

**UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE
PORTO ALEGRE – UFCSPA
PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA**

Luiz Gustavo dos Anjos Borges

**Microbioma e diversidade bacteriana
do trato respiratório superior em
pacientes infectados pelo vírus
Influenza A**

UFCSPA
Universidade Federal de Ciências da Saúde
de Porto Alegre

**Porto Alegre
2016**

Luiz Gustavo dos Anjos Borges

Microbioma e diversidade bacteriana do trato respiratório superior em pacientes infectados pelo vírus Influenza A

Tese submetida ao Programa de
Pós-Graduação em Patologia da
Fundação Universidade Federal de
Ciências da Saúde de Porto Alegre
como requisito para a obtenção do
grau de Doutor

Orientadora: Dra. Ana Beatriz Gorini da Veiga
Co-orientador: Dr. Fabrício Souza Campos

**Porto Alegre
2016**

“If gripe condemns,
the secondary infections execute.”

Louis Cruveilhier

(1873 – 1950)

Aos meus pais,
que nunca me mostraram como desistir -
mas sempre,
como mudar, desviar, variar,
substituir, alterar, transformar
...

Agradecimentos

À minha orientadora, ANA. Por me dar a oportunidade de fazer parte do que é ser cientista, pela oportunidade de fazer muito “do que eu quis” e, pela oportunidade de fazer tudo, daquilo que foi possível fazer, durante estes quatro anos. Por ter confiado em minhas ideias e por sempre acreditar que é possível transformar ou construir, mesmo quando tudo parece estar na contramão. Obrigado por sempre estar disposta a ouvir, orientar e, quando possível, atender minhas solicitações.

Ao meu co-orientador FABRÍCIO, por ter me recebido no Labvir. Pela paciência em passar seus conhecimentos, mesmo quando o mundo parecia estar sobre seus ombros. Por estar sempre disposto a contribuir para a ciência e, principalmente, para a virologia, lamentavelmente marginalizada nas instituições de ensino/pesquisa deste Estado e deste país. Espero que este nosso encontro possa ter sido apenas o início de muitos trabalhos “viroológicos”.

Aos professores do Labvir, Dra. ANA CLÁUDIA e Dr. PAULO, por aceitarem minha presença no Labvir. Obrigado por me darem a oportunidade de trabalhar com virologia, como um virologista! Por me mostrarem as atividades de pesquisa e, principalmente, a importância de um verdadeiro virologista. Tenho orgulho de ter feito parte deste grupo, mesmo que como membro externo.

Aos Professores do Icahn Scholl of Medicine at Mount Sinai, Dr. ADOLFO GARCÍA-SASTRE e Dr. RANDY ALBRECHT que permitiram que por rápidos e intensos sete meses, eu entrasse no “mundo do vírus influenza”. Aos demais pesquisadores da instituição, Dr. SHASHANK, Dr. JUAN, Dra. MAITE, Dr. GIORGI, Dr. GUOJUN, Dra. TERESA, Dr. MICHAEL, Dra. SARA, Dr. VICTOR,

Dra. LUPITA, pelas orientações, pelos ensinamentos e pela gentileza. Tenham certeza que a saudade que hoje sinto é sinal de que há muitas lembranças boas que permanecem em minha memória.

Aos demais professores que proporcionaram meu crescimento como pesquisador, em especial Dr. HUGO (UFRGS) e Dr. ALEXANDRE (FIOCRUZ-MG). Seus ensinamentos me fizeram enxergar além e contribuíram significativamente para a minha visão como pesquisador.

Ao Programa de Pós Graduação em Patologia, aos professores que sempre deram o seu melhor à cada aula. Obrigado MARISTELA e LUCIANI que sempre estiveram dispostas a dar toda a atenção e resolver problemas, muitas vezes insolucionáveis.

À Dra. TATIANA (LACEN) que sempre me orientou, aconselhou e incentivou. Espero que possamos manter uma parceria duradoura. Sem sua contribuição nada teria acontecido.

Aos colegas Dr. FERNANDO (japa), FERNANDO (finoketti), THALITA, JÚ, que durante estes quatro anos compartilharam não só toda a dificuldade de um pesquisador em formação, mas também as alegrias e conquistas de cada dia.

Aos demais alunos, em especial aos persistentes que ainda sonham com a virologia. A AMANDA que persiste nos estudos sobre o vírus influenza. A dificuldade de trabalhar com um vírus tão difícil quanto é o vírus influenza não será em vão. O futuro é um tanto quanto promissor. Mantenha a convicção, mesmo quando tudo parece incerto. Mantenha o foco.

À esposa Adri e à Dra. ADRIANA por estar ao meu lado no melhor e no pior. Por ser minha orientadora nos finais de semana, feriados e quando todos já estão sonhando com o dia seguinte. Por aceitar meus argumentos e não

desistir da ciência, mesmo quando não há como ter razão para ser pesquisador em um país como o nosso. Fé na ciência e nos nossos!

À minha família que me deu tudo e um pouco mais! ARTHUR, ROSANE, JULI, DESSA, AMELIE, HELENA, DULCE, ANTÔNIO ... Aos que estão e aos que estiveram.

Aos meus amigos, sem exceção!

À todos!

Sumário

1. Introdução	13
1.1. O vírus influenza	15
1.1.1. Hemaglutinina (HA)	19
1.1.2. Infecção pelo IAV(H1N1)pdm09	21
1.2. Microbioma humano	22
1.2.1. Microbioma da nasofaringe humana	26
1.3. Coinfecção vírus-bactéria	28
1.4. Microbioma da nasofaringe de pacientes infectados pelo IAV(H1N1)pdm09	30
1.5. Referências Bibliográficas	32
2. Objetivos	37
3. Artigos Científicos Redigidos em Inglês	
3.1. “Chronic pneumopathy associated with lower bacterial diversity on upper respiratory tract of patients infected by IAV(H1N1)pdm09”	38
3.2. “Bacterial community in the nasopharynx of hospitalized patients infected and non-infected by influenza A virus”	65
4. Considerações Finais	92
5. Anexos	
5.1. Anexo I – Genomic analysis of pandemic and post-pandemic influenza A pH1N1 viruses isolated in Rio Grande do Sul, Brazil	99
5.2. Anexo II – Polymerase activity of influenza virus (H1N1)pdm09 ribonucleoprotein reassortment with H7N9 human influenza virus	110
5.3. Anexo III – Parecer do Comitê de Ética em Pesquisa	125
5.4. Anexo IV – Ficha de Investigação	128

Lista de Abreviaturas

AS: Ácido Siálico

AUR: *Staphylococcus aureus*

HA: hemaglutinina

HA0: hemaglutinina imatura

HA1: subunidade 1 da hemaglutinina

HA2: subunidade 2 da hemaglutinina

HAE: *Haemophilus influenzae*

HFR: *Hospitalization Fatality Risk*

IAV: Influenza A Vírus

IAV(H1N1)pdm09: Vírus Influenza A H1N1 da linhagem pandêmica de 2009

ITS: *Internal Transcribed Spacer*

LACEN-RS: Laboratório Central do Estado do Rio Grande do Sul

M1: proteína de matriz 1

M2: proteína de matriz 2

MCAT: *Moraxella catarrhalis*

NA: neuraminidase

NEP: *Nuclear Exportation Protein*

NP: nucleoproteína

NS1: proteína Não Estrutural 1

NS2: proteína Não Estrutural 2

OMS: Organização Mundial da Saúde

OTU: *Operational taxonomic unit*

PA: *Polymerase Acid*

PB1: *Polymerase Basic 1*

PB2: *Polymerase Basic 2*

PCR: *Polymerase Chain Reaction*

PGM: *Personal Genome Machine*

PNE: *Streptococcus pneumoniae*

RISA: *Ribosomal Intergenic Spacer Analysis*

SARI: *Severe Acute Respiratory Infection*

SPSS: *Statistic Package for the Social Sciences*

SRAG: Síndrome Respiratória Aguda Grave

TBE: Tris-Borato EDTA

UFCSPA: Universidade Federal de Ciências da Saúde de Porto Alegre

UPGMA: *Unweighted Pair Group Method with Arithmetic mean*

URT: *Upper Respiratory Tract*

WHO: *World Health Organization*

Resumo

Introdução:

O vírus influenza A (IAV) é um dos principais agentes etiológicos da síndrome respiratória aguda grave (SRAG) em humanos, podendo causar pandemias. A mais recente pandemia por IAV ocorreu em 2009, sendo ocasionada pelo subtipo H1N1. Frequentemente é reportada uma associação positiva na interação entre bactérias patogênicas e vírus respiratórios nas infecções do trato respiratório, o que, por sua vez, tem relação com os sintomas e o desfecho da SRAG.

Objetivos:

Investigar o microbioma bacteriano do trato respiratório superior em pacientes infectados pela linhagem IAV pandêmica de 2009.

Material e Métodos

Um total de 144 amostras de aspirado de nasofaringe coletadas em 2012 e enviadas para o LACEN-RS para diagnóstico viral foram selecionadas para o estudo. Todas as amostras foram provenientes de pacientes hospitalizados por apresentarem SRAG. Realizou-se PCR espécie específico para a identificação de bactérias patogênicas, método de *fingerprinting* por PCR para observação da diversidade bacteriana e, por fim, sequenciamento de alto desempenho para identificar a composição do microbioma bacteriano.

Resultados

A PCR identificou a presença de bactérias patogênicas em mais de dois terços das amostras testadas. No entanto, a presença bacteriana não esteve associada à presença de IAV pandêmico. A PCR *fingerprinting* resultou na identificação da menor diversidade bacteriana nos pacientes positivos para o influenza A pandêmico com pneumopatia crônica quando comparados àqueles sem a mesma. O microbioma bacteriano de pacientes positivos para o IAV pandêmico apresentou maior frequência de sequências bacterianas do filo Firmicutes, enquanto que o microbioma de pacientes negativos apresentaram maior frequência para o filo Proteobacteria.

Conclusões

Os pacientes com doença crônica do trato respiratório infectados pelo IAV estão mais propensos à baixa diversidade bacteriana e, consequentemente, à dominância de linhagens potencialmente patogênicas. O IAV(H1N1)pdm09 é um importante modulador da composição do microbioma favorecendo, o gênero *Streptococcus* em detrimento aos gêneros pertencentes ao filo Proteobacteria.

Palavras-chave

Metabarcoding; bacterioma; *RISA*; Pandemia; IAV(H1N1)pdm09;

1. Introdução

A gripe ou influenza é uma doença respiratória aguda causada pelos vírus influenza A ou B. A influenza sazonal apresenta um período de incubação de 1 a 4 dias, podendo ser mais extenso em crianças (WHO, 2011). A gravidade clínica da infecção varia desde uma infecção assintomática até uma pneumonia viral importante, podendo causar a morte do indivíduo. Complicações comuns ocasionadas pela infecção pelo influenza incluem pneumonia secundária causada por bactérias, otite média em crianças e a exacerbação de doenças crônicas (CEVS, 2011).

O aumento da circulação do vírus influenza na população está diretamente associado ao aumento de doenças respiratórias agudas, do número de visitas médicas e de hospitalizações, e dos casos de morte. Esta associação dá a este agente etiológico um caráter relevante em saúde pública. Estima-se que durante períodos não pandêmicos, a taxa de infecção por influenza entre adultos seja entre 5 e 15% (SVS, 2012).

A vigilância do influenza no Estado do Rio Grande do Sul utilizou diferentes estratégias para controlar os possíveis surtos de gripe pelo vírus Influenza A pandêmico [IAV(H1N1)pdm09]. Foram empregadas as investigações de surtos em instituições/comunidades fechadas, o controle de síndromes gripais em unidades sentinelas e o controle dos casos hospitalizados por síndrome respiratória aguda grave (SRAG). A triagem de caso suspeito foi definida pela presença de sinais e sintomas de SRAG, que incluem febre, tosse e dispneia. Além disso, exames diagnósticos para o vírus influenza foram empregados seguindo a recomendação da Organização

Mundial da Saúde (OMS) (WHO, 2011), além do diagnóstico diferencial para observar a existência de coinfeção por outros vírus respiratórios, como o vírus sincicial respiratório (VSR), o adenovírus e o parainfluenza .

Em 2009, o IPB/LACEN/RS recebeu 5.286 amostras de casos suspeitos de Influenza A. Destas, 2.109 (39,9%) foram positivas para o IAV(H1N1)pdm09 e 523 (9,9%) para a linhagem sazonal que, até então, circulava na população (CEVS, 2009), sendo uma proporção de 4:1 entre casos de linhagem pandêmica e de linhagem sazonal. Estes dados mostraram que, embora ainda houvesse uma parcela do vírus sazonal causando importante infecção, o IAV(H1N1)pdm09 apresentava um domínio das infecções respiratórias agudas por influenza durante o ano pandêmico.

Em 2010, após o primeiro ano de vacinação contra a linhagem pandêmica, não se detectou no Estado nenhum caso de infecção respiratória aguda pelo novo subtipo viral. Possivelmente, este dado esteja relacionado à obtenção da meta de vacinação de 80% da população (Luna e cols., 2014). Em agosto deste mesmo ano, a OMS anunciou o fim do período pandêmico e o início da fase pós-pandêmica (CEVS, 2011).

Os casos de infecção respiratória aguda pelo IAV(H1N1)pdm09 tornaram a ser detectadas em 2011. Durante aquele ano, novamente se observou a co-circulação dos vírus IAV(H1N1)pdm09 (107 casos, 7,5% dos casos de SRAG) e influenza A sazonal (182 casos, 12,7% dos casos de SRAG), em todas as faixas etárias da população (CEVS, 2011). Este dado descaracterizou uma contínua dominância da linhagem pandêmica sobre a linhagem sazonal que havia sido observada em 2009 e reforçou a importância

da manutenção das campanhas vacinais pós período pandêmico, bem como o acompanhamento dos subtipos circulantes (CEVS, 2011).

O terceiro ano do período pós-pandêmico foi marcado por uma vigilância mais experiente para o controle dos novos casos de infecção por IAV(H1N1)pdm09. A prática da triagem de novos casos suspeitos por SRAG, adquirida desde o ano pandêmico, ocasionou o aumento do número de notificações em 2012. No Brasil, foram observados 20.539 casos de SRAG. Destes, 19,5% foram confirmados como sendo ocasionados pelo vírus influenza. Observou-se uma dominância dos casos de IAV(H1N1)pdm09 (65% dos casos) sobre os casos de vírus influenza A sazonal H1N1 (SVS, 2012). No Rio Grande do Sul, foram confirmados 522 casos (13,2% dos casos de SRAG) de IAV(H1N1)pdm09 com 67 mortes (28,9% dos casos de morte com SRAG). Em torno de 34% dos casos de morte ocorreram entre pacientes que apresentavam comorbidades, como pneumopatia crônica, cardiopatia, obesidade, doença renal ou *diabetes mellitus* (CEVS, 2012).

1.1. O vírus influenza

O vírus influenza está classificado dentro da família *Orthomyxoviridae*. Os vírus desta família são envelopados e caracterizados por apresentarem genoma segmentado de RNA fita simples e senso negativo. O genoma é utilizado como molde para o RNA mensageiro e para a síntese da fita complementar. O vírion possui aspecto pleomórfico, na grande maioria com partículas de diâmetro entre 80-120 nm (Cheung e Poon, 2007), mas pode se apresentar de forma alongada, atingindo até 300 nm (Arias e cols., 2009).

Filogeneticamente, existem três principais tipos virais: influenza A, influenza B e influenza C. Enquanto este último, que ocorre principalmente em humanos, não causa danos significativos à saúde e apresenta pequena variação antigênica, o influenza A e o influenza B causam a influenza viral humana, com altas taxas de morbidade e mortalidade (Frank, 2002; Hilleman, 2002).

O influenza A é o maior responsável por infecções em humanos, estando envolvido na ocorrência de pandemias. Este tipo viral infecta também aves e outros mamíferos como suínos, cavalos, martas, baleias e focas. (Cheung e Poon, 2007; Taubenberger e Kash, 2010).

O genoma total do vírus influenza A possui 13.6 Kb, sendo dividido em 8 segmentos de RNA genômico (Figura 1) que codificam 11 proteínas principais, além de outras proteínas acessórias (Cheung e Poon, 2007; Arias e cols., 2009; Vasin e cols., 2014). Cada segmento possui nas extremidades 5' e 3' uma região não traduzida de tamanho variável contendo sinais de replicação e encapsidação viral.

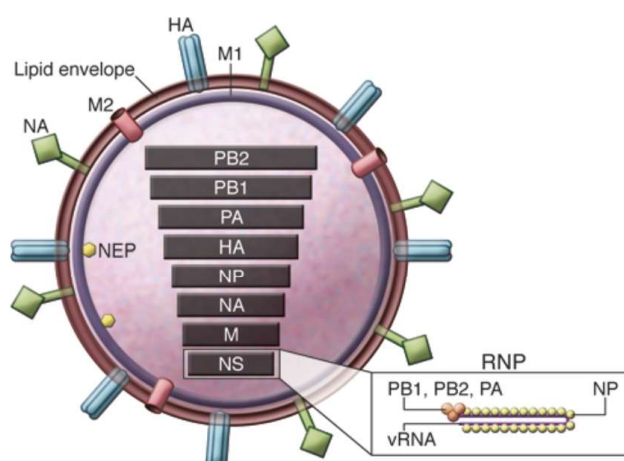


Figura 1 – Partícula do vírus influenza A. A representação mostra genoma viral segmentado, proteínas estruturais e não estruturais. Em destaque o complexo ribonucleoproteína (RNP).

Fonte: Tscherne e García-Sastre, 2011

Cada um dos oito segmentos de RNA do vírus influenza A possui um gene que codifica pelo menos uma proteína. O segmento 1 do genoma viral codifica uma das subunidades da polimerase viral, a polimerase básica 2 (PB2). Estudos sugerem que esta proteína possa ser a maior determinante do controle da patogenicidade do vírus influenza A (Shinya e cols., 2004; Zhou e cols., 2011). Os segmentos 2 e 3 do genoma codificam, respectivamente, a polimerase básica 1 (PB1) e a polimerase ácida (PA), subunidades da RNA polimerase viral. A subunidade PB1 está ligada à PA na porção amino-terminal e à PB2 na porção carboxi-terminal, sendo responsável tanto pelo acoplamento das três subunidades da polimerase quanto pela função catalítica da polimerização do RNA (Cheung e Poon, 2007). Algumas linhagens virais também codificam uma proteína de 90 aminoácidos chamada de PB1-F2, que é reconhecida como um fator indutor de apoptose por indução de estresse oxidativo celular (Schnitzler e Schnitzler, 2009), contribuindo indiretamente nos estágios de replicação viral e no aumento da gravidade da doença (Shin e cols., 2015).

A PA é a menor subunidade da RNA polimerase viral. Essa proteína contém sinal de localização nuclear e é essencial para a atividade de transcrição e replicação. Sua expressão na célula, na ausência das demais subunidades que compõem a polimerase, pode causar indução de lise celular (Cheung e Poon, 2007).

O segmento 4 do genoma viral codifica a hemaglutinina (HA), uma glicoproteína de superfície responsável pela ligação ao ácido siálico presente nos receptores das células do epitélio respiratório do hospedeiro e pela fusão do vírus à membrana celular (Cheung e Poon, 2007). O segmento 5 codifica a

nucleoproteína (NP), uma proteína envolvida nas etapas de transcrição e replicação do genoma viral, além de encapsidar o genoma viral (Arias e cols., 2009). O segmento 6 codifica a neuraminidase (NA), uma glicoproteína que atua na liberação da nova partícula viral através da clivagem do resíduo de ácido siálico do receptor celular da célula hospedeira infectada, tornando a nova partícula viral apta a infectar novas células. Devido ao seu importante papel na multiplicação viral, a NA é alvo de drogas antivirais, as quais bloqueiam o sítio de atividade enzimática, inibindo a neuraminidase (Moscona, 2005).

O segmento 7 do influenza A codifica duas proteínas: a proteína de matrix 1 (M1) e a proteína de matrix 2 (M2). A M1 promove a interação do complexo ribonucleoproteína (RNP) com a membrana lipídica da célula hospedeira durante a montagem viral, fornecendo a estrutura do vírus. A M2 é uma proteína transmembrana que forma canais iônicos para a regulação do pH viral, atuando tanto em etapas da entrada quanto do brotamento viral (Rossman e Lamb 2011). O segmento 8 do genoma do influenza A é responsável por codificar a proteína não-estrutural 1 (NS1) e a proteína não-estrutural 2 (NS2). A NS1 está envolvida em processos de evasão viral do sistema imune do hospedeiro. A NS2 está envolvida na sinalização de exportação nuclear do RNA viral (Cheung e Poon, 2007; Rossman e Lamb, 2011).

Baseado na variação da estrutura antigênica das glicoproteínas de superfície HA e NA, o vírus influenza A é dividido em subtipos. Atualmente são conhecidos 18 diferentes HA e 11 diferentes NA (Tong et al., 2012; Neumann e Kawaoka, 2015). A grande maioria destes subtipos podem ser encontrados em

aves aquáticas, o que sugere que estes animais sejam os reservatórios naturais deste vírus. As mutações que ocorrem nos genes dessas glicoproteínas podem determinar a efetividade do sistema imune do hospedeiro no reconhecimento antigênico da variante. Por outro lado, a recombinação ou rearranjo destas diferentes estruturas antigênicas pode ser determinante para o sucesso da infecção viral. Apenas os subtipos H1N1, H2N2, H3N2, H5N1, H7N7, H7N9 e H9N2 foram isolados em humanos, indicando que há uma importante restrição na interação subtipo viral-hospedeiro (Cheung e Poon, 2007).

1.1.1. Hemaglutinina (HA)

A glicoproteína de superfície HA é responsável pela ligação do vírus influenza a receptores da superfície celular do hospedeiro e pela mediação da fusão da membrana viral à membrana do hospedeiro, a fim de promover a endocitose do capsídeo viral. A HA é o principal alvo de neutralização viral por anticorpos. A alta pressão imunológica sobre a molécula HA é um importante direcionador e a maior causa da alta taxa de mutação desta região viral, mutação esta que é estimada em uma substituição de base no gene HA por geração viral (Webster e cols., 1992; Cheung e Poon, 2007; King e cols., 2011).

