, 42% vs female, 58%). The majority (38, 84.4%) of patients were hospitalized with severe influenza disease, and 7 (18.4%) patients died during hospitalization. Comorbidity and risk factors were present in 40% of patients with available data (Table1). No patient had cancer, autoimmune disease, kidney disease, chronic heart disease or asthma and only three patients received the influenza vaccine (2009 recommendations). Furthermore, 81.2% of patients had X-ray results compatible with pneumonia (data not shown).

Table 1. Distribution of risk factors and comorbidities among groups considering disease severity.

Condition/risk factor (available patient data)	Total (n=45)*	Mild cases (n=7)	Severe cases (n=31)	Deceased (n=7)
Pregnancy (42)	5	1(2.4%)	4(9.5%)	-
Diabetes Mellitus (39)	1	-	1(2.6%)	-
Obesity (39)	2	1(2.6%)	1(2.6%)	-
Immunosuppression (39)	1	-	-	1(2.6%)
Neurological Disease (39)	2	-	1(2.6%)	1(2.6%)
Down´s syndrome (39)	1	-	-	1(2.6%)
COPD[1] (39)	5	-	4(10.2%)	1(2.6%)
Age years (median)	34.4	31.0	30.5	40.5

*Total number of patients included in the study. 1: Chronic Obstructive Pulmonary Disease

Clinical Background of Patients: from full genome sequencing

Among patients whose samples had sequencing of influenza A(H1N1)pdm09 virus genome (n=56), the age median was 22 years old with 12 (21.4%) representing children with less than 2 years and 6 (10.7%) representing over 60 years old patients; underlying medical conditions were present in 19 patients (33.9%). Gender distribution was 25 (44.6%) male vs 31 (55%) female; 4 (7%) were outpatients and 52 (93%) patients were hospitalized with severe clinical picture, of which 9 (17.3%) patients died during hospitalization. The risk group represents 66% of all patients.

Cytokines/Chemokines Responses Influenza A and Clinical Correlations

Comparison of expression levels of each cytokine/chemokine (fold increase in expression related to control group “0”) in the 3 groups of severity, are shown in Figure 1 and Figure 2, and in the heatmap on supplementary material S1. Outpatients (mild cases) were called group 1; group 2 were severe hospitalized patients, group 3 consisted of deceased patients who died during hospitalization due to complications of IAV infection. The cytokines/chemokines response patterns in groups of higher severity, that is hospitalized and deceased, showed increased expression of the pro-inflammatory cytokines IL6 (p=0.025), IL-10 (p=0.000), IL-4 (p=0.004), IL-15 (p=0.020) and TNF-α (p=0.001), and the chemokines IL-8 (p=0.000), IP-10 (p=0.000), MIP1-α (p=0.026) and MCP-1 (p=0.001) than healthy controls. The increased concentrations of these pro-inflammatory cytokines and chemokines are in agreement with other studies which showed equivalent profile correlated with disease severity and outcomes, as reviewed in Ramos et al. [4]. Our study adds that high-expression of another cytokine, IL-4, an inductor of TH2 response, may be a predictor of critical condition (fatal cases), along with TNF-α, IL-8 and IL-15. The latter also has a significant role in ARDS, which is characterized by large influx of neutrophils to the lung during severe influenza [17]. Some of the cytokines released (e.g. IL-6 and IL-10) might further dysregulate or inhibit the adaptive immunity, thus playing a role in disease severity [18].

Types I and II Interferon levels (IFN-α and IFN-γ, respectively) in severity group were similar to healthy control group, though a tendency to lower concentrations of IFN-γ was observed in hospitalized patients (Figure 1). These cytokines play crucial roles in the activation of an antiviral state, in activating the cellular TH1 responses, and regulating B cell functions. The deficit of production of IFN-γ may partly explain the severity of cases of influenza in the study area, which has one of the highest death rates in the country, along with its neighboring states of Santa Catarina and Paraná [19].

Cytokine/chemokine activation was decreased in outpatients (group 1) when compared with the healthy control group, hospitalized and fatal patients (group 0, 2 and 3, respectively), as is the case of IL-4 (p=0.022), IL-8 (p=0.04), TNF-α (p=0.038), IL-15 (p=0.02); notably, all 45 patients presented suppressed expression of IL-5 and GM-CSF, and no expression of IL1- β, IL-2 and IL-12 (p40).

A lower expression of chemokine MCP-1 was independently associated with pregnancy (p=0.024); this chemokine plays an important role in migration and infiltration of

monocytes, memory T cells and NK cells [4]. Patients who are hospitalized showed higher levels of serum TNF-α concentrations than patients who were not hospitalized (p=0.024). Other conditions/comorbidities were less present, therefore no statistical association with cytokines was found. Nevertheless, MCP-1 serum concentrations were found to correlate significantly with cough (p=0.024), MIP1-α was correlated with chills (p=0.041) and sore throat (p=0.024), IL-15 with rhinorrhea (p=0.046) and IL-8 with sore throat (0.039) and dyspnea (p=0.010). This last symptom has been associated with severity and adverse clinical outcome in cases of influenza [20].

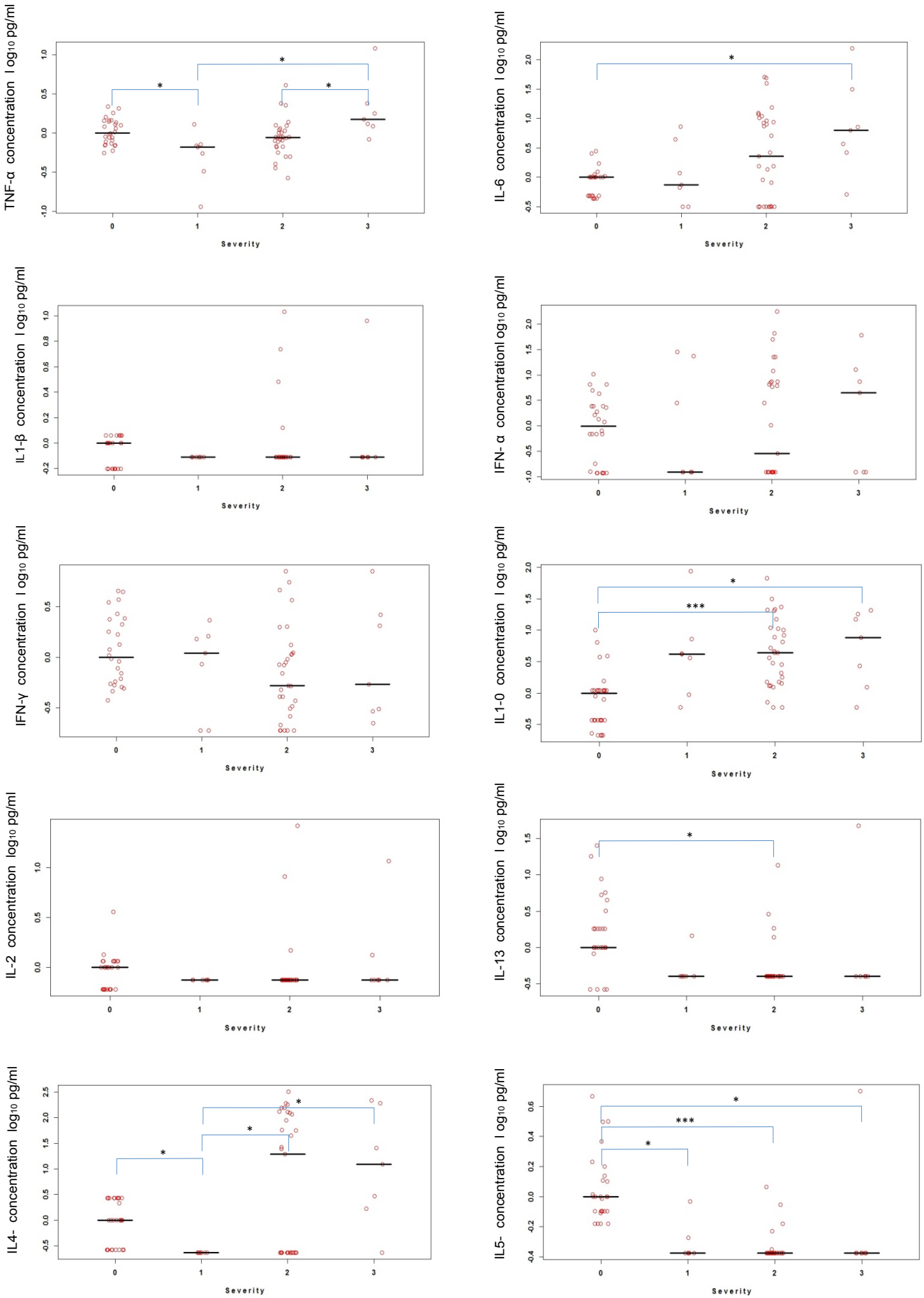


Figure 1. Dot Plots of normalized serum levels (pg/mL) of cytokines TNF-α, IL6β, IL1, IFN-γ, IFN-α, IL-10, IL-2, IL-13, IL-4, IL-5 for patient groups according to disease severity: control (0), outpatients (1), hospitalized severe patients (2) and deceased (3). *P<0.05, **P<0.001, ***P<0.0001: Kluskal Wallis NP test by Dunn´s multiple comparison test.

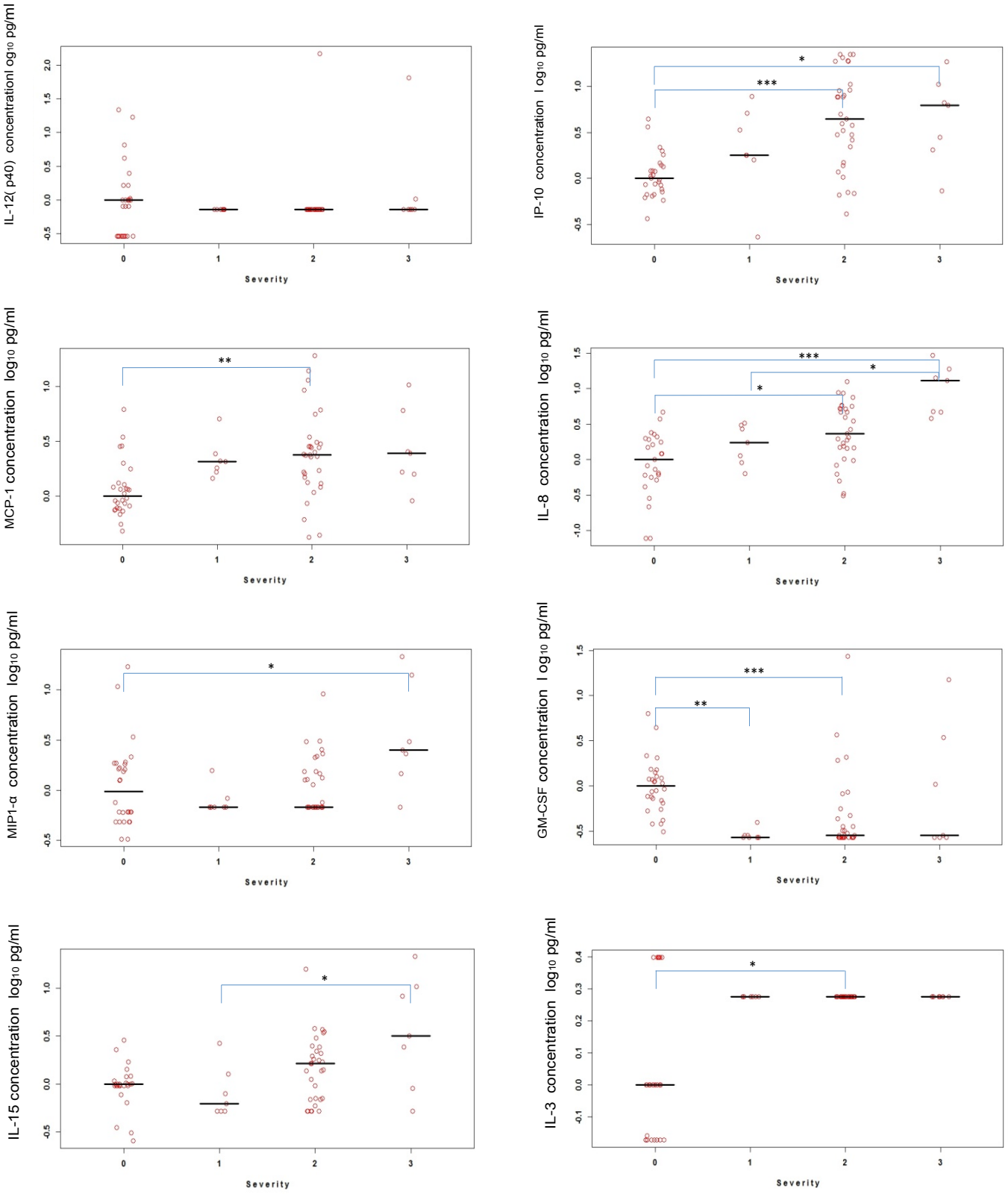


Figure 2. Dot Plots of normalized serum levels (pg/mL) of cytokines/chemokines IL-12 p40, IP-10, MCP-1, IL-8, MIP1-α, GM-CSF, IL-15, IL-3 for the patient groups according to disease severity: control (0), outpatients (1), hospitalized severe patients (2) and deceased (3). *P<0.05, **P<0.001, ***P<0.0001: Kluskal Wallis NP test followed by Dunn´s multiple comparison test.

Phylogenetics of Influenza A Virus in South America along years 2009 and 2015

Since 2009, the South and Southeast regions of Brazil have had the highest number of IAV cases in the country [11]. Brazil is a country that borders many South American countries, with heavy inter-traffic of people. A maximum-likelihood phylogenetic tree of whole genomes of influenza A (H1N1)pdm09 was constructed (Figure 3), including 51 samples sequenced in this study and 200 representative strains from other South American regions circulating during 2009-2015.

All sequences from the 2009 pandemic clustered together, presenting a pattern indicative of rapid increase in genetic diversity in the absence of strong selective pressures. Interestingly, a 2011 virus sequence from a deceased case (female, 48 years old obese patient) was clustered among the 2009 samples and shows an outlier behavior, mainly due to specific amino acid substitutions unique of this sample (Tables 2–8).

Noteworthy, other strains from later years were clustered among the 2009 samples: one sequence of 2014 and three of 2015 are grouped to the strains of the pandemic period. These viruses infected newborns and infant patients from a children's Hospital in Porto Alegre. This finding is interesting because the evolution of the strains of Influenza tends to supplant the strains related to previous epidemics and can change the strategy for composition of the next vaccine. The remaining of strains from past seasons indicates that there is a need for constant surveillance of circulating strains for better orientations regarding the composition of influenza vaccines.

In contrast with the pandemic period, post-pandemic A (H1N1)pdm09 viruses isolated since 2011 have exhibited a ladder-like topology, characteristic of viruses subject to continuous antigenic drift, typical of human seasonal influenza viruses. During this period distinct clusters of isolates from Rio Grande do Sul are observed, according to each season, with more than 80% of isolates within the cluster sampled in this geographical region. This temporal segregation of Brazilian samples may suggest a tendency of positive selection of A (H1N1)pdm09 with better adaptation to propagate than the predecessor strains [21].

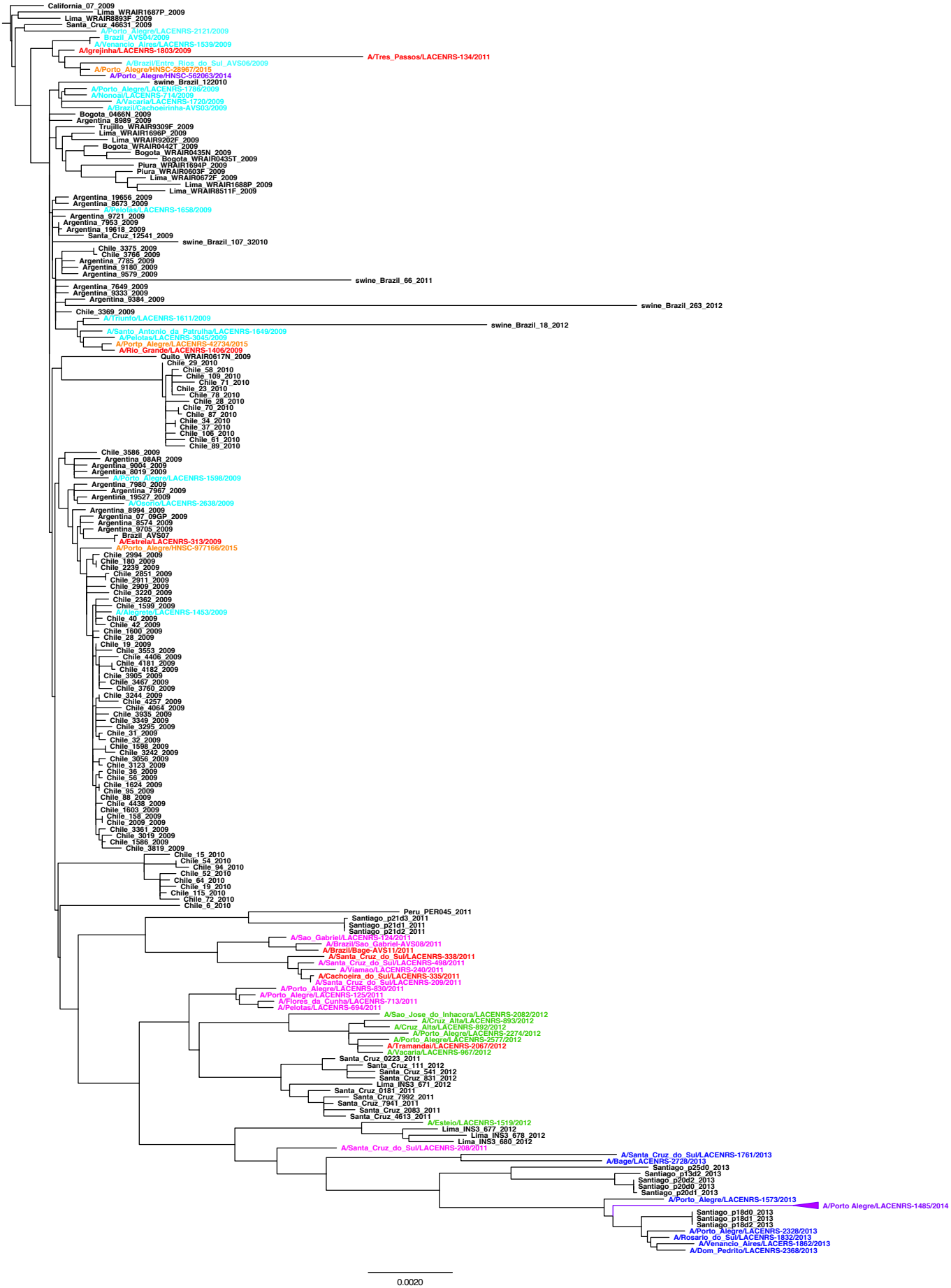


Figure 3. Whole coding genome Mmaximum likelihood phylogenetic tree of IAV whole genome, including sequences from South America and sequences from samples analyzed in study, which are with the study viral strains labeled according to year of appearance to distinguish independent and cluster occurrences. The deceased cases are labeled with in red. Viruses are colour-coded by year of collection as follows: 2009 in cyan, 2011 in magenta, 2012 in blue, 2013 green, 2014 in violet and 2015 in orange. Scale bar represents number of substitutions per site.