A HA é traduzida na forma precursora HA0, necessitando de processamento pós-tradução. O processamento da HA0 ocorre em três etapas, que envolvem clivagem proteolítica (Kim e cols., 2013), glicosilação (Medina e cols., 2013) e acilação de ácidos graxos (Veit e cols., 2013). Em uma última etapa de processamento, é necessário que a HA0 seja clivada por ação de

proteases, gerando duas novas subunidades. O processo de clivagem ocorre no meio extracelular por proteínas exógenas e é relativamente instável em pH ácido. A subunidade HA1 possui aproximadamente 324 aminoácidos e cadeias variáveis de carboidratos, e a subunidade HA2 possui aproximadamente 222 aminoácidos, cadeias variáveis de carboidratos e três resíduos de palmitato. Esta clivagem final é um pré-requisito para a maturação da HA do vírus influenza A (Webster e cols., 1992; Cheung e Poon, 2007; King e cols., 2011; Lu e cols., 2012).

Ao longo do processo de maturação, a molécula de HA forma homotrímeros. Cada molécula de HA consiste de uma porção globular suportada por uma haste. A porção globular é formada exclusivamente pela subunidade HA1. O sítio de ligação ao receptor de membrana da célula hospedeira está localizado no domínio da HA1. A especificidade desta ligação determina a afinidade de certos subtipos virais do vírus influenza ao hospedeiro. A HA reconhece as moléculas de ácido siálico (AS) no receptor de superfície da célula hospedeira. Os ASs são monossacarídeos de nove carbonos comumente encontrados na extremidade terminal de muitos conjugados de glicol, geralmente ligadas à galactose através de ligações $\alpha 2,3$ ou $\alpha 2,6$ (Jongkon e cols., 2009; Ma e cols., 2009). Moléculas HA que contêm um resíduo glutamina na posição 226 possuem preferência por ligações ao AS $\alpha 2,3$, encontrado em células do tecido gástrico de aves. A presença de moléculas de leucina na posição 226 da molécula de HA dão especificidade para a ligação $\alpha 2,6$, encontrada em células do tecido traqueal humano (Webster e cols., 1992; Cheung e Poon, 2007; King e cols., 2011).

1.1.2. Infecção pelo IAV(H1N1)pdm09

As pandemias tipicamente envolvem o surgimento ou ressurgimento de uma HA, marcando o predomínio do novo subtipo viral e o desaparecimento do subtipo viral anterior. Cerca de 68 anos é o tempo estimado entre pandemias de mesmo subtipo, o que possibilita previsões sobre quando ocorrerão os próximos eventos (Hilleman, 2002). Esse intervalo sugere que seja necessário aproximadamente 68 anos para que ocorra uma queda de imunidade na população humana como um todo, permitindo que o vírus ganhe novo acesso ao hospedeiro e se estabeleça novamente na população (Hilleman, 2002). Por exemplo, as pandemias de 1889 e de 1957 foram causadas pelo vírus influenza A H2N2; a de 1900, pelo H3N8, sendo que a H3 reapareceu somente 68 anos depois, no vírus H3N2, que causou a pandemia de Hong Kong em 1968. A pandemia de 1918-1919, mais conhecida como gripe Espanhola, foi causada pelo H1N1, o qual parou de circular na população com o surgimento do H2N2 que se estabeleceu em 1957 (Hilleman, 2002). Um novo H1N1 potencialmente pandêmico voltou a circular na população em 2009 causando a mais recente pandemia por influenza. O subtipo pandêmico se contrapôs a uma linhagem H1N1 que a alguns anos estava presente na população (Tscherne e García-Sastre, 2011).

A gripe por IAV acomete principalmente crianças e idosos, apresentando uma curva de distribuição por faixa etária da população geralmente em forma de V. Nas pandemias, como foi o caso da gripe espanhola e da pandemia de 2009, a letalidade também é elevada entre pessoas da faixa etária de 20-40 anos (Hilleman, 2002; Hsieh e cols., 2006; Gorini da Veiga e cols., 2012).

Clinicamente, os pacientes infectados pelo IAV apresentam, em geral, febre alta, tosse seca, calafrios e dificuldade respiratória, podendo evoluir para pneumonia. Alguns casos também descrevem diarreia e vômitos, dores de cabeça e de garganta, tosse sanguinolenta e, nos casos mais graves, morte do paciente. A gripe espanhola foi mais agressiva, causando um quadro de prostração crescente, epistaxe e pneumonia, além de uma maior susceptibilidade a infecções bacterianas (Hsieh e cols., 2006).

1.2. Microbioma humano

O primeiro estudo da microbiota humana foi realizado por Antonie van Leewenhoek em 1680 (Ursell et al., 2012). Atualmente, a avaliação do microbioma humano está em evidência, e o entendimento da inter-relação micro-organismo–hospedeiro tem evoluído rapidamente nos últimos anos.

Alguns conceitos ainda são vistos de maneira sobreposta ou mesmo trocados nos estudos publicados. No entanto, a disponibilidade de novas tecnologias laboratoriais, e o avanço da bioinformática e dos bancos de dados tem contribuído para a obtenção de novos achados e para o aumento do interesse da comunidade científica pelo estudo da inter-relação entre micro-organismos e hospedeiro. Consequentemente, os conceitos que permeiam este campo de estudo tem sido melhor estabelecidos.

O termo microbiota é definido como o conjunto de micro-organismos (unidades taxonômicas microbianas) em um ambiente ou amostra, enquanto que microbioma é definido como o conjunto de genes de micro-organismos em um ambiente ou amostra (Ley e cols., 2008; Ursell e cols., 2012). Outros

conceitos importantes são a metagenômica e a *metabarcoding*. O primeiro refere-se à caracterização do DNA total de uma amostra pela aplicação de técnicas de sequenciamento total (*shotgun*), enquanto que o segundo refere-se à caracterização do DNA de uma amostra utilizando genes marcadores, como os genes ribossomais (Ursell e cols., 2012).

Neste contexto, análises utilizando *metabarcodes* apresentam-se como estratégias adequadas para caracterizar a composição e a dinâmica das comunidades microbianas, pois permitem a caracterização dos microrganismos ali presentes, sem necessidade de cultivo. Isto proporciona a coleta de dados em grande escala, de forma padronizada e comparável, em ambientes diversos (Hugenholtz e Tyson, 2008). No âmbito deste tipo de abordagem, a análise da região codificadora da subunidade menor do gene ribossomal (16S para procariotos e 18S para eucariotos) tem se tornado um dos métodos mais eficientes para a identificação de organismos. Estes marcadores se revelam poderosos para a caracterização da diversidade de linhagens filogenéticas presentes em um determinado ambiente, e também servem como base para o delineamento de varreduras genômicas empregando múltiplos segmentos, ou para a reconstrução de genomas completos ou vias metabólicas presentes no local. A escala desta caracterização é particularmente grande quando aplicada uma estratégia de sequenciamento de DNA de alto desempenho (*deep sequencing*), o qual gera milhares de fragmentos do gene de interesse simultaneamente, permitindo a obtenção de informações detalhadas sobre a população microbiana de uma determinada amostra (Cristescu, 2014).

Além do uso de metagenômica para estudos ecológicos, a Análise Intergênica de Espaçadores Ribossomais (RISA) é uma importante ferramenta

molecular utilizada em estudos de microbiologia ambiental e clínica (Borneman e Triplett, 1997). Esta técnica se baseia na amplificação por PCR de uma região do gene ribossomal entre o 16S e o 23S, chamada de região espaçadora, que possui heterogeneidade significativa na sequência de nucleotídeos e no comprimento. Isso permite uma visualização da diversidade microbiana em uma amostra levando em conta o tamanho dos fragmentos gerados por PCR e também o número de fragmentos que aparecem em uma amostra (Fisher e Triplett, 1999; Scanlan e cols., 2008; Flight e cols., 2015).

A forma dominante de interação entre humanos e micro-organismos é aquela que beneficia o hospedeiro, também conhecida como relação comensal, e também aquela em que ambos se beneficiam, conhecida como relação simbiótica (Blaser e Falkow, 2009). O microbioma humano facilita a obtenção de energia dos alimentos, fornece fatores acessórios de crescimento, estimula os sistemas imunológicos inato e adaptativo, e fornece resistência à colonização de patógenos invasores (Blaser e Falkow, 2009). Esta interação foi formada ao longo da evolução humana e pode ser considerada bastante individualizada.

Atualmente, entende-se que, com o passar do tempo, um microbioma cerne foi formado em humanos. Este microbioma cerne foi levemente alterado com o passar das gerações, principalmente devido a mudanças ambientais e comportamentais da população. Até o momento, é estabelecido que antes do nascimento os humanos são livres de qualquer micro-organismo. Rapidamente após o nascimento se inicia a formação de comunidades microbianas nos mais diversos ambientes do organismo (Dominguez-Bello e cols., 2010; de Steenhuijsen Piters e cols., 2015). No entanto, é possível que a formação das

primeiras comunidades se iniciem ainda durante a gestação (Koleva e cols., 2015). A formação da primeira composição do microbioma de cada um destes ambientes depende de diversos fatores, como a forma de nascimento, a dieta, o histórico genético e outros fatores ambientais (Blaser e Falkow, 2009). Com o passar dos anos, o microbioma adquire uma composição específica com diferenças interindividuais estáveis (Palmer e cols., 2007).

O microbioma humano é formado por diferentes e numerosas populações bacterianas. No entanto, é dominado por apenas 4 dos 50 filos bacterianos conhecidos – Firmicutes, Bacteroidetes, Actinobacteria e Proteobacteria (Blaser e Falkow, 2009). A composição do microbioma é tão específica que é capaz de diferenciar-se de qualquer outro microbioma de qualquer outra espécie de mamífero. Estas características demonstram o poder seletivo durante a formação do microbioma que, em indivíduos saudáveis, é amplamente dependente da relação micro-organismo–hospedeiro (Ley et al., 2008).

A estabilidade do microbioma depende de um estado de equilíbrio entre os micro-organismos que compõem o microbioma e o hospedeiro. Blaser e Kirschner (2007) propuseram que cada ecossistema contém comunidades que contribuem no controle de uma outra comunidade. Isto é, o estado de equilíbrio de uma população microbiana é controlador de outra população dentro de um mesmo microbioma, gerando uma cadeia de controle que pode explicar o equilíbrio e a proteção contra bactérias patogênicas.

1.2.1. Microbioma da nasofaringe humana

O microbioma do trato respiratório humano ainda foi pouco explorado se compararmos com os estudos referentes ao microbioma intestinal. No entanto, assim como para o intestino, sabe-se que o nascimento tem importante relação com a composição do microbioma da nasofaringe (Dominguez-Bello e cols., 2010).

Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria e Cyanobacteria são filos comumente encontrados na nasofaringe (Bogaert e cols., 2011). As primeiras populações microbianas a colonizar a nasofaringe pertencem a gêneros normalmente encontrados na pele (*Staphylococcus*, *Streptococcus* e *Dolosigranulum*) ou na vagina materna (*Lactobacillus*, *Prevotella*, *Atopobium* e *Sneathia*) (Figura 2). Ainda, outras populações, como a *Moraxella*, o *Corynebacterium* e o *Dolosigranulum*, podem ser associadas à amamentação materna (Biesbroek e cols., 2014; de Steenhuijsen Piters e cols., 2015).

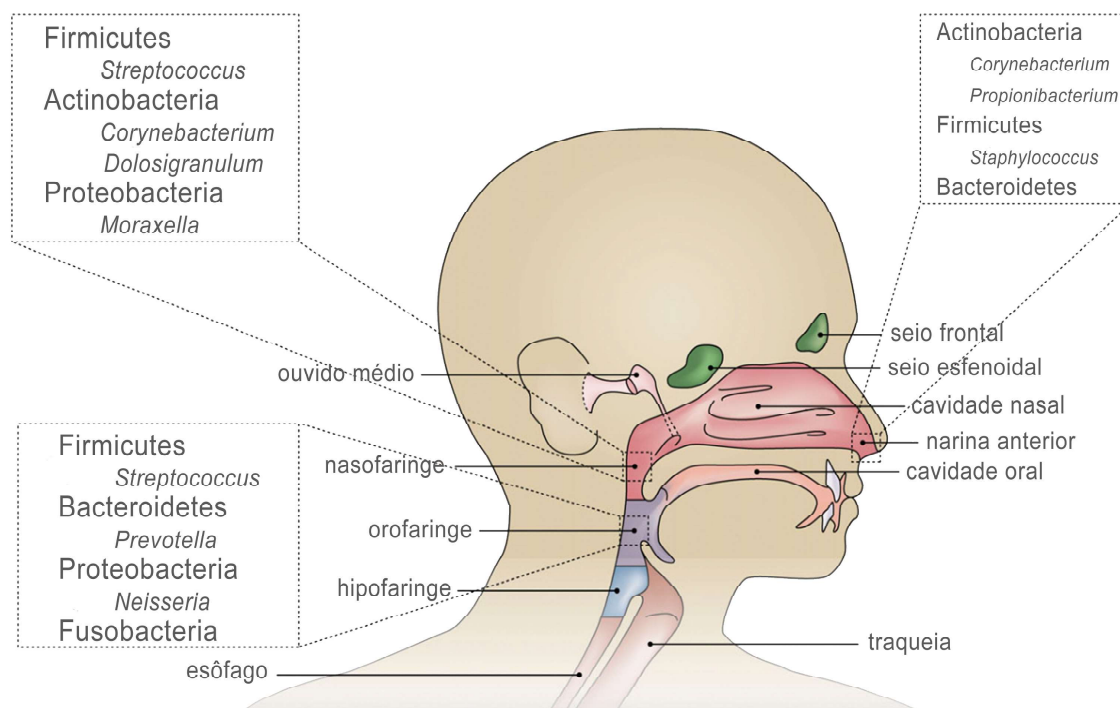


Figura 2 – Principais filos bacterianos que compõem o microbioma da Narina anterior, Nasofaringe e Orofaringe humana. A representação mostra a localização anatômica dos diferentes ambientes e particularidades da composição da comunidade bacteriana de cada um deles. Os filos bacterianos aparecem descritos em ordem de ocorrência na população. São indicados os gêneros mais frequentes entre os filos.

Adaptado de: de Steenhuijsen Piters, 2015.

Mudanças são observadas na composição do microbioma com o passar dos primeiros anos de vida. Algumas espécies microbianas desaparecem, outras iniciam a colonização do trato respiratório, e pelo menos até os dois anos de vida o microbioma ainda é diferente do encontrado na vida adulta (Biesbroek e cols., 2014). Após estabelecido o primeiro microbioma da nasofaringe, a tendência é o alcance do equilíbrio das populações e a confirmação da estabilidade da comunidade. Neste estágio, de maneira geral, o microbioma é composto por uma ampla variedade de micro-organismos que se misturam entre comensais e potencialmente patogênicos. *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* e *Staphylococcus aureus* são algumas das espécies patogênicas mais comuns a colonizar a

nasofaringe em humanos livres de doença (Bogaert e cols., 2004; Margolis e cols., 2010).

Bogaert e cols. (2004) observaram que crianças saudáveis com 3 anos de idade apresentavam um pico de incidência de 55% de colonização por *S. pneumoniae*. No mesmo estudo, se demonstrou que a incidência desta diminuiu para 8% em crianças com 10 anos de idade.

Biesbroek e cols. (2014) observaram ausência do gênero *Prevotella* em crianças, entre o período de 1,5 e 24 meses após o nascimento. No mesmo estudo, se demonstrou que quando a colonização inicial do trato respiratório foi composto por um perfil dominado por *Moraxella* e *Corynebacterium* ou *Dolosigranulum* e *Corynebacterium*, o microbioma foi marcado pela maior estabilidade com o passar do tempo. No entanto, quando a colonização inicial foi composta por um perfil dominado por *Streptococcus* e *Haemophilus*, o microbioma foi marcado por um padrão de frequentes mudanças durante o passar do tempo.

1.3. Coinfecção vírus-bactéria

A detecção viral na nasofaringe é tão frequente quanto a presença de bactérias patogênicas. Alguns autores tem reportado a co-presença destes patógenos e a possível associação existente entre eles para o desfecho da doença (van den Bergh e cols., 2012; Bosch e cols., 2013). No entanto, é difícil dissociar a causa do desfecho da doença, uma vez que a mortalidade por pneumonia e influenza incluem a morte por infecção viral primária e a morte por

infecção bacteriana secundária. Isto se deve à dificuldade de se estabelecer distinção entre as causas (Thompson e cols., 2003).

Por mais que uma infecção viral por influenza A possa ser por si só causadora de doença grave, o risco de complicações e de mortalidade aumenta na presença de uma bactéria patogênica. A sugestão de que a infecção viral predispõe à doença bacteriana foi primeiro atribuída ao médico francês Laennec, em 1803, que observou o aumento do número de casos de pneumonia durante períodos de epidemia de influenza (McCullers, 2006). Desde então, a interação entre bactérias patogênicas e vírus respiratórios durante o desenvolvimento de infecção viral é frequentemente reportada (Brockson e cols., 2012; van den Bergh e cols., 2012; Bosch e cols., 2013; Leung e cols., 2013).

Wei e cols. (2015), reportaram 17% de casos de coidentificação vírus-bactérias patogênicas entre 3181 crianças hospitalizadas devido à infecção respiratória aguda. No entanto, o mesmo estudo demonstrou não haver influência no desfecho da doença. Damasio e cols. (2015), detectaram a presença de coinfeção em 45% dos pacientes analisados, sendo que 40% estavam infectados pelo IAV(H1N1)pdm09. No mesmo estudo, não se observou nenhuma influência da presença de coinfeção sobre o período de hospitalização, sobre a severidade ou sobre a taxa de mortalidade.

Estudos têm demonstrado uma associação positiva para doença em infecções pelo vírus influenza na presença de algumas espécies bacterianas, como o *S. pneumoniae*, o *H. influenzae* e o *S. aureus* (Bosch e cols., 2013). Já van den Bergh e cols. (2012), reportaram forte associação positiva entre o *S. aureus*, o *S. pneumoniae* e o vírus influenza.

Bactérias coinfectantes do trato respiratório podem ser responsáveis pela excreção de proteases, contribuindo para a ativação da HA viral e, conseqüentemente, para o aumento de partículas virais aptas a ingressarem nas células hospedeiras. Proteases microbianas podem, também, promover a produção de proteases pelo hospedeiro nos locais de infecção; por exemplo, plasmina, trombina e calicreína pela presença de estáfilo e estreptoquinase (Mancini e cols., 2005).

Tashiro e cols. (1987a), reportaram que algumas linhagens de *Staphylococcus aureus* são capazes de secretar proteases que promovem a ativação da HA do vírus influenza, exercendo decisiva influência no desfecho da infecção em camundongos. No mesmo ano, Tashiro e cols (1987b), reportaram uma serino protease bacteriana com atividade de ativação da HA, semelhante à tripsina e à plasmina. Uma importante especificidade envolvendo tanto a linhagem bacteriana produtora da protease quanto a HA ativada com sucesso foram determinadas no mesmo estudo.

1.4. Microbioma da nasofaringe de pacientes infectados por IAV(H1N1)pdm09

O vírus influenza A é, dentre os vírus respiratórios que infectam humanos, possivelmente o mais habilidoso na arte de se modificar a fim de evitar o ataque do sistema imunológico hospedeiro. Tendo em vista o grande número de casos estimados de infecções humanas por este vírus, pode-se dizer que uma infecção pelo vírus influenza raramente causa doença em pessoas saudáveis, exceto quando há o envolvimento de linhagens virais pandêmicas.

Inclusive, é possível detectar vírus influenza em pessoas portadoras assintomáticas (Melchior e cols., 2015).

Estudos envolvendo amostras de pacientes mortos durante pandemias por influenza mostraram que grande parte dos óbitos podem ser atribuídos à coinfeção bacteriana. Linhagens virais mais agressivas foram associadas ao *S. pneumoniae* (Morens e cols., 2008) enquanto linhagens mais brandas foram associadas ao *S. aureus* (McCullers, 2006).

O trato respiratório superior é a via de entrada dos vírus respiratórios. Nas vias aéreas é que as partículas virais do influenza entram em contato com as células do hospedeiro e estabelecem ligação e adesão vírus-célula via receptores específicos. Neste ambiente, o vírus precisa encontrar condições favoráveis para progredir e estabelecer sua infecção. No entanto, é importante observar que neste ambiente há estabelecido pelo menos duas complexas relações de constante conflito: (a) entre o sistema imune do hospedeiro e bactérias potencialmente patogênicas e (b) entre populações bacterianas que compartilham o microbioma local.

Assim sendo, o estudo do microbioma da nasofaringe pode esclarecer alguns aspectos que envolvem a coinfeção vírus-bactéria e o desfecho da doença em pacientes saudáveis e em portadores de doenças crônicas.

Referências

- Arias CF, Escalera-Zamudio M, Soto-Del Río MDLD, Cobián-Güemes AG, Isa P, López S. Molecular anatomy of 2009 influenza virus A (H1N1). *Arch Med Res.* 2009;40(8):643–54.
- Biesbroek G, Tsvitvadze E, Sanders EAM, Montijn R, Veenhoven RH, Keijser BJF, et al. Early respiratory microbiota composition determines bacterial succession patterns and respiratory health in children. *Am J Respir Crit Care Med.* 2014;190(11):1283–92.
- Blaser MJ, Falkow S. What are the consequences of the disappearing human microbiota? *Nat Rev Microbiol.* Nature Publishing Group; 2009;7(12):887–94.
- Blaser MJ, Kirschner D. The equilibria that allow bacterial persistence in human hosts. *Nature.* 2007;449(7164):843–9.
- Bogaert D, De Groot R, Hermans PWM. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. *Lancet Infect Dis.* 2004;4(3):144–54.
- Bogaert D, Keijser B, Huse S, Rossen J, Veenhoven R, van Gils E, et al. Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis. *PLoS One.* 2011;6(2):e17035.
- Borneman J, Triplett EW. Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. *Appl Environ Microbiol.* 1997;63(7):2647–53.
- Bosch AATM, Biesbroek G, Trzcinski K, Sanders EAM, Bogaert D. Viral and bacterial interactions in the upper respiratory tract. *PLoS Pathog.* 2013;9(1):e1003057.
- Brockson ME, Novotny LA, Jurcisek JA, McGillivray G, Bowers MR, Bakaletz LO. Respiratory syncytial virus promotes *Moraxella catarrhalis*-induced ascending experimental otitis media. *PLoS One.* 2012;7(6):e40088.
- CEVS. Boletim Influenza A(H1N1) de 25 de novembro de 2009. Porto Alegre; 2009. Disponível em: https://www.google.com.br/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=0ahUKEwi8krqaxaXKAhXCI5AKHYnDq8QFggiMAA&url=http%3A%2F%2Fwww1.saude.rs.gov.br%2Fdados%2F1275051726093BOLETIM%252025.11.09.pdf&usg=AFQjCNFfRhiikQkd2l3UmAQ4kj2zP_ixOA&bvm=bv.111396085,d.Y2l. Acesso em : 15 dez. 2015.
- CEVS. Boletim Epidemiológico. Porto Alegre; 2011. Disponível em: http://www.saude.rs.gov.br/upload/1337355126_v.13,%20n.3,set.,%202011.pdf. Acesso em: 15 dez. 2015.
- CEVS. Divisão de Vigilância Epidemiológica. Influenza no Rio Grande do Sul em 2012. Disponível em: http://www.saude.rs.gov.br/upload/1366813096_Boletim%20final%20Influenza%20no%20RS%202012.pdf. Acesso em: 15 dez. 2015.
- Cheung TKW, Poon LLM. Biology of influenza a virus. *Ann N Y Acad Sci.* 2007;1102:1–25.

Cristescu ME. From barcoding single individuals to metabarcoding biological communities: towards an integrative approach to the study of global biodiversity. *Trends Ecol Evol.* 2014;29:566–571.

Damasio GAC, Pereira LA, Moreira SDR, Duarte dos Santos CN, Dalla-Costa LM, Raboni SM. Does virus-bacteria coinfection increase the clinical severity of acute respiratory infection? *J Med Virol.* 2015;87(9):1456–61.

de Steenhuijsen P, Sanders EAM, Bogaert D. The role of the local microbial ecosystem in respiratory health and disease. *Philos Trans R Soc B Biol Sci.* 2015;370(1675):20140294.

Deem MW, Pan K. The epitope regions of H1-subtype influenza A, with application to vaccine efficacy. *Protein Eng Des Sel.* 2009;22:543–6.

Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc Natl Acad Sci.* 2010;107(26):11971–5.

Fisher MM, Triplett EW. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Appl Environ Microbiol.* 1999;65(10):4630–4636.

Flight WG, Smith A, Paisey C, Marchesi JR, Bull MJ, Norville PJ, et al. Rapid Detection of emerging pathogens and loss of microbial diversity associated with severe lung disease in cystic fibrosis. *J Clin Microbiol.* 2015;53(7):2022–9.

Frank SA. 2002. *Immunology and evolution of infectious disease.* Princeton University Press, 348p.

Gorini da Veiga AB, Kretzmann NA, Corrêa LT, Goshiyama AM, Baccin T, Ardenghi P, et al. Viral load and epidemiological profile of patients infected by pandemic influenza A (H1N1) 2009 and seasonal influenza A virus in southern Brazil. *J Med Virol.* 2012;84(3):371–379.

Hilleman MR. Realities and enigmas of human viral influenza: pathogenesis, epidemiology and control. *Vaccine.* 2002; 20(25-26):3068–87.

Hsieh Y-C, Wu T-Z, Liu D-P, Shao P-L, Chang L-Y, Lu C-Y, et al. Influenza pandemics: past, present and future. *J Formos Med Assoc.* 2006;105(1):1–6.

Hugenholtz P, Tyson GW. Microbiology: metagenomics. *Nature.* 2008;455(7212):481–3.

Jongkon N, Mokmak W, Chuakheaw D, Shaw PJ, Tongsimma S, Sangma C. Prediction of avian influenza A binding preference to human receptor using conformational analysis of receptor bound to hemagglutinin. *BMC Genomics.* 2009;10Suppl.3:S24.

Kim JI, Lee I, Park S, Hwang M-W, Bae J-Y, Lee S, et al. Genetic requirement for hemagglutinin glycosylation and its implications for influenza A H1N1 virus evolution. *J Virol.* 2013;87(13):7539–49.

King MD, Guentzel MN, Arulanandam BP, Bodour A a, Brahmakshatriya V, Lupiani B, et al. Effects of bacterial microflora of the lower digestive tract of free-range waterfowl on influenza virus activation. *Appl Environ Microbiol.* 2011;77(12):4119–25.

- Koleva PT, Kim JS, Scott JA, Kozyrskyj AL. Microbial programming of health and disease starts during fetal life. *Birth Defects Res. C Embryo Today*. 2015;105(4):265-77.
- Leung RKK, Zhou JW, Guan W, Li SK, Yang ZF, Tsui SKW. Modulation of potential respiratory pathogens by pH1N1 viral infection. *Clin Microbiol Infect*. 2013;19(10):930-5.
- Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JI. Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat Rev Microbiol*. 2008;6(10):776-88.
- Lu X, Shi Y, Gao F, Xiao H, Wang M, Qi J, *et al*. Insights into avian influenza virus pathogenicity: the hemagglutinin precursor HA0 of subtype H16 has an alpha-helix structure in its cleavage site with inefficient HA1/HA2 cleavage. *J Virol*. 2012;86(23):12861-70.
- Luna EJdeA, Gattás VL, Campos SRdeSLdaC. Efetividade da estratégia brasileira de vacinação contra influenza: uma revisão sistemática. *Epidemiol e Serviços Saúde*. 2014;23(3):559-76.
- Ma W, Lager KM, Vincenta L, Janke BH, Gramer MR, Richt JA. The role of swine in the generation of novel influenza viruses. *Zoonoses Public Health*. 2009;56(6-7):326-37.
- Mancini DAP, Mendonça RMZ, Dias ALF, Mendonça RZ, Pinto JR. Co-infection between influenza virus and flagellated bacteria. *Rev Inst Med Trop Sao Paulo*. 2005;47(5):275-80.
- Margolis E, Yates A, Levin BR. The ecology of nasal colonization of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus*: the role of competition and interactions with host's immune response. *BMC Microbiol*. 2010;10(1):59.
- McCullers JA. Insights into the interaction between influenza virus and pneumococcus. *Clin Microbiol Rev*. 2006;19(3):571-82.
- Medina RA, Stertz S, Manicassamy B, Zimmermann P, Sun X, Albrecht RA, *et al*. Glycosylations in the globular head of the hemagglutinin protein modulate the virulence and antigenic properties of the H1N1 influenza viruses. *Sci Transl Med*. 2013; 5(187):187ra70.
- Melchior TB, Perosa AH, Camargo CN, Granato C, Bellei N. Influenza virus prevalence in asymptomatic and symptomatic subjects during pandemic and postpandemic periods. *Am J Infect Control*. 2015;43(5):460-4.
- Morens DM, Taubenberger JK, Fauci AS. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. *J Infect Dis*. 2008;198(7):962-70.
- Moscona A. Neuraminidase inhibitors for influenza. *N Engl J Med*. 2005;353(13):1363-73.
- Nelson M, Spiro D, Wentworth D, Beck E, Fan J, Ghedin E, *et al*. The early diversification of influenza A/H1N1pdm. *PLoS Curr*. 2009;1.
- Neumann G, Kawaoka Y. Transmission of influenza A viruses. *Virology*. 2015;479-480:234-246.

Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the human infant intestinal microbiota. *PLoS Biol.* 2007;5(7):e177.

Rossman JS, Lamb RA. Influenza virus assembly and budding. *Virology.* 2011;411(2):229–36.

Sant'Anna FH, Borges LGA, Fallavena PRV, Gregianini TS, Matias F, Halpin RA, *et al.* Genomic analysis of pandemic and post-pandemic influenza A pH1N1 viruses isolated in Rio Grande do Sul, Brazil. *Arch Virol* 2013;159:621–30.

Scanlan PD, Shanahan F, Clune Y, Collins JK, O'Sullivan GC, O'Riordan M, *et al.* Culture-independent analysis of the gut microbiota in colorectal cancer and polyposis. *Environ Microbiol.* 2008;10(3):789–98.

Schnitzler SU, Schnitzler P. An update on swine-origin influenza virus A/H1N1: a review. *Virus Genes.* 2009;39(3):279–92.

Shin N, Pyo C-W, Jung KII, Choi S-Y. Influenza A virus PB1-F2 is involved in regulation of cellular redox state in alveolar epithelial cells. *Biochem Biophys Res Commun.* 2015;459(4):699–705.

Shinya K, Hamm S, Hatta M, Ito H, Ito T, Kawaoka Y. PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. *Virology.* 2004;320(2):258–66.

SVS. Secretaria de Vigilância em Saúde. Informe técnico de influenza. 1. ed. – Brasília, 2012. Disponível em:

<http://portalsaude.saude.gov.br/images/pdf/2014/maio/22/informe-influenza-2009-2010-2011-220514.pdf> . Acesso em: 02 out. 2015.

SVS. Secretaria de Vigilância em Saúde. Boletim informativo de influenza: 2012. Brasília, 2013. Disponível em:

<http://portalsaude.saude.gov.br/images/pdf/2014/maio/22/boletim-influenza-se52de2012-220514.pdf> . Acesso em: 5 nov. 2015.

Tashiro M, Ciborowski P, Klenk HD, Pulverer G, Rott R. Role of *Staphylococcus* protease in the development of influenza pneumonia. *Nature.* 1987a;325(5):536–7.

Tashiro M, Ciborowski P, Reinacher M, Pulverer G, Klenk HD, Rott R. Synergistic role of staphylococcal proteases in the induction of influenza virus pathogenicity. *Virology.* 1987b;157(2):421–30.

Taubenberger JK, Kash JC. Influenza virus evolution, host adaptation, and pandemic formation. *Cell Host Microbe.* 2010;7(6):440–51.

Tscherne, DM, García-Sastre, A. Virulence determinants of pandemic influenza viruses. *J. Clin. Invest.* 2011;121:6–13.

Thompson WW, Shay DK, Weintraub E, Brammer L, Cox N, Anderson LJ. Mortality associated with influenza and respiratory syncytial virus in the United States. *JAMA.* 2003;289(2):179–86.

Tong S, Li Y, Rivallier P, Conrardy C, Castillo DAA, Chen L-M, *et al.* A distinct lineage of influenza A virus from bats. *Proc Natl Acad Sci.* 2012;109(11):4269–74.

Ursell LK, Metcalf JL, Parfrey LW, Knight R. Defining the human microbiome. *Nutr Rev.* 2012;70(Suppl 1):S38–44.

Vasin AV, Temkina OA, Egorov VV, Klotchenko SA, Plotnikova MA, Kiselev OI. Molecular mechanisms enhancing the proteome of influenza A viruses: an overview of recently discovered proteins. *Virus Res* 2014;185:53–63.

van den Bergh MR, Biesbroek G, Rossen JWA, de Steenhuijsen Piters WAA, Bosch AATM, van Gils EJM, *et al.* Associations between pathogens in the upper respiratory tract of young children: interplay between viruses and bacteria. *PLoS One*. 2012;7(10):e47711.

Veit M, Serebryakova MV, Kordyukova LV. Palmitoylation of influenza virus proteins. *Biochem Soc Trans*. 2013;41(1):50–5.

Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. *Microbiol Rev*. 1992;56(1):152–79.

Wei L, Liu W, Zhang X-A, Liu E-M, Wo Y, Cowling BJ, *et al.* Detection of viral and bacterial pathogens in hospitalized children with acute respiratory illnesses, Chongqing, 2009-2013. *Medicine (Baltimore)*. 2015;94(16):e742.

WHO. Manual for the laboratory diagnosis and virological surveillance of influenza. WHO, editor. Geneva;2011.

Zhou B, Li Y, Halpin R, Hine E, Spiro DJ, Wentworth DE. PB2 residue 158 is a pathogenic determinant of pandemic H1N1 and H5 influenza A viruses in mice. *J Virol*. 2011;85(1):357–65.

2. Objetivos

Tendo como foco investigar o microbioma do trato respiratório superior de pacientes infectados pela linhagem pandêmica 2009 do vírus influenza A da população do estado do Rio Grande do Sul, os objetivos deste estudo foram:

- a) Determinar a diversidade bacteriana do trato respiratório superior de diferentes grupos de pacientes hospitalizados por Síndrome Respiratória Aguda Grave (SRAG) no Estado do Rio Grande do Sul;
- b) Identificar a presença de coinfeção entre influenza A H1N1 linhagem pandêmica e bactérias patogênicas no trato respiratório superior de pacientes hospitalizados;
- c) Identificar o perfil do microbioma de pacientes hospitalizados com doença respiratória aguda por vírus influenza A da linhagem pandêmica (H1N1) 2009, utilizando o método de *metabarcoding* com sequenciamento de alto desempenho.

3. Artigo científico redigido em inglês

3.1. “Chronic pneumopathy associated with lower bacterial diversity on upper respiratory tract of patients infected by IAV(H1N1)pdm09”

Luiz Gustavo dos Anjos Borges; Adriana Giongo; Tatiana Schäffer Gregianini; Paulo Michael Roehe; Ana Cláudia Franco; Fabrício Souza Campos; Ana Beatriz Gorini da Veiga

Submetido ao periódico *PLoS One*

**Chronic pneumopathy associated with lower bacterial diversity on upper
respiratory tract of patients infected by IAV(H1N1)pdm09**

L.G.A. Borges¹; A. Giongo²; T.S. Gregianini³; P.M. Roehe⁴; A.C. Franco⁴; F.S. Campos⁴; A.B.G.Veiga^{1*}

Affiliations:

¹Laboratório de Biologia Molecular, Programa de Pós-Graduação em Patologia, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) - CEP 90050-170 – Porto Alegre, RS, Brazil.

²Instituto do Petróleo e dos Recursos Naturais (IPR), Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) - CEP 90619-900 – Porto Alegre, RS, Brazil.

³Laboratório de Virologia, Instituto de Pesquisas Biológicas – Laboratório Central do Estado do Rio Grande do Sul (IPB-LACEN-RS) – CEP 90610-000 – Porto Alegre, RS, Brazil.

⁴Laboratório de Virologia, Departamento de Microbiologia, Imunologia e Parasitologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul (UFRGS) – CEP 90050-170 – Porto Alegre, RS, Brazil.

* Corresponding author: Ana B. G. Veiga

R. Sarmiento Leite, 245 / Sala 309, Centro – CEP 90050-170 – Porto Alegre-RS, Brazil,

Phone: +55 51 33038763

FAX: +55 51 33038061

E-mail:

anabgv@ufcspa.edu.br

Keywords: influenza virus; RISA; nasopharynx; *Streptococcus pneumoniae*; *Haemophilus influenzae*; *Moraxella catarrhalis*; *Staphylococcus aureus*.

Abstract

Influenza A virus (IAV) infection causes significant morbidity and mortality worldwide. The coinfection with potentially pathogenic bacteria as well as the presence of comorbidities has been reported to be associated with increased virulence and complications for the viral disease. The molecular fingerprint method RISA is based on the amplification of the bacterial intergenic region located between the 16S and 23S rRNA gene. The aim of the present study was to verify the bacterial diversity in the upper respiratory tract of patients infected by the pandemic strain of influenza A virus (IAV(H1N1)pdm09) with and without chronic pneumopathy (CP). Nasopharyngeal samples from 144 patients were enrolled in the study. Species-specific PCRs were performed to identify *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, and *Haemophilus influenzae*. RISA was also performed to find the bacterial community profile and measure the diversity using ecological data analyses. The microbial diversity observed in IAV(H1N1)pdm09 patients ($H' = 3.438$) when compared to non-IAV(H1N1)pdm09 patients ($H' = 3.361$) did not present statistical difference. Samples of IAV(H1N1)pdm09 patients presenting CP displayed lower bacterial diversity ($H' = 3.223$) when compared to samples from IAV(H1N1)pdm09 patients without CP ($H' = 3.455$). The *Streptococcus pneumoniae* was identified in 62% of samples, while 26% presented *Moraxella catarrhalis*. Lower frequencies were observed for *Haemophilus influenzae* (17%), and *Staphylococcus aureus* (12%). As a conclusion, it was verified a high frequency of potentially pathogenic bacteria in the nasopharynx of patients. Our findings suggest that IAV(H1N1)pdm09 patients with CP may present a profile of lower bacterial diversity than IAV(H1N1)pdm09 patients without CP.

Introduction

Acute lower respiratory infections are the second most common cause of illness affecting people at different ages and socioeconomic groups, having *Influenza A Virus* (IAV) as its major contributor of illness. It is estimated that 5-10% of the world's population is infected each year by IAV [1]. Influenza disease is characterized by non-specific acute symptoms like fever, myalgia, headache and pneumonia in several cases [2], but also asymptomatic individuals have been identified [3]. More than 50,000 cases of IAV infection were reported in Brazil during the last pandemic period, most of them by pandemic strain of *Influenza A virus* (IAV(H1N1)pdm09). The *Influenza A virus* can be responsible for increasing the hospitalization up to 33% for children and up to 9% for adults presenting chronic pneumopathy (CP) [4]. Moreover, comorbidities may contribute to enhancing the severity of IAV-associated infections [5], as well as coinfection by pathogenic bacteria [6].

Species of potentially bacterial pathogens like *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Staphylococcus aureus* can colonize the human upper respiratory tract [7]. Each one of those species may be present in a healthy upper respiratory tract as part of a stable bacterial community or promoting a process of rupture of the bacterial community and leading to a respiratory disease, such as a secondary infection of the lower respiratory tract in patients infected by IAV [6]. In fact, a positive association has been already demonstrated between IAV and *S. pneumoniae* [8].

Bacterial diversity has been studied in representative human samples from the gastrointestinal system, skin, and more recently, the upper respiratory tract [9–11]. Analyses of the bacterial community inhabiting the upper respiratory tract of

IAV infected patients might give insights on influenza disease outcome. Microbial diversity can be assessed using a molecular fingerprint method called ribosomal intergenic spacer analysis (RISA) which is based on the amplification of the bacterial intergenic region located between the 16S and 23S rRNA gene [12–15]. RISA is broadly applied for microbial community analysis and it is a culture independent method. The aim of the present study was identify and measure the bacterial diversity in nasopharynx of patients infected by IAV(H1N1)pdm09 with and without chronic pneumopathy.

Material and methods

Ethics Statement

Experiments were performed in compliance with relevant laws and in accordance with the ethical standard of the Declaration of Helsinki. All ethical issues were previously approved by the Research Ethics Committee of Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA).

Influenza is a notifiable disease, and all samples used in this study have been collected for health surveillance, diagnosis and treatment of the patients, without written Informed Consent. Nevertheless, the acquisition of the samples adheres to the regulations and ethical guidelines for the protection of human subjects of research in Brazil, and all research activities involving human subjects were conducted according to these ethical principles. The samples are labeled in a coded, de-identified manner. All patient identifiers – name, date of birth, address, and other information that are confidential – are not disclosed, and researchers have access only to information important for the objectives of the study, such as clinical symptoms and demographical data (age, gender).

Biological samples and DNA extraction

A total of 144 nasopharyngeal aspirates from patients with severe acute respiratory infection (SARI = fever $>38^{\circ}\text{C}$, cough, and dyspnea) were enrolled in the study. All samples were collected in hospital units of Rio Grande do Sul State, southern Brazil, during 2012 and sent to the State Central Laboratory (LACEN-RS). Patients were hospitalized for presenting SARI symptoms. Moreover, each sample was provided with laboratorial results for IAV(H1N1)pdm09 – following the WHO/CDC RT-PCR protocol [16] – and a

clinical record data form. Samples were divided between samples from patients presenting IAV(H1N1)pdm09 infection and samples from patients presenting non-IAV(H1N1)pdm09 infection. The information of CP from clinical record data form of patients presenting IAV(H1N1)pdm09 infection were used to evaluated the results of analysis.

Samples were stored at -80°C until required for further laboratory analyses. Each sample was thawed on ice and DNA was extracted using QIAamp DNA mini Kit (QIAGEN) according to the manufacturer's instructions. DNA was quantified using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and then stored at -20°C for further analysis.

Ribosomal Intergenic Spacer Analysis (RISA)

The universal primer set 1406F (5'TGYACACACCGCCCGT3') and 23Sr (5'GGGTTBCCCCATTCTRG3') [17] was used for RISA profile. Reactions included an initial denaturation at 94°C for 5 min; 35 cycles of amplification at 94°C for 20 s, 50°C for 30 s, and 72°C for 1 min; 72°C for 5 min for final extension. PCR-RISA reaction mixture was prepared with 1X PCR buffer (Invitrogen), 3 mM MgCl₂, 200 µM of each dNTP, 0.8 µM of each primer, 2.5 U of recombinant Taq DNA Polymerase, and 2 µL (10-50 ng) of template DNA in a final reaction volume of 25 µL. PCR products were loaded in a 1.7% agarose gel and electrophoresis was carried out at 85 V for 180 min (6.5 V cm⁻¹) in 0.5X TBE buffer. The size of PCR fragments stained with 0.7 µg mL⁻¹ of ethidium bromide was estimated using molecular weight markers (GeneRuler 100 pb DNA ladder from Thermo Fisher Scientific; and 1 kb DNA ladder from New England Biolabs Inc.).

RISA Fingerprint analyses

Pattern of banding and genetic distance between samples were analyzed using PAST v3.10 [18]. Each band into the fingerprint of each sample was considered an operational taxonomic unit (OTU) represented by the molecular weight demonstrated on the agarose gel. The intensity of bands was not considered to differentiate patterns between samples. Presence and absence of each band was converted into a binary matrix comparing each PCR product and similarity measure between samples was estimated by Dice coefficient [19]. The samples were clustered by unweighted pair group method using arithmetic average (UPGMA) algorithm. Richness was determined by Margalef's index and diversity of microbial community was examined by Shannon index (H') [20]. Dominance (D) was observed using Simpson's diversity index ($D = 1/\text{Simpson index}$). Compare diversities module from PAST v3.10 software was used to check differences between groups' diversity indices. Relative abundance was expressed as frequency of different OTUs in each group.