Mutation Mapping

The full-genome sequences of the 56 isolates included in this study were compared with the archetype strain A/California/07/2009 in search for mutation distribution patterns as well as the appearance of new mutations along the post-pandemic period, as depicted in Tables 2–8 where sequences of 11 proteins were aligned (HA, NA, PA, PA-X, PB1, PB2, NS1, NS2, NP, M1, M2).

In our study only viruses sequences recovered directly from clinical samples were included, thus avoiding substitutions that might have been introduced by passaging in cultured cells. Detect signs of antigenic drift, genetic and antigenic analyses have been extensively performed for influenza A (H1N1)pdm09 isolates obtained from 2009 to 2013 [22-24]. These authors found various mutations at the antigenic sites of post-pandemic isolates compared with the influenza A (H1N1)pdm09; however, antigenic differences from the A/California/07/2009 vaccine strain were not found when using ferret antiserum against A/California/7/2009 [22;24].

Analysis of amino acid substitutions revealed important differences between strains from the pandemic and the post-pandemic periods for eleven viral proteins, with a significant accumulation of both season markers and single substitutions intra-strain along the years with a higher average of accumulation of substitutions per sites in the year of 2013 ($p<0.05$). Noteworthy, in 2012 and 2013 the mortality rate (MR=0.61 and MR=0.65, respectively) was higher in Rio Grande do Sul when compared to others years (MR average of 0.14 for years 2011, 2014 and 2015) (.

Considering the mutations observed in the genome along the years, all eight segments displayed continuous mutations reflecting constant evolution of the virus. As expected, such evolution is most pronounced for segments 4 and 6, which code for the surface immunogenic glycoproteins HA and NA, respectively (see Table 2 and Figure S1 for HA, Table 3 and Figure S2 for NA; unpublished data).

HA presented 45 substitutions sites in post-pandemic strains versus 17 sites in pandemic strains, and NA presented 30 vs 13 substitutions sites, respectively. Of 33 sequenced samples from the post-pandemic period, 31 (93.9%) had the amino acid substitution HA-E391K, and 27 (81.8%) had the HA-D114N substitution (Table 2). These substitution sites are epitopes of antibody recognition. Changes in these amino acids have been found to confer a replicative advantage in mouse bronchioles, but its effect in the pandemic strain is yet unknown [25]. Other important HA mutations are highlighted in Table 2; among these, 6 are located in antigenic sites Ca2 (P154S, A156D, D239G), Sa (K180N/I, S200P) and Sb (S202P). Changes in these sites have been reported in the literature, suggesting an association to antigenic drifts and generation of escape mutants [26]. Interestingly, mutations in Sa K180Q/I co-occurred with Sb S202T and D114N; all these sites are antibody receptor-sites and can generate a escape mutant, that is, the mutant HA can no longer be recognized by host immune system. [26;27]. The mutation D239D has been found to alter the host cell receptor specificity of HA, so that besides human alpha-2,6 sialic acid it can also bind avian-like alpha-2,3 sialic acid receptors, which are more common in ciliated human cells of the lower respiratory tract [27;28]. This mutant strain was present on two fatal cases in our study (total of 9 fatality cases). These patients had not

received influenza vaccine, one was a 23 years old pregnant patient from the 2009 pandemic period, and the other case was a 48 years old obese patient from 2011. In contrast, this mutation was not founded in the 2012–2015 isolates neither in mild case.

Other mutations found in HA were single sites without previous annotations of virulence or antigenic effects. Among these are amino acid substitutions T277T/D, T298S, T316I, N337S, and A302G. We also found the S92T mutation, which has been associated with host specificity shift in H5N1 influenza A viruses [29]. Our analyses show that different seasons are marked by the appearance of specific mutations, such as I233V and V266L in 2011; V6A, N55D, V190I and N277T/D in 2012; K180Q/I, A273T, K300E and E516K in 2013. All mutations that characterized the season strains and/or the grouping in the phylogenetic tree of HA, and also its locations in the molecular structure of the protein are depicted in Figure S1 (A) and (B), respectively.

Comparison of the NA gene of influenza A(H1N1)pdm09 viruses from post-pandemic years with the vaccine strain shows amino acid substitutions V241I and N369K (Table 3 and S3). These mutations are predicted to compensate for the negative effects of the H275Y amino acid substitution [30]. Four amino acid changes characterized the 2013 season viruses: I34V, N200S, I321V and K432E. In turn, 2012 was characterized by one substitution site, the I467V, and 2011 viruses had 2 distinguished substitution sites, S95N and I193V, although only 40% of the strains had these markers. Most of the post-pandemic period strains carried the N44S mutation, which creates or loses a putative new potential N-glycosylation site; modification that changes glycosylation motifs may also affect antigenicity and other properties of NA [30]. More over, one 2011 strain and one 2013 strain had a mutation in the antigenic site 222, with the substitution of Asparagine to Aspartic Acid; this mutation has been shown to evolve during generation of escape mutants of IAV H3N2 [31]. Another important mutation found in two strains of 2012 and 2013 seasons was N386K, a permissive mutation related with the glycosylation motif, and accessible on the protein surface [32]. In a local context, this mutation is unique for our study region. This mutation had been detected in others countries like Australia and Japan, but not in the Americas [30]. The various single intra-strain mutations are depicted in Table 3. Mutations associated with drug resistance (p.e. H275Y and I223R) were not found in the samples analyzed in this study. The mutations that characterized each seasons strains and/or the grouping in the phylogenetic tree, based on NA sequences, and also its locations in protein structure are depicted in fig S2 (A) and (B), respectively.

Tables 4–8 depict amino acid substitutions observed in the other 9 IAV proteins based on sequences obtained from the samples. Notably, some amino acid substitutions are shared by strains from the post-pandemic period (shown in gray boxes), suggesting they were well established in the population, while other amino acid substitutions are markers of viruses from a specific season.

The polymerase protein PA, which is one of the determinants of viral replicative capacity, showed a total of 36 amino acid substitutions among the post-pandemic strains versus 9 amino acid substitutions sites in the pandemic period. Mutations V100I and K361R characterized the 2013 viruses, while H297Y and P400L characterized the 2012 viruses; the

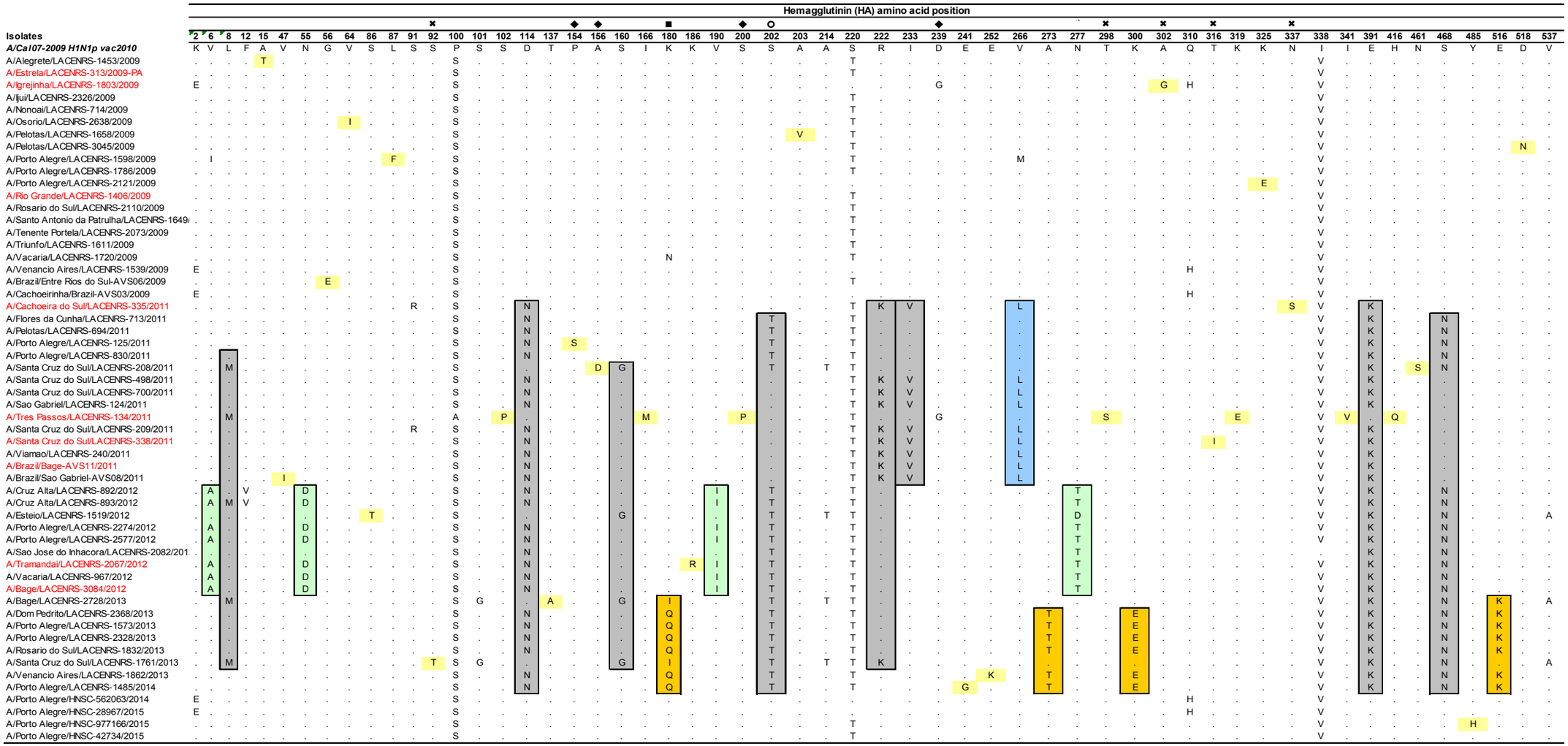
2011 viruses had 2 distinguished substitutions sites, V14I and S225G. The latter, along with site 400 of the 2012 strains and a combination of other mutations have been associated with virulence; for example, L400S is a host-specificity marker and S225G is a T-cell epitope presented by MHC-II molecules [33;34]. In the PA-X protein, the 2011 viruses had the substitution V14I, and the 2013 viruses had V100I, which is related to be a marker of adaptation to the host in pandemic strains [33]. Unique mutations in the PB1 protein were seen only in the 2011 viruses: V113A, K327R and I606V. Overall, persistent season markers are typically found in RNA replication complex proteins and can be located in protein binding domains. These regions may directly influence the RNP replication complex, or they may enhance replication through the interaction with host factors.

As mentioned above, 2013 was the year with the highest number of accumulated mutations (data not shown). Among the markers for strains from this year are the amino acid substitutions R54K and M66I in the PB2 protein; E55K, K131E and N2015S in NS1; N29S and T48A in NEP; A22T and S483N in NP; M192V and K230R in M1; and D21G in M2.

No amantadine-sensible strain was found among the samples. Furthermore, there was no association of specific mutations with the fatal cases in our study, but some mutations were strain-specific. Indeed some single mutations present in fatal-cases are worth of investigation as to their phenotype, such as HA-T298S, HA-A302G, HA-T316I, HA-N337N; NA-S364G, NA-I195V; PB2-K147T, NS1-I128M and M1-T150I.

There are many mutation sites in the genomic dataset, both season marker or single intra-strain markers, that have not yet been tested for biological effects, and their role in infectivity and virulence should be clarified. Also relevant is the fact of mutations occurring in combination and/or being unique to specific influenza seasons, which should also be further analyzed. This sporadic enrichment of amino acids present in human-hosted viruses may indicate that influenza A(H1N1)pdm09 have made modest adaptations to their new hosts in the recent history, but may have become even more virulent over the years.

Table 2. Amino acid substitutions in sequences of the hemagglutinin (HA) protein of the samples in relation to the archetype strain A/California/07/2009



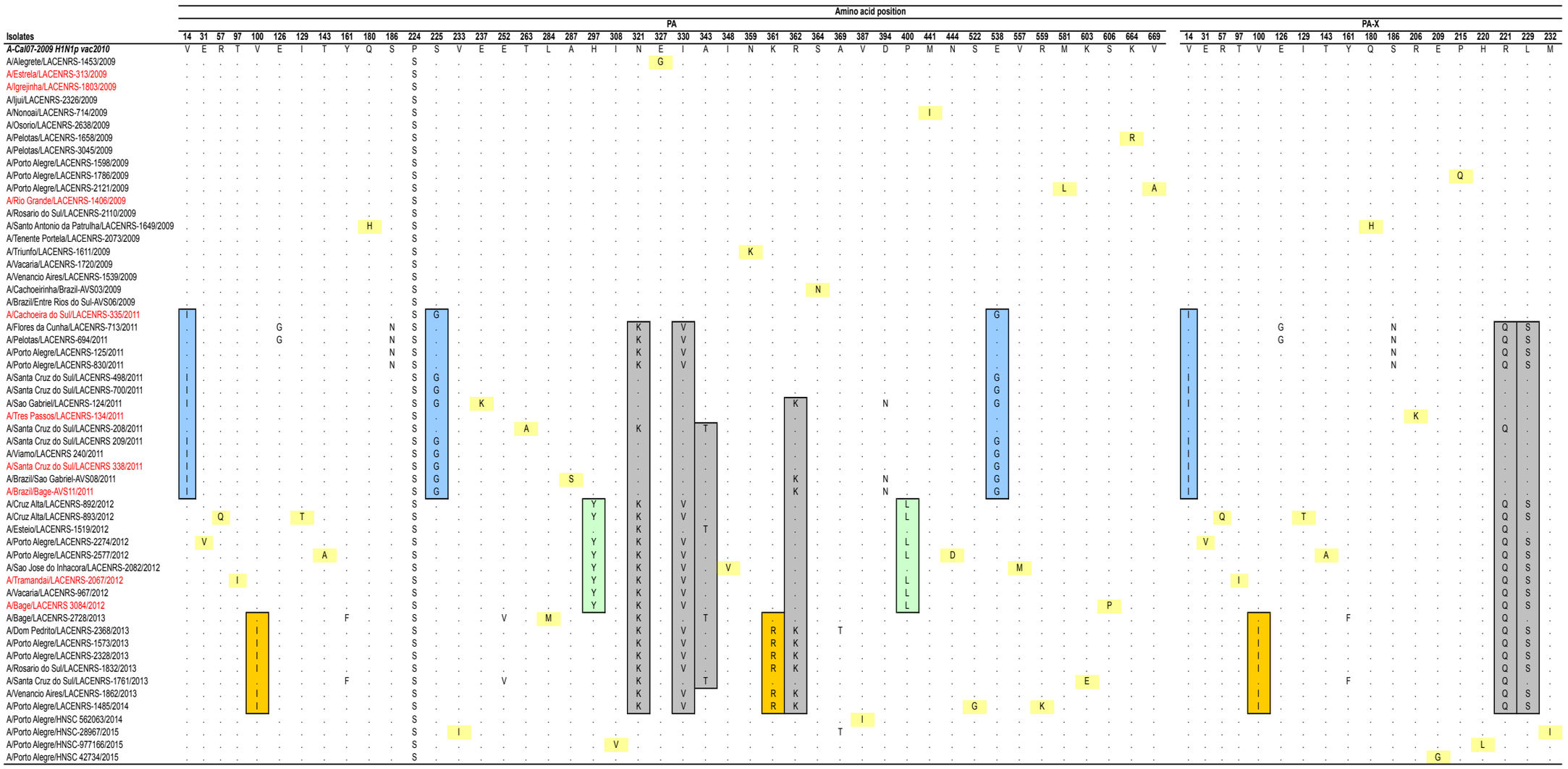
Alignment of the hemagglutinin (HA) vs the archetype sequence A/California/07/2009. Residues within antigenic sites are market with "◆" to Ca2,"■" to Sa and "O" to Sb. The taxa in red are deceased cases. The mutations that characterized the post-pandemic period are shaded in grey, the mutations consensus for 2011 season is blue, for 2012 are green and 2013 are range. The intra-strain single mutations are yellow, and the mutations marked with "✖" are the local unique mutations

Table 3. Amino acid substitutions in sequences of the neuraminidase (NA) protein of the samples in relation to the archetype strain A/California/07/2009.

	Neuraminidase (NA) amino acid position																																							
Isolates	**16**	**26**	**34**	**40**	**41**	**42**	**43**	**44**	**57**	**64**	**79**	**81**	**83**	**88**	**95**	**106**	**127**	**157**	**188**	**193**	**195**	**200**	**222**	**241**	**248**	**264**	**269**	**284**	**321**	**322**	**339**	**364**	**366**	**369**	**386**	**396**	**399**	**432**	**449**	**466**	**467**
A/Cal07-2009H1N1pvac2010-NA	T	I	I	L	G	N	Q	N	E	Q	S	V	V	N	S	V	L	T	I	I	I	N	N	V	N	V	M	D	I	F	S	S	S	N	N	I	W	K	N	T	I
A/Alegrete/LACENRS-1453/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Estrela/LACENRS-313/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.
A/Igrejinha/LACENRS-1803/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Ijui/LACENRS-2326/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	L	.	.	.	.	.	.	.	.	.	I	.
A/Nonoai/LACENRS-714/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Osorio/LACENRS-2638/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Pelotas/LACENRS-1658/2009	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Pelotas/LACENRS-3045/2009	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-1598/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-1786/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-2121/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Rio Grande/LACENRS-1406/2009	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Rosario do Sul/LACENRS-2110/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Santo Antonio da Patrulha/LACENRS-1649/2009	.	M	.	.	.	.	R	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Tenente Portela/LACENRS-2073/2009	.	.	.	.	.	.	.	.	.	.	.	.	M	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Triunfo/LACENRS-1611/2009	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Vacaria/LACENRS-1720/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Venacio Aires/LACENRS-1539/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Cachoeirinha/Brazil-AVS03/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Brazil/Entre Rios do Sul-AVS06/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Cachoeira do Sul/LACENRS-335/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	I	.	.	.	V	.	.	.	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.
A/Flores da Cunha/LACENRS-713/2011	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.
A/Pelotas/LACENRS-694/2011	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-125/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-830/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS-498/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	I	.	.	.	V	.	.	D	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS-700/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	I	.	.	.	V	.	.	.	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.
A/Sao Gabriel/LACENRS-124/2011	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	I	.	A	.	.	.	.	.	I	D	.	.	.	.	.	.	.	.	K	.	M	.	.	.	.	.
A/Tres Passos/LACENRS-134/2011	.	.	.	I	.	.	.	.	.	.	.	.	A	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
A/Santa Cruz do Sul/LACENRS-208/2011	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS-209/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	I	.	.	.	V	.	.	.	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.
A/Viamao/LACENRS-240/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	I	.	.	.	V	.	.	.	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS-338/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	I	.	.	.	V	V	.	.	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.
A/Brazil/Sao Gabriel-AVS08/2011	.	.	.	I	.	.	.	.	.	.	.	A	.	.	.	I	.	A	.	.	.	.	.	I	D	.	.	.	.	.	.	.	.	K	.	M	.	.	.	.	.
A/Brazil/Bage-AVS11/2011	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	I	.	A	.	.	.	.	.	I	D	.	.	.	.	.	.	.	.	K	.	M	.	.	.	.	.
A/Cruz Alta/LACENRS-892/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	I	D	.	.	.	.	.	.	.	.	K	K	.	.	.	.	.	V
A/Cruz Alta/LACENRS-893/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	I	D	I	.	.	.	.	L	.	.	K	.	.	.	.	.	.	V
A/Esteio/LACENRS-1519/2012	.	.	.	.	R	.	.	S	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-2274/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	V
A/Porto Alegre/LACENRS-2577/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	V
A/Sao Jose do Inhacora/LACENRS-2082/2012	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	I	.	.	T	.	.	.	.	I	D	.	.	.	.	.	.	.	N	K	.	.	.	.	.	.	V
A/Tramandai/LACENRS-2067/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	V
A/Vacaria/LACENRS-967/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	V
A/Bage/LACENRS-3084/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	V
A/Bage/LACENRS-2728/2013	.	.	.	V	.	.	.	S	.	L	P	.	.	.	.	.	.	.	.	.	.	S	D	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.
A/Dom Pedrito/LACENRS-2368/2013	.	.	V	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	I	D	.	.	.	V	.	.	.	.	K	.	.	.	E	.	.	.
A/Porto Alegre/LACENRS-1573/2013	.	.	V	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	I	D	.	.	.	V	.	.	.	.	K	.	.	.	E	.	.	.
A/Porto Alegre/LACENRS-2328/2013	.	.	V	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	I	D	.	.	.	V	.	.	.	.	K	.	.	.	E	.	.	.
A/Rosario do Sul/LACENRS-1832/2013	.	.	V	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	I	D	.	.	.	V	.	.	.	.	K	.	.	.	E	.	.	.
A/Santa Cruz do Sul/LACENRS-1761/2013	.	.	.	V	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	I	D	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.
A/Venacio Aires/LACENRS-1862/2013	.	.	V	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	I	D	.	.	.	V	.	.	.	.	K	.	.	.	E	.	.	.
A/Porto Alegre/LACENRS-1485/2014	.	.	V	I	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	I	D	I	.	.	V	.	.	.	.	K	K	.	R	E	.	.	.
A/Porto Alegre/HNSC-562063/2014	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/HNSC-28967/2015	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/HNSC-977166/2015	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/HNSC-42734/2015	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Alignment of the protein neuraminidase (NA) strains with the sequence of archetype strain A/California/07/2009. The taxa in red are deceased cases. The mutations that characterized the post pandemic period are shaded in grey. The mutations consensus for 2011 season is blue, for 2012 are green, 2013 orange. The intra-strain single mutations are yellow.

Table 4. Amino acid substitutions in sequences of the polymerase basic protein PA and PA-X proteins of the samples in relation to the archetype strain A/California/07/2009.



Alignment of the protein acid PA and PA-X of isolates with the sequence of archetype strain A/California/07/2009. The taxa in red are deceased cases. The mutations that characterized the post pandemic period are gray. The mutations consensus of the 2011 strains is blue, for 2012 are green and for 2013 are orange. The intra-strain single mutations are yellow.

Table 5. Amino acid substitutions found in sequences of polymerase basic protein PB1 of the samples in relation to the archetype strain A/California/07/2009

	Amino acid position PB-1																																				
Isolates	**14**	**20**	**80**	**97**	**111**	**113**	**154**	**164**	**171**	**197**	**205**	**260**	**317**	**327**	**363**	**374**	**375**	**384**	**393**	**397**	**435**	**451**	**456**	**529**	**563**	**566**	**587**	**606**	**618**	**619**	**637**	**646**	**654**	**667**	**698**	**715**	**753**
A-Cal07-2009 H1N1p vac2010	A	T	S	E	M	V	G	I	M	K	I	R	M	R	K	A	S	S	R	I	I	V	H	V	R	T	V	I	D	D	I	M	S	I	K	V	L
A/Alegrete/LACENRS-1453/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Estrela/LACENRS-313/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.
A/Igrejinha/LACENRS-1803/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Ijui/LACENRS-2326/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Nonoai/LACENRS-714/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Osorio/LACENRS-2638/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Pelotas/LACENRS-1658/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Pelotas/LACENRS-3045/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	N	.	.	.	.
A/Porto Alegre/LACENRS-1598/2009	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-1786/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-2121/2009	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Rio Grande/LACENRS-1406/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
A/Rosario do Sul/LACENRS-2110/2009	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Santo Antonio da Patrulha/LACENRS-1649/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
A/Tenente Portela/LACENRS-2073/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Triunfo/LACENRS-1611/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	.	.	.	.	.	.	.	.	.	.
A/Vacaria/LACENRS-1720/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Venancio Aires/LACENRS-1539/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Cachoeirinha/Brazil-AVS03/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Brazil/Entre Rios do Sul-AVS06/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Cachoeira do Sul/LACENRS-335/2011	.	.	.	.	.	A	.	.	.	.	.	.	.	.	R	.	.	.	.	.	T	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.
A/Flores da Cunha/LACENRS-713/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.
A/Pelotas/LACENRS-694/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.
A/Porto Alegre/LACENRS-125/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	F
A/Porto Alegre/LACENRS-830/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.
A/Santa Cruz do Sul/LACENRS-498/2011	.	.	.	.	.	A	.	.	.	.	.	.	.	.	R	.	.	.	.	.	T	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS-700/2011	.	.	.	.	.	A	.	.	.	.	.	.	.	.	R	.	.	.	.	.	T	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.
A/So Gabriel/LACENRS 124/2011	.	.	.	.	.	A	.	.	.	.	.	.	.	.	R	.	.	.	.	.	T	.	.	M	.	.	.	V	.	.	.	.	.	.	.	.	.
A/Tres Passos/LACENRS-134/2011	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS-208/2011	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	M	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	F
A/Santa Cruz do Sul/LACENRS 209/2011	.	.	.	.	.	A	.	.	.	.	.	.	.	.	R	.	.	.	.	.	T	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.
A/Viamo/LACENRS 240/2011	.	.	.	.	.	A	.	.	.	.	.	.	.	.	R	.	.	.	.	.	T	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS 338/2011	.	.	.	.	.	A	.	.	.	.	.	.	.	.	R	.	.	.	.	.	T	.	.	M	.	.	.	V	.	.	.	.	.	.	.	.	.
A/Brazil/Sao Gabriel-AVS08/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Brazil/Bage-AVS11/2011	.	.	.	.	.	A	.	.	.	R	.	.	.	.	R	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Cruz Alta/LACENRS-892/2012	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
A/Cruz Alta/LACENRS-893/2012	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	M	.
A/Esteio/LACENRS-1519/2012	.	I	.	.	.	.	.	V	.	.	.	.	.	K	.	S	.	.	.	M	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-2274/2012	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.
A/Porto Alegre/LACENRS-2577/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/So Jos do Inhacor/LACENRS-2082/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Tramandai/LACENRS-2067/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Vacaria/LACENRS-967/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Bage/LACENRS 3084/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Bage/LACENRS-2728/2013	.	.	.	.	.	.	D	.	.	.	.	K	.	.	.	.	.	.	.	M	T	.	Y	.	.	.	.	.	G	.	.	.	.	.	.	.	.
A/Dom Pedrito/LACENRS-2368/2013	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	M	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-1573/2013	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	N	.	.	M	T	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.
A/Porto Alegre/LACENRS-2328/2013	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	M	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Rosario do Sul/LACENRS-1832/2013	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	M	T	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS-1761/2013	.	.	.	K	.	.	D	.	.	.	.	.	.	.	.	.	.	P	.	M	T	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Venancio Aires/LACENRS-1862/2013	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	M	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-1485/2014	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	M	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/HNSC 562063/2014	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/HNSC-28967/2015	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/HNSC-977166/2015	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
A/Porto Alegre/HNSC 42734/2015	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.

Alignment of the protein basic PB1 of isolates with the sequence of archetype strain A/California/07/2009 The taxa in red are deceased cases. Mutations that characterized the post-pandemic period are shaded in grey; mutations common only in 2011 viruses are blue. The intra-strain single mutations are shown in yellow.

Table 6. Amino acid substitutions found in sequences of PB2 protein of the samples in relation to the archetype strain A/California/07/2009

	Amino acid position PB-2																																									
Isolates	12	23	54	60	65	66	82	90	94	107	141	154	157	164	184	191	192	195	251	266	293	297	299	303	339	344	354	394	402	405	411	451	453	456	480	507	526	559	667	680	716	731
A-Cal07-2009 H1N1p vac2010	S	T	R	D	D	M	N	M	L	S	I	L	K	M	A	E	E	D	R	I	R	I	R	T	K	V	I	I	M	S	I	I	S	N	V	Q	K	I	V	D	L	V
A/Alegrete/LACENRS-1453/2009	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Estrela/LACENRS-313/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.
A/Igrejinha/LACENRS-1803/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.
A/Ijui/LACENRS-2326/2009	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Nonoai/LACENRS-714/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Osorio/LACENRS-2638/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Pelotas/LACENRS-1658/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Pelotas/LACENRS-3045/2009	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-1598/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-1786/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-2121/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.
A/Rio Grande/LACENRS-1406/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Rosario do Sul/LACENRS-2110/2009	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.
A/Santo Antonio da Patrulha/LACENRS-1649/	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Tenente Portela/LACENRS-2073/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Triunfo/LACENRS-1611/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Vacaria/LACENRS-1720/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Venancio Aires/LACENRS-1539/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Cachoeirinha/Brazil-AVS03/2009	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Brazil/Entre Rios do Sul-AVS06/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
A/Cachoeira do Sul/LACENRS-335/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	M	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Flores da Cunha/LACENRS-713/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	M	L	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.
A/Pelotas/LACENRS-694/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	M	L	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-125/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	M	L	.	.	.	.	.	.	S	.	.	.	.	.	.	F	.
A/Porto Alegre/LACENRS-830/2011	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	M	L	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS-498/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	M	L	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS-700/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	M	L	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.
A/So Gabriel/LACENRS 124/2011	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Tres Passos/LACENRS-134/2011	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	T	.	.	.	.	.	.	.	.	.	.	.	I
A/Santa Cruz do Sul/LACENRS-208/2011	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	K	N	.	.	K	.	.	.	.	M	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
A/Santa Cruz do Sul/LACENRS 209/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	M	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Viamo/LACENRS 240/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	M	L	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS 338/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	M	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Brazil/Sao Gabriel-AVS08/2011	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Brazil/Bage-AVS11/2011	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Cruz Alta/LACENRS-892/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	M	L	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.
A/Cruz Alta/LACENRS-893/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	K	.	.	M	L	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.
A/Esteio/LACENRS-1519/2012	T	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	M	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-2274/2012	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	M	L	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-2577/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	R	M	L	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.
A/So Jos do Inhacor/LACENRS-2082/2012	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	K	.	.	M	L	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.
A/Tramandai/LACENRS-2067/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	M	L	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.
A/Vacaria/LACENRS-967/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	M	L	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.
A/Bage/LACENRS 3084/2012	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	K	.	.	M	L	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.
A/Bage/LACENRS-2728/2013	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	K	.	.	.	.	M	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
A/Dom Pedrito/LACENRS-2368/2013	.	.	K	.	.	I	.	.	.	.	.	.	.	.	.	.	.	N	.	.	K	.	.	.	.	M	L	.	.	.	.	.	.	.	.	.	.	.	I	.	.	I
A/Porto Alegre/LACENRS-1573/2013	.	.	K	.	.	I	.	.	.	.	.	.	.	.	.	.	.	N	.	.	K	.	.	.	.	M	L	T	.	.	.	.	.	.	.	.	.	.	.	.	.	I
A/Porto Alegre/LACENRS-2328/2013	.	.	K	.	.	I	.	.	.	.	.	.	.	.	.	.	.	N	.	.	K	.	.	.	.	M	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
A/Rosario do Sul/LACENRS-1832/2013	.	.	K	.	.	I	.	.	.	.	.	.	.	.	.	.	.	N	.	.	K	.	.	.	.	M	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
A/Santa Cruz do Sul/LACENRS-1761/2013	.	.	.	.	.	.	.	.	M	.	.	.	.	.	.	K	.	N	.	.	K	.	.	.	.	M	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
A/Venancio Aires/LACENRS-1862/2013	.	.	K	.	.	I	.	.	.	.	.	.	.	.	.	.	.	N	.	.	K	.	.	.	.	M	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
A/Porto Alegre/LACENRS-1485/2014	.	.	K	.	.	I	.	.	.	.	.	.	.	.	.	.	.	N	.	.	K	.	.	.	.	M	L	.	.	.	.	.	P	.	.	.	.	.	.	.	.	I
A/Porto Alegre/HNSC 562063/2014	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/HNSC-28967/2015	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/HNSC-977166/2015	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/HNSC 42734/2015	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Alignment of the protein basic PB2 of isolates with the sequence of archetype strain A/California/07/2009. The taxa in red are deceased cases. The mutations that characterized the post-pandemic period are gray; mutations common only in 2013 viruses are in orange. The intra-strain single mutations are shown in yellow.

Table 7. Amino acid substitutions found in sequences of non-structural proteins NS1 and NS2, and of nucleoprotein NP of the samples in relation to the archetype strain A/California/07/2009.

	Amino acid position																																							
	NS1																		NEP								NP													
Isolates	**2**	**55**	**90**	**91**	**98**	**111**	**122**	**123**	**128**	**129**	**131**	**137**	**149**	**173**	**178**	**202**	**205**	**219**	**2**	**22**	**29**	**48**	**67**	**81**	**89**	**115**	**22**	**34**	**100**	**105**	**119**	**122**	**126**	**152**	**256**	**297**	**373**	**425**	**483**	**498**
A-Cal07-2009 H1N1p vac2010	D	E	L	S	M	I	A	I	I	V	K	I	A	D	V	A	N	K	D	G	N	T	E	E	A	A	A	G	V	M	V	L	G	R	L	S	T	V	N	S
A/Alegrete/LACENRS-1453/2009	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Estrela/LACENRS-313/2009	.	.	.	.	I	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Igrejinha/LACENRS-1803/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Ijui/LACENRS-2326/2009	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Nonoai/LACENRS-714/2009	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Osorio/LACENRS-2638/2009	.	.	.	Y	.	.	.	V	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Pelotas/LACENRS-1658/2009	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Pelotas/LACENRS-3045/2009	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-1598/2009	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-1786/2009	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-2121/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Q	.	.	.	.	I	.	.	.
A/Rio Grande/LACENRS-1406/2009	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Rosario do Sul/LACENRS-2110/2009	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Santo Antonio da Patrulha/LACENRS-1649/2009	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Tenente Portela/LACENRS-2073/2009	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Triunfo/LACENRS-1611/2009	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Vacaria/LACENRS-1720/2009	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Venancio Aires/LACENRS-1539/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	E	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Cachoeirinha/Brazil-AVS03/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	I	.	.	Q	.	.	.	.	.	.	T	.
A/Brazil/Entre Rios do Sul-AVS06/2009	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Cachoeira do Sul/LACENRS-335/2011	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	I	.	I	Q	.	.	.	.	.	.	.	.
A/Flores da Cunha/LACENRS-713/2011	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Pelotas/LACENRS-694/2011	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-125/2011	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-830/2011	N	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	I	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS-498/2011	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS-700/2011	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Sao Gabriel/LACENRS-124/2011	.	.	.	.	.	.	V	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Tres Passos/LACENRS-134/2011	.	.	.	.	.	.	.	V	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	S	I	.	.	Q	.	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS-208/2011	.	.	I	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS-209/2011	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	I	.	I	Q	.	.	.	.	.	.	.	.
A/Viamo/LACENRS-240/2011	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS-338/2011	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Brazil/Sao Gabriel-AVS08/2011	.	.	.	.	.	.	V	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Brazil/Bage-AVS11/2011	.	.	.	.	.	.	V	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Cruz Alta/LACENRS-892/2012	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Cruz Alta/LACENRS-893/2012	.	.	.	.	.	.	.	V	.	L	.	.	.	.	.	.	.	E	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Esteio/LACENRS-1519/2012	.	.	I	.	.	.	.	V	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	S	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-2274/2012	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-2577/2012	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Sao Jose do Inhacora/LACENRS-2082/2012	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Tramandai/LACENRS-2067/2012	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Vacaria/LACENRS-967/2012	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Bage/LACENRS-3084/2012	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Bage/LACENRS-2728/2013	.	.	I	.	.	T	V	V	.	.	.	.	.	N	.	.	S	.	.	.	.	A	K	.	T	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	N
A/Dom Pedrito/LACENRS-2368/2013	.	K	I	.	.	.	.	V	.	.	E	.	.	.	.	.	S	.	.	.	S	A	.	K	.	.	T	.	I	.	.	Q	.	.	.	.	.	.	.	N
A/Porto Alegre/LACENRS-1573/2013	.	K	I	.	.	.	.	V	.	.	E	.	.	.	.	.	S	.	.	.	S	A	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	I	.	N
A/Porto Alegre/LACENRS-2328/2013	.	K	I	.	.	.	.	V	.	.	E	.	.	.	.	.	S	.	.	.	S	A	.	.	.	.	T	.	I	.	.	Q	.	.	.	.	.	.	.	N
A/Rosario do Sul/LACENRS-1832/2013	.	K	I	.	.	.	.	V	.	.	E	.	.	.	.	.	S	.	.	.	S	A	.	.	.	.	T	.	I	.	.	Q	.	.	.	.	.	.	.	N
A/Santa Cruz do Sul/LACENRS-1761/2013	.	.	I	.	.	T	.	V	.	.	.	T	.	.	.	.	S	.	.	.	.	A	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	N
A/Venancio Aires/LACENRS-1862/2013	.	K	I	.	.	.	.	V	.	.	E	.	.	.	.	.	S	.	.	.	S	A	.	.	.	.	T	.	I	.	.	Q	.	.	.	.	.	.	.	N
A/Porto Alegre/LACENRS-1485/2014	.	K	I	.	.	.	.	V	.	.	E	.	.	.	.	.	S	.	.	.	S	A	.	.	.	.	T	.	I	T	.	Q	.	.	.	.	.	.	.	N
A/Porto Alegre/HNSC-562063/2014	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	K	.	T	.	.	.	.
A/Porto Alegre/HNSC-28967/2015	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Porto Alegre/HNSC-977166/2015	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.
A/Porto Alegre/HNSC-42734/2015	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	Q	.	.	.	.	.	.	.	.

Alignment of the non-structural proteins NS1 and NS2 of isolates with the sequence of archetype strain A/California/07/2009. The taxa in red are deceased cases. The mutations that characterized the post -pandemic period are gray; the mutations consensus of the 2013 strains is orange. The intra-strain single mutations are yellow.

Table 8. Amino acid substitutions found in sequences of the matrix proteins M1 and M2 of the samples in relation to the archetype strain A/California/07/2009.