Species-specific PCR identification

A multiplex PCR was performed using species-specific forward primers for the 16S rRNA gene sequences of *Haemophilus influenzae* (5'CGTATTATCGGAAGATGAAAGTGC3'), *Streptococcus pneumoniae* (5'AAGGTGCACTTGCATCACTACC3'), *Moraxella catarrhalis* (5'CCCATAAGCCCTGACGTTAC3'), and a universal reverse primer (5'CTACGCATTTACCGCTACAC3') for all three species [21]. The primer sequences were verified on the Ribosomal Database Project (RDP,

<http://rdp.cme.msu.edu/>). The reaction mixture consisted of 1X PCR buffer (Invitrogen), 1.6 mM of MgCl₂, 200 µM of each dNTP, 0.24 µM of each forward primer, 0.48 µM of the reverse primer, 1 U of Platinum Taq DNA Polymerase, and 2 µL (5 ng) of template DNA in a final volume of 25 µL. Initial DNA denaturation at 94°C for 5 min was followed by 30 cycles at 94°C for 30 s, 53°C for 30 s, and 72°C for 45 s. A final extension was performed at 72°C for 2 min. The products of reactions were loaded in a 3% agarose gel.

A set of primers (F5'ATCACAAACAGATAACGGCG3' and R5'CGTAAATGCACTTGCTTCAGG3') was used to amplify a region of the *nucA* gene for identify *Staphylococcus aureus* on the samples [22]. The PCR mixture consisted of 1X PCR buffer (Invitrogen), 1.6 mM of MgCl₂, 200 µM of each dNTP, 0.4 µM of each primer, 1 U of Platinum Taq DNA Polymerase, and 2 µL (5 ng) of template DNA in a final volume of 25 µL. Initial DNA denaturation at 94°C for 5 min was followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s; and final extension at 72°C for 2 min. The products of reactions were loaded in a 1% agarose gel.

Statistical analysis

Statistical analysis was performed using SPSS 20.0. Data were presented as relative frequencies (%) or median (IQR - interquartile range). The χ^2 test was used to observe differences. Values were considered statistically significant when $p < 0.05$. We used Odds Ratio (OR) under 95% of confidence interval to explore a possible association between IAV(H1N1)pdm09 infection and the presence of potentially pathogenic bacteria.

Results

Samples were obtained from 144 patients presenting age between <1 and 86 and median of 38 (11.75 – 55) years old. The dataset was constituted of 52.8% female and 47.2% male patients. Among them, 15.3% declared smoking. A total of 52.1% were infected by IAV(H1N1)pdm09 and 19.4% of them had chronic pneumopathy. The chest X-ray information showed abnormalities in 95.8% of the patients tested (n=93) (Table 1).

Table 1. Demographic and clinical characteristics of patients groups

Group		IAV(H1N1)pdm09 ^a	non-IAV(H1N1)pdm09 ^b	Total ^c
	n	75(52.1)	69(47.9)	144
Age (%)				
	≤5	15 (20.0)	18 (26.1)	33 (22.9)
	6-14	3 (4.0)	1 (1.4)	4 (2.8)
	15-24	3 (4.0)	5 (7.2)	8 (5.6)
	25-59	46 (61.3)	35 (50.7)	81 (56.3)
	>60	8 (10.7)	10 (14.5)	18 (12.5)
Gender (%)				
	Male	33 (44.0)	35 (50.7)	68 (47.2)
	female	42 (56.0)	34 (49.3)	76 (52.8)
Chronic Pneumopathy (%)				
	n	72	62	134
	missed	3	7	10
		20 (27.8)	6 (9.7)	26 (19.4)
Smoking (%)				
	n	71	66	137
	missed	4	3	7
		12 (16.9)	9 (13.6)	21 (15.3)
Chest x-Ray (%)				
	n	48	45	93
	missed	27	24	51
	Normal	2 (4.2)	2 (4.4)	4 (4.2)
	Interstitial infiltrate	22 (45.8)	24 (53.3)	46 (49.5)
	Consolidation	12 (25.0)	8 (17.8)	20 (21.5)
	Mixed	10 (20.8)	7 (15.6)	17 (18.3)
	Other	2 (4.2)	4 (8.9)	6 (6.5)

(a) – Samples of IAV(H1N1)pdm09 patients; (b) – Samples of non-

IAV(H1N1)pdm09 patients; (c) – Samples of all patients.

Bacterial pattern by RISA

Each bacterial population was successfully analyzed by the RISA methodology. Amplification using primers 1406F and 23SR generated from 1 to 22 fragments ranging between 300 and 3,000 bp. All fragments were considered in the analysis. The occurrence of fragments was more frequent in the range between 500 bp and 1,000 bp and the fragment with 525 bp appeared in 56% of all samples. The patterns of amplification were observed to be reproducible by replicates of control.

Dendrograms of cluster analysis using UPGMA algorithm based in the bacterial pattern of samples by RISA are showed (Figs 1-3). The similarity using Dice's coefficient measured 0.606 of correlation between samples (Fig 1). When the dendrograms were construct separately for samples of IAV(H1N1)pdm09 and non-IAV(H1N1)pdm09 patients, the measures were 0.660 and 0.594, respectively (Fig 2). Finally, when the Dice's coefficient was applied for samples of IAV(H1N1)pdm09 patients with CP and without CP the similarity measured was 0.765 and 0.680 respectively (Fig 3).

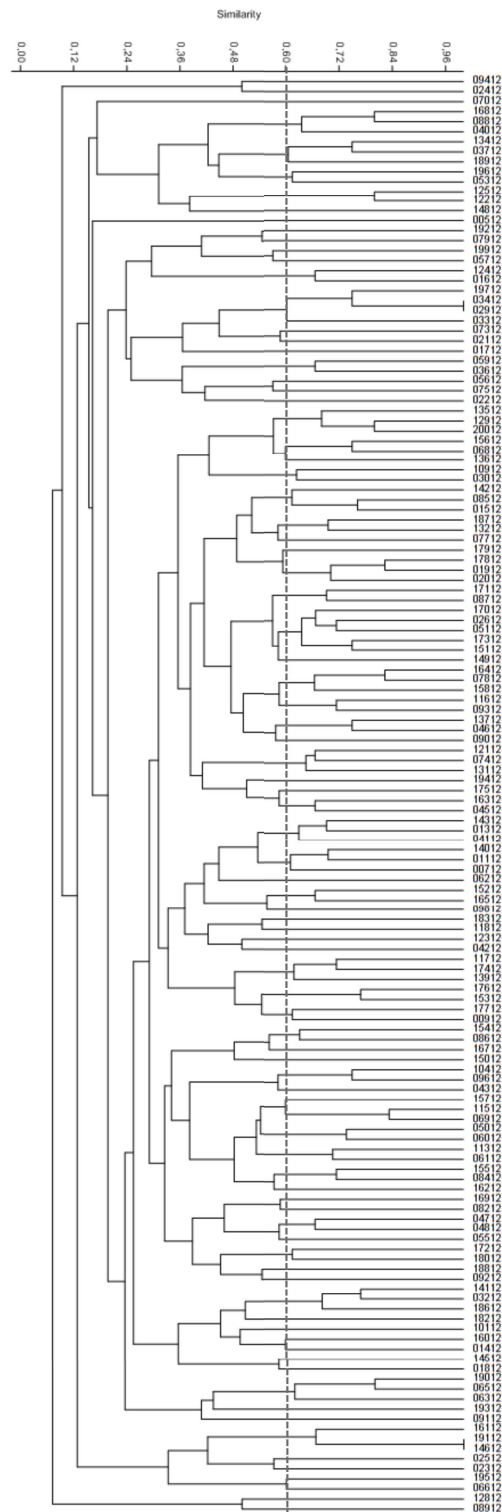
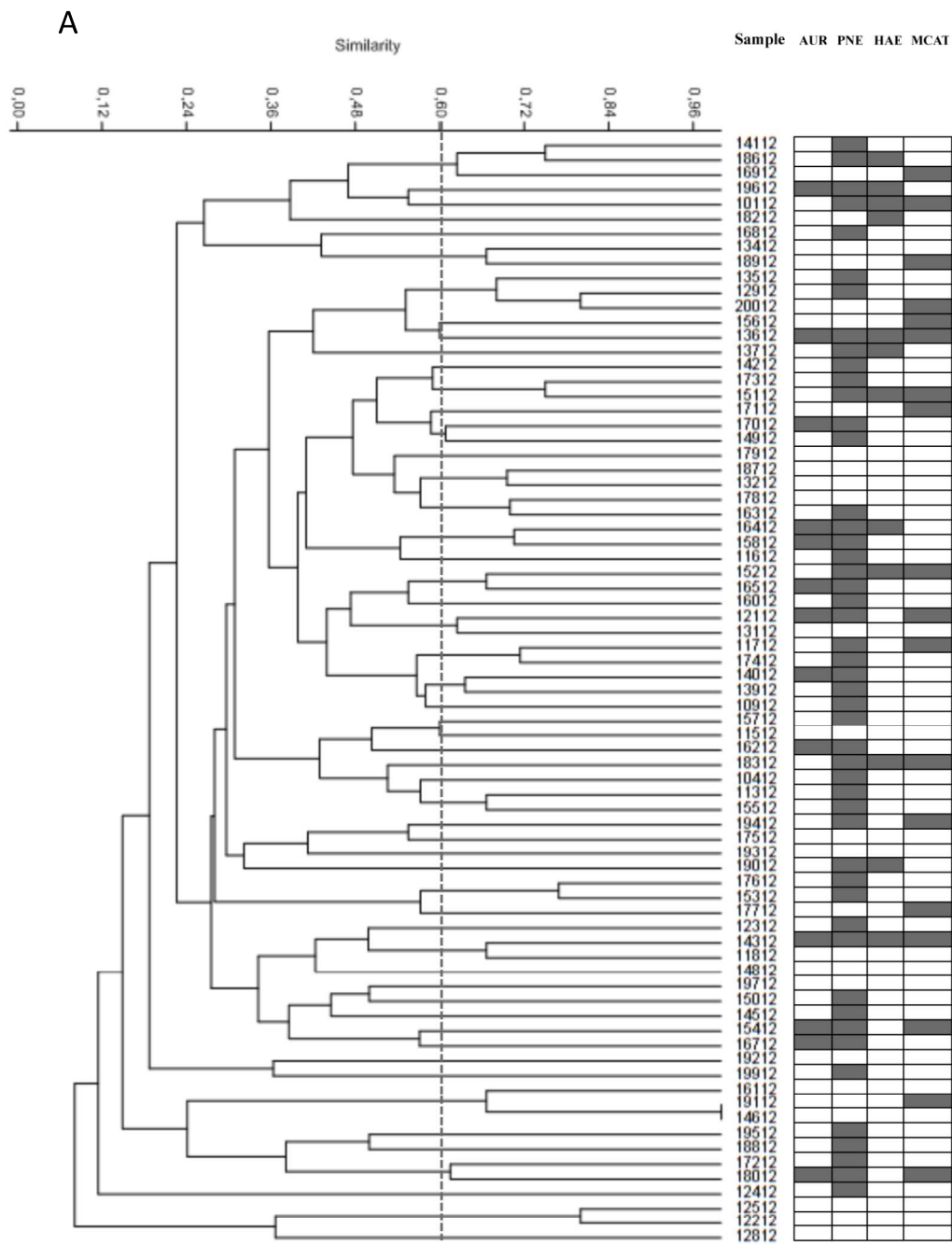


Fig 1. Dendrogram of similarity profiles for bacterial communities from nasopharyngeal samples by RISA. Constructed using UPGMA algorithm and Dice's coefficient of similarity. Dashed line indicates 60% similarity. The Dice's correlation for the 144 samples of nasopharynx was 0.606.



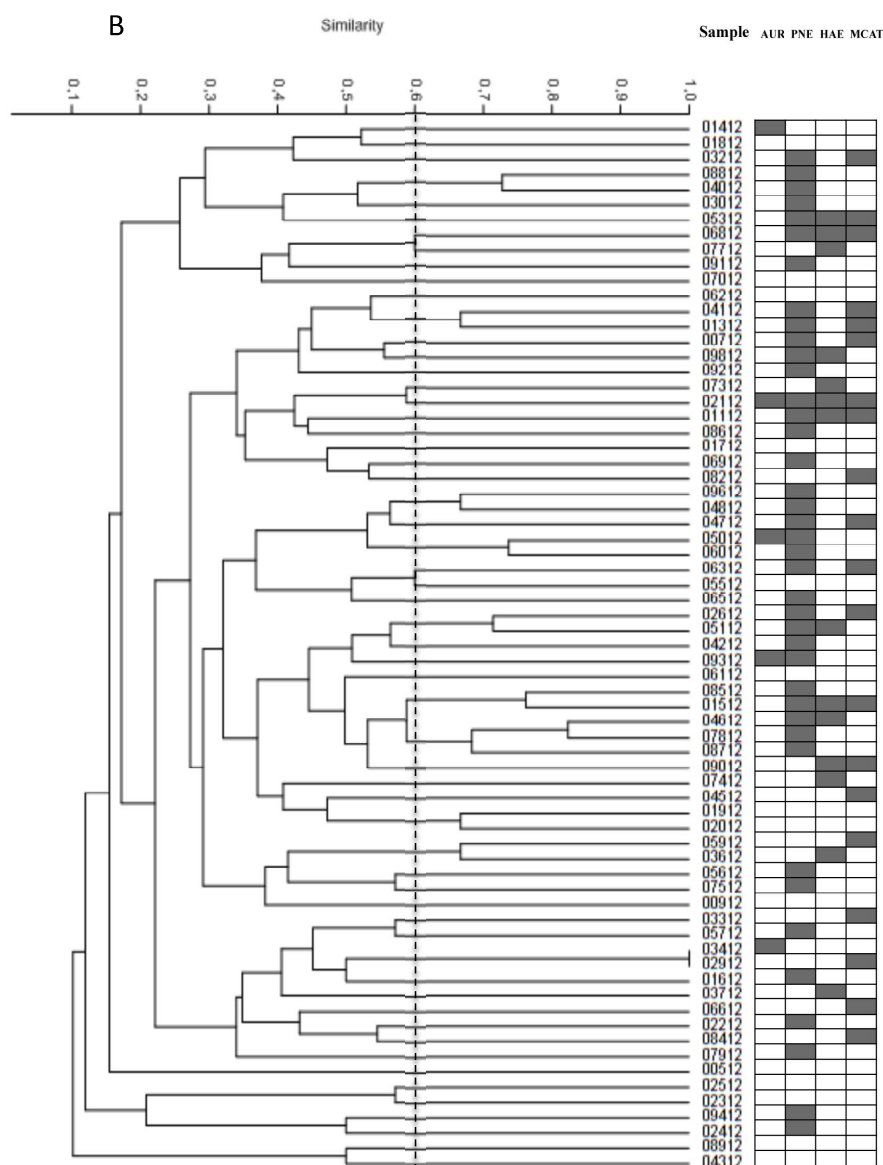


Fig 2. Dendrogram of similarity profiles for bacterial communities from nasopharyngeal of patients (A) IAV(H1N1)pdm09 and (B) non-IAV(H1N1)pdm09. Constructed using UPGMA algorithm and Dice's coefficient of similarity. Dashed line indicates 60% similarity. The Dice's correlation for the samples of nasopharynx was (A) – 0.660; (B) – 0.594. The presence of bacterial specie in the sample is highlighted in black at the table. AUR = *Staphylococcus aureus*; PNE = *Streptococcus pneumoniae*; HAE = *Haemophilus influenzae*; MCAT = *Moraxella catarrhalis*. Dashed line indicates 60% similarity.

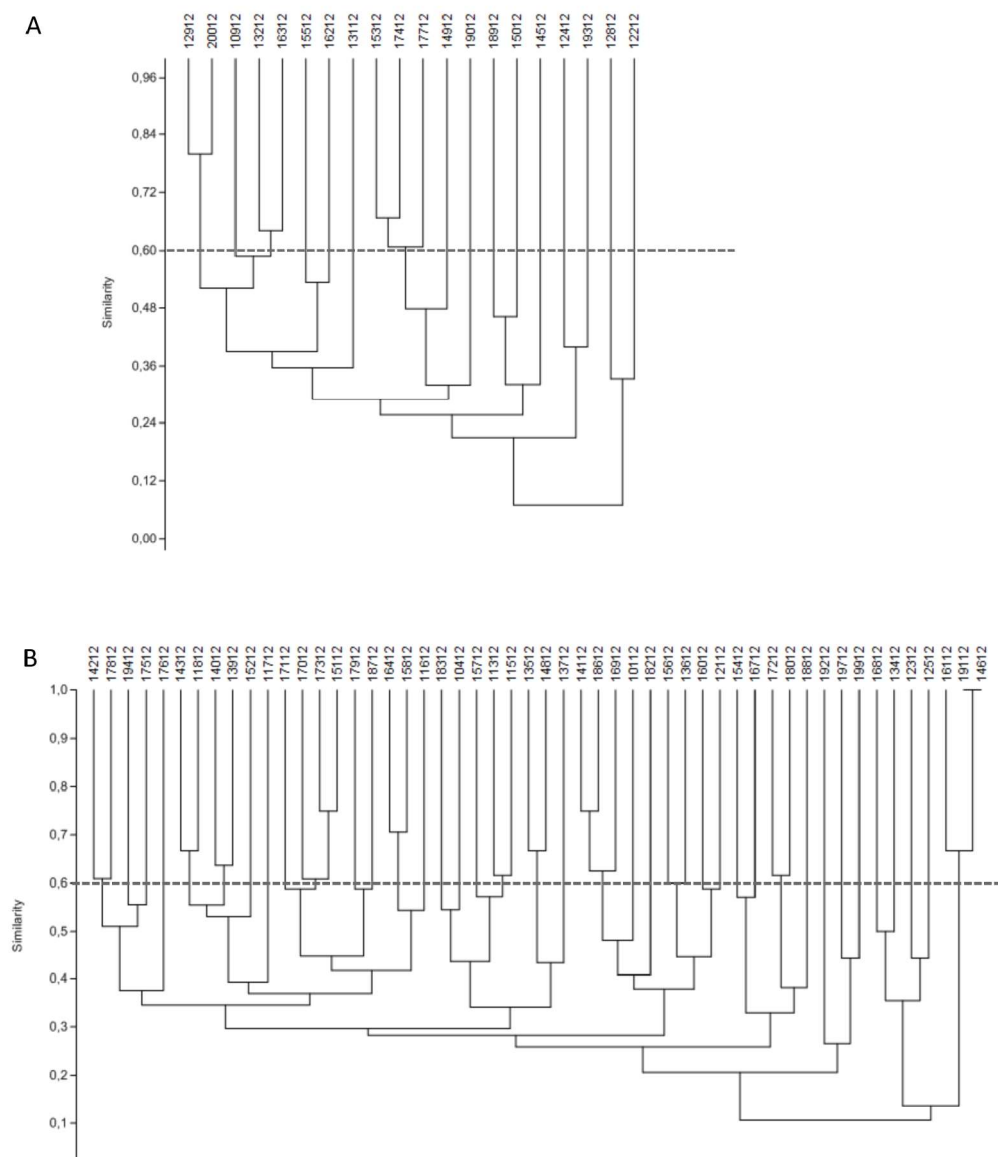


Fig 3. Dendrogram of similarity profiles for bacterial communities from nasopharyngeal of patients IAV(H1N1)pdm09 (A) with chronic pneumopathy and (B) without chronic pneumopathy. Constructed using UPGMA algorithm and Dice's coefficient of similarity. Dashed line indicates 60% similarity. The Dice's correlations for the samples of nasopharynx were (A) – 0.765; (B) – 0.680.

Bacterial diversity in IAV(H1N1)pdm09 patients

The Shannon index (H') was used in order to measure the diversity among each bacterial population, (Table 2). The diversity was $H'= 3.429$ for all samples. A higher measure of diversity was observed for samples of IAV(H1N1)pdm09 patients ($H'= 3.438$) when compared with samples of non-IAV(H1N1)pdm09 ($H'= 3.361$). Samples of patients from samples of IAV(H1N1)pdm09 with CP showed lower bacterial diversity ($H'= 3.223$) when compared with samples from patients without CP ($H'= 3.455$).

There is no significant difference ($p= 0.068$) on bacterial diversity when samples of non-IAV(H1N1)pdm09 with and without CP ($H'= 2.583$ and $H'= 3.362$, respectively) were compared (data not shown).

Table 2. Ecological group-specific analysis: bacterial diversity

Group ^a	n	Shannon (H')		Margalef's richness		Dominance (D)	
		(CI 95%)	p value ^b	(CI 95%)	p value ^b	(CI 95%)	p value ^b
all samples	144	3.429 (3.371-3.445)		5.602 (5.315-5.602)		0.037 (0.036 - 0.040)	
IAV(H1N1)pdm09	75	3.438 (3.344-3.456)	0.060 ^c	6.116 (5.646-6.116)	0.359 ^c	0.037 (0.036 - 0.043)	0.362 ^c
non-IAV(H1N1)pdm09	69	3.361 (3.260-3.374)		5.857 (5.206-5.857)		0.039 (0.038- 0.045)	
IAV(H1N1)pdm09 with CP	20	3.223 (2.976-3.208)	0.023 ^d	6.292 (4.871-6.089)	0.897 ^d	0.047 (0.046 - 0.064)	0.058 ^d
IAV(H1N1)pdm09 without CP	52	3.455 (3.351-3.463)		6.442 (5.781-6.442)		0.036 (0.035 - 0.042)	

(a) – Group of samples enrolled in each ecological diversity analyses; (b) – Bootstrapped comparison of diversity indices; (c) – p -value from diversity analysis comparison between samples of IAV(H1N1)pdm09 and non-IAV(H1N1)pdm09 patients; (d) – p -value from diversity analysis comparison between samples IAV(H1N1)pdm09 with CP and without CP patients. Significant difference between groups' diversity indices when $p<0.05$.

The relative abundance of OTU showed difference of frequencies between samples of IAV(H1N1)pdm09 with and without CP (Fig 4). The OTUs 825 and

1375 were abundant between samples of patients without CP and absent between samples of patients with CP. In lower proportion another OTUs presented difference on frequency between groups and some of those were also absent in samples of CP patients.