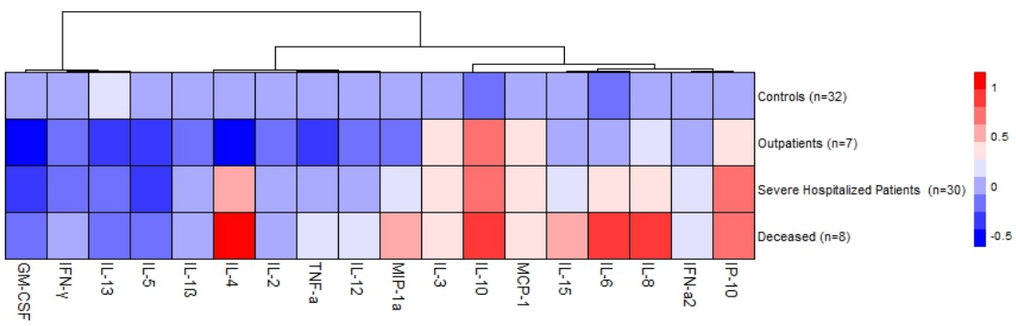
	Amino acid position														
	M1								M2						
Isolates	**30**	**80**	**150**	**181**	**191**	**192**	**219**	**230**	**11**	**13**	**16**	**21**	**23**	**27**
A-Cal07-2009 H1N1p vac2010	S	V	T	L	Q	M	I	K	T	S	E	D	S	V
A/Alegrete/LACENRS-1453/2009	N	.	.	.	.	.	.	.	.	.	G	.	.	.
A/Estrela/LACENRS-313/2009	.	.	I	.	.	.	.	.	.	.	.	.	.	.
A/Igrejinha/LACENRS-1803/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Ijui/LACENRS-2326/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Nonoai/LACENRS-714/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Osorio/LACENRS-2638/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Pelotas/LACENRS-1658/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Pelotas/LACENRS-3045/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-1598/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-1786/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-2121/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Rio Grande/LACENRS-1406/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Rosario do Sul/LACENRS-2110/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Santo Antonio da Patrulha/LACENRS-1649/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Tenente Portela/LACENRS-2073/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Triunfo/LACENRS-1611/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Vacaria/LACENRS-1720/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Venancio Aires/LACENRS-1539/2009	.	.	.	.	.	.	.	.	.	.	.	G	.	.
A/Cachoeirinha/Brazil-AVS03/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Brazil/Entre Rios do Sul-AVS06/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Cachoeira do Sul/LACENRS-335/2011	.	.	.	.	.	.	V	.	.	.	.	.	.	.
A/Flores da Cunha/LACENRS-713/2011	.	I	.	.	.	.	.	.	.	.	.	.	.	.
A/Pelotas/LACENRS-694/2011	.	I	.	.	.	.	.	.	.	.	.	.	.	.
A/Tres Passos/LACENRS-134/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-125/2011	.	I	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-830/2011	.	I	.	.	.	.	.	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS-498/2011	.	.	.	.	.	.	V	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS-700/2011	.	.	.	.	.	.	V	.	.	.	.	.	.	.
A/Sao Gabriel/LACENRS-124/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS-208/2011	.	I	.	.	.	.	.	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS 209/2011	.	.	.	.	.	.	V	.	.	.	.	.	.	.
A/Viamo/LACENRS 240/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Santa Cruz do Sul/LACENRS 338/2011	.	.	.	.	.	.	V	.	.	.	.	.	.	.
A/Brazil/Sao Gabriel-AVS08/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Brazil/Bage-AVS11/2011	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Cruz Alta/LACENRS-892/2012	.	I	.	M	.	.	.	.	.	.	.	.	.	.
A/Cruz Alta/LACENRS-893/2012	.	I	.	M	.	.	.	.	.	.	.	.	.	.
A/Esteio/LACENRS-1519/2012	.	I	.	.	.	.	.	.	.	N	.	.	.	.
A/Porto Alegre/LACENRS-2274/2012	.	I	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/LACENRS-2577/2012	.	I	.	.	.	.	.	.	.	.	.	.	.	.
A/Sao Jose do Inhacora/LACENRS-2082/2012	.	I	.	.	.	.	.	.	.	.	.	.	.	I
A/Tramandai/LACENRS-2067/2012	.	I	.	.	.	.	.	.	.	.	.	.	.	.
A/Vacaria/LACENRS-967/2012	.	I	.	.	.	.	.	.	.	.	.	.	.	.
A/Bage/LACENRS 3084/2012	.	I	.	.	.	.	.	.	.	.	.	.	.	.
A/Bage/LACENRS-2728/2013	.	I	.	.	H	.	.	R	.	.	.	G	.	.
A/Dom Pedrito/LACENRS-2368/2013	.	I	.	.	.	V	.	R	.	.	.	G	.	.
A/Porto Alegre/LACENRS-1573/2013	.	I	.	.	.	V	.	R	.	.	.	G	.	.
A/Porto Alegre/LACENRS-2328/2013	.	I	.	.	.	V	.	R	.	.	.	G	N	.
A/Rosario do Sul/LACENRS-1832/2013	.	I	.	.	.	V	.	R	.	.	.	G	.	.
A/Santa Cruz do Sul/LACENRS-1761/2013	.	I	.	.	N	.	.	R	.	.	.	G	.	.
A/Venancio Aires/LACENRS-1862/2013	.	I	.	.	.	V	.	R	.	.	.	G	.	.
A/Porto Alegre/LACENRS-1485/2014	.	I	.	.	.	V	.	R	I	.	.	G	.	.
A/Porto Alegre/HNSC 562063/2014	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/HNSC-28967/2015	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/HNSC-977166/2015	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Porto Alegre/HNSC 42734/2015	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Alignment of the matrix proteins M1 and M2 of isolates with the sequence of archetype strain A/California/07/2009. The taxa in red are deceased cases. The mutations that characterized the post-pandemic period are gray; the mutations consensus of the 2011strains is blue and the 2013 mutations consensus is orange. The intra-strain single mutations are yellow.

Conclusion

This study attempted to summarize some of cytokine response patterns identified in patients with different clinical manifestations of influenza A cases. Beyond that, viral genomic characteristics in patients infected by influenza A(H1N1)pdm09, including hospitalized and deceased patients were accessed. Our observations provide evidence that cytokines IL-4, TNF-α, and chemokine IL-8 may be markers of disease severity since the levels of these were up-regulated in severe cases. Pathogenesis of severe influenza is complex, involving interactions between host and viral factors, e.g. innate and adaptive responses, prior or cross-immunity in addition to comorbidities. Pathogen features like complex genetic background, virulence, immune evasion, tropism can be accessed by whole genome. We investigated a total of 56 influenza A(H1N1)pdm09 from different years (2009 to 2015) to map the full spectrum of mutations. We showed the accumulation of mutations and the appearance of mutant strains along the years and the putative sites of selective pressure (epitopes sites) or founder effect, identified as homogeneous clusters in phylogenetic trees. This, however, requires further mutation experimental testing, with an ideal animal model, to study distinct phenotypes and transmission capacity, disease and vaccine efficacies.

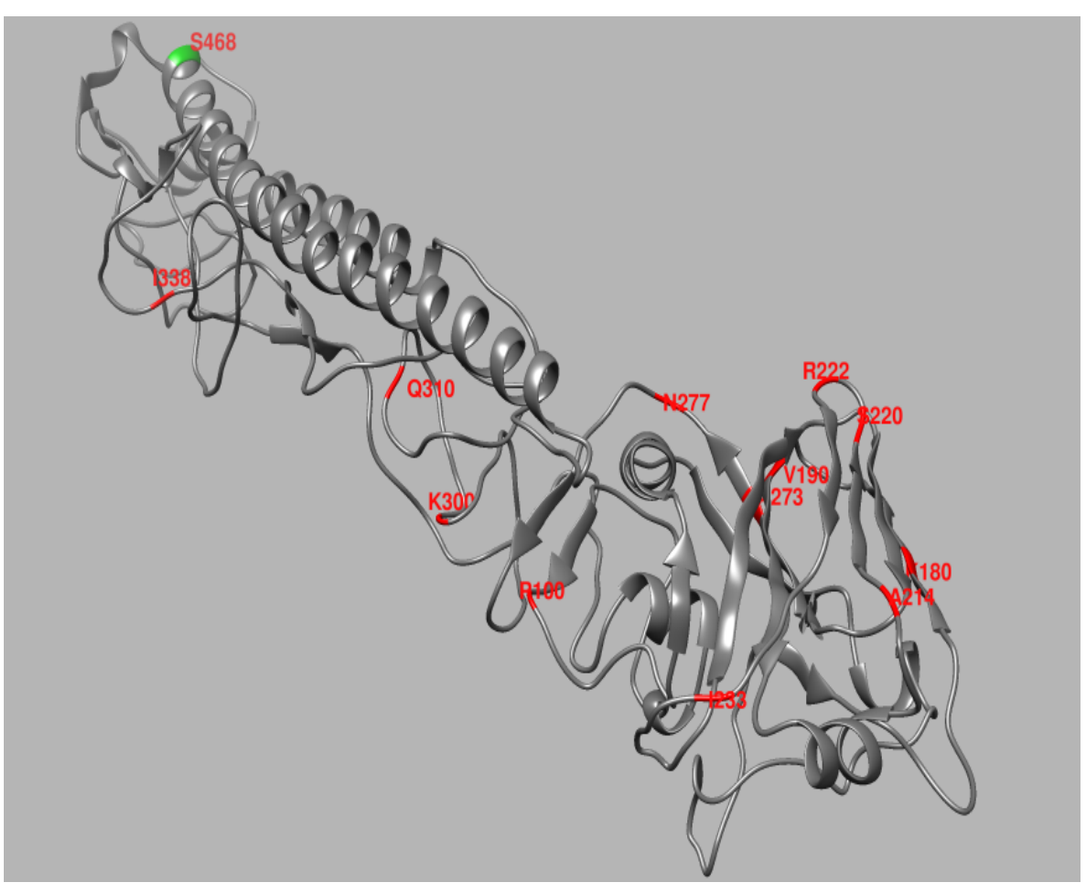
Supplementary Information



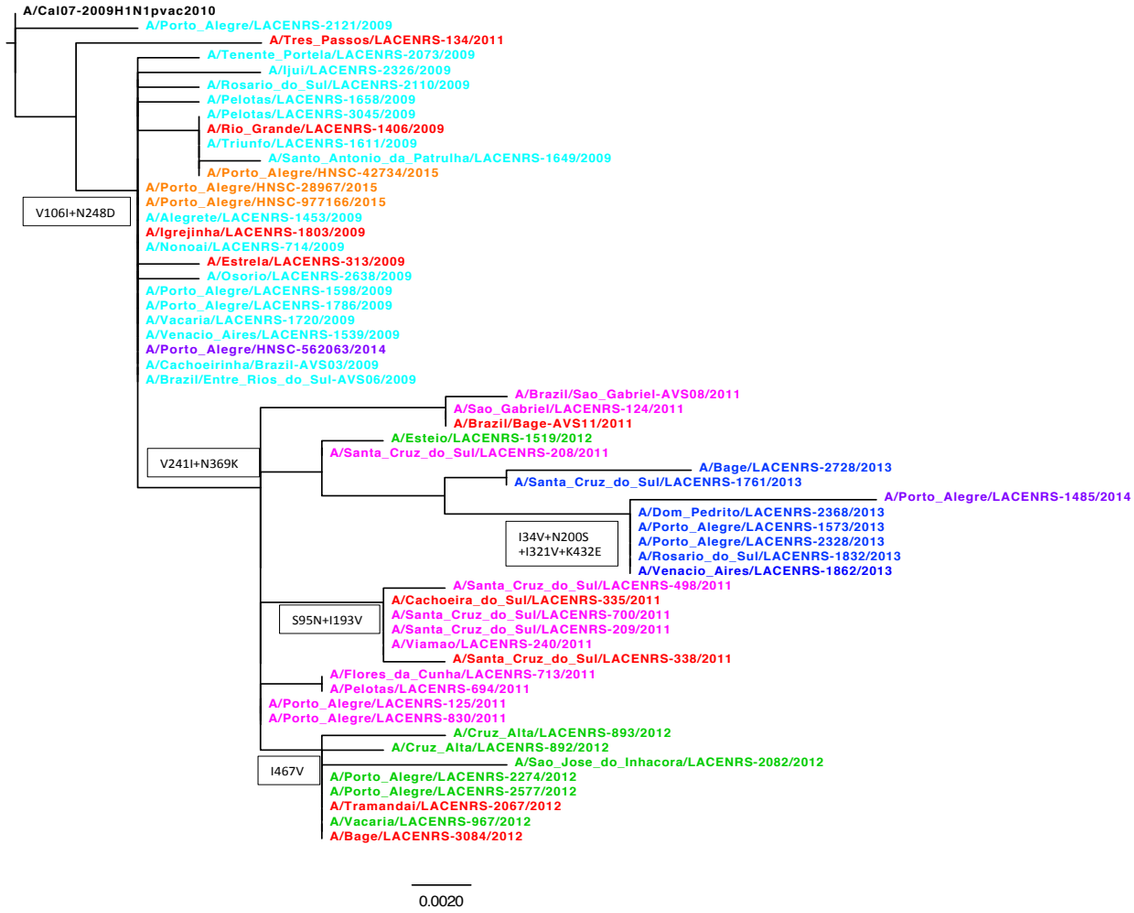
S1 Fig. Heatmap: analysis of the cytokines/chemokines relative concentrations by Pearson distances with ward hierarchical clustering-column, comparing the different groups of patients.

S2 Fig. (A) ML-phylogenetic tree of segment 4, that codes for HA, of influenza A (H1N1)pdm09 virus isolates aligned with archetype strain A/California/07/2009 and mapping of mutations possibly associated with each cluster. Year of sample collection are marked by different colors.

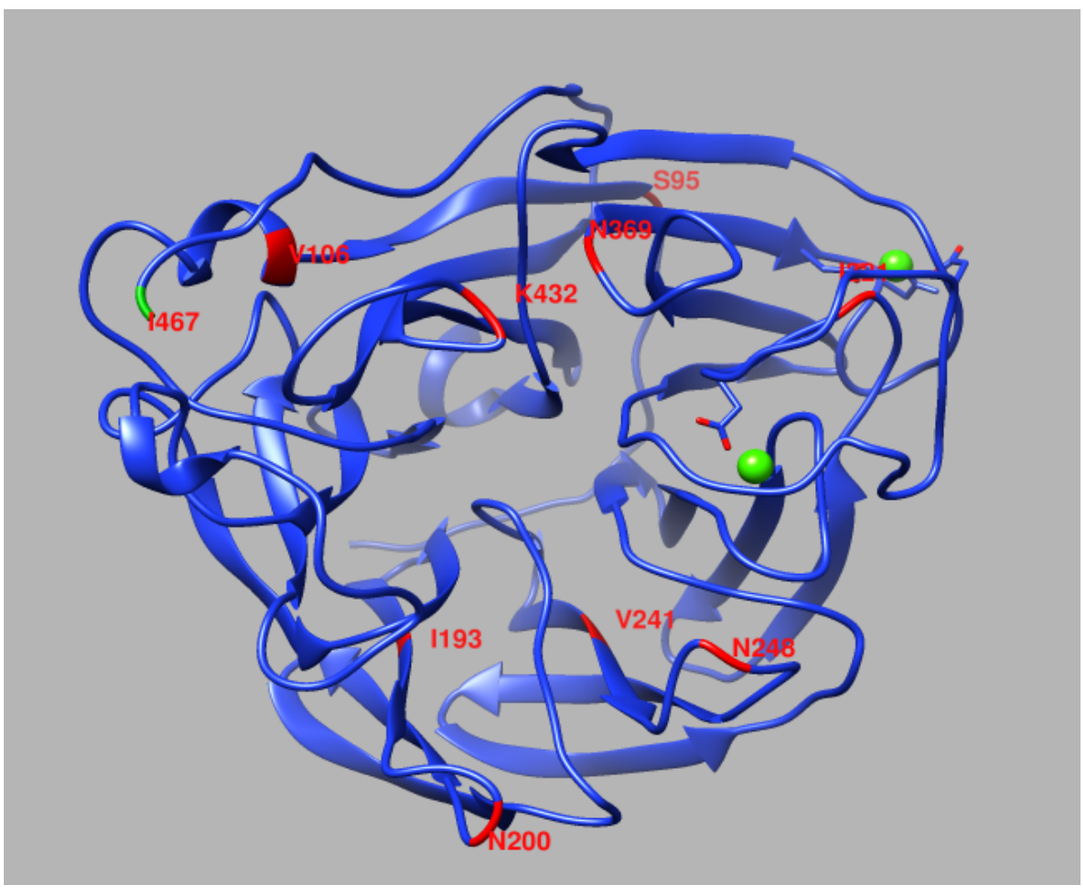
S2 Fig. (B) Position of mutations that characterized the phylogenetic clades of this study in viral protein structure of hemmagglutinin (HA) monomer modeled onto the Cal/09 HA (PDB 3LZG). Most mutations are located in the globular domain than stem of protein.



S3 Fig. (A) NA ML-phylogenetic tree relating segments of influenza A (H1N1)pdm09 virus isolates aligned with archetype strain A/California/07/2009 and mapping the mutations possibly associated with cluster. Year of sample collection are marked by different colors.



S3 Fig. (B) Positions of mutations that characterized phylogenetic clades of our study in viral protein structure of neuraminidase (NA) monomer modeled onto the Cal/09 HA (PDB 4QNP) in complex with CD6 monoclonal antibody. All the mutations are onto the catalytic domain of the protein



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7. Considerações finais e perspectivas

Muitos são os fatores que podem contribuir para a patogenia da infecção pelo vírus influenza A, e estes estão relacionados tanto ao hospedeiro quanto ao patógeno. Com base nisto, nosso objetivo inicial foi avaliar quais características clínicas e epidemiológicas poderiam estar relacionadas a determinado subtipo do vírus influenza A – H1N1 pandêmico de 2009 ou H3N2 sazonal – e qual a prevalência destes dois subtipos na população da nossa região, além de avaliar se a carga viral e/ou características demográficas poderiam estar associados à gravidade da doença, comparando o período pandêmico com o período pós-pandêmico.

No período pandêmico vimos que não houve associação do subtipo viral com os casos de óbitos, embora a carga viral tenha sido mais elevada nas amostras positivas para o subtipo pandêmico; já no período pós-pandêmico, o número de óbitos foi maior em pacientes hospitalizados com diagnóstico de influenza A pandêmico comparado ao subtipo sazonal, embora a carga viral avaliada no ano pós-pandêmico de 2011 tenha sido significativamente maior nas cepas sazonais. A partir desses dados, sugerimos que outros fatores, que não apenas a quantidade de vírus presente no trato nasofaríngeo, podem estar interagindo para caracterizar a gravidade da doença, como por exemplo, a resposta imune inata e condições clínicas adjacentes, ou, ainda, fatores de virulência adquiridos por pressão seletiva do vírus frente à resposta imune. Assim, como segundo objetivo, avaliamos o perfil de resposta imune inata para um grupo de pacientes da primeira onda da pandemia de 2009, e encontramos um perfil de resposta entre os diferentes grupos classificados de acordo com a gravidade da doença. Os pacientes

que foram a óbito apresentaram maior expressão sérica de IL-4, IL-8, IL-15 e TNF-α. Este perfil poderá impactar no manejo clínico futuro de pacientes com níveis séricos exacerbados destes marcadores imunológicos.

Além dos fatores do hospedeiro, analisamos o repertório genético do vírus, através do sequenciamento de alta performance, diretamente das amostras clínicas de pacientes do Rio Grande do Sul, uma das regiões brasileiras mais afetadas pela epidemia anual de influenza e com taxas de mortalidade mais elevadas em comparação a outras regiões do Brasil.

Estudos de epidemiologia molecular do influenza A no nosso estado são escassos, assim como em toda América Latina, portanto o presente estudo contribuiu com uma amostragem significativa para a vigilância das modificações que o vírus sofreu ao longo do tempo (2009 a 2015), além de ter analisado a possível aquisição de novos fatores de virulência, representados pelas mutações pontuais nas 8 proteínas virais. Não encontramos associação entre estas mutações e os caso de óbitos desta casuística. Por outro lado, encontramos mutações comprovadamente associadas a casos de óbitos em outras regiões geográficas, como a mutação D239G na hemaglutinina, presente em 2 dos 9 casos de óbitos aqui analisados. De modo geral, houve um aumento do número de mutações ao longo do período de 2011 a 2015, sendo algumas destas mutações associadas às diferentes epidemias anuais.

É importante enfatizar que não só a vigilância epidemiológica em humanos contribui para a vigilância do vírus influenza, mas a detecção e caracterização dos vírus circulante em animais domésticos e selvagens é de fundamental importância para a detecção e mapeamento de novos vírus,

uma vez que a chance de ocorrer o fenômeno de recombinação nestes reservatórios virais é iminente e constante. Outro aspecto a ser considerado quando do seguimento da vigilância clínico-epidemiológica do influenza é a diferença no padrão de expressão da resposta imunológica nos indivíduos, principalmente num contexto de pós-pandemia e co-circulação de distintas linhagens virais. O acompanhamento da atividade das cepas de influenza A circulantes tanto em humanos como em outros hospedeiros, portanto, é importante para estudos de evolução e dinâmica viral e para o controle da doença.

8. Resultados não publicados

O desenvolvimento de doença grave em alguns indivíduos diagnosticados com o vírus da gripe A humana pode ser modulada pelo genótipo e por modificações do genoma viral durante o processo de infecção, modificações que podem incrementar sua virulência. Por esse motivo, faz-se necessário isolar o vírus das amostras clínicas destes pacientes e testá-los em modelo animal. Pensando em uma perspectiva de análise destes genótipos virais e suas mutações pontuais, nós realizamos o cultivo viral, utilizando o protocolo sugerido pela OPAS (Organização Pan-Americana da Saúde). Foram cultivadas um total de 68 amostras de aspirado nasofaringe. Ao final, obtivemos 21 isolados virais, sendo 11 isolados virais de amostras de pacientes diagnosticados com H1N1 pandêmico de 2009 e 10 isolados virais de amostras identificadas como Influenza A H3N2. Estes isolados virais estão armazenados à -80ºC na Universidad Católica de Chile e poderão ser testadas em projetos de pesquisa futuros.

9. Anexos

9.1. Artigo publicado I

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Research Article Open Access

Epidemiological Profile of Influenza A Cases in Southern Brazil in the Post-Pandemic Period

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Abstract

Influenza viruses are highly contagious and circulate in all geographical regions. During the 2009 pandemics caused by influenza A(H1N1), the State of Rio Grande do Sul (RS) was the first to detected A(H1N1) cases. In 2010, a broad vaccination program was applied in RS when 44.9% of the population joined the program. During the 2011, a total of 1,433 samples were sent to the Central Laboratory in Porto Alegre (LACEN-RS) for viral detection by qRT-PCR. Only 107 (7.5%) cases of the A(H1N1) virus were confirmed versus 182 (12.7%) cases of seasonal influenza A. The incidence of both influenza types virus was higher in patients aged 0-10 years old. The median viral load was higher in patients infected with seasonal, in comparison to those infected with A(H1N1) virus contrary of pandemic period. In 2011 most of the patients that were infected by influenza A virus (79%, p<0.001), did not receive vaccine. The presence of fever, cough, dyspnea, myalgia and rhinorrhea were the most frequent symptoms (positivity >60%). Furthermore in 2011 only patients infected by pandemic virus died (12.9%, p=0.001) in contrast with 2009 pandemic period when 6% of patients infected by pandemic virus died. In other hand in the whole population (5.3%) the mortality rate was similar that observed in pandemic period (5.9%). These analyses about epidemiological and molecular data provide important scenery about the characteristics of the host-pathogen interaction after massive exposure during pandemic period.

Keywords: Influenza A viruses; Viral load; Post pandemic period; Vaccine

Introduction

Influenza viruses are highly contagious and circulate in all geographical regions. Although it causes mild symptoms in the majority of cases, illnesses can result in hospitalizations and deaths mainly among high-risk groups (the very young, elderly or chronically ill patients). Worldwide, these annual epidemics result in about three to five million cases of severe illness, and about 250,000 to 500,000 deaths [1].

In spite of the constant concern of authorities and the establishment of health surveillance systems, influenza A viruses are known for its tendency to antigenic variation, which can be quick and unpredictable [2]. Intense selection from the host immune system drives antigenic change in influenza virus, resulting in continuous replacement of circulating strains [3]. In Brazil, during the 2009 pandemics caused by influenza A(H1N1)pdm09 virus, over 80,000 cases of severe acute respiratory infection (SARI) cases were reported, among which 27,850 cases were confirmed as pandemic influenza. Hospitals and health care services were overcrowded and unprepared for such a large number of cases, especially in the South, where 18,349 cases were confirmed and mortality rates reached 2.32 (per 100.000 inhabitants); the State of Rio Grande do Sul (RS) alone confirmed 2,109 influenza A(H1N1) pdm09 case [4]. In 2010, a massive vaccination program was applied in RS when 4,898,723 people (44.9% of the population) were vaccinated. As a consequence, the A(H1N1)pdm09 virus was not detected in 2010 in RS, but reemerged in 2011 and has since then been increasingly circulating in the population, together with seasonal H3N2 influenza. Co-circulation of viral subtypes has been observed since the pandemic period [5]. Past the pandemic period, after August of 2010, the behavior of influenza A(H1N1)pdm09 virus is like that of a seasonal virus [1].

Brazil has a Program of Surveillance of Influenza since 1999 and has as goal the identification of the viral subtype, including emergent strains. Meanwhile, investigation of biological and clinical aspects, such as viral loads and correlation with aspects of the host is needed for the continuity of influenza A virus control, prevention and treatment of infected patients. Concerned with these issues, our group has started a continuous research on the molecular epidemiology of influenza A in RS, combining molecular analyses based on qRT-PCR, genome sequencing of viral samples, quantitation of viral loads, with clinical data of patients. Accordingly, we investigated influenza A cases in the State of Rio Grande do Sul during the year of 2009, showing that viral loads were much higher in patients infected by the pandemic influenza A (H1N1)2009 than with seasonal influenza A virus; furthermore, relative viral load correlated positively with symptoms such as chills, myalgia and rhinorrhea [5]. Viremia in patients infected by different influenza A subtypes depends on several factors that interfere with

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virus–host interaction; mutations that lead to antigenic changes, the host's immunity and genetic background, as well as vaccination status are factors that contribute to the virus fitness and influence viral loads.

In this study we analyzed the relative viral loads observed in patients infected with either A(H1N1)pdm09 virus or seasonal influenza A viruses in the State of Rio Grande do Sul, Southern Brazil, during 2011 and correlate the results with the clinical and demographical data in the post- pandemic period.

Materials and Methods

Study subjects, clinical data and biological samples

During the epidemiological weeks 1-52 (January-December) of 2011, 1,501 cases of severe acute respiratory infection were reported in the State of Rio Grande do Sul (RS), Southern Brazil (estimated population: 10.7 million people). Of these, nasopharyngeal aspirate samples were collected from 1,433 patients, together with the *Influenza Notification Form* (demographic characteristics, date of notification, date of onset of symptoms, acute respiratory infection symptoms, co-morbidities, smoking habits, pregnancy status, vaccination status, and X-ray results). The form is filled out by the attending physician/nurse, at time of collection at the respective health unit, however some data might be neglected.

All 1,433 samples and forms were sent to the State Central Laboratory (LACEN-RS) for real time reverse transcription-polymerase chain reactions (qRT-PCR) analysis. Samples were identified either as pandemic influenza A or seasonal influenza A according to the qRT-PCR results (see below). All experiments were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki. The Research Ethics Committee of UFCSPA approved this study.

Identification and quantitation of influenza A virus

RNA was extracted from 1,433 nasopharyngeal aspirate samples using the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. qRT-PCR was performed using the SuperScript-III Platinum One-Step Quantitative kit and the Influenza A (H1N1) Primer and Probe Set (Invitrogen-Life Technologies, Carlsbad, CA) as described elsewhere. Briefly, reactions were performed using 0.5 ml of SSIII/Platinum Taq Mix, 1 mM of each primer, 250 nM of probe, 12.5 µl 2X Master Mix, 5 µl of RNA sample and water, to a final volume of 25 µl. This method is based on a panel of 4 primers/probe sets: InfA is designed for universal detection of type A influenza viruses; swInfA detects all swine influenza A viruses; swH1 detects swine H1 influenza; and RNP targets the human RNase P gene and serves as an internal positive control for human nucleic acid. Therefore, if a sample was amplified with all the sets, it was considered pandemic influenza A. Samples that were positive only with InfA and RNP were considered seasonal influenza A. Samples that amplified only with RNP were considered negative for influenza A [5]. All PCR reactions were performed in a Step One Plus thermocycler (Applied Biosystems-Life Technologies, Carlsbad, CA). The following reaction conditions were applied: 50°C for 30 min; 95°C for 2 min; 45 cycles at 95°C for 15 s and 55°C for 35 s.

The relative viral load in each sample was calculated with the threshold cycle (CT) value based on the $2^{-\Delta CT}$ method [6]. The CT values obtained in the qRT-PCR with the InfA and RNP primer/probe sets (target and reference genes respectively) were used for delta calculation ($\Delta CT = CT_{InfA} - CT_{RNP}$).

Statistical analysis

Only samples with complete epidemiological data (n=88 for influenza A(H1N1)pdm09 and n=149 for seasonal influenza A) were included in statistical analyses to compare viral loads and clinical data from patients infected by pandemic or seasonal influenza. Regarding clinical conditions such as co morbidities, smoking habit and disease evolution, the total number of patients analyzed was 80 and 120 for pandemic and seasonal influenza A, respectively.

Descriptive statistics was used to summarize the data. Categorical data were treated with chi- square and Exact Fisher test, while continuous variables were compared using Mann–Whitney test, Md (P25-P75). Values were considered statistically significant when $P<0.05$.

Results

Influenza A identification by qRT-PCR

In 2010, the first post-pandemic year in Brazil, no cases of influenza A(H1N1)pdm09 virus were confirmed. Nevertheless, the virus reemerged in May 2011 and has since then circulated in the human population. Accordingly, 1,501 cases of SARI were notified in 2011 in RS. Of these, 1,433 nasopharyngeal aspirates were collected and sent to the Central Laboratory in Porto Alegre (LACEN-RS) for viral detection by qRT-PCR. A total of 107 (7.5%) cases of influenza A(H1N1)pdm09 virus were confirmed versus 182 (12.7%) cases of seasonal influenza A. Further analyses showed the seasonal influenza A being of the H3N2 subtype (data not shown). The highest influenza activity occurred in June, with concomitant circulation of both A(H1N1)pdm09 and H3 subtypes. The seasonal influenza was detected during epidemiological weeks 1-32, with five positive outbreaks in different institutions. The A(H1N1)pdm09 circulation persisted until the epidemiological week 44, especially after the seasonal influenza decline.

Samples negative for influenza A (n=1,144) were investigated for other respiratory viruses. Respiratory Syncytial Virus accounted for 20.1% of the influenza-negative SARI cases, and influenza B, adenovirus and parainfluenza (1, 2 and 3) viruses accounted for a total of 4.4% of these cases. In 67% of SARI cases, no respiratory viruses were found.

Characteristics of patients infected with influenza A in rio grande do sul

Complete *Influenza Notification Forms* were available for 88 and 149 pandemic and seasonal influenza A confirmed cases, respectively. However, clinical conditions such as comorbidities, smoking habit and disease evolution (death) were available only for 80 pandemic and 120 seasonal influenza-infected patients.

As shown in Table 1, the age distribution of patients infected by pandemic and seasonal influenza A in the year 2011 was homogenous. Among patients infected with A(H1N1)pdm09 virus, 53.4% were male and among patients infected with seasonal virus, 52.3% were female.

The frequency of pandemic and seasonal influenza A infection varied significantly among age groups (p=0.01), and the highest frequency of both subtypes was in patients aged 0-10 years (39.8% and 27.3%, respectively). When the distribution of the type of influenza in each age group was compared, the highest difference between seasonal

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and pandemic influenza was in patients in the age group 31-40 years old (14.1% and 4.5%, respectively) (Table 1 and Figure 1). When age groups that are more susceptible to infection (0-10 years old and ≥ 60 years old) are combined, a total incidence of about 46.7% is observed for both A(H1N1)pdm09 and seasonal influenza A viral infection.

Regarding the vaccination status of the patients, in the year 2011 most of the patients that were infected by influenza A virus (79%, p<0.001) did not receive vaccine (Table 1).

All samples examined in this study were from patients presenting SARI with at least one of the symptoms: fever, cough, chills, dyspnea, sore throat, arthralgia, myalgia, conjunctivitis, rhinorrhea, diarrhea, as depicted in Table 2. The presence of fever, cough, dyspnea, myalgia and rhinorrhea were the most frequent symptoms both in patients infected by pandemic as well as by seasonal influenza A (positivity >60%) and conjunctivitis was the least frequent symptom, presented by 6.7% and 15.6% of patients with seasonal and pandemic influenza A, respectively. Notably, fever, dyspnea and conjunctivitis showed a positive correlation with infection by A(H1N1)pdm09 virus (p<0.05).

Characteristic	Seasonal (n=149)[a]	Pandemic (n=88)[a]	*p*
Age group (years)			
0-10	35 (27.3%)	35 (39.8%)	*p=0.01
11-20	13 (10.2%)	11 (12.5%)	
21-30	12 (9.4%)	13 (14.8%)	
31-40	18 (14.1%)	4 (4.5%)	
41-50	8 (6.3%)	11 (12.5%)	
51-60	17 (13.3%)	8 (9.1%)	
61-70	16 (12.5%)	6 (6.8%)	
71-80	7 (5.5%)	0	
81-90	2 (1.6%)	0	
Gender			
Female	78 (52.3%)	41 (46.6%)	*p=0.235
Male	71 (47.7%)	47 (53.4%)	
Viral load			
(Median [p25-p75])	1.86 (0.06-157.58)	0.05 (0.002-2.44)	#p<0.001
Vaccine			
Yes	33 (28.9%)	8 (9.5%)	*p=0.001
No	81 (71.1%)	76 (90.5%)	

[a]Vaccination status was not informed for all patients
*Statistically significant by Chi-Square Test
#Statistically significant by Mann Whitney U Test

Table 1: Demographic characteristics of patients infected by seasonal and pandemic Influenza A virus.

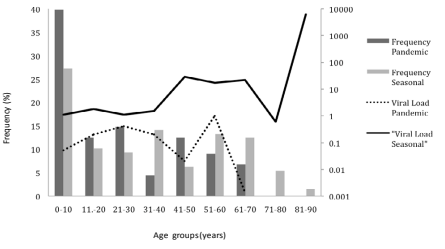

Figure 1: Frequency of pandemic and seasonal influenza A virus in each age group. Relative viral loads are shown in logarithmic scale.

Symptom	Frequencies		
	Seasonal (n=149)[a]	Pandemic (n=88)[a]	*p* Seasonal/Pandemic
Fever			
Yes	120 (93.8%)	87 (100%)	*p=0.017
No	8 (6.2%)	0	
Cough			
Yes	125 (97.7%)	85 (98.8%)	p=0.532
No	3 (2.3%)	1 (1.2%)	
Chills			
Yes	55 (50.4%)	36 (50%)	p=0.952
No	54 (49.6%)	36 (50%)	
Dyspnea			
Yes	90 (72%)	73 (84.9%)	*p=0.028
No	35 (28%)	13 (15.1%)	
Sore Throat			
Yes	40 (37.1%)	28 (41.2%)	p=0.583
No	68 (62.9%)	40 (58.8%)	
Arthralgia			
Yes	42 (39.6%)	22 (33.8%)	p=0.449
No	64 (60.4%)	43 (66.2%)	
Myalgia			
Yes	64 (60.9%)	45 (66.2%)	p=0.487
No	41 (39.1%)	23 (33.8%)	
Conjunctivitis			
Yes	8 (6.7%)	12 (15.6%)	*p=0.045
No	111 (93.3%)	65 (84.4%)	
Rhinorrhea			
Yes	72 (64.9%)	50 (62.5%)	p=0.670
No	49 (35.1%)	30 (37.5%)	
Diarrhea			
Yes	22 (20.2%)	15 (18.5%)	p=0.999
No	97 (79.8%)	66 (81.5%)	

[a]Not all symptoms were available for all patients
*Statistically significant by Chi-Square Test

Table 2: Frequencies of acute respiratory infection symptoms in patients infected by pandemic and seasonal influenza A virus.

In addition to symptoms attributable to SARI, other clinical manifestations that represent risk factors to this acute respiratory disease were observed in patients infected with both seasonal and pandemic virus. The frequency of each symptom was lower than the frequency of SARI symptoms with cardiopathy and pneumopathy being the most frequent comorbidities observed (Table 3). The distribution between the two types of virus was homogenous.

Only patients infected by the pandemic virus died (12.9%, p=0.001), showing an overall frequency of 5.4%.

Relative viral load and demographic data

In order to compare data from the post pandemic periods with that obtained during the pandemic period according to variables such as age, gender, clinical condition or severity of symptoms, viremia was calculated for each patient. Analysis or relative viral loads in seasonal and pandemic influenza A samples was based on the $2^{-\Delta CT}$ method [6], considering the CT values obtained in qRT-PCR with the infA primer/probe set [5].

The median relative viral load was higher in patients infected with seasonal virus in comparison to those infected by A(H1N1)pdm09 virus (1.86[0.06-157.58] vs. 0.05[0.0020-2.44], p<0.001) (Table 1).

When median viral load was compared between different age

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groups for pandemic or seasonal influenza cases, no differences were observed (Table 4). On the other hand, the highest viral loads for pandemic influenza occurred in the age group 41-70 years, followed by the age group 21-30 years (Figure 1); regarding seasonal influenza, the highest viral loads occurred in patients older than 70 years, followed by the age groups 41-50 years and 61-70 years (Table 4).

The median viral loads between females and males infected by seasonal influenza A virus were statistically different, with females displaying higher viral loads in relation to male (11.72[0.15-340.35] versus 0.81[0.02-47.3], p=0.02). As for patients infected by pandemic influenza A, no significant differences were found in viral loads between genders.

Viral load did not show statistically significant association with any respiratory infection symptom. Nevertheless, considering other clinical conditions such as immunosuppression and metabolic disease, a positive association between high viral loads and these conditions was observed within seasonal influenza group only (Table 4).

Discussion

Past the pandemic period when a new influenza A virus was introduced worldwide, a strong surveillance was applied to monitoring the circulation of respiratory viruses in Brazil. Prevention actions coordinated by the Ministry of Health to contain the flu, including fast diagnosis and treatment of patients, were boosted all over the country, especially in the state of Rio Grande do Sul (RS), where high incidence of SARI is observed. A massive vaccination program was applied in RS beginning in April 2010, when 44.9% of the population was vaccinated; in that year, which was the first post-pandemic year in Brazil, no cases of influenza A virus were confirmed. Nevertheless, the virus reemerged in May 2011 and since then both A(H1N1)pdm09 and seasonal influenza A have been co-circulating with influenza B virus.