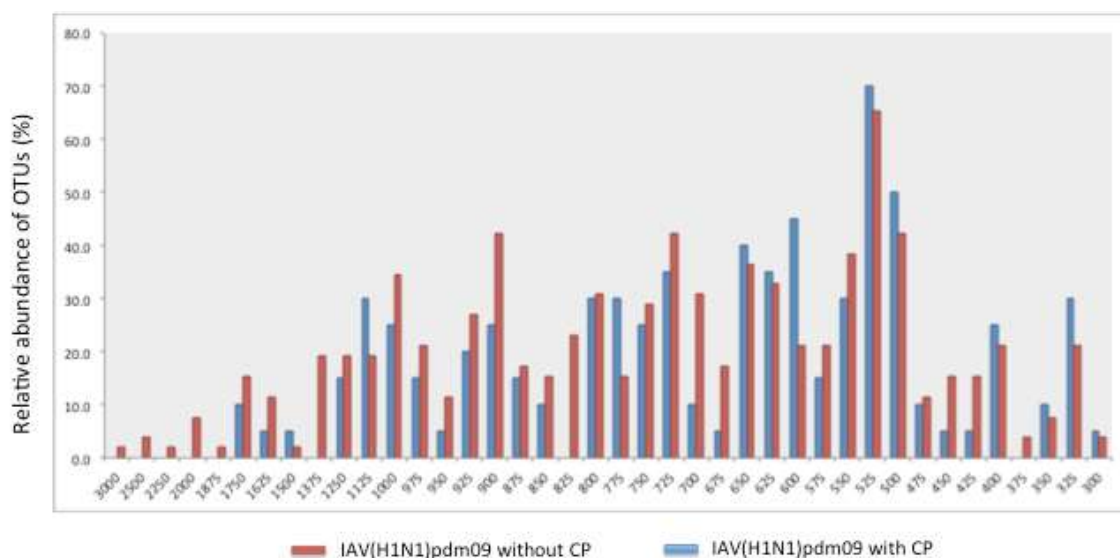


Fig 4. Relative abundance of OUTs from samples of IAV(H1N1)pdm09 patients using RISA. Red bars represent samples of patients IAV(H1N1)pdm09 without chronic pneumopathy; Blue bars represent samples of patients IAV(H1N1)pdm09 with chronic pneumopathy.

Specie specific bacteria identification

Identification of specific bacteria was done in a multiplex PCR where the products in the gel appeared as three DNA bands with sizes close to 522 bp, 481 bp and 234 bp for *H. influenza*, *S. pneumoniae* and *M. catarrhalis*, respectively. Other specific PCR for *S. aureus* produced a single band with approximate 244 bp. Results of PCR amplifications were accepted when positive and negative reaction controls were validated.

At least one of specie those potential pathogenic bacteria tested was positive for 77% of nasopharyngeal samples. *Streptococcus pneumoniae* was identified on 90 samples (62.5%). There were 25 (17.4%) and 38 (26.4%) positive PCRs for *H. influenzae* and *M. catarrhalis*, respectively. The less prevalent was *S. aureus*, present in 18 samples (12.5%) (Table 3).

Table 3. Prevalence of potentially pathogenic bacteria in the nasopharynx of IAV(H1N1)pdm09 patients and non-IAV(H1N1)pdm09 patients.

Age years	Groups	Samples n	Species			
			<i>S. aureus</i> n (%)	<i>S. pneumoniae</i> n (%)	<i>H. Influenzae</i> n (%)	<i>M. catarrhalis</i> n (%)
0-5	All samples ^a	33	5 (15.1)	22 (66.6)	11 (33.3)	18 (54.5)
	IAV(H1N1)pdm09 ^b	15	3 (20)	8 (53.3)	5 (33.3)	8 (53.3)
	non-IAV(H1N1)pdm09 ^c	18	2 (11.1)	14 (77.7)	6 (33.3)	10 (55.5)
6-17	All samples ^a	9	3 (33.3)	8 (88.8)	3 (33.3)	2 (22.2)
	IAV(H1N1)pdm09 ^b	5	3 (60)	5 (100)	1 (20)	1 (20)
	non-IAV(H1N1)pdm09 ^c	4	0 (0)	3 (75)	2 (50)	1 (25)
≥18	All samples ^a	102	10 (9.8)	60 (58.8)	11 (10.7)	18 (17.6)
	IAV(H1N1)pdm09 ^b	55	7 (12.7)	36 (65.4)	6 (10.9)	9 (16.3)
	non-IAV(H1N1)pdm09 ^c	47	3 (6.3)	24 (51)	5 (10.6)	9 (19.1)

(a) – Samples of all patients; (b) – Samples of IAV(H1N1)pdm09 patients; (c) – Samples of non-IAV(H1N1)pdm09 patients.

Co-presence of bacteria was observed in samples. Among the 144 samples, 23 (16%) were co-positive for both *M. catarrhalis* and *S. pneumoniae*. Similarly, *H. influenzae* and *S. pneumoniae* were co-identified in 19 (13%) samples. There were also 16 (11%) co-positives for *S. aureus* and *S. pneumoniae*, what correspond to 88.8% of all *S. aureus* identifications. *Staphylococcus aureus* was not co-identified together with *M. catarrhalis* nor *H. influenzae* when *S.*

pneumoniae was absent (Fig 5). Results of triple co-positivity on the multiplex PCR were evidenced for 8 (5%) samples.

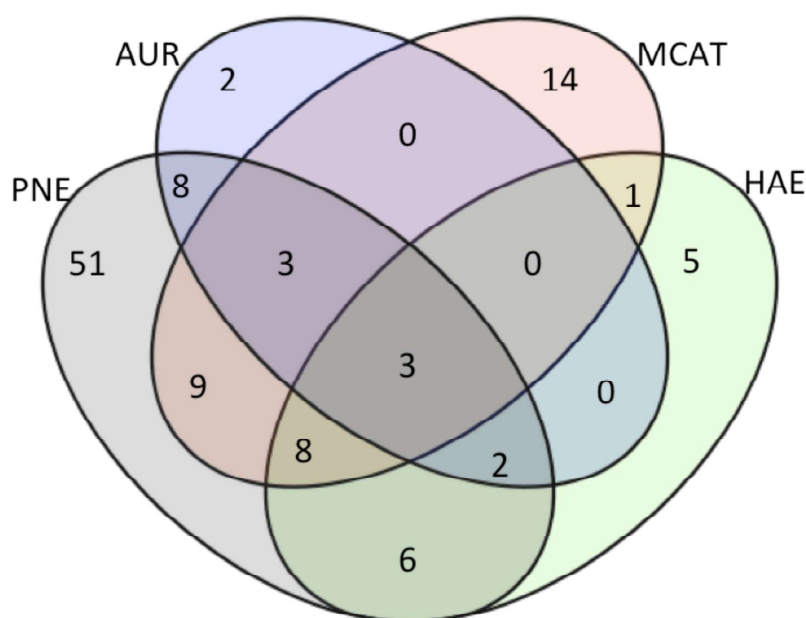


Fig 5. Venn diagram showing the co-occurrence of potentially pathogenic bacteria in nasopharynx samples of IAV(H1N1)pdm09 and non-IAV(H1N1)pdm09 patients. (AUR) – *Staphylococcus aureus*; (PNE) – *Streptococcus pneumoniae*; (MCAT) – *Moraxella catarrhalis*; (HAE) – *Haemophilus influenzae*.

Discussion

The stability in a biological community results from cooperation among several interactions. Nevertheless competition factors between two populations tend to eliminate one of the populations from the common habitat [23]. In the respiratory tract the mechanisms by which viruses influence bacterial colonization on host are diverse and some of them are well known [6]. Both positive and negative associations between virus and bacteria have been

broadly described as an important cause of a secondary bacterial infection outcome [6,24]. Study about the Spanish Flu has shown that most of the deaths observed during the pandemic in 1918 and 1919 were due to secondary pneumococcal pneumonia [25]. On the other hand, recent studies have identified as asymptomatic carriers of influenza virus as asymptomatic carriers of bacterial pathobionts [3,11]. In this context the diversity of bacterial community in patients with influenza constitute a rich source of information to understand the infection process.

The present study measured bacterial diversity in nasopharyngeal samples of patients with IAV(H1N1)pdm09 infection associated or not to chronic pneumopathy status. Using the RISA method, the bacterial diversity measured was similar for IAV(H1N1)pdm09 or non-IAV(H1N1)pdm09 patients. Most of the clusters displayed around 60% of similarity or lower with IAV(H1N1)pdm09 and non-IAV(H1N1)pdm09 patients belonging to the same cluster (Fig 1). Even when the coefficient of similarity is taken into account, both groups seem to be close. Therefore we did not observe a specific RISA pattern to differentiate IAV infected from IAV non-infected individuals.

The samples of patients IAV(H1N1)pdm09 with CP presented higher similarity of bacterial community than the similarity observed on samples of IAV(H1N1)pdm09 without CP. Moreover, when we observed the Shannon's diversity and the Margalef's richness measures we found lower diversity and lower richness on IAV(H1N1)pdm09 with CP compared to patients without CP. The low richness and diversity might suggest the presence of a bacterial dominance in the upper respiratory tract of IAV(H1N1)pdm09 with CP patients, as measured on Dominance index. Accordingly, IAV(H1N1)pdm09 with CP

patients have shown less frequency of OTUs as opposed to IAV(H1N1)pdm09 without CP patients. Indeed, some OTUs that lack on IAV(H1N1)pdm09 with CP patients, such as OTU 825, and others OTUs that are plentiful, such as OTU 600, suggesting loss of bacterial population and dominance of specific bacteria. Studies have suggested an association between lung diseases such as chronic obstructive pulmonary disease or asthma and disturbed microbiome [10,26]. Microbial agents such as *Staphylococcus aureus* and *Haemophilus influenzae* are frequently recovered in cultures from samples of patients with chronic pneumopathy, being considered causes of disease complications [26]. In the present study, *S. aureus* and *H. influenzae* were co-identified in the nasopharynx of patients with IAV with and without CP. Considering that the absence of CP is an apparent condition of stability on a bacterial community, our results suggest that both species can be present in healthy patients; therefore the presence of pathogenic bacteria species alone may not be taken as a parameter for bacterial community stability, but for specific bacterial populations that constitute the community.

Virus-bacteria coinfection has been shown to play a role in predisposing individuals to secondary infections. Coinfection of respiratory syncytial virus (RSV) and *H. influenzae* predispose *M. catarrhalis* of upper respiratory tract to invade middle ear of host and cause infection [24]. Also, influenza virus can increase bacterial colonization when in coinfection with *Streptococcus pneumoniae* [8]. Both pathogens might be involved in a process of microbial community breakdown by inducing changes in the host immune response. Indeed, positive association between *S. aureus* and influenza virus infection was reported in the upper respiratory tract of patients with acute respiratory

illness [27]. In our study, there was no difference in the identification of *S. pneumoniae* between IAV(H1N1)pdm09 and non-IAV(H1N1)pdm09 ($p=0.576$), neither of *S. aureus* ($p=0.115$), *H. influenzae* ($p=0.654$) and *M. catarrhalis* ($p=0.625$) between both groups. Nevertheless, a difference for *S. aureus* cases was observed ($p=0.036$) in a positive association with the presence of *S. pneumoniae* (OR= 4.574, 1.203-17.392). Thus, coinfection of influenza A virus and *S. pneumoniae* might be responsible for increase occurrence of *S. aureus*. Previous studies comparing the bacterial diversity in patients infected and in patients not infected by IAV did not show significant differences [9,11]. In accordance with that, the diversity of microbial community were also similar in both groups ($p=0.060$) of present study; notwithstanding, our study found significant differences on bacterial diversity between IAV(H1N1)pdm09 with CP and non-IAV(H1N1)pdm09 patients without CP ($p=0.023$).

The present study has some limitations, such as limited information about the smoking habit or the specific chronic pneumopathy of each patient, then it was not possible to associate bacteria species identified and specific disease outcomes. Another limitation is to be a retrospective study that include only samples of patients with SARI illness and do not assess samples of healthy individuals. Indeed, a prospective study may strengthen the results. Nevertheless, our findings suggest that upper respiratory tract infected by IAV might disrupt bacterial community equilibrium and that a low bacterial diversity in these patients might be associated to chronic pneumopathy.

Conclusion

RISA method has been effective to demonstrate that IAV infected patients presenting chronic pneumopathy have differences in bacterial diversity of the upper respiratory tract when compared to IAV without chronic pneumopathy patients. No difference on bacterial diversity between IAV(H1N1)pdm09 and non-IAV(H1N1)pdm09 patients was observed. Finally, the presence of bacteria pathobionts might not be associated to a higher chance of IAV(H1N1)pdm09 infection, on the other hand a lower bacterial diversity in IAV-infected patients might be associated with dominance of bacteria that cause chronic pneumopathy.

Acknowledgments

We specially thank Sergio Kakuta Kato from NUPESQ/UFCSPA for the advise on statistic analyses.

References

1. World Health Organization. A Manual for Estimating Disease Burden Associated with Seasonal Influenza. Geneva: 2015.
2. Garg S, Jain S, Dawood FS, Jhung M, Pérez A, D'Mello T, *et al.* Pneumonia among adults hospitalized with laboratory-confirmed seasonal influenza virus infection—United States, 2005–2008. *BMC Infect Dis* 2015;15:369.
3. Melchior TB, Perosa AH, Camargo CN, Granato C, Bellei N. Influenza virus prevalence in asymptomatic and symptomatic subjects during pandemic and postpandemic periods. *Am J Infect Control* 2015;43:460–4.
4. Griffin MR, Coffey CS, Neuzil KM, Mitchel EF, Wright PF, Edwards KM.

- Influenza- and Respiratory Syncytial Virus–Related Morbidity in Chronic Lung Disease. *Arch Intern Med* 2002;162:1229–36.
5. Gorini da Veiga AB, Kretzmann NA, Corrêa LT, Goshiyama AM, Baccin T, Ardenghi P, *et al.* Viral load and epidemiological profile of patients infected by pandemic influenza a (H1N1) 2009 and seasonal influenza a virus in southern Brazil. *J Med Virol* 2012;84:371–9.
 6. Bosch AATM, Biesbroek G, Trzcinski K, Sanders EAM, Bogaert D. Viral and Bacterial Interactions in the Upper Respiratory Tract. *PLoS Pathog* 2013;9:e1003057.
 7. Margolis E, Yates A, Levin BR. The ecology of nasal colonization of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus*: the role of competition and interactions with host's immune response. *BMC Microbiol* 2010;10:59.
 8. Nakamura S, Davis KM, Weiser JN. Synergistic stimulation of type I interferons during influenza virus coinfection promotes *Streptococcus pneumoniae* colonization in mice. *J Clin Invest* 2011;121:3657–65.
 9. Chaban B, Albert A, Links MG, Gardy J, Tang P, Hill JE. Characterization of the Upper Respiratory Tract Microbiomes of Patients with Pandemic H1N1 Influenza. *PLoS One* 2013;8:e69559.
 10. Erb-Downward JR, Thompson DL, Han MK, Freeman CM, McCloskey L, Schmidt LA, *et al.* Analysis of the lung microbiome in the “healthy” smoker and in COPD. *PLoS One* 2011;6:e16384.
 11. Lemon KP, Klepac-Ceraj V, Schiffer HK, Brodie EL, Lynch S V, Kolter R. Comparative analyses of the bacterial microbiota of the human nostril and oropharynx. *MBio* 2010;1:e00129–10.

12. Kovacs A, Yacoby K, Gophna U. A systematic assessment of automated ribosomal intergenic spacer analysis (ARISA) as a tool for estimating bacterial richness. *Res Microbiol* 2010;161:192–7.
13. Scanlan PD, Shanahan F, Clune Y, Collins JK, O'Sullivan GC, O'Riordan M, *et al.* Culture-independent analysis of the gut microbiota in colorectal cancer and polyposis. *Environ Microbiol* 2008;10:789–98.
14. Flight WG, Smith A, Paisey C, Marchesi JR, Bull MJ, Norville PJ, *et al.* Rapid Detection of Emerging Pathogens and Loss of Microbial Diversity Associated with Severe Lung Disease in Cystic Fibrosis. *J Clin Microbiol* 2015;53:2022–9.
15. Roesch LFW, Lorca GL, Casella G, Giongo A, Naranjo A, Pionzio AM, *et al.* Culture-independent identification of gut bacteria correlated with the onset of diabetes in a rat model. *ISME J* 2009;3:536–48.
16. WHO. Manual for the laboratory diagnosis and virological surveillance of influenza. Geneva: 2011.
17. Borneman J, Triplett EW. Molecular microbial diversity in soils from eastern Amazonia: Evidence for unusual microorganisms and microbial population shifts associated with deforestation. *Appl Environ Microbiol* 1997;63:2647–53.
18. Hammer O, Harper D, Ryan P. Past: Paleontological Statistics Software Package For Education And Data Analysis. *Paleontol Electronica* 2001;4:1–9.
19. Dice LR. Measures of the Amount of Ecologic Association Between Species. *Ecology* 1945;26:297–302.
20. Shannon CE. A mathematical theory of communication. *Bell Syst Tech J*

- 1948;27:379–423, 623–56.
21. Hendolin PH, Markkanen A, Ylikoski J, Wahlfors JJ. Use of multiplex PCR for simultaneous detection of four bacterial species in middle ear effusions. *J Clin Microbiol* 1997;35:2854–8.
 22. Brakstad OG, Aasbakk K, Maeland JA. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. *J Clin Microbiol* 1992;30:1654–60.
 23. Atlas RM, Bartha R. Structure of Microbial Communities. In: *Microbial Ecology: Fundamentals and Applications*. Redwood City, CA: The Benjamin/Cummings Publishing Company Inc.; 1993. page 140–5.
 24. Brockson ME, Novotny LA, Juncisek JA, McGillivray G, Bowers MR, Bakaletz LO. Respiratory syncytial virus promotes *Moraxella catarrhalis*-induced ascending experimental otitis media. *PLoS One* 2012;7:e40088.
 25. Morens DM, Taubenberger JK, Fauci AS. Predominant Role of Bacterial Pneumonia as a Cause of Death in Pandemic Influenza: Implications for Pandemic Influenza Preparedness. *J Infect Dis* 2008;198:962–70.
 26. Han MK, Huang YJ, Lipuma JJ, Boushey HA, Boucher RC, Cookson WO, *et al.* Significance of the microbiome in obstructive lung disease. *Thorax* 2012;67:456–63.
 27. van den Bergh MR, Biesbroek G, Rossen JWA, de Steenhuijsen Piters WAA, Bosch AATM, van Gils EJM, *et al.* Associations between pathogens in the upper respiratory tract of young children: interplay between viruses and bacteria. *PLoS One* 2012;7:e47711.

3.2.“Bacterial community in the nasopharynx of hospitalized patients infected and non-infected by influenza A virus”

Luiz Gustavo dos Anjos Borges; Adriana Giongo; Leandro Mattos Pereira ; Fernanda Pedone Valdez; Fernanda J Trindade; Tatiana Schäffer Gregianini; Paulo Michael Roehe; Ana Cláudia Franco; Fabrício Souza Campos; Ana Beatriz Gorini da Veiga

Submetido ao periódico *Microbiome*

Bacterial community in the nasopharynx of hospitalized patients infected and non-infected by influenza A virus

Luiz G A Borges¹, Adriana Giongo², Leandro M Pereira³, Fernanda P Valdez⁴,
Fernanda J Trindade⁴, Tatiana S Gregianini⁵, Paulo M Roehe⁶; Ana C Franco⁶;
Fabrício S Campos⁶; Ana B G Veiga^{1*}

Affiliations:

¹Laboratório de Biologia Molecular, Programa de Pós-Graduação em Patologia,
Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), R.
Sarmiento Leite, 245 / 309, Centro – CEP 90050-170 – Porto Alegre, RS, Brazil.
luizgaborges@gmail.com; anabgv@ufcspa.edu.br

²Instituto do Petróleo e dos Recursos Naturais (IPR), Pontifícia Universidade
Católica do Rio Grande do Sul (PUCRS), Av. Ipiranga, 6681 / Prédio 96J,
Partenon – CEP 90619-900 – Porto Alegre, RS, Brazil.
adriana.giongo@pucrs.br

³Laboratório de Virologia Parasitária, Faculdade de Biociências, Pontifícia
Universidade Católica do Rio Grande do Sul (PUCRS), Av. Ipiranga, 6681 /
Prédio 12C, Partenon – CEP 90619-900 – Porto Alegre, RS, Brazil.
mattoslmp@gmail.com

⁴Laboratório de Biologia Genômica e Molecular, Faculdade de Biociências,
Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Av. Ipiranga,
6681 / Prédio 12C, Partenon – CEP 90619-900 – Porto Alegre, RS, Brazil.
fefapedone@gmail.com; f.fertrindade@gmail.com

⁵Laboratório de Virologia, Instituto de Pesquisas Biológicas – Laboratório
Central do Estado do Rio Grande do Sul (IPB-LACEN-RS), Av. Ipiranga, 5400,
Jardim Botânico – CEP 90610-000 – Porto Alegre, RS, Brazil.

tatiana-gregianini@fepps.rs.gov.br

⁶Laboratório de Virologia, Departamento de Microbiologia, Imunologia e Parasitologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul (UFRGS), R. Sarmiento Leite, 500 / 315, Centro – CEP 90050-170 Porto Alegre, RS, Brazil.

proehe@gmail.com; anafranco.ufrgs@gmail.com; camposvet@gmail.com

* Corresponding author: Ana B. G. Veiga

Abstract

Background: The microbiome of the upper respiratory tract may be disrupted during infection by viral pathogens. Influenza A virus causes severe acute respiratory infection with high morbidity and mortality worldwide and the disease outcome may be associated to changes in the microbiome profile of infected patients. We aimed to identify traits into the bacterial community structure in the nasopharynx of hospitalized patients with severe acute respiratory infection infected and not infected by influenza A pandemic virus.

Methods: A total of 12 nasopharyngeal aspirate samples from patients with acute respiratory infection were enrolled in the study. Using a metabarcoding approach we assessed the bacterial community based on the 16S rRNA gene. Results from the Ion Torrent PGM sequencing were submitted to a quality control for the minimum Phred score of 30 using PRINSEQ. Good quality sequences were analyzed using QIIME v1.8.0.

Results: No statistic differences were observed for Shannon ($p=1.000$), chao1 ($p=0.631$) or Simpson_e ($p=0.522$) between patients IAV infected and IAV non-infected. Unassigned sequences were observed in all samples with frequency of 6.4% (5.31 / IQR 2.71 – 7.06). In general, the frequencies of phyla sequences were variable between samples, with Proteobacteria (28.3 / IQR 0.8 – 75.8), Firmicutes (13.5 / IQR 2.7 – 40) and Bacteroidetes (17.3 / IQR 0.8 – 56.1) being the dominant phyla. Differences on frequencies of Proteobacteria ($p=0.01$) and Firmicutes were observed between samples of IAV infected and IAV non-infected patients.

Conclusions: The present study has shown differences in the bacterial community of patients hospitalized with severe acute respiratory infection infected and not infected by influenza A virus.

Keywords – Microbiome; Influenza A Virus; SARI; co-infection; Upper Respiratory Tract

Background

The microbiome of the human upper respiratory tract (URT) is susceptible to changes during imbalanced states. Influenza A virus (IAV) entrance into the host epithelial cells of the URT may disrupt the microbiome stability promoting the outgrowth of pathogenic bacteria [1].

IAV is among the most common and major causes of human respiratory infection presenting high morbidity and mortality. The virus is responsible for hundreds of thousands of hospitalizations every year, mainly during a pandemic but also in non-pandemic periods [2]. Hospitalized patients with flu disease present a variety of nonspecific symptoms that are also presented by patients with other respiratory diseases. While those symptoms are commonly considered when measuring the severity of the disease, hospitalization fatality risk (HFL) has been underexplored and underestimated [3]. HFL can define the probability of death among IAV cases and is considered a better way to predict the severity of the disease and to avoid worse outcomes [3]. Nevertheless, others measure need to be included once around 12% of those pandemic IAV patients admitted in the hospital require high dependency or intensive care and up to 5% of them eventually die [4].

Studies of the URT's bacteriome have shown the importance of bacterial community structure analyses on IAV infected patients [5–7]. At birth, bacteria colonize the nasopharynx as well as other compartments of the respiratory tract compartments [8]. The URT is colonized by a wide variety of commensal and pathogenic microorganisms that constitute the nasopharynx microbiome. Furthermore, it has been reported that the bacterial community from the lower respiratory tract (LRT) is indistinguishable from the community structure from

the URT [9], what makes the use of nasopharynx samples highly qualified to screening for respiratory tract infections.

In balanced states the human microbiome is considered beneficial to the host [10]. Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria and Cyanobacteria are common phyla found in URT of healthy people in variable proportions [11]. Even pathogenic bacteria can be present in established communities. Eventually, a specific ecological perturbation can change the bacterial community structure leading to local or systemic infection by both bacterial and viral pathogens [12, 13].

Some chronic and acute diseases have been associated to decrease or to increase bacteria of specific phyla [13]. However, alterations at a lower taxonomic level such as genera or species might be more informative about the bacterial profile that can be associated to a viral infection. The abundance of the genera *Pseudomonas* and *Prevotella* has been reported to change in patients infected by IAV [14]. Moreover specific and commensal bacteria like *Streptococcus pneumoniae* have been reported to establish a mutually beneficial relationship with influenza virus [15] leading patients to a bad outcome of respiratory disease.

The 16S rRNA metabarcoding is a gene marker technique that has become an important approach for the rapid identification and classification of bacterial communities from environmental and clinical samples [16, 17]. High-throughput sequencing takes the strength of the highly conserved 16S rRNA gene for phylogenetic assignments within bacterial communities from any environment. It is proper to produce many thousands of small 16S rRNA gene fragments per sample.

The aim of the present study was to identify differences in the bacterial community structure in the URT of hospitalized patients with severe acute respiratory infection (SARI) either infected or not infected by the pandemic lineage of influenza A virus (IAV[H1N1]pdm09).

Methods

Ethics Statement

Experiments were performed in compliance with relevant laws and in accordance with the ethical standard of the Declaration of Helsinki. All ethical issues were previously approved (document number 1774/12) by the Research Ethics Committee of Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA).

Biological samples and DNA extraction

The twelve samples enrolled in the study were nasopharyngeal aspirates from a biorepository of the State Central Laboratory (LACEN-RS). All samples were collected in hospital units of Rio Grande do Sul, southern Brazil, during 2012, from patients hospitalized with SARI and suspected of being infected by IAV. Demographic and clinical information including outcome disease and viral diagnostic [18] data of patients were obtained for analysis. Samples were considered “positive samples” when positives for IAV(H1N1)pdm09, while “negative samples” were those negative for all influenza A and B viruses. Patients were not vaccinated for IAV and did not present comorbidities like chronic pneumopathy or chronic heart disease. They were not infected or co-

infected by respiratory virus other than IAV(H1N1)pdm09. The samples were stored at -80°C until required for laboratory analyses.

Each sample was thawed on ice and DNA was extracted using QIAamp DNA mini Kit (QIAGEN) according to the manufacturer's instructions. DNA was quantified in a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and then stored at -20°C for further analysis.

High-throughput sequencing

The set of primers 515F / 806R was used to amplify ~291 bp from the V3-V4 hypervariable region of the prokaryotic 16S rRNA gene [19]. The PCR conditions used were one initial denaturation step at 94°C for 45 s; 30 cycles including denaturation for 45 s at 94°C, annealing for 45 s at 50°C, and extension for 1 min at 72°C; one final extension step for 7 min at 72°C. PCR amplicons were purified using solid-phase reversible immobilization (SPRI) paramagnetic bead technology (Agencount AMPure XP; Beckman Coulter). After fluorometric quantification (Qubit; Invitrogen), 100 ng of DNA were used for libraries construction following the Ion Plus Fragment Library Kit manufacturer's recommendations (Thermo Fisher Scientific). Normalization was done using Ion Library Equalizer Kit (Thermo Fisher Scientific). Then, Ion One Touch 2 system was used for template preparation and enrichment of barcoded libraries. Finally, a multiplexed sequencing run was performed on an Ion Torrent Personal Genome Machine (PGM) System (Thermo Fisher Scientific) using Ion 316 Chip kit v2. Sequencing results were deposited in the National Center for Biotechnology Information (NCBI) under BioProject ID 000000.

High-throughput sequencing analysis

First, all 16S rRNA reads produced by high-throughput sequencing were submitted to a quality control which retains sequences with minimum length of 100 bp and trimmed to remove low quality bases for the minimum Phred score of 30 using PRINSEQ [20]. Also, replicated sequences were identified and sorted by decreasing read abundance and then filtered to exclude singletons using USEARCH v7.0.1090 [21]. Clusters were assembled using a minimum identity of 99% and chimeras were removed using RDP reference database [22]. The taxonomic assignment was obtained using QIIME v1.8.0 [23] and Operational Taxonomic Units (OTUs) were selected based on 97% sequence similarity. Taxonomic data was generated through the classification algorithm using the 97% OTUs version of GreenGenes 13.8 [24]. The default parameters of QIIME v1.8.0 were used for the alignment of OTUs (pyNAST) and for generate of phylogenies (FastTree). Rarefactions of the OTU table were performed to a maximum subsampling depth of 4700 sequences. Alpha diversity metrics were calculated using QIIME v1.8.0. Multiple rarefactions were performed for chao1 (estimates species richness), and Shannon (the entropic information of the abundances of observed OTUs), and Simpson_e (evenness). The beta diversity analysis was calculated using unweighted UniFrac. The Principal Coordinates Analysis (PCoA) was generated to observe differences between groups and the results were visualized using EMPeror software [25].

Statistical analysis

We used SPSS 20.0 to perform statistical analysis. Data were presented as relative frequency or median and interquartile ranges (IQR). Mann-Whitney test

was used to compare the diversity of groups. Values were considered statistically significant when $p < 0.05$.

Results

Samples and databank

Nasopharyngeal samples of twelve hospitalized patients presenting flu-like symptoms were enrolled in the study. According to the clinical databank, patients showed no associated comorbidities. Samples had been collected between days 0 to 2 after hospitalization and were sent to the laboratory for influenza virus diagnosis. Half of the samples ($n=6$) selected were positive to IAV(H1N1)pdm09, whereas the other half ($n=6$) consisted of IAV negative (Table 1).

Table 1 – Demographic and clinical characteristics for influenza diagnosis and disease outcome of hospitalized patient enrolled in the study

Samples ^a	Age	Gender	IAV(H1N1)pdm09 ^b	Outcome
IP1	38	Female	Positive	Death
IP2	29	Female	Positive	Cure
IP3	43	Male	Positive	Cure
IP4	2	Female	Positive	Death
IP5	52	Female	Positive	Death
IP6	28	Female	Positive	Death
IN1	47	Female	Negative	Death
IN2	54	Male	Negative	Cure
IN3	60	Male	Negative	Cure
IN4	<1	Female	Negative	Cure
IN5	<1	Female	Negative	Cure
IN6	34	Female	Negative	Cure

a - IP = identification of positive samples for influenza A virus pandemic 2009 strain; IN = identification of negative samples for influenza A virus pandemic 2009 strain.

b - IAV identification test was performed at LACEN-RS following the WHO/CDC RT-PCR protocol [18].

Sequencing data and estimation of bacterial diversity

DNA sequencing yielded 876,246 bacterial sequences (mean length 184 bp) from the amplified V3-V4 region of the 16S rRNA gene. The quality control removed bad quality bases and short reads resulting in 673,186 good quality sequences with mean length of 187 bp. Eventually, 2,543 OTUs from twelve samples were analyzed. Rarefaction curves of observed OTUs in all 12 samples reached a plateau between 43 and 290 OTUs at the maximum depth (Figure 1).

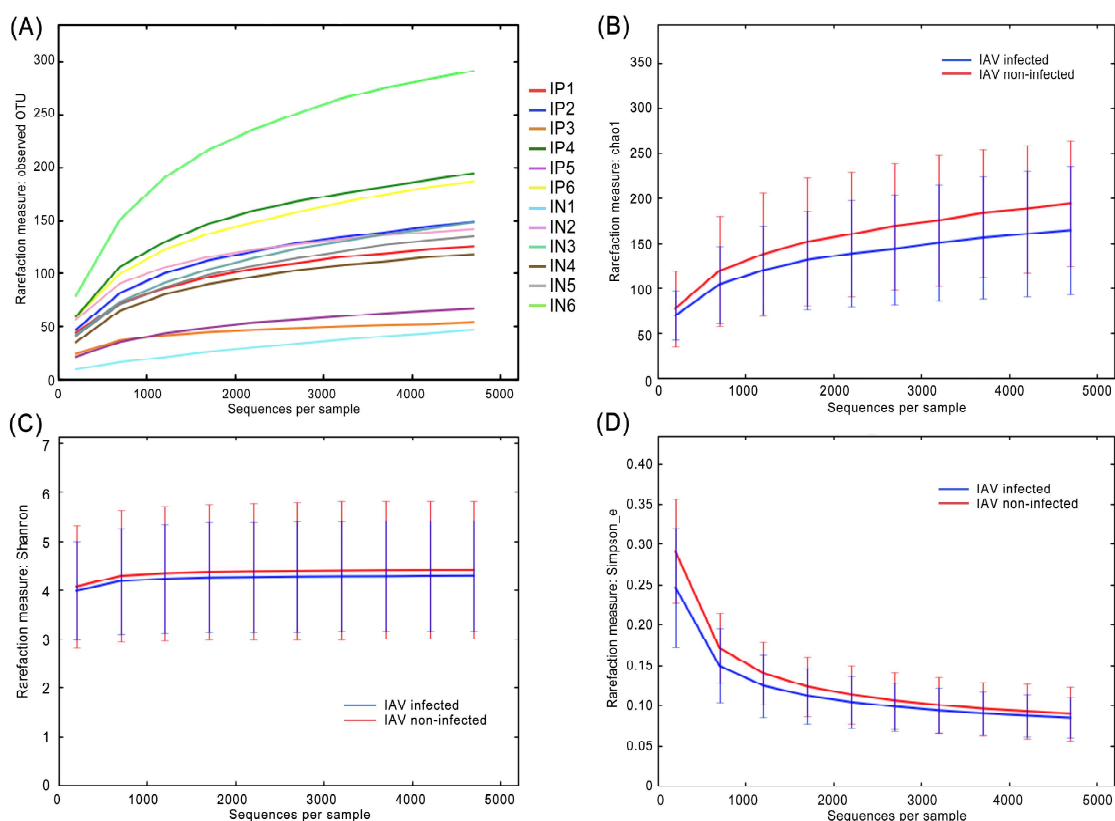


Figure 1 – Rarefaction plots and diversity indexes. Sampling maximum depth of 4700 sequences. Bar indicate standard deviation. (A) Rarefaction curves of observed OTUs from all 12 samples. (B) Chao1 index for IAV infected and IAV non-infected groups of samples. (C) Shannon index for IAV infected and IAV non-infected groups of samples. (D) Simpson's index for IAV infected and IAV non-infected groups of samples.

non-infected groups of samples. (D) Simpson_e index for IAV infected and IAV non-infected groups of samples.

Ecological data were plotted for IAV infected and IAV non-infected groups measuring the Shannon (H') index for bacterial diversity and chao1 index for bacterial richness (Figure 1). No statistic differences were observed for Shannon ($p=1.000$), chao1 ($p=0.631$) or Simpson_e ($p=0.522$) between IAV infected and IAV non-infected samples (Table 2).

Table 2 – Ecological measures of the bacterial community of the nasopharynx of patients with severe acute respiratory infection

	Samples	OTU ^a	Shannon ^b	chao1 ^c	Simpson_e ^d
IAV infected samples	IP1	189	4.732	212.214	0.066
	IP2	217	4.654	244.067	0.048
	IP3	90	2.883	102.750	0.043
	IP4	316	5.382	350.182	0.053
	IP5	123	2.645	139.667	0.028
	IP6	317	5.570	349.523	0.077
IAV non-infected samples	IN1	49	1.698	142.000	0.047
	IN2	203	5.375	242.667	0.102
	IN3	268	4.817	297.129	0.058
	IN4	186	3.832	202.235	0.032
	IN5	244	4.608	309.045	0.049
	IN6	341	6.241	378.500	0.084

a - Operational Taxonomic Units; b - diversity index; c - richness index; d - evenness index

Taxonomic identification

The classification of sequences revealed frequency of gene markers of nine bacterial phyla (Figure 2). The number of assigned phyla decreased to six phyla

when frequencies lower than one percent (1%) was compressed for analyses. Unassigned sequences were observed in all samples with frequency of 6.4% (5.31 / IQR 2.71 – 7.06). In general, the frequencies of sequences in phyla were variable between samples, with Proteobacteria (28.3 / IQR 0.8– 75.8), Firmicutes (13.5 / IQR 2.7 - 40) and Bacteroidetes (17.3 / IQR 0.8 - 56.1) being by far the dominant phyla. The comparison between IAV infected and IAV non-infected patients showed significant differences in the frequencies of sequences of two phyla: phylum Proteobacteria was more frequent ($p=0.01$) in IAV non-infected patients, while sequences of phylum Firmicutes were more frequent ($p=0.01$) in samples from IAV infected group (Table 3).

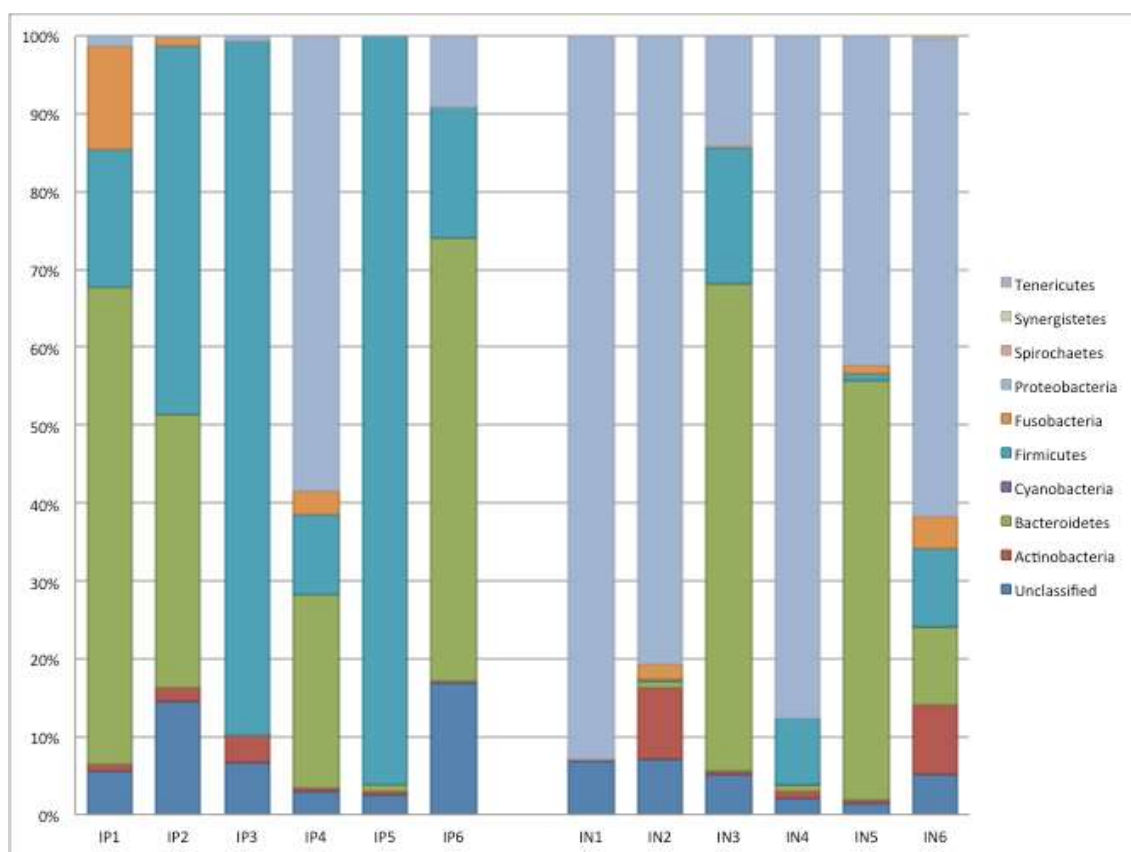


Figure 2 – Relative abundance of genome sequences assigned to bacterial phyla on samples of IAV infected and IAV non-infected group. IP = identification of influenza positive samples; IN = identification of influenza negative samples.

Similar trends occurred at the class level (Table 3). A total of nineteen bacterial classes were detected among the samples. Sequences from Alpha, Beta and Gammaproteobacteria were significantly more abundant in influenza negative samples, than in influenza positive samples ($p=0.004$, $p=0.025$, $p=0.025$; respectively). At the Order level, frequencies of six orders were significantly different in a total of thirty-one identified. At the Family level, sequences of eleven families presented differences in a total of sixty-four sequences assigned at family taxonomic level.

Table 3 – Frequencies of bacterial OTUs with statistical difference between IAV infected and IAV non-infected groups of hospitalized patients with severe acute respiratory infection

Taxon level	Operational Taxonomic Unit (OTU)			IAV infected		IAV non-infected		pvalue ^d
	Total ^a	Total ^b	Taxons	Frequency ^c (%)	Median (IQR)	Frequency ^c (%)	Median (IQR)	
Phylum	9	6	Proteobacteria	11.6	0.99 (0.23 - 21.4)	63.20	70.98 (35.34 - 89.02)	$p = 0.01$
			Firmicutes	46.3	32.54 (15.13 - 90.97)	6.10	4.60 (0.22 - 11.78)	$p = 0.01$
Class	19	10	Alphaproteobacteria	0.03	0.005 (0.00 - 0.06)	6.74	4.09 (0.79 - 11.86)	$p = 0.004$
			Betaproteobacteria	1.45	0.04 (0.00 - 2.19)	3.96	0.97 (0.26 - 9.94)	$p = 0.025$
			Gamaproteobacteria	9.88	0.33 (0.007 - 19.01)	51.78	44.31 (33.18 - 82.44)	$p = 0.025$
Order	31	17	Burkholderiales	0.01	0.00 (0.00 - 0.02)	3.75	0.95 (0.18 - 9.13)	$p = 0.003$
			Rhizobiales	0.03	0.00 (0.00 - 0.05)	4.81	0.74 (0.22 - 10.15)	$p = 0.008$
			Rhodobacterales	0.00	0.00 (0.00 - 0.00)	1.29	0.57 (0.23 - 2.18)	$p = 0.002$
			Sphingomonadales*	0.00	0.00 (0.00 - 0.00)	0.25	0.12 (0.01 - 0.56)	$p = 0.007$
			Xanthomonadales	0.00	0.00 (0.00 - 0.75)	7.73	30.93 (12.78 - 82.16)	$p = 0.007$
			Pseudomonadales	0.43	0.00 (0.00 - 0.00)	41.72	0.57 (0.06 - 19.67)	$p = 0.006$
Family	64	39	Nocardiaceae	0.00	0.00 (0.00 - 0.002)	2.77	0.54 (0.32 - 7.07)	$p = 0.003$
			Rhizobiales family	0.00	0.00 (0.00 - 0.00)	0.69	0.04 (0.02 - 1.55)	$p = 0.002$
			Bradyrhizobiaceae	0.00	0.00 (0.00 - 0.002)	0.82	0.19 (0.17 - 2.07)	$p = 0.004$
			Hyphomicrobiaceae	0.00	0.00 (0.00 - 0.00)	0.46	0.60 (0.02 - 0.73)	$p = 0.002$
			Phyllobacteriaceae	0.00	0.00 (0.00 - 0.00)	0.66	0.34 (0.04 - 1.42)	$p = 0.007$
			Rhizobiaceae*	0.00	0.00 (0.00 - 0.002)	0.99	0.02 (0.007 - 1.52)	$p = 0.020$
			Rhizobiales family (other)	0.00	0.00 (0.00 - 0.00)	0.33	0.07 (0.007 - 0.55)	$p = 0.007$
			Rhodobacteraceae	0.00	0.00 (0.00 - 0.00)	1.29	0.57 (0.23 - 2.18)	$p = 0.002$
			Comamonadaceae	0.00	0.00 (0.00 - 0.00)	2.60	0.91 (0.14 - 4.86)	$p = 0.002$
			Xanthomonadaceae	0.00	0.00 (0.00 - 0.00)	7.73	0.57 (0.06 - 19.67)	$p = 0.003$
			Pseudomonadaceae	0.01	0.00 (0.00 - 0.02)	23.78	19.93 (0.55 - 44.31)	$p = 0.007$
Genus	110	52	<i>Rhodococcus</i>	0.00	0.00 (0.00 - 0.002)	2.77	0.07 (0.00 - 10.61)	$p = 0.003$
			Rhizobiales genus (other)	0.00	0.00 (0.00 - 0.00)	0.69	0.04 (0.02 - 1.55)	$p = 0.002$
			Bradyrhizobiaceae genus	0.00	0.00 (0.00 - 0.00)	0.56	0.11 (0.01 - 1.42)	$p = 0.007$
			<i>Devosia</i>	0.00	0.00 (0.00 - 0.00)	0.33	0.05 (0.01 - 0.54)	$p = 0.002$
			<i>Phyllobacterium</i>	0.00	0.00 (0.00 - 0.00)	0.51	0.29 (0.01 - 0.54)	$p = 0.007$
			<i>Agrobacterium</i>	0.00	0.00 (0.00 - 0.002)	0.99	0.02 (0.007 - 1.52)	$p = 0.020$
			Rhizobiales genus (Other)	0.00	0.00 (0.00 - 0.00)	0.33	0.07 (0.007 - 0.55)	$p = 0.007$
			Rhodobacteraceae genus	0.00	0.00 (0.00 - 0.00)	0.34	0.21 (0.06 - 0.54)	$p = 0.002$
			<i>Paracoccus</i>	0.00	0.00 (0.00 - 0.00)	0.95	0.06 (0.02 - 1.75)	$p = 0.002$
			<i>Acidovorax</i>	0.00	0.00 (0.00 - 0.00)	0.94	0.28 (0.05 - 1.80)	$p = 0.002$
			<i>Hylemonella</i>	0.00	0.00 (0.00 - 0.00)	1.31	0.06 (0.02 - 2.79)	$p = 0.002$
			Comamonadaceae genus (Other)	0.00	0.00 (0.00 - 0.00)	0.33	0.14 (0.02 - 0.72)	$p = 0.002$
			Xanthomonadaceae genus	0.00	0.00 (0.00 - 0.00)	4.86	0.35 (0.04 - 12.50)	$p = 0.007$
			<i>Pseudomonas</i>	0.01	0.00 (0.00 - 0.02)	23.34	19.75 (0.54 - 44.14)	$p = 0.003$
			<i>Stenotrophomonas</i>	0.00	0.00 (0.00 - 0.00)	2.87	0.22 (0.01 - 7.16)	$p = 0.007$

a – Total number of observed OTUs; b – Total number of observed OTUs with frequency superior than 1%; c – Relative frequency of each taxon in all IAV infected samples or IAV non-infected samples; d – Mann-Whitney test used to calculate the p -value; * - Taxon with frequency <1% in all samples enrolled in the study.