Characteristic	Frequencies		
	Seasonal (n=149)[a]	Pandemic (n=88)[a]	*P* Seasonal/Pandemic
Cardiopathy			
Yes	16 (13.7%)	6 (7.5%)	p=0.177
No	101 (86.3%)	74 (92.5%)	
Pneumopathy			
Yes	14 (11.2%)	11 (13.6%)	p=0.737
No	103 (88.8%)	70 (86.4%)	
Renal			
Yes	2 (1.6%)	0	p=0.238
No	114 (91.9%)	80 (100%)	
Immunosuppression			
Yes	4 (3.4%)	5 (6.2%)	p=0.350
No	113 (96.6%)	75 (93.8%)	
Smoking habit			
Yes	12 (10.2%)	8 (10.0%)	p=0.969
No	106 (89.8%)	72 (90.0%)	
Metabolic disease			
Yes	8 (6.7%)	2 (2.5%)	p=0.181
No	111 (93.3%)	78 (97.5%)	
Hemoglobinopathy			
Yes	3 (2.6%)	0 (0%)	p=0.149
No	114 (97.4%)	80 (100%)	
Death			
Yes	0	11 (12.9%)	*p=0.001
No	120 (100%)	74 (87.1%)	

[a]Not all characteristics were available for all patients
*Statistically significant by Chi-Square Test

Table 3: Frequencies of clinical conditions and disease evolution in patients infected by pandemic and seasonal influenza A virus.

Characteristic	Viral Load		
	Seasonal (n=128) $Md_{(P25-75)}$	Pandemic (n=88) $Md_{(P25-75)}$	*P* Seasonal/Pandemic
Gender			
Male	0.81 (0.02-47.3)	0.1(0.0005-1.9)	*p=0.02/0.86
Female	11.72 (0.15-340.35)	0.04 (0.007-3.4)	
Age group (years)			
0-10	1.1 (0.2-90.6)	0.05(0.03-8.0)	#p=0.1/0.49
11-20	1.9 (0.4-1280.0)	0.2 (0.0005-10.5)	
21-30	1.1 (0.1-71.3)	0.4 (0.01-3.7)	
31-40	1.5 (0.02-170.2)	0.2 (0.03-0.1)	
41-50	28.8 (0.5-411.8)	0.02 (0.0006-0.1)	
51-60	17.1 (0.4-1676.5)	1.0 (0.02-5.4)	
61-70	21.6 (1.1-524.2)	0.0013 (0.0-2.0)	
71-80	0.6 (0.2-0.9)	-	
81-90	6238.3 (891-.)	-	
Comorbidity			
Immunosuppression			
Yes	438.6 (30.8-12495.9)	0.114 (.0001-14.9)	#p=0.049/0.87
No	1.87 (0.06-163.2)	0.101 (0.003-3.3)	
Metabolic disease			
Yes	1503.9 (8.2-18024.6)	4.23 (3.53-.)	#p=0.025/0.175
No	1.9 (0.07-168.9)	0.089 (0.002-2.7)	

*statistically significant by Chi-Square Test
Results by Mann Whitney U Test

Table 4: Viral loads in patients infected by pandemic and seasonal influenza A virus distributed by gender and age group. Statistically significant clinical conditions are also shown.

In the present study, virologic and epidemiological data was described for 237 patients who were diagnosed with pandemic (n=88) and seasonal (n=149) influenza A viruses infection in 2011. Analyses combining epidemiological and molecular data provide important information about the disease, including characteristics of the host-pathogen interaction after massive exposure during pandemic periods. The viral loads, vaccine and epidemiological data for patients infected by influenza A viruses during the whole year were analyzed. Nasopharyngeal aspirates were collected from patients in RS presenting with symptoms of acute respiratory infection and tested by qRT-PCR. Only 7.5% cases of A(H1N1)pdm09 virus were confirmed versus 12.7% of seasonal influenza A (either H1N1 or H3N2). In the last publication describing the 2009 pandemics in southern Brazil found that, among patients presenting SARI, 30% were positive for A(H1N1)pdm09 and 5.5% were positive for seasonal influenza [5]. According to Dapat et al. [7] decrease in the number of clinical cases may be attributed to an increase in antibody levels against the A(H1N1)pdm09 virus in the community. In the case of Brazil, such a decrease can also be attributed to the vaccination campaign.

In the present study, the frequency of infection varied significantly among age groups. The highest frequency of infection was observed in patients between 0 and 10 years old in both seasonal (27.3%) and pandemic (39.8%) groups. This is quite different from the pandemic period, in which the highest frequency was in the age groups 21-30 years old (26.3% seasonal and 25.7% pandemic influenza) and 31-40 years old (21.1% seasonal and 19.3% pandemic influenza) [5]. This might result from a combination of acquired immunity, vaccination

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and also a public health program focused on educative information such as hand washing and other preventive behaviors. Infections leading to death were observed only in patients infected with the pandemic virus (12.9%); this frequency was higher when compared with the 2009 pandemic period when 5.9% of patients infected with A(H1N1)pdm09 died. This result suggests that the pandemic virus could have acquired additional pathogenicity, overcoming the host's immunity. On the other hand, considering the whole population presenting SARI, the mortality rate (5.3%) was similar to that observed in the pandemic period.

The fact that no death cases were observed among patients infected by seasonal influenza A suggests that the public health policies were considerably efficient and the patient management was more appropriate in the post-pandemic period, including extensive antiviral treatment and prophylaxis. Nevertheless, 79% of the patients that were infected by influenza A virus did not receive vaccine, a fact that reveals a gap in the vaccination program in that year.

Further studies are needed in order to infer possible molecular changes in the viral genome that could be associated with differences in its pathogenicity during the pandemic and the post- pandemic periods. A study that analyzed samples from a different region of Brazil aiming the monitoring of antiviral resistance to neuraminidase inhibitors showed that the prevalence of mutants did not support the detection of resistance strains of A(H1N1)pdm09 virus during 2009-2010 [8]. Another study that analyzed only samples from RS found a low prevalence of the mutations H275Y and S247N of the NA protein in strains circulating between 2009-2011 [9]. In a recent study with Brazilian clinical samples, Ferreira et al. showed a significant association between the D239G substitutions in the haemagglutinin (HA) gene of pandemic influenza A H1N1 virus with mortality [10]. In the present study, seasonal influenza A virus was detected co- circulating with the pandemic strain throughout the whole year of 2011, corroborating other studies that suggest that influenza genetic diversity is generated continually in tropical regions [11]. The next step of this study will be to evaluate how viral genome alterations may be associated with drug resistance and factors of virulence.

During the post-pandemic period, viremia was higher in patients infected by seasonal influenza A, as opposed to the pandemic period, when viral loads were higher in patients infected by the pandemic virus and is in accordance with other study realized in China with samples collected out of the pandemic period [5,12]. Even though vaccination could contribute to lower viremia, most of the patients with pandemic virus infection (90.5%) were not vaccinated; regardless of they displayed low viremia, a high rate of mortality was observed among these patients (12.9%).

In this present study were analyzed samples from patients presenting acute respiratory infection symptoms during the post-pandemic period. Therefore, most patients presented fever, cough, dyspnea, myalgia and rhinorrhea; conjunctivitis was the least frequent symptom. Considering all the mentioned symptoms, fever, dyspnea and conjunctivitis showed a positive correlation with infection by A(H1N1) pdm09 virus. Unlike the pandemic period, no significant association was found between these symptoms and viremia [5]; in contrast, when other clinical conditions are taken into account, a positive association was found between high viral loads and immunosuppression as well as metabolic disease in patients infected by seasonal influenza A. Lee et al. [13] report a correlation between viral loads and systemic comorbidities emphasizing that the use of systemic corticosteroids to treat concomitant medical conditions during influenza infection is associated with a slow decrease in viremia. These can explain the association between immunosuppression and higher viral load within the seasonal group. Yet these data should be interpreted cautiously due to the varied quality of data regarding underlying diseases and small numbers of complete *Influenza Notification Forms*.

As a matter of fact, one of the limitations of this study was that the *Influenza Notification Form* was not uniformly filled out by the different health units' clinician/nurse. Therefore, some demographic and clinical data were missing, reducing our sample number – of 107 samples positive for pandemic influenza A and 182 samples positive for seasonal influenza A samples, only 88 and 149 had complete forms for analyses, respectively, which means a loss of approximately 18%. Finally, vaccination status was also not informed for all patients and this information is of major interest to evaluate the vaccine's efficacy and for public health measures.

In summary, the data demonstrated marked differences among pandemic and post-pandemic periods about dynamics of host, environment and pathogens. Molecular characteristics such as virus' genome sequencing should be considered in future strategies to understand viral pathogenesis. Brazilian surveillance has been effective to implement policies of outbreaks contention through vaccination programs and antiviral treatment offered by the public health system. These strategies include improvement of laboratory capacity, information about prevention, availability of prophylaxis treatment and global molecular studies.

Grant Sponsors

CAPES (MEC, Brazilian Government); Graduate Program in Pathology – UFCSPA; FEPPS-RS.

Conflict of Interest

The authors of this study declare they do not have any kind of conflict of interest.

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9.2. Parecer CEP UFCSPA

UFCSPA

COMISSÃO CIENTÍFICA E COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE

COMITÊ DE ÉTICA EM PESQUISA - CEP
UFCSPA

O Comitê de Ética em Pesquisa da UFCSPA, registrado na Comissão Nacional de Ética em Pesquisa (CONEP) sob o nº 075/05 em 23/07/04, analisou o Projeto:

Projeto: 10-691 **Versão do Projeto:** **Versão do TCLE:**

Pesquisadores:
ANA BEATRIZ GORINI DA VEIGA
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PEDRO ALVES D'AZEVEDO
TATIANA SCHAFFER GREGLANINI
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Título: EMPREGO DE FERRAMENTAS DE BIOINFORMÁTICA PARA O ESTUDO DE VÍRUS PATOGÊNICOS HUMANOS- ANÁLISE FILOGÊNICA E RELAÇÃO ENTRE CARGA VIRAL E EVOLUÇÃO DO QUADRO CLÍNICO.

Esse projeto foi aprovado em seus aspectos éticos e metodológicos conforme as Resoluções 196/09 e demais Resoluções complementares. Toda e qualquer alteração do projeto, assim como eventos adversos graves, deverão ser comunicados a este CEP. Os TCLE, quando necessários, somente poderão ser utilizados após prévia e explícita aprovação (carimbo) de sua redação por este CEP".

Porto Alegre, 15 de dezembro de 2010.

José Geraldo Vernet Taborda
Coordenador do CEP/FFFCMPA

9.3. Parecer CEP HNSC

GHC Grupo Hospitalar Conceição

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100% SUS

Vinculados ao Ministério da Saúde - Decreto nº 99.244/90

O Comitê de Ética em Pesquisa do Grupo Hospitalar Conceição (CEP/GHC), que é reconhecido pela Comissão Nacional de Ética em Pesquisa (CONEP)/MS desde 31/10/1997, pelo Office For Human Research Protections (OHRP)/USDHHS, como Institutional Review Board (IRB0001105) e pelo FWA - Federalwide Assurance (FWA 00000378), em 03 de agosto de 2015, reavaliou o seguinte projeto de pesquisa:

Projeto: 14199 **Versão do Projeto:** **Versão do TCLE:**

Pesquisadores:
ANA BEATRIZ GORINI DA VEIGA
TATIANA SCHÄFFER GREGIANINI
NÉLSON ALEXANDRE KRETZMANN FILHO
TATIANA GASPERIN BACCIN

Título: O vírus influenza A no Rio Grande do Sul.

Documentação: Aprovada
Aspectos Metodológicos: Adequados
Aspectos Éticos: Adequados

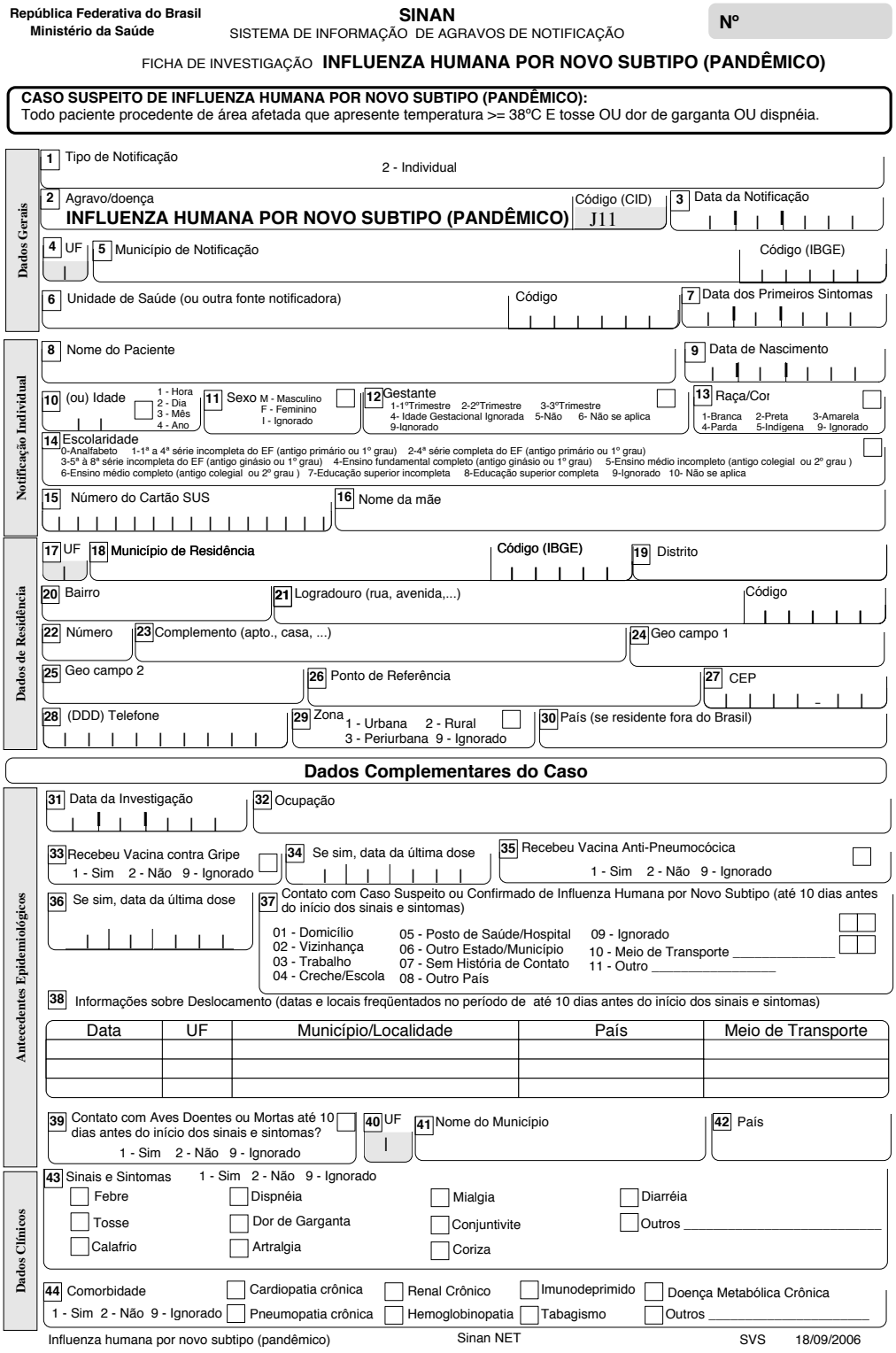
Parecer final: Este projeto de pesquisa, bem como o Termo de Consentimento Livre e Esclarecido (se aplicável), por estar de acordo com as Diretrizes e Normas Internacionais e Nacionais e complementares do Conselho Nacional de Saúde, especialmente a Resolução 466/12, obteve o parecer de APROVADO(S) neste CEP.

O Pesquisador responsável deve encaminhar dentro dos prazos estipulados, o(s) relatório(s) parcial(ais) e/ou final ao Comitê de ética em Pesquisa do GHC e o Centro de Resultados onde foi desenvolvida a pesquisa.

Porto Alegre, 04 de agosto de 2015.

Daniel Demétrio Faustino da Silva
Coordenador-geral do CEP-GHC

9.4. Ficha epidemiológica



República Federativa do Brasil
Ministério da Saúde

SINAN
SISTEMA DE INFORMAÇÃO DE AGRAVOS DE NOTIFICAÇÃO

Nº

FICHA DE INVESTIGAÇÃO **INFLUENZA HUMANA POR NOVO SUBTIPO (PANDÊMICO)**

CASO SUSPEITO DE INFLUENZA HUMANA POR NOVO SUBTIPO (PANDÊMICO):
Todo paciente procedente de área afetada que apresente temperatura >= 38ºC E tosse OU dor de garganta OU dispnéia.

Dados Gerais

1 Tipo de Notificação — 2 - Individual

2 Agravo/doença — **INFLUENZA HUMANA POR NOVO SUBTIPO (PANDÊMICO)** — Código (CID) J11 — 3 Data da Notificação

4 UF — 5 Município de Notificação — Código (IBGE)

6 Unidade de Saúde (ou outra fonte notificadora) — Código — 7 Data dos Primeiros Sintomas

Notificação Individual

8 Nome do Paciente — 9 Data de Nascimento

10 (ou) Idade — 1 - Hora 2 - Dia 3 - Mês 4 - Ano — 11 Sexo M - Masculino F - Feminino I - Ignorado — 12 Gestante 1-1ºTrimestre 2-2ºTrimestre 3-3ºTrimestre 4- Idade Gestacional Ignorada 5-Não 6- Não se aplica 9-Ignorado — 13 Raça/Cor 1-Branca 2-Preta 3-Amarela 4-Parda 5-Indígena 9- Ignorado

14 Escolaridade 0-Analfabeto 1-1ª a 4ª série incompleta do EF (antigo primário ou 1º grau) 2-4ª série completa do EF (antigo primário ou 1º grau) 3-5ª à 8ª série incompleta do EF (antigo ginásio ou 1º grau) 4-Ensino fundamental completo (antigo ginásio ou 1º grau) 5-Ensino médio incompleto (antigo colegial ou 2º grau) 6-Ensino médio completo (antigo colegial ou 2º grau) 7-Educação superior incompleta 8-Educação superior completa 9-Ignorado 10- Não se aplica

15 Número do Cartão SUS — 16 Nome da mãe

Dados de Residência

17 UF — 18 Município de Residência — Código (IBGE) — 19 Distrito

20 Bairro — 21 Logradouro (rua, avenida,...) — Código

22 Número — 23 Complemento (apto., casa, ...) — 24 Geo campo 1

25 Geo campo 2 — 26 Ponto de Referência — 27 CEP

28 (DDD) Telefone — 29 Zona 1 - Urbana 2 - Rural 3 - Periurbana 9 - Ignorado — 30 País (se residente fora do Brasil)

Dados Complementares do Caso

Antecedentes Epidemiológicos

31 Data da Investigação — 32 Ocupação

33 Recebeu Vacina contra Gripe 1 - Sim 2 - Não 9 - Ignorado — 34 Se sim, data da última dose — 35 Recebeu Vacina Anti-Pneumocócica 1 - Sim 2 - Não 9 - Ignorado

36 Se sim, data da última dose

37 Contato com Caso Suspeito ou Confirmado de Influenza Humana por Novo Subtipo (até 10 dias antes do início dos sinais e sintomas)
01 - Domicílio 02 - Vizinhança 03 - Trabalho 04 - Creche/Escola 05 - Posto de Saúde/Hospital 06 - Outro Estado/Município 07 - Sem História de Contato 08 - Outro País 09 - Ignorado 10 - Meio de Transporte _____ 11 - Outro _____

38 Informações sobre Deslocamento (datas e locais freqüentados no período de até 10 dias antes do início dos sinais e sintomas)

Data	UF	Município/Localidade	País	Meio de Transporte

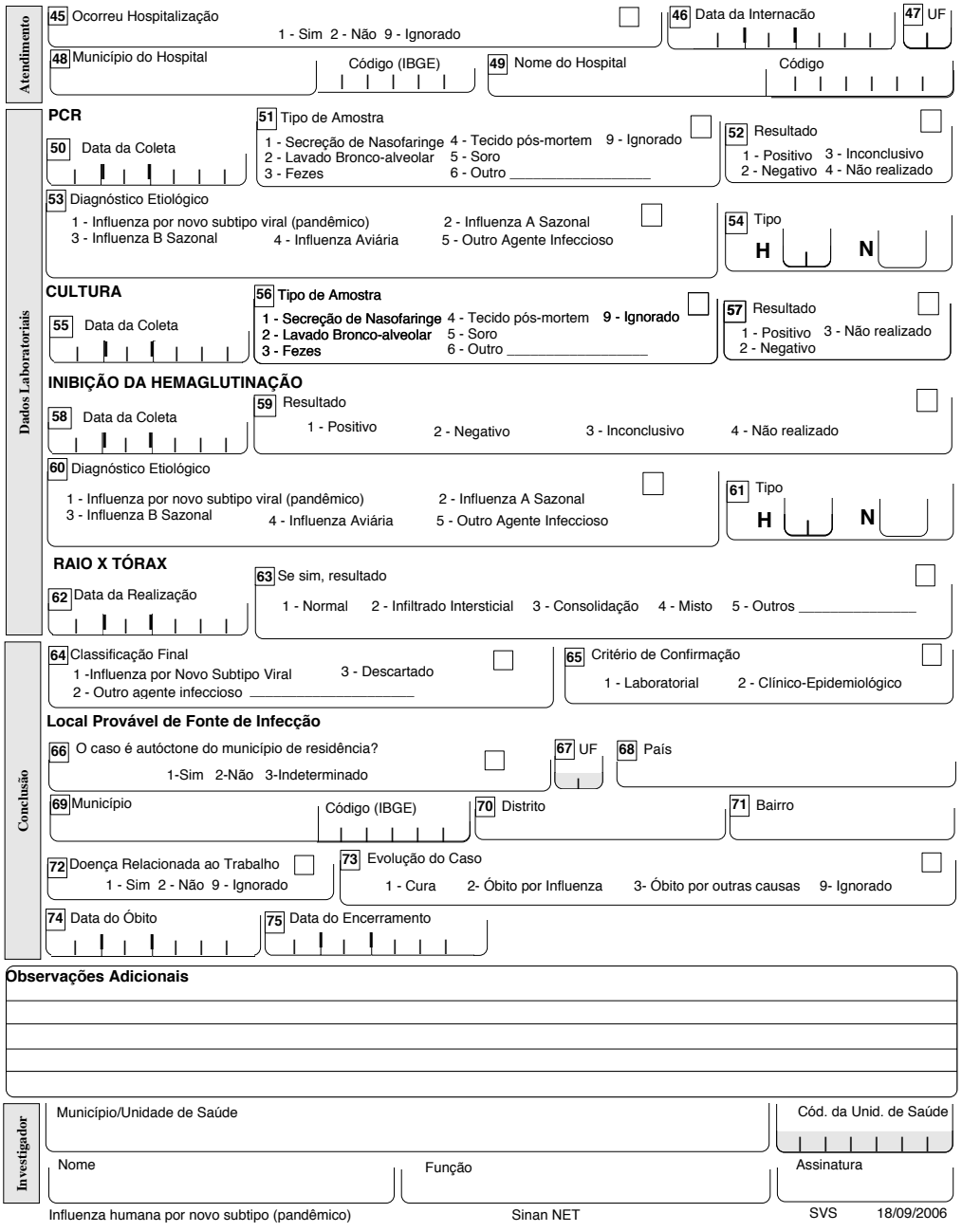
39 Contato com Aves Doentes ou Mortas até 10 dias antes do início dos sinais e sintomas? 1 - Sim 2 - Não 9 - Ignorado — 40 UF — 41 Nome do Município — 42 País

Dados Clínicos

43 Sinais e Sintomas 1 - Sim 2 - Não 9 - Ignorado
Febre — Dispnéia — Mialgia — Diarréia
Tosse — Dor de Garganta — Conjuntivite — Outros _____
Calafrio — Artralgia — Coriza

44 Comorbidade 1 - Sim 2 - Não 9 - Ignorado
Cardiopatia crônica — Renal Crônico — Imunodeprimido — Doença Metabólica Crônica
Pneumopatia crônica — Hemoglobinopatia — Tabagismo — Outros _____

Influenza humana por novo subtipo (pandêmico) Sinan NET SVS 18/09/2006



Atendimento

45 Ocorreu Hospitalização ☐
1 - Sim 2 - Não 9 - Ignorado

46 Data da Internacão

47 UF

48 Município do Hospital

Código (IBGE)

49 Nome do Hospital

Código

Dados Laboratoriais

PCR

50 Data da Coleta

51 Tipo de Amostra ☐
1 - Secreção de Nasofaringe 4 - Tecido pós-mortem 9 - Ignorado
2 - Lavado Bronco-alveolar 5 - Soro
3 - Fezes 6 - Outro _____

52 Resultado ☐
1 - Positivo 3 - Inconclusivo
2 - Negativo 4 - Não realizado

53 Diagnóstico Etiológico ☐
1 - Influenza por novo subtipo viral (pandêmico) 2 - Influenza A Sazonal
3 - Influenza B Sazonal 4 - Influenza Aviária 5 - Outro Agente Infeccioso

54 Tipo
H N

CULTURA

55 Data da Coleta

56 Tipo de Amostra ☐
1 - Secreção de Nasofaringe 4 - Tecido pós-mortem 9 - Ignorado
2 - Lavado Bronco-alveolar 5 - Soro
3 - Fezes 6 - Outro _____

57 Resultado ☐
1 - Positivo 3 - Não realizado
2 - Negativo

INIBIÇÃO DA HEMAGLUTINAÇÃO

58 Data da Coleta

59 Resultado ☐
1 - Positivo 2 - Negativo 3 - Inconclusivo 4 - Não realizado

60 Diagnóstico Etiológico ☐
1 - Influenza por novo subtipo viral (pandêmico) 2 - Influenza A Sazonal
3 - Influenza B Sazonal 4 - Influenza Aviária 5 - Outro Agente Infeccioso

61 Tipo
H N

RAIO X TÓRAX

62 Data da Realização

63 Se sim, resultado ☐
1 - Normal 2 - Infiltrado Intersticial 3 - Consolidação 4 - Misto 5 - Outros _____

Conclusão

64 Classificação Final ☐
1 -Influenza por Novo Subtipo Viral 3 - Descartado
2 - Outro agente infeccioso _____

65 Critério de Confirmação ☐
1 - Laboratorial 2 - Clínico-Epidemiológico

Local Provável de Fonte de Infecção

66 O caso é autóctone do município de residência? ☐
1-Sim 2-Não 3-Indeterminado

67 UF

68 País

69 Município

Código (IBGE)

70 Distrito

71 Bairro

72 Doença Relacionada ao Trabalho ☐
1 - Sim 2 - Não 9 - Ignorado

73 Evolução do Caso ☐
1 - Cura 2- Óbito por Influenza 3- Óbito por outras causas 9- Ignorado

74 Data do Óbito

75 Data do Encerramento

Observações Adicionais

Investigador

Município/Unidade de Saúde

Cód. da Unid. de Saúde

Nome

Função

Assinatura

Influenza humana por novo subtipo (pandêmico) Sinan NET SVS 18/09/2006

9.5. Artigo publicado II: este artigo foi publicado no decorrer do ingresso da aluna no doutorado, o qual é parte do projeto 10.691, com aprovação do CEP citado no item 8.2.

JMV-11-2597.R2(23198)

Journal of Medical Virology 9999:1–9 (2011)

Viral Load and Epidemiological Profile of Patients Infected by Pandemic Influenza A (H1N1) 2009 and Seasonal Influenza A Virus in Southern Brazil

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[4]*Instituto de Pesquisas Biológicas-Laboratório Central/Fundação Estadual de Produção e Pesquisa em Saúde-Rio Grande do Sul (IPB-LACEN/FEPPS-RS), Porto Alegre-RS, Brazil*

Correlation between virologic profile and clinical features of patients infected by influenza virus provides important information for epidemiological control and clinical management of future disease outbreaks. Samples from patients in Southern Brazil, from June to December 2009, were examined and the viral load was correlated with epidemiological data. All samples were analyzed by qRT-PCR for detection of the 2009-pandemic Influenza A (H1N1). Relative viral loads were assessed based on the $2^{-\Delta CT}$ method and epidemiological data were obtained for each patient, following ethical policies. A total of 933 samples were positive for pH1N1 (2009) influenza; 172 were positive for seasonal influenza A; 13 were undetermined; 1992 samples were negative for influenza A. Combined molecular and epidemiological data were available for 38 seasonal and 198 pandemic samples. The median viral load was higher in pandemic than in seasonal influenza samples; in patients infected with pH1N1 (2009), viral load associated positively with chills, myalgia and rhinorrhea, and negatively with dyspnea, but no association was observed with other symptoms, nor with clinical conditions such as pregnancy, smoking, immunodepression and co-morbidities. Regarding patients infected with seasonal influenza, viral loads did not show statistically significant association with any of the symptoms. This is the first study in Brazil that examines epidemiological and molecular data from the 2009 influenza pandemic. The results may serve as a basis for developing strategies to control human-to-human infection and viral dissemination, and for implementing effective measures and public health policies against future novel disease outbreaks. ***J. Med. Virol. 9999:1–9, 2011.***

KEY WORDS: acute respiratory infection; influenza-like symptoms; influenza pandemic; relative viral load; $2^{-\Delta CT}$ method

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Ana Beatriz Gorini da Veiga and Nélson Alexandre Kretzmann contributed equally to this work.

The authors of this study declare they do not have any kind of conflict of interest.

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INTRODUCTION

Influenza A are negative-sense single-stranded RNA ((−)ssRNA) viruses of the genera *Influenzavirus A*, which belongs to the *Orthomyxoviridae* virus family, and is known for causing severe respiratory illness in humans. According to the World Health Organization (WHO), 214 countries and territories had reported influenza cases caused by the pandemic influenza A (pH1N1) 2009 virus—pH1N1 (2009)—until July 2010, including over 18,239 deaths [WHO, 2010]. In Brazil, the highest incidence and mortality rates (66.2 and 2.3 per 100,000 inhabitants, respectively) occurred in the South, with 18,349 confirmed cases. In the state of Rio Grande do Sul, the first case was registered in the epidemiological week 18; owing to the Corpus Christi holiday—during which many people traveled abroad, either to neighbor countries such as Argentina, Uruguay, Paraguay and Chile, or to USA and Europe—the number of cases increased significantly after epidemiological week 23, reaching its peak at epidemiological week 31 and decreasing after that. A total of 2,074 cases were confirmed in Rio Grande do Sul by March 2010 [CEVS, 2009]. Recently, 97 new cases of patients infected with the pH1N1 (2009) have been confirmed in Rio Grande do Sul in 2011, including 13 deaths [CEVS, 2011].

While in countries of the northern hemisphere the dominant circulating influenza subtype in 2009 was the pH1N1 (2009) virus, in South American countries co-circulation of seasonal subtypes (mainly H3N2) was also observed [WHO, 2010]. Identification of the viral subtype and quantitation of viral loads are important for a proper diagnosis and treatment of infected patients. In addition, information on circulating influenza A subtypes may be useful in conducting studies on viral reassortment and prediction of novel emerging pathogenic viruses, as well as for implementing adequate health control measures [Schrauwen et al., 2011].

With the announcement of the pandemic influenza A by the World Health Organization in June 2009, the Health Secretary in Rio Grande do Sul took several measures to control the disease; concerning diagnosis and treatment, such measures included analysis of all samples collected from patients with acute respiratory infection symptoms throughout the State and prescription of oseltamivir [CEVS, 2009]. Even though medical bulletins have been released, the most recent data have not been analyzed and there are no studies relating the viral loads and clinical data found of these patients in Rio Grande do Sul.

This study examined the relative viral loads observed in patients infected with either pH1N1 (2009) or seasonal influenza A viruses in the State of Rio Grande do Sul, Southern Brazil and correlate the results with the clinical and demographical data. The main differences between the present findings and those reported by similar studies in other geographical regions are discussed.

MATERIALS AND METHODS

Study Subjects, Clinical Data, and Biological Samples

During the 2009 influenza pandemic, nasopharyngeal aspirate samples were collected from all patients presenting with an acute respiratory infection in health units throughout Rio Grande do Sul state, Southern Brazil (estimated population: 10.7 million people). For each patient, a clinical form was filled out by the attending physician/nurse, at time of collection [Ministério da Saúde, 2010]. The following data were obtained: demographic characteristics, date of notification, date of onset of symptoms, acute respiratory infection symptoms, co-morbidities, smoking habits, pregnancy status, and X-ray results (when available). All samples and forms were sent to a central laboratory (LACEN-RS) for real time reverse transcription-polymerase chain reactions (qRT-PCR) analysis. Only samples with complete epidemiological data were included in this study. Samples were identified either as pandemic influenza A ($n = 198$) or seasonal influenza A ($n = 38$), according to the qRT-PCR results (see below). All experiments were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki; this study was approved by the Research Ethics Committee of UFCSPA, and written consent was obtained from each patient.

Identification and Quantitation of Influenza A Virus

RNA was extracted from clinical samples using the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. qRT-PCR was performed using the SuperScriptIII Platinum One-Step Quantitative kit and the Influenza A (H1N1) Primer and Probe Set (Invitrogen-Life Technologies, Carlsbad, CA) as described elsewhere [WHO, 2009]. Briefly, reactions were performed using 0.5 μl of SSIII/Platinum Taq Mix, 1 μM of each primer, 250 nM of probe, 12.5 μl 2X Master Mix, 5 μl of RNA sample and water, to a final volume of 20 μl. This method is based on a panel of 4 primers/probe sets: InfA is designed for universal detection of type A influenza viruses; swInfA detects all swine influenza A viruses; swH1 detects swine H1 influenza; and RNP targets the human RNase P gene and serves as an internal positive control for human nucleic acid. Therefore, if a sample was amplified with all the sets, it was considered pandemic influenza A. Samples that were positive only with InfA and RNP were considered seasonal influenza A. Samples that amplified only with RNP were considered negative for influenza A. All PCR reactions were performed in a Step One Plus thermocycler (Applied Biosystems-Life Technologies, Carlsbad, CA). The following conditions were applied: 50°C for 30 min, 95°C for 2 min, and 45 cycles at 95°C for 15 s and 55°C for 35 s.

The relative viral load in each sample was calculated with the threshold cycle (CT) value based on the $2^{-\Delta CT}$ method [Livak and Schmittgen, 2001[Q3]]. The CT values obtained in the qRT-PCR with the InfA and RNP primer/probe sets (target and reference genes, respectively) were used for delta calculation ($\Delta CT = CT_{InfA} - CT_{RNP}$).

Statistical Analysis

Descriptive statistics was used to summarize the data. Categorical data were treated with chi-square and Fisher test, while continuous variables were compared using Mann–Whitney test, Md (P25-P75). Values were considered statistically significant when $P < 0.05$.

RESULTS

Influenza A Identification by qRT-PCR

Over 8,000 patients presented with an acute respiratory infection in the State of Rio Grande do Sul state, during epidemiological weeks 16–46 in 2009. A total of 5,506 respiratory samples were sent to the Central Laboratory in Porto Alegre (LACEN-RS) for viral detection, with 3,108 samples being analyzed by qRT-PCR. Nine hundred and thirty-three samples were positive for pH1N1 (2009); 172 samples were positive for seasonal influenza A. The results of 13 samples were undetermined. The other 1,990 samples were negative for influenza. Combined molecular and epidemiological data were available for only 198 pandemic and 38 seasonal influenza samples.

Relative Viral Load Determination

Analysis of relative viral loads in seasonal and pandemic influenza A samples was based on the $2^{-\Delta CT}$ method [Livak and Schmittgen, 2001]. The median relative viral load in samples positive for pandemic (H1N1) 2009 was 4.9453 (0.1698–47.5496), while in seasonal influenza A samples the median relative viral load was 0.0024 (0.0004–0.019).

Characteristics of Patients Infected With Influenza A in Rio Grande do Sul

Table I describes the characteristics of patients infected by pandemic or seasonal influenza A virus in the State of Rio Grande do Sul state. The highest incidence for pH1N1 (2009) influenza virus infection was observed in patients aged 21–30 years-old, followed by the age groups 31–40 and 11–20 years-old. For seasonal influenza, the highest incidence was also in patients in the age group 21–30 years old, followed by the age groups 31–40 and 41–50 years-old. Among the patients infected with pH1N1 (2009), 56.4% were females; the largest difference in gender distribution was observed in the 21–30 years-old group, in which 69.6% of patients were female (data not shown). In the case of seasonal influenza A virus, 65.8% of the infected patients were females.

TABLE I. Characteristics of Patients Infected by Pandemic Influenza A and Seasonal Influenza A Virus

Characteristic	Frequencies	
	Pandemic (n = 198)	Seasonal (n = 38)
Gender		
Female	56.4	65.8
Male	43.6	34.2
Age group (years)		
0–10	9.6	5.3
11–20	17.9	10.5
21–30	25.7	26.3
31–40	19.3	21.1
41–50	13.3	18.4
51–60	11.0	10.5
61–70	3.2	5.3

All samples examined in this study were from patients who sought medical assistance during 2009 in Rio Grande do Sul presenting at least one of the following influenza-like symptoms: fever, cough, chills, dyspnea, sore throat, arthralgia, myalgia, conjunctivitis, rhinorrhea. The occurrence of these symptoms was observed in patients infected by both pandemic and seasonal influenza, as depicted in Figure 1A. The distribution of these symptoms in each group of patients is shown in Table II.

In addition to symptoms attributable to acute respiratory infection, other clinical manifestations and underlying diseases such as diarrhea, pneumopathies, nephropathies, immunodepression, metabolic disease, smoking habit and pregnancy were also observed in pandemic and seasonal influenza-infected patients. However, the frequency of these symptoms was lower than the frequency of acute respiratory infection symptoms, as shown in Figure 1B and Table III. On the other hand, heart disease was observed only among patients infected by pandemic influenza.

The frequency of death was similar in the pandemic (6%) and the seasonal (5.9%) influenza groups.

Viral Load and Demographic Data of Infected Patients

In order to verify a possible correlation between viremia in the patients and severity of illness—if viral titers in the samples vary according to the patient's age, gender, clinical condition or severity of the illness—viral load data were compared with epidemiological data available for each patient.