At 97% similarity, the sequences matched 110 different OTUs in which 52 (47.2%) had frequencies above 1% of the total reads. Ten of the twelve samples had at least one third of the total reads composed by a predominant genus. In general, the most abundant bacterial genera found were *Prevotella* (15.8%), *Pseudomonas* (11.7%) and *Streptococcus* (9.5%). At all taxonomic levels, the sequences that could not be classified to known taxa ranged from 1.41% to 16.93% of the total reads and no significant differences have been observed. Significant differences were evidenced in fifteen genera (Table 3). Sequences of bacterial genus *Pseudomonas* was not present on samples of IAV infected patients, while it was found in high frequency among samples of IAV non-infected patients (Figure 3). The genus *Streptococcus* was found in all samples with relative frequency over than 1%, except in the sample IN1.

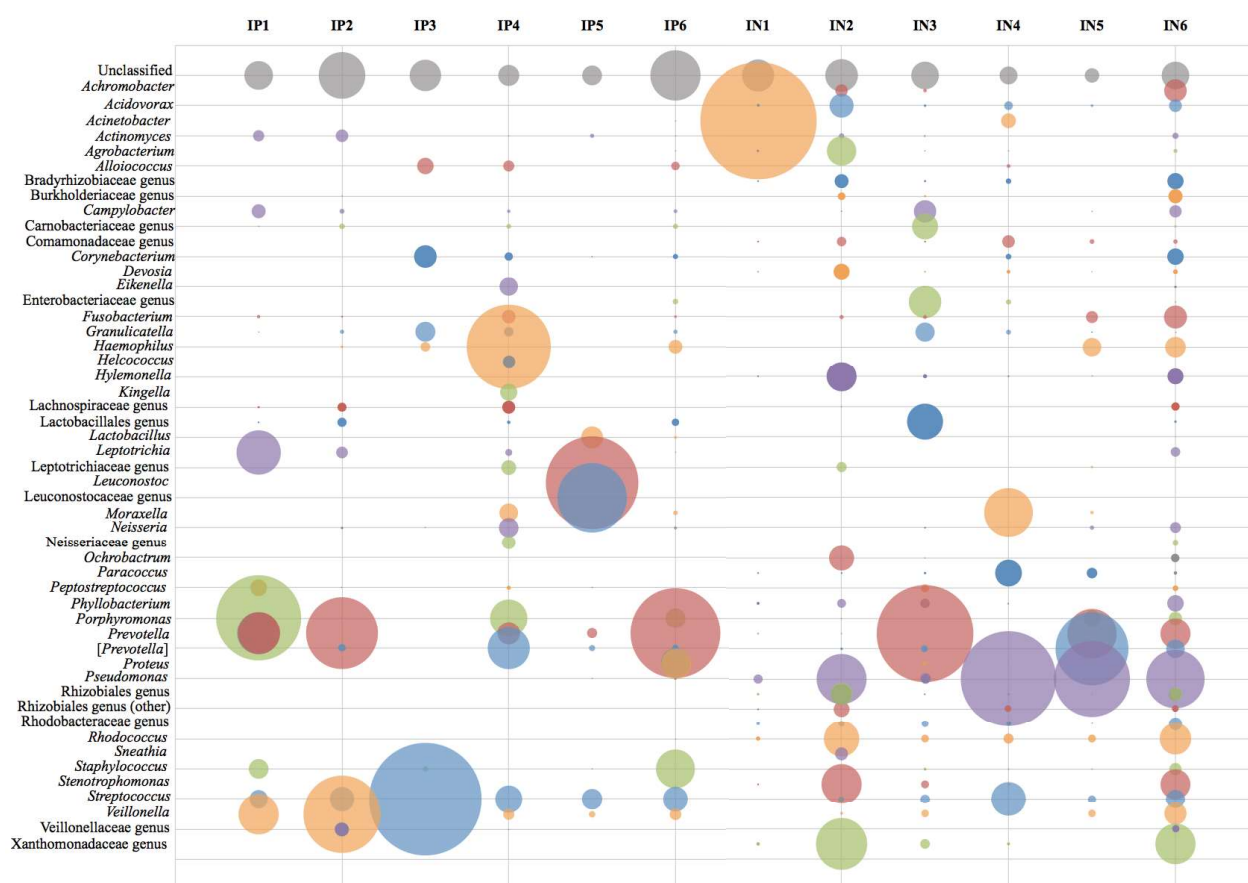


Figure 3 – Relative abundance of OTUs sequences assigned at 97% of similarity across 12 samples. The circle dots size represents the relative abundance of each OTU with frequency higher than 1%.

The pairwise value using unweighted UniFrac and PCoA to cluster samples based on sequence information revealed an association between the bacterial diversity and IAV infection profile (Figure 4). Individual samples of each IAV infected and IAV non-infected plotted fell into two separated clusters suggesting that the bacterial community in nasopharynx of both groups of patients might be distinct. When the three most abundant phyla and genera were plotted on the PCoA the result suggested that phylum Proteobacteria and genus *Pseudomonas* were associated to IAV non-infected patients. In opposite,

phylum Firmicutes and genus *Streptococcus* were associated to IAV infected patients.

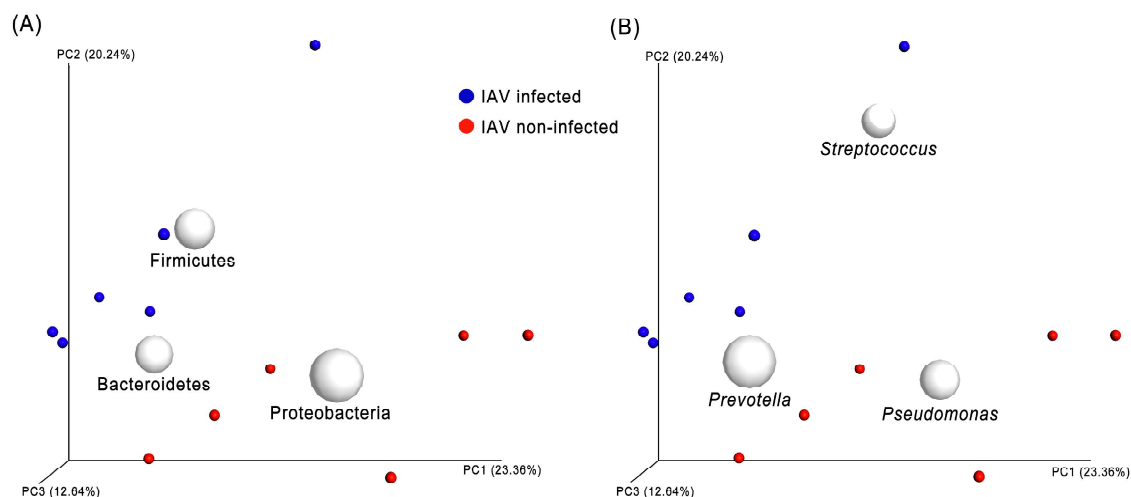


Figure 4 – Principal Coordinate Analysis (PCoA) profile of bacterial diversity across samples using unweighted UniFrac. Red dots represent IAV non-infected samples; Blue dots represent IAV infected samples; White dots represent taxa. (A) – Plot of three principal coordinate axes for PCoA with all 12 samples and the three more frequent phyla. (B) – Plot of three principal coordinate axes for PCoA with all 12 samples and the three more frequent genera.

Discussion

Major changes have been observed in the bacterial community of the URT of patients after the beginning of an infectious disease process induced by a virus [1, 26]. Once the patient is hospitalized for a severe acute respiratory infection, the changes in the microbiome seem to be more specific, consequently it may be possible to observe and to differentiate groups of patients with similar symptoms based on their URT microbiome.

The present study aimed to identify differences in the bacterial community in nasopharyngeal samples of hospitalized patients with SARI, either infected and not infected by IAV(H1N1)pdm09. Patients were not vaccinated for IAV and did not present comorbidities like chronic pneumopathy or chronic heart disease. They were not infected or co-infected by respiratory virus other than IAV(H1N1)pdm09.

The performance of metabarcoding protocol established for this study was extremely effective. The high throughput sequencing yielded a mean of 56,000 good sequences per sample, which was higher than the numbers reported by other similar studies [7, 11, 26]. The fact that a high number of sequences were obtained here most probably contributed to our findings regarding the differences observed between groups for all taxonomic levels.

The average of OTUs obtained was very similar between the IAV infected group (1,252) and the IAV non-infected patients (1,291). Each group had one sample with low abundance of OTUs, meaning a lower bacterial richness and dominance of specific bacteria. Notably, IP3 (from IAV infected group) and IN1 (from IAV non-infected group) samples showed low number of OTUs with dominance of genera *Streptococcus* (84%) and *Acinetobacter* (91%),

respectively. The patient of sample IN1 had a bad outcome of acute respiratory disease most probably by *Acinetobacter* infection. In opposite, IP3 patient had better outcome of acute respiratory disease even co-infected by IAV(H1N1)pdm09 and bacteria of genus *Streptococcus*.

The diversity measured using the Shannon index has shown no statistic differences between groups. Even for richness or evenness indexes, no differences were observed. Studies comparing patients infected and not infected by respiratory viruses report no differences in the Shannon's diversity index [7, 26], however, when the acute viral infection is associated to specific bacteria present on the microbiome, statistically significant differences are found for the diversity index [27, 28].

Actinobacteria has been reported as the most frequent phylum in healthy people, while Firmicutes is the most frequent phylum in the nose microbiome of hospitalized patients [29]. Therefore, it was not a surprise to find relatively low frequencies of Actinobacteria in the microbiomes, once all samples were from hospitalized patients. On the other hand, we found a higher frequency of sequences belonging to the phylum Firmicutes in the IAV infected group than in the IAV non-infected patients, even though all patients were hospitalized. The differences between our results and those reported by Frank et al. [29] might be due to differences in study design: they analyzed nares' microbiome, which might be less stable than the nasopharyngeal microbiome; also, we analyzed influenza A infection status of the patients, while those examined bacterial infections; finally, was not reported clinical information, neither the reason of hospitalization for the patients enrolled in that study.

In the present study, the major positive contributor for a high frequency of Firmicutes in the group of IAV infected patients was the genus *Streptococcus*, which presented high frequencies in all samples. This genus has been reported as an important agent of secondary pneumoniae in IAV patients [30] and benefited/benefiter in an interrelation with IAV [15], as well as an important controller over URT colonization of pathogenic species into the genus [30, 31]. Notably, *Streptococcus* was also present in almost all samples of IAV non-infected patients, and the difference between groups was not significant ($p = 0.055$).

The phylum Proteobacteria was the most frequent in the IAV non-infected hospitalized patients. Differently from what we have found, some studies have reported a high frequency of Proteobacteria in patients infected by IAV [5, 7]. Many factors might be responsible for the discordance found, including differences in the platform used for deep sequencing, protocols using pooled samples, protocols using processing for enrichment of genome, amount and origin of samples, and DNA extraction method applied [32]. Others Proteobacteria genera than *Pseudomonas* might be involved in acute respiratory infection. Recently, Lo et al [33] identified and isolated new species of phylum Proteobacteria causing acute respiratory disease in healthy patients. Those species were identified as belonging to the Order Rhizobiales. The referred order presented an average frequency of 4.81% among IAV non-infected patients and 0.03% among IAV infected patients sequences on the present study.

An association between influenza A and *Pseudomonas* has been previously reported, suggesting that IAV infection may facilitate the establishment of

pathogenic bacteria in the lower respiratory tract [14]. In contrast, in our study we have not found evidences of such association. As a matter of fact, the genus *Pseudomonas* was identified in very low frequency (less than 1%) in two (IP5 and IP6) of six samples of IAV infected patients, while all samples from IAV non-infected patients showed high frequencies of sequences assign for this genus. One of limitations of our study is the relatively small number of individuals sampled. Probably it will be important to extend the analysis.

Our results demonstrate that it is possible to differentiate the bacterial profile between patients with acute respiratory infection both infected and not infected by IAV using metabarcoding deep-sequencing analyses of nasopharyngeal samples based on the Ion Torrent PGM platform. Moreover, based on our findings suggest that the presence of *Streptococcus* is not an indicative of a bad outcome in IAV infected patients, and also suggest that *Pseudomonas* and IAV are not associated in SARI hospitalized patients. Finally, the present study is the first report using the Ion Torrent PGM platform for analyses of URT microbiome, showing that this is a powerful technique for the characterization of the human nasopharyngeal microbiome.

Conclusion

The present study has shown that it is possible to identify differences in the bacterial community between SARI hospitalized patients with and without IAV acute infection. *Streptococcus* is highly frequent in nasopharynx of both groups studied while *Pseudomonas* might be the mainly discordant genus in the

nasopharyngeal microbiome of SARI in patients IAV infected and IAV non-infected.

Acknowledgments

This research was supported by grants and fellowships of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Ministry of Education, Brazil) and Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS, RS, Brazil).

References

1. Cauley LS, Vella AT: **Why is coinfection with influenza virus and bacteria so difficult to control?** *Discov Med* 2015, **19**:33–40.
2. Taubenberger JK, Kash JC: **Influenza Virus Evolution, Host Adaptation, and Pandemic Formation.** *Cell Host Microbe* 2010, **7**:440–451.
3. Wong JY, Kelly H, Cheung C-MM, Shiu EY, Wu P, Ni MY, Ip DKM, Cowling BJ: **Hospitalization Fatality Risk of Influenza A(H1N1)pdm09: A Systematic Review and Meta-Analysis.** *Am J Epidemiol* 2015, **182**:294–301.
4. Nguyen-Van-Tam JS, Openshaw PJM, Hashim A, Gadd EM, Lim WS, Semple MG, Read RC, Taylor BL, Brett SJ, McMenamin J, Enstone JE, Armstrong C, Nicholson KG: **Risk factors for hospitalisation and poor outcome with pandemic A/H1N1 influenza: United Kingdom first wave (May-September 2009).** *Thorax* 2010, **65**:645–651.
5. Greninger AL, Chen EC, Sittler T, Scheinerman A, Roubinian N, Yu G, Kim

- E, Pillai DR, Guyard C, Mazzulli T, Isa P, Arias CF, Hackett J, Schochetman G, Miller S, Tang P, Chiu CY: **A Metagenomic Analysis of Pandemic Influenza A (2009 H1N1) Infection in Patients from North America.** *PLoS One* 2010, **5**:e13381.
6. Ichinohe T, Pang IK, Kumamoto Y, Peaper DR, Ho JH, Murray TS, Iwasaki A: **Microbiota regulates immune defense against respiratory tract influenza A virus infection.** *Proc Natl Acad Sci U S A* 2011, **108**:5354–9.
7. Chaban B, Albert A, Links MG, Gardy J, Tang P, Hill JE: **Characterization of the Upper Respiratory Tract Microbiomes of Patients with Pandemic H1N1 Influenza.** *PLoS One* 2013, **8**:e69559.
8. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, Knight R: **Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns.** *Proc Natl Acad Sci* 2010, **107**:11971–11975.
9. Charlson ES, Bittinger K, Haas AR, Fitzgerald AS, Frank I, Yadav A, Bushman FD, Collman RG: **Topographical continuity of bacterial populations in the healthy human respiratory tract.** *Am J Respir Crit Care Med* 2011, **184**:957–63.
10. Blaser MJ, Falkow S: **What are the consequences of the disappearing human microbiota?** *Nat Rev Microbiol* 2009, **7**:887–894.
11. Bogaert D, Keijser B, Huse S, Rossen J, Veenhoven R, van Gils E, Bruin J, Montijn R, Bonten M, Sanders E: **Variability and Diversity of Nasopharyngeal Microbiota in Children: A Metagenomic Analysis.** *PLoS One* 2011, **6**:e17035.
12. Bosch AATM, Biesbroek G, Trzcinski K, Sanders EAM, Bogaert D: **Viral**

and Bacterial Interactions in the Upper Respiratory Tract. *PLoS Pathog* 2013, **9**:e1003057.

13. de Steenhuijsen Piters WAA, Sanders EAM, Bogaert D: **The role of the local microbial ecosystem in respiratory health and disease.** *Philos Trans R Soc B Biol Sci* 2015, **370**:20140294.

14. Leung RKK, Zhou JW, Guan W, Li SK, Yang ZF, Tsui SKW: **Modulation of potential respiratory pathogens by pH1N1 viral infection.** *Clin Microbiol Infect* 2013, **19**:930–935.

15. Short KR, Habets MN, Hermans PW, Diavatopoulos DA: **Interactions between *Streptococcus pneumoniae* and influenza virus: a mutually beneficial relationship?** *Future Microbiol* 2012, **7**:609–624.

16. The Human Microbiome Project Consortium: **Structure, function and diversity of the healthy human microbiome.** *Nature* 2012, **486**:207–214.

17. Cristescu ME: **From barcoding single individuals to metabarcoding biological communities: towards an integrative approach to the study of global biodiversity.** *Trends Ecol Evol* 2014, **29**:566–571.

18. WHO: ***Manual for the Laboratory Diagnosis and Virological Surveillance of Influenza.*** Geneva; 2011.

19. Bates ST, Berg-Lyons D, Caporaso JG, Walters WA, Knight R, Fierer N: **Examining the global distribution of dominant archaeal populations in soil.** *ISME J* 2011, **5**:908–17.

20. Schmieder R, Edwards R: **Quality control and preprocessing of metagenomic datasets.** *Bioinformatics* 2011, **27**:863–864.

21. Edgar RC: **Supplementary Material - Search and clustering orders of magnitude faster than BLAST.** *Bioinformatics* 2010, **26**:2460–2461.

22. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, Tiedje JM: **Ribosomal Database Project: data and tools for high throughput rRNA analysis.** *Nucleic Acids Res* 2014, **42**:D633–D642.
23. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich K, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone C a, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R: **QIIME allows analysis of high-throughput community sequences data.** *Nat Methods* 2010, **7**:335–336.
24. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL: **Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB.** *Appl Environ Microbiol* 2006, **72**:5069–5072.
25. Vázquez-Baeza Y, Pirrung M, Gonzalez A, Knight R: **EMPeror: a tool for visualizing high-throughput microbial community data.** *Gigascience* 2013, **2**:16.
26. Allen EK, Koeppe AF, Hendley JO, Turner SD, Winther B, Sale MM: **Characterization of the nasopharyngeal microbiota in health and during rhinovirus challenge.** *Microbiome* 2014, **2**:22.
27. Yi H, Yong D, Lee K, Cho Y-J, Chun J: **Profiling bacterial community in upper respiratory tracts.** *BMC Infect Dis* 2014, **14**:583.
28. Flight WG, Smith A, Paisey C, Marchesi JR, Bull MJ, Norville PJ, Mutton KJ, Webb AK, Bright-Thomas RJ, Jones AM, Mahenthiralingam E: **Rapid Detection of Emerging Pathogens and Loss of Microbial Diversity**

Associated with Severe Lung Disease in Cystic Fibrosis. *J Clin Microbiol* 2015, **53**:2022–2029.

29. Frank DN, Feazel LM, Bessesen MT, Price CS, Janoff EN, Pace NR: **The Human Nasal Microbiota and Staphylococcus aureus Carriage.** *PLoS One* 2010, **5**:e10598.

30. Morens DM, Taubenberger JK, Fauci AS: **Predominant Role of Bacterial Pneumonia as a Cause of Death in Pandemic Influenza: Implications for Pandemic Influenza Preparedness.** *J Infect Dis* 2008, **198**:962–970.

31. Cremers AJ, Zomer AL, Gritsfeld JF, Ferwerda G, van Hijum SA, Ferreira DM, Shak JR, Klugman KP, Boekhorst J, Timmerman HM, de Jonge MI, Gordon SB, Hermans PW: **The adult nasopharyngeal microbiome as a determinant of pneumococcal acquisition.** *Microbiome* 2014, **2**:44.

32. Delmont TO, Robe P, Clark I, Simonet P, Vogel TM: **Metagenomic comparison of direct and indirect soil DNA extraction approaches.** *J Microbiol Methods* 2011, **86**:397–400.