The median viral load was higher in patients infected with pandemic H1N1 virus, in comparison to those infected by seasonal influenza A (4.9453 [0.1698–47.5496] vs. 0.0024 [0.0004–0.0190]). The highest viral loads for pandemic influenza occurred in the age group 50–59 years, followed by age groups 60–69 and 10–19 years old. In the case of seasonal

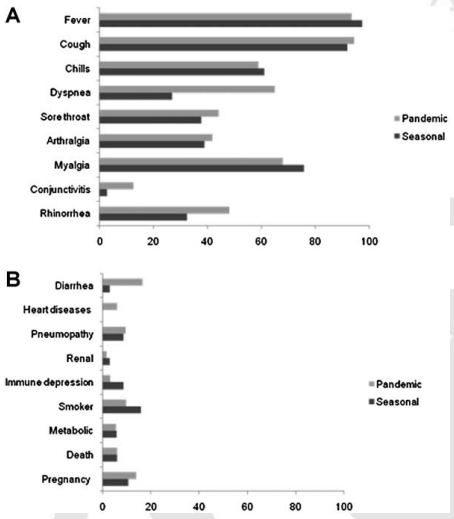

Fig. 1. Frequency of acute respiratory infection symptoms (**A**) and other clinical manifestations (**B**) in patients infected by pandemic influenza A and seasonal influenza A virus.

influenza, the highest viral loads occurred in patients in the age group 40–59 years, followed by the age group 0–9 (Table IV and Figure 2). The median viral loads were not statistically different between females and males, neither between age groups.

Patients with pandemic influenza who sought medical care during the first 48 hr of the onset of symptoms had higher viral loads; in other words, viral loads were associated inversely with the time taken from the onset of symptoms to sample collection (onset hours) (Table IV).

Viral Load and Clinical Profile

As shown in Table V, the patients infected with pH1N1 (2009) the viral load associated positively with the presence of chills ($P = 0.010$), myalgia ($P = 0.007$), and rhinorrhea ($P = 0.039$), while a negative association was observed with dyspnea ($P = 0.02$); no association was found between viral loads and fever, cough, sore throat, arthralgia or conjunctivitis in this group of patients. In patients with seasonal influenza, the viral load did not show statistically significant association with any respiratory infection symptom.

With regards to other clinical conditions such as pregnancy, smoking habits, immunodepression, and comorbidities, no association was found between viral loads and any of the symptoms in either group of patients (Table VI).

TABLE II. Frequencies of Acute Respiratory Infection Symptoms in Patients Infected by Pandemic Influenza A and Seasonal Influenza A Virus

	Frequencies	
Symptom	Pandemic (n = 198)	Seasonal (n = 38)
Fever		
Yes	93.5	97.3
No	6.5	2.7
Cough		
Yes	94.5	91.9
No	5.5	8.1
Chills		
Yes	58.9	61.1
No	41.1	38.9
Dyspnea		
Yes	64.9	27.0
No	35.1	73.0
Sore Throat		
Yes	44.2	37.8
No	55.8	62.2
Arthralgia		
Yes	41.9	38.9
No	58.1	61.1
Myalgia		
Yes	68	75.7
No	32	24.3
Conjunctivitis		
Yes	12.6	2.8
No	87.4	97.2
Rhinorrhea		
Yes	48.1	32.4
No	51.9	67.6

TABLE III. Frequencies of Clinical Manifestations and Evolution of Patients Infected by Pandemic Influenza A and Seasonal Influenza A Virus

	Frequencies	
Characteristic	Pandemic (n = 198)	Seasonal (n = 38)
Diarrhea		
Yes	16.5	2.9
No	83.5	97.1
Heart diseases		
Yes	6.0	0
No	94.0	100
Pneumopathy		
Yes	9.5	8.6
No	90.5	91.4
Renal		
Yes	1.5	2.9
No	98.5	97.1
Immunodepression		
Yes	3.0	8.6
No	97.0	91.4
Smoking habit		
Yes	9.8	15.8
No	90.2	84.2
Metabolic disease		
Yes	5.5	5.7
No	94.5	94.3
Death		
Yes	6.0	5.9
No	94.0	94.1
Pregnancy		
Yes	13.8	10.5
No	86.2	89.5

TABLE IV. Viral Loads[Q4] and Demographic Characteristics of Patients Infected by Pandemic Influenza A and Seasonal Influenza A Virus

Characteristic	Viral load		
	Pandemic (n = 198) Md ($_{P25-P75}$)	Seasonal (n = 38) Md ($_{P25-P75}$)	*P* Pandemic/Seasonal
Gender			
Female	4.7 (0.2–39.6)	0.002 (0.0007–0.03)	0.816/0.415
Male	5.5 (0.2–103.7)	0.001 (0.0002–0.01)	
Age group (years)			
0–10	1.1 (0.2–119.5)	0.006 (0.002–0.01)	0.663/0.377
11–20	7.5 (0.7–161.9)	0.002 (0.0003–0.02)	
21–30	5.7 (0.5–73.7)	0.003 (0.0002–0.8)	
31–40	1.0 (0.01–47.8)	0.001 (0.0006–0.05)	
41–50	5.4 (0.02–75.8)	0.01 (0.001–0.03)	
51–60	10.2 (0.6–39.4)	0.01 (0.003–0.7)	
61–70	8.0 (0.2–18.3)	0.0002 (0.0001–0.0004)	
71–80	—	0.0001 (0.0001–0.0001)	
Onset of symptoms (days)			
<1	7.3 (0.2–180.1)	0.0001 (0.0001–0.0001)	0.003*/0.885
1	14.3 (0.6–161.2)	0.01 (0.0004–4.24)	
2	20.32 (2.3–221.1)*	0.001 (0.0003–35.7)	
3	3.1 (0.1–43.5)	0.002 (0.0002–0.008)	
4	2.0 (0.04–12.0)	0.002 (0.0003–19.4)	
>5	0.6 (0.1–14.6)	0.003 (0.001–0.03)	

DISCUSSION

During the 2009 influenza pandemic, over 2,000 confirmed cases of pH1N1 (2009) infections were recorded in Rio Grande do Sul state, Southern Brazil. Owing to the emergency of medical care during the pandemic, health measures were focused on diagnosis and treatment of these patients, with little attention being given for molecular and epidemiological studies. In this study, virologic and epidemiological data are described for 236 patients who were diagnosed with influenza A virus infection during the pandemic (H1N1) 2009, including 198 patients who were infected by the pH1N1 virus. These results provide important information about the virus, the severity of the disease, and the characteristics of the host–pathogen interaction. Furthermore, this type of analysis may be employed for establishing public health measures to control the spread of infection and disease outbreaks. Since Rio Grande do Sul was the first state to report the 2009 pandemic influenza in Brazil and the number of cases increased rapidly, preventive measures were adopted by the Government, such as: cancellation of classes in Elementary and Middle Schools, restriction of visits to patients in hospital, establishment of a military front next to the main hospital care in the state, the introduction of alcohol gel for hand hygiene in public and private places, among others [Capelozzi et al., 2010; Jiménez et al., 2010; Donalisio et al., 2011].

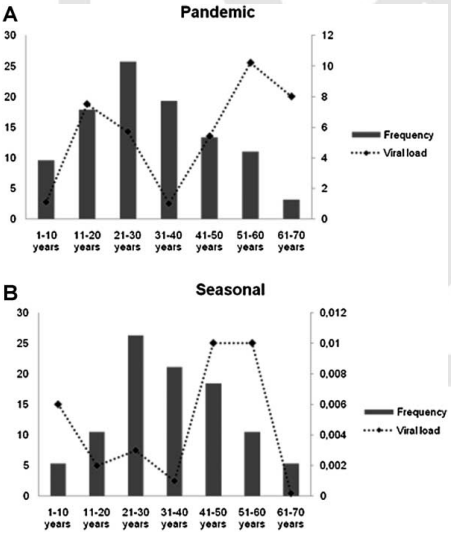

Fig. 2. Viral load in patients infected by pandemic influenza A (**A**) and seasonal influenza A (**B**) virus, distributed by age group.

This study correlates viral loads and epidemiological data for patients infected by influenza virus during the 2009 pandemic, from the epidemiological weeks 16–46. Nasopharyngeal aspirates were collected from patients in Rio Grande do Sul presenting with symptoms of acute respiratory infection and tested by qRT-PCR. Approximately 30% of the samples were positive for pH1N1 (2009) influenza and 5.5% were positive for seasonal influenza (either H1N1 or H3N2). Few reports differentiate the frequency of pandemic versus seasonal influenza during the 2009

TABLE V. Correlations Between Viral Loads and Acute Respiratory Infection Symptoms in Patients Infected by Pandemic Influenza A and Seasonal Influenza A Virus

Symptom	Viral load		
	Pandemic (n = 198)	Seasonal (n = 38)	*P*
	Md ($_{P25-P75}$)	Md ($_{P25-P75}$)	Pandemic/Seasonal
Fever			
Yes	5.0 (0.2–46.3)	0.002 (0.0004–0.02)	0.791/0.261
No	7.5 (0.01–694.6)	0.0002 (0.0002–0.0002)	
Cough			
Yes	5.2 (0.2–46.3)	0.002 (0.0004–0.02)	0.731/1.000
No	1.0 (0.02–563.0)	0.008 (0.00007–0.02)	
Chills			
Yes	12.0 (0.8–83.8)*	0.002 (0.0003–0.01)	0.010*/0.256
No	1.4 (0.02–42.3)	0.003 (0.001–0.8)	
Dyspnea			
Yes	1.2 (0.1–41.7)*	0.002 (0.0004–0.01)	0.002*/0.437
No	12.04 (1.5–172.6)	0.005 (0.001–1.5)	
Sore throat			
Yes	7.2 (0.6–87.2)	0.002 (0.0003–0.01)	0.346/0.684
No	4.62 (0.17–42.33	0.002 (0.0004–0.02)	
Arthralgia			
Yes	7.1 (0.6–52.7)	0.002 (0.0002–0.01)	0.262/0.299
No	4.0 (0.17–45.2)	0.003 (0.0005–0.04)	
Myalgia			
Yes	8.3 (0.6–125.2)*	0.004 (0.0006–0.03)	0.007*/0.096
No	1.1 (0.1–21.3)	0.0004 (0.0001–0.003)	
Conjunctivitis			
Yes	13.3 (2.6–166.4)	0.0001 (0.0001–0.0001)	0.089/0.163
No	4.6 (0.1–45.4)	0.002 (0.0004–0.02)	
Rhinorrhea			
Yes	7.7 (0.6–75.0)*	0.02 (0.0002–11.2)	0.039*/0.218
No	2.6 (0.1–42.6)	0.001 (0.0004–0.008)	

pandemic period in Brazil. According to the Health Surveillance Secretary in Brazil, among the acute respiratory infection cases registered in 2009 in the country until the epidemiological week 47, 34.5% were positive for pH1N1 (2009) influenza, while 2.7% were positive for seasonal influenza; in the south, 40.9% and 1.7% of the acute respiratory infection cases were positive for pandemic and seasonal influenza A, respectively [SVS, 2009]. The most recent international publication describing the 2009 influenza pandemic in Brazil reports the situation from epidemiological week 16 to epidemiological week 33 [Oliveira et al., 2009]. Even though the number of cases in Rio Grande do Sul reduced after epidemiological week 31 (first week of August), severe acute respiratory infection cases caused by pandemic influenza were reported in the state until December 2009 [SVS, 2009]. Thus, this study contributes with additional data on these cases.

In the present study, 56.4% of the patients infected by pandemic influenza were females, which is in accordance with the national surveillance report (56.5% female) [Oliveira et al., 2009] and with the 2010 demographic census of the State of Rio Grande do Sul (53.1% females) [IBGE, 2010]. However, when comparing the sex and age average, 21–30 years old, it appears that the incidence of pH1N1 (2009) infections in women is higher than the average population [IBGE, 2010]. There was a high frequency of infection in patients between 20 and 40 years old in both pandemic and seasonal influenza groups; considering younger patients (<20 years old), pandemic cases were more common than seasonal infections. These findings are in agreement with other epidemiological studies worldwide [Oliveira et al., 2009; Vaillant et al., 2009]. Approximately 5.96% of the pH1N1 (2009) cases analyzed in this study progressed to death, which is in accordance to the national (5.8%) and worldwide reports for mortality of 2–9% during the 2009 influenza pandemic [SVS, 2009]. A similar mortality rate was observed in the present study regarding seasonal influenza infection. It has been well documented and discussed elsewhere that, despite the negative impacts that an influenza pandemic has on global health and economy, mortality by influenza virus is higher in interpandemic years due to infection by seasonal influenza [Hilleman, 2002].

It has been reported that, in individuals infected by influenza virus, the highest viral loads and viral shedding are observed within 1–2 days after onset of acute respiratory infection symptoms [Lau et al., 2010]. Accordingly, in the group of patients infected by pandemic influenza the highest viral loads were detected in samples collected after two days of onset of symptoms, and in the group of seasonal influenza the highest viral loads were in samples collected after 24 hr of the onset of symptoms. In a study comparing viral loads in patients with seasonal and pandemic

TABLE VI. Correlations Between Viral Loads and Clinical Characteristics of Patients Infected by Pandemic Influenza A and Seasonal Influenza A Virus

Characteristic	Viral load		
	Pandemic (n = 198)	Seasonal (n = 38)	*P*
	Md ($_{P25-P75}$)	Md ($_{P25-P75}$)	Pandemic/Seasonal
Diarrhea			
Yes	6.2 (0.5–38.0)	0.0002 (0.0002–0.0002)	0.848/0.198
No	5.2 (0.2–55.2)	0.002 (0.0004–0.02)	
Heart diseases			
Yes	2.6 (0.1–15.9)	—	0.410/—
No	5.2 (0.2–48.4)	0.00005 (0.00005–0.00005)	
Pneumopathy			
Yes	4.6 (0.1–132.6)	0.002 (0.0002–0.02)	0.874/0.929
No	5.4 (0.2–47.8)	0.001 (0.0001–0.02)	
Renal			
Yes	18.3 (11.8–xx[Q5])	38.9 (38.9–38.9)	0.189/0.113
No	4.7 (0.2–47.9)	0.002 (0.0004–0.02)	
Immunodepression			
Yes	4.8 (1.1–315.6)	0.002 (0.0002–0.02)	0.948/0.768
No	5.4 (0.2–48.2)	0.003 (0.0004–0.02)	
Smoking habit			
Yes	4.2 (0.02–39.6)	0.01 (0.0004–0.03)	0.726/0.423
No	6.5 (0.2–60.7)	0.002 (0.0002–0.02)	
Metabolic			
Yes	3.4 (0.01–16.0)	7.0 (0.01–14.0)	0.336/0.201
No	5.4 (0.2–53.0)	0.002 (0.0004–0.02)	
Death			
Yes	5.8 (0.2–48.1)	0.003 (0.0004–0.02)	0.142/0.341
No	0.5 (0.03–13.0)	0.001 (0.0002–0.001)	
Pregnancy			
Yes	17.0 (0.2–160.6)	0.03 (0.008–35.7)	0.510/0.51
No	5.4 (0.2–45.0)	0.002 (0.0003–0.01)	

influenza, To et al. [2010] found higher viral loads in the former group, and in both groups viral load decreased gradually after onset of symptoms [To et al., 2010]. The present study reports higher viral loads in pandemic influenza-infected patients than in those with seasonal influenza virus; the difference between results described in the current study and those from To et al. [2010] relies in the fact that their seasonal influenza samples were from archived respiratory specimens from 2007, while in the present study, both seasonal and pandemic samples were collected during the 2009 influenza pandemic. On the other hand, another study found no significant differences in viral loads among samples positive for pH1N1 (2009) virus, seasonal H1N1 and seasonal H3N2 collected during the 2009 pandemic [To et al., 2010]. As will be discussed further on, viremia in patients infected by different influenza A subtypes depends on several factors that interfere with virus–host interaction.

This study examined samples from patients that sought health emergency units in Rio Grande do Sul during the 2009 influenza pandemic presenting acute respiratory infection symptoms, so fever and cough were observed in most of the patients, regardless of the virus subtype. However, no association between mean viral loads and these symptoms was observed. On the other hand, results show an association between viral loads in pandemic cases and chills, myalgia, rhinorrhea, and dyspnea. Regarding other clinical characteristics, no association was found in this study, not even between viral loads and pregnancy, immunodepression or death. While some studies report no statistical correlation between viral loads and acute respiratory infection symptoms [To et al., 2010], others report a correlation between viral loads and systemic comorbidities [Lee et al., 2009].

Lee et al. [2009] emphasize that the use of systemic corticosteroids to treat concomitant medical conditions during influenza infection is associated with a slow viral decrease and, thus, to a higher viral load in these patients [Lee et al., 2009]. The present study did not evaluate the use of any medication by the infected patients, which could influence viral shedding. On the other hand, the use of antiretroviral drugs by immunosuppressed patients may lower viral loads, which could explain the lack of association between viral loads and immunosuppression in this study.

In a recent study in pregnant patients infected by the pH1N1 (2009) virus in Porto Alegre, no correlation was found between pregnancy or comorbidities and the risk of being admitted to the Intensive Care Unit (ICU); no viral loads were examined in the study [Jiménez et al., 2010]. Accordingly, no association between pregnancy and viral load, neither between pregnancy and death was found (data not shown).

The pathogenicity and replicative fitness of influenza A viruses may vary significantly among different

viral clades of the same subtype [Memoli et al., 2009]. Mutations in proteins involved in viral infection and replication may account for an enhanced replicative fitness, which plays an important role in viral evolution. Considering that the immune response and genetic background of the host are also key factors influencing viral evolution, the viral–host interaction is a complex process that depends on and involves the biology of both pathogen and patient; thus, parameters such as viral load may vary between different human populations, epidemic seasons and viral subtypes. Therefore, it is not surprising that studies that examine viral loads in samples from groups of patients infected by different influenza viruses from different geographical regions find diverging results. Nevertheless, such epidemiological and molecular data help understanding local and global viral dynamics.

One limitation of this study is that, since it examined data already collected from health units throughout the State of Rio Grande do Sul, it was not possible to require physicians and nurses to complete the Pandemic Human Influenza Investigation Forms, so some information has not been provided. In addition, owing to technical limitations such as the number of staff members and thermocyclers available for real time RT-PCR, the molecular analyses were performed in separate laboratory units and most of the information from qRT-PCR was not available. Therefore, even though over 3,000 samples were analyzed by qRT-PCR and that 933 samples were positive for pandemic and 172 samples were positive for seasonal influenza, combined information on viral loads and epidemiological data was available for only 198 pandemic and 38 seasonal influenza samples.

Since the aim of this study was to analyze and compare the relative viral loads of seasonal or pandemic influenza A in the samples, only the InfA gene was used as target gene. Nonetheless, the CT values obtained in the qRT-PCR for amplification of the swInfA and the swH1 genes are also being examined (data not shown); since they may give different relative viral loads from those obtained with the InfA gene, comparison of the three markers in the same samples is informative and could have an application in finding the best parameter to be taken into account when studying pH1N1 (2009) infection in a specific patient population.

Finally, this is the first study conducted in Brazil that analyzes and makes correlations between epidemiological and molecular data from the 2009 influenza pandemic. Descriptions of antigenicity and other aspects of viral phenotype, such as virulence or transmissibility, improve understanding of the constraints on viral evolution [Ferguson et al., 2003]. Most importantly, considering that 97 cases of influenza A pH1N1 (2009) infections have been confirmed in patients in Rio Grande do Sul in 2011, including 13 deaths [CEVS, 2011], the present results serve as a basis for developing strategies to control human-to-human infection and viral dissemination, and for implementing effective measures and public health policies against future novel disease outbreaks. Collaborations with the Ministry of Health in Brazil and the Health Surveillance Unit of the state of Rio Grande do Sul have been established aiming the improvement of the influenza A database in our region. Furthermore, in 2010 the Central Laboratory of the State (LACEN-RS) contracted staff scientists to work on the molecular diagnosis of viral pathogens such as influenza A, dengue and Yellow fever, which will help in future epidemiological and molecular studies.

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