33. Lo S-C, Li B, Hung G-C, Lei H, Li T, Zhang J, Nagamine K, Tsai S, Zucker MJ, Olesnicky L: **Isolation and characterization of two novel bacteria *Afipia cberi* and *Mesorhizobium hominis* from blood of a patient afflicted with fatal pulmonary illness.** *PLoS One* 2013, **8**:e82673.

4. Considerações Finais

Há muitos anos, junto ao início do século 20, quando agentes infecciosos filtráveis foram cientificamente comprovados serem causadores de doença em humanos, o mundo dos vírus foi desvelado. Infelizmente, o vírus em questão era o da febre amarela. Caso contrário, poder-se-ia ter poupado algumas vidas durante a gripe espanhola de 1918. Foi somente nos anos 1930s que o pesquisador Richard E. Shope demonstrou que a doença mais devastadora do século 20, a gripe, era causada por um vírus.

O mesmo pesquisador que identificou o vírus influenza pela primeira vez demonstrou a existência da cooperação vírus-bactéria na patogênese, injetando o *Haemophilus influenza* e o vírus influenza concomitantemente em um animal experimental. Desde então, o vírus influenza A foi constantemente estudado e também temido nos diversos novos episódios de pandemia.

A primeira pandemia do século XXI foi causada pelo vírus influenza A subtipo H1N1 - coincidentemente, o mesmo subtipo viral que se estima ter causado mais de 1,5 milhões de mortes quase um século antes, durante a Gripe espanhola de 1918 (Morens e cols., 2008). No Brasil, a pandemia por IAV(H1N1)pdm09 foi controlada de maneira eficiente e o ano pandêmico finalizou com 2060 óbitos, correspondendo a 4% dos casos de SRAG confirmados (SVS, 2012). No Rio Grande do Sul, houveram 298 (8,31%) óbitos entre os casos confirmados de SRAG por IAV(H1N1)pdm09 (CEVS, 2012).

Ao ingressar na fase pós-pandêmica, observou-se uma queda no número de casos de infecção por influenza A. No entanto, quando analisamos a estratificação dos dados, por Estado, os números mostram um padrão

incomum para o Rio Grande do Sul: entre 2010 e 2012, observa-se uma curva ascendente de casos de SRAG por IAV(H1N1)pdm09, mesmo mantendo os esforços de vigilância do influenza: foram 0 (2010), 103 (2011) e 522 (2012) casos confirmados para IAV(H1N1)pdm09. Em outros Estados do país, por outro lado, observa-se uma curva descendente no número de casos de SRAG por IAV(H1N1)pdm09 entre 2009 e 2011 (SVS, 2012), com um aumento abrupto de casos nos estados de Minas Gerais, São Paulo, Paraná e Santa Catarina em 2012 (SVS, 2013).

Um estudo envolvendo a análise do genoma do vírus IAV(H1N1)pdm09 foi realizado por Sant'Anna e cols. (2013), na tentativa de encontrar possíveis explicações para a epidemia no Estado. No estudo em questão, o RNA viral foi sequenciado e, posteriormente, foram verificadas as mutações presentes nos oito segmentos do vírus, além da eficácia vacinal. Os resultados obtidos demonstraram que pelo menos duas linhagens evolutivas do subtipo pandêmico co-circularam na população do Estado e que, de acordo com as sequências genômicas das amostras de 2011, os vírus que circularam no período pós-pandêmico derivaram do mesmo ancestral formador do clado 7 (Nelson e cols., 2009). Além disso, observou-se uma queda significativa na eficiência da vacina ($p = 2,285 \times 10^{-5}$) para as sequências virais de 2011, tomando-se como base o estudo das mutações dos aminoácidos dos cinco epitopos da subunidade H1 da hemaglutinina (Deem e Pan, 2009). Estes achados indicaram que houve um escape viral representado pela linhagem do clado 7, podendo esta ser uma das explicações para o aumento do número de casos durante o período pós-pandêmico. O estudo foi publicado por Sant'Anna e cols (2013), na revista Archives of Virology (Anexo I).

O estudo publicado mostrou a importância do desenvolvimento de projetos utilizando amostras locais e impulsionou as pesquisas relacionadas ao influenza A em nosso Estado, uma vez que a posição geográfica e as condições climáticas tornam-no propício para epidemias por vírus respiratórios. Neste sentido, o nosso grupo de pesquisa estabeleceu parceria com a *Icahn School of Medicine at Mount Sinai Hospital* (MSSM), através de uma colaboração entre a Dr^a Ana Beatriz Gorini da Veiga (UFCSPA) e o Dr. Adolfo García-Sastre (MSSM), além da obtenção de projeto contemplado pela Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS) para dar continuidade ao estudo da evolução do vírus influenza A.

As explicações inicialmente obtidas a partir desses resultados foram um tanto quanto satisfatórias. No entanto, tendo em vista a natureza viral e as características da via de infecção em questão, não seria sensato assumirmos a adaptação viral como única explicação para os achados epidemiológicos na população do Estado. O trato respiratório é uma das principais portas de entrada de micro-organismos e principalmente um ambiente complexo de interação micro-organismo–hospedeiro. Bosch e cols. (2013), descrevem com clareza diferentes mecanismos de interação vírus-bactéria que interferem na colonização do trato respiratório superior. Em um primeiro momento, a presença de um micro-organismo potencialmente patogênico, como é o caso do IAV, é fundamental para a ocorrência da doença respiratória. No entanto, é visível que outros fatores que permitam o estabelecimento e que favoreçam o domínio do ambiente por outros patobiontes são determinantes para a evolução e gravidade do quadro clínico.

Verificar a inter-relação vírus-bactéria foi a meta planejada para a continuação deste estudo. Dentro das possibilidades metodológicas disponíveis para o trabalho, estabeleu-se o uso de ensaios *in vitro* que possibilitassem identificar a diversidade bacteriana e a co-presença das principais bactérias envolvidas em infecções do trato respiratório humano. A técnica de *fingerprinting* utilizando a amplificação de regiões ITS do genoma bacteriano é bastante informativa e amplamente utilizada em estudos de comunidades bacterianas (Borneman and Triplett, 1997; Scanlan e cols., 2008; Flight e cols., 2015). Os resultados demonstraram a prevalência de *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Moraxella catarrhalis* e *Haemophilus influenzae*, quatro das principais bactérias patogênicas do trato respiratório superior dos pacientes, e a diversidade bacteriana em presença ou ausência do vírus IAV(H1N1)pdm09 e na presença ou ausência de pneumopatia crônica. Constatou-se que mais de dois terços das amostras (112/144) apresentaram ao menos uma das quatro espécies bacterianas pesquisadas. No entanto, a presença não esteve associada ao IAV(H1N1)pdm09. Da mesma forma, não foi identificada diferença na diversidade bacteriana entre as populações com presença ou ausência do vírus IAV(H1N1)pdm09. Apesar disso, constatamos que há diferença na diversidade bacteriana quando se estabelece uma associação entre infecção aguda por IAV(H1N1)pdm09 e pneumopatia crônica, o que indica que a comunidade bacteriana é diretamente reduzida pela presença de doença respiratória crônica e pode favorecer a presença viral.

A partir dos resultados obtidos, nota-se que a observação da diversidade bacteriana em pacientes assintomáticos é o contraponto que falta para determinar se há alteração de diversidade entre pacientes positivos para o

IAV(H1N1)pdm09. Por se tratar de um estudo retrospectivo com amostras de pacientes doentes triados pela sintomatologia, não foi possível incluir no estudo um grupo de pacientes assintomáticos positivos para o IAV(H1N1)pdm09. Este foi, possivelmente, o primeiro estudo de diversidade bacteriana em pacientes pneumopatas crônicos com diagnóstico positivos para o vírus influenza A. Estes resultados foram submetidos para publicação na revista PlosOne.

Determinar diferenças entre o trato respiratório superior de pacientes IAV(H1N1)pdm09 positivos e negativos ainda seria possível se conseguíssemos identificar as bactérias do microbioma destes pacientes. Assim, empregamos o método de sequenciamento de alto desempenho utilizando o 16S como gene marcador (*metabarcoding*). A composição do microbioma de 12 pacientes com e sem infecção por IAV(H1N1)pdm09, hospitalizados por doença respiratória aguda, foi obtida. Mesmo utilizando uma metodologia mais robusta e informativa do que o estudo anterior, confirmou-se que não há diferença de diversidade microbiana entre os grupos. Por outro lado, a composição do microbioma diferenciou os dois grupos em todos os níveis taxonômicos.

As diferenças de frequências entre os filos Firmicutes e Proteobacteria foram evidentes entre os grupos. No entanto, constatou-se que a diferença observada para o filo Firmicutes pareceu ser às custas de gêneros do filo Proteobacteria. A ausência de infecção por IAV(H1N1)pdm09 pareceu estar em direção oposta à presença de Proteobacteria e favoreceu o aumento de frequência de gêneros que compõem o filo Firmicutes, com predominância do gênero *Streptococcus*. A única diferença observada para gêneros não pertencente ao filo Firmicutes ou Proteobacteria foi o gênero *Rhodococcus*,

pertencente ao filo Actinobacteria. Este se encontrou aumentado entre as amostras negativas para IAV(H1N1)pdm09.

Durante o período do doutorado, houveram oportunidades de intercâmbios e estágios em diversos laboratórios. Todos foram importantes para a experimentação de novas metodologias de estudo. O primeiro estágio realizado foi no laboratório de Bioinformática Estrutural da Universidade Federal do Rio Grande do Sul (UFRGS), por intermédio do professor Dr. Hugo Verli. Neste local, realizou-se treinamento de bioinformática para o estudo de moléculas proteicas *in silico*. O segundo estágio foi direcionado ao aprendizado de técnicas de isolamento e amplificação do vírus influenza em células. Este foi realizado no laboratório de imunologia de doenças virais do Centro de Pesquisas René Rachou, por intermédio do Dr. Alexandre de Magalhães Vieira Machado. Dentre todas as oportunidades, as atividades realizadas durante o doutorado sanduíche na *Icahn School of Medicine at Mount Sinai* (Nova Iorque, EUA) merecem destaque. Os estudos realizados no laboratório de virologia do Professor Dr. A. García-Sastre foram relacionados à infectividade do IAV, utilizando metodologias de biologia molecular recombinante. As metodologias até então não são empregadas pelo nosso grupo e serão de grande valia para estudos futuros. Os experimentos foram realizados com sucesso e os resultados estão sumarizados no Anexo II.

O presente estudo foi aprovado pelo Comitê de Ética em Pesquisa (CEP) da UFCSPA, sob o parecer número 1774/12 (Anexo III), e contribuiu para o entendimento epidemiológico da pandemia de influenza A ocorrida em 2009 no Estado. Dados clínicos identificados na Ficha de Investigação (Anexo IV) de cada paciente foram utilizadas respeitando o sigilo de identificação do

paciente. Ficou evidenciado que os pacientes com doença crônica do trato respiratório infectados por IAV(H1N1)pdm09 estão mais propensos à baixa diversidade bacteriana e à dominância por linhagem potencialmente patogênica frequentemente encontrada no microbioma destes pacientes. Esta pode ser uma condição favorável para infecção bacteriana secundária. O IAV(H1N1)pdm09 é um importante modulador da composição do microbioma, favorecendo o gênero *Streptococcus* em detrimento aos gêneros pertencentes ao filo Proteobacteria. Estes achados colaboram principalmente para o entendimento de fatores prognósticos de epidemias e pandemias por IAV (H1N1). Entretanto, como esperado, um doutorado é pouco para responder tantas dúvidas e novas questões que vão sendo levantadas a partir dos resultados obtidos. A continuidade das pesquisas nessa área dependem do uso de metodologias mais complexas e, conseqüentemente, de estrutura laboratorial específica para estudos de Virologia. Sabe-se que ainda são poucos os laboratório deste tipo no país. Assim, este trabalho também contribui para aumentar as redes de colaboração e os esforços científicos para a pesquisa sobre o vírus influenza A no Brasil.

5. Anexos

5.1. Anexo I

**“Genomic analysis of pandemic and post-pandemic influenza A
pH1N1 viruses isolated in Rio Grande do Sul, Brazil”**

Fernando Hayashi Sant`Anna; Luiz Gustavo dos Anjos Borges; Paulo Roberto Vargas Fallavena; Tatiana Shäeffer Gregianini; Fernanda Matias; Rebecca A. Halpin; David E Wentworth; Pedro Alves d`Azevedo; Ana Beatriz Gorini da Veiga;

Publicado no periódico Archives of virology

Genomic analysis of pandemic and post-pandemic influenza A pH1N1 viruses isolated in Rio Grande do Sul, Brazil

F. H. Sant'Anna · L. G. A. Borges · P. R. V. Fallavena ·
T. S. Gregianini · F. Matias · R. A. Halpin · D. Wentworth ·
P. A. d'Azevedo · A. B. G. Veiga

Received: 8 July 2013 / Accepted: 14 September 2013
© Springer-Verlag Wien 2013

Abstract During the 2009 influenza A pH1N1 pandemics in Brazil, the state that was most affected was Rio Grande do Sul (RS), with over 3,000 confirmed cases, including 298 deaths. While no cases were confirmed in 2010, 103 infections with 14 deaths by pH1N1 were reported in 2011. Genomic analysis of the circulating viruses is fundamental for understanding viral evolution and supporting vaccine development against these pathogens. This study investigated whole genomes of six pH1N1 virus isolates from pandemic and post-pandemic periods in RS, Brazil. Phylogenetic analysis using the concatenated genome segments demonstrated that at least two lineages of the virus co-circulated in RS during the 2009 pandemic period. Moreover, our analysis showed that the post-pandemic

pH1N1 virus from 2011 constitutes a distinct clade whose ancestor belongs to clade 7. All six isolates contained amino acid substitutions in their proteins when compared to the archetype strains California/04/2009 and California/07/2009. The 2011 isolates contained more amino acid substitutions, and most of their genes were under purifying selection. Based on the amino acid substitutions in HA epitopes from strains isolated in RS, Brazil, *in silico* analysis predicted a decrease in vaccine efficacy against post-pandemic strains (median 31.562 %) in relation to pandemic ones (median 39.735 %).

Introduction

Influenza A virus is a pathogen that causes thousands of human deaths every year. In April 2009, a new H1N1 influenza A virus strain (pH1N1) emerged in North America and rapidly disseminated around the globe, culminating in the first influenza pandemic of the 21st century [1]. By the end of the pandemic period, the pH1N1 strain was responsible for more than 18,000 deaths worldwide [2]. Comprehensive phylogenetic analysis demonstrated that this virus originated from multiple reassortment events, and although it most likely arose in pigs, it also contained genes from viruses that infect birds and humans [3, 4].

Gradual genetic changes in influenza A viruses culminate in their ability to evade recognition by the immune system and therefore allow their constant circulation among human populations [5]. As demonstrated by Nelson et al. [6], at least seven distinct pH1N1 influenza A virus lineages circulated around the globe in the first months of the pandemic period. Considering its ability to mutate rapidly, the major concern was that the virus

Electronic supplementary material The online version of this article (doi:10.1007/s00705-013-1855-8) contains supplementary material, which is available to authorized users.

F. H. Sant'Anna · L. G. A. Borges · P. R. V. Fallavena ·
F. Matias · P. A. d'Azevedo · A. B. G. Veiga (✉)
Graduate Program in Pathology, Laboratory of Molecular
Microbiology and Laboratory of Molecular Biology,
Universidade Federal de Ciências da Saúde de Porto Alegre
(UFCSPA), R. Sarmento Leite, 245 / sala 309-Centro, Porto
Alegre, RS 90050-170, Brazil
e-mail: anabgv@ufcspa.edu.br

F. H. Sant'Anna
e-mail: fhsantanna@yahoo.com.br

T. S. Gregianini
Laboratory of Virology, 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

R. A. Halpin · D. Wentworth
The J. Craig Venter Institute, Rockville, MD, USA

would acquire mutations that could lead to greater transmissibility and pathogenicity, and also to drug resistance [7–9]. For monitoring these evolutionary changes, genome sequencing is a valuable and accessible approach. Genomic sequences provide fundamental information about the chronological and geographical distribution of the strains, and these data support appropriate vaccine development.

Rio Grande do Sul (RS) was the state that was most affected during the pandemic period in Brazil, with over 3,000 confirmed pH1N1 cases and almost 300 reported deaths [10, 11]. A vaccination campaign started in March of 2010 as a measure to control influenza virus infections, and no cases of pH1N1 were confirmed in 2010. On the other hand, over 100 cases occurred in 2011, including 14 deaths [10, 11].

Studies concerning the molecular evolution of this strain in Brazil are scarce. This shortcoming is even worse in the current post-pandemic period, since there are no data available concerning the evolution of the established pH1N1 viruses. Consequently, this lack of information can compromise local public-health vaccination policies.

In this study, six pH1N1 influenza A genomes – four pandemic and two post-pandemic isolates from RS, Brazil – were analyzed. Phylogenetic analysis using the concatenated genome segments was performed to determine the lineages of these isolates. Also, amino acid substitutions in the viral proteins were mapped, and the efficacy of the vaccine against all isolates was predicted. Finally, the type of natural selection that the isolates were subjected to was evaluated.

Materials and methods

Biological samples, virus identification, and genome sequencing

Nasopharyngeal aspirate samples were collected from patients with acute respiratory infection in the state of Rio Grande do Sul, Southern Brazil, during 2009 and 2011 (Online Resource, Table S1). In order to identify the virus, all samples were analyzed by real-time PCR at the State Central Laboratory (LACEN-RS) as described by Veiga et al. [12]. All ethical issues were approved by the Research Ethics Committee of Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA).

Twelve pH1N1 samples with high viral load, eight from the pandemic and four from the post-pandemic period, were sent to the J. Craig Venter Institute. Some of them had an excess of mucus and did not homogenize properly with the viral transport media. Therefore, after RNA extraction,

only six samples were viable for amplification by multi-segment RT-PCR [13]. Subsequently, whole-genome sequencing was performed on these samples, and the sequences were deposited in GenBank (accession numbers are listed in Table 1).

Sequence alignment and concatenation

Sequences of influenza A virus strains were retrieved from the Influenza Research Database (<http://www.fludb.org/>), and their accession numbers are listed in Table 1. The genomic segments and genes were aligned using MUSCLE [14], embedded in MEGA 5 software [15]. Amino acid substitutions were visually inspected using the sequences of archetype strains California/04/2009 and California/07/2009 as references. Aligned segment sequences were concatenated using Sequence Matrix (<http://code.google.com/p/sequencematrix/>).

Phylogenetic analysis

For phylogenetic analyses, the aligned sequences were first evaluated using the FindModel software (<http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html>) in order to identify the evolutionary model that best fit the sequence dataset. Subsequently, phylogenetic analysis was performed on the Phylogeny.fr platform (www.phylogeny.fr) [16]. Phylogenetic trees were reconstructed using the maximum-likelihood method implemented in the PhyML program (v3.0 aLRT). 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).

Detection of adaptive evolution

A codon-based Z-test for detection of positive and purifying selection within viral sequences using the Nei-Gojobori method was conducted in MEGA 5 software [15]. The numbers of synonymous (dS) and nonsynonymous substitutions (dN) per site of each gene from RS influenza A virus pH1N1 isolates in relation to its counterparts from the archetype strains California/04/2009 and California/07/2009 were assessed. The difference between the dS and dN of each gene was computed, indicating the type of adaptive evolution. $dN > dS$ indicates positive selection, and $dS > dN$ indicates purifying selection. The variance of the difference was computed using the bootstrap method (1000 replicates).

Table 1 Accession numbers of the segment sequences of pH1N1 strains utilized for whole-genome phylogeny

Strain	Accession number							
	Segment 1	Segment 2	Segment 3	Segment 4	Segment 5	Segment 6	Segment 7	Segment 8
Arizona/01/2009	GQ200241	GQ377066	GQ377065	GQ117067	GQ117063	GQ117064	GQ117066	GQ117065
Beijing/3/2009	GQ225378	GQ225379	GQ225380	GQ225381	GQ225382	GQ225383	GQ225384	GQ225385
Brazil/AVS03/2009	CY120722	CY120721	CY120720	CY120715	CY120718	CY120717	CY120716	CY120719
Brazil/AVS04/2009	CY120730	CY120729	CY120728	CY120723	CY120726	CY120725	CY120724	CY120727
Brazil/AVS06/2009	CY120738	CY120737	CY120736	CY120731	CY120734	CY120733	CY120732	CY120735
Brazil/AVS07/2009	CY120746	CY120745	CY120744	CY120739	CY120742	CY120741	CY120740	CY120743
Brazil/AVS08/2011	CY120754	CY120753	CY120752	CY120747	CY120750	CY120749	CY120748	CY120751
Brazil/AVS11/2011	CY120762	CY120761	CY120760	CY120755	CY120758	CY120757	CY120756	CY120759
California/04/2009	FJ966079	FJ915189	FJ969515	GQ280797	FJ969512	FJ915186	FJ969513	FJ969514
California/06/2009	FJ966963	FJ966965	FJ966964	FJ966960	FJ966961	FJ971075	FJ966962	FJ971074
California/07/2009	CY121687	FJ966978	FJ966977	CY121680	GQ338390	CY121682	CY121681	CY121684
California/14/2009	GQ117035	GQ117034	GQ117037	GQ117040	GQ117033	GQ117036	GQ117039	FJ969538
Himeji/1/2009	GQ267838	GQ267837	GQ267836	GQ261272	GQ267834	GQ261273	GQ267833	GQ267835
Italy/127/2009	GQ392026	GQ392027	GQ392028	GQ392029	GQ392030	GQ392031	GQ392032	GQ392033
Jiangsu/1/2009	GQ433894	HM754227	GQ433896	HM754224	GQ433897	GQ433898	GQ433899	GQ433900
Korea/01/2009	GQ160811	GQ160813	GQ160812	GQ131023	GQ131024	GQ132185	GQ131025	GQ131026
Mexico/4108/2009	GQ379815	GQ149652	GQ149661	GQ149662	GQ149667	GQ149666	GQ149690	GQ379814
Minnesota/02/2009	GQ117070	GQ117069	GQ457488	GQ338364	GQ117068	GQ117071	GQ117073	GQ117072
New York/21/2009	GQ200246	GQ200247	GQ200249	GQ200250	GQ200248	GQ200251	GQ338353	GQ200252
New York/3199/2009	CY041209	CY041208	CY041207	CY041202	CY041205	CY041204	CY041203	CY041206
New_York/3177/2009	CY041604	CY041603	CY041602	CY041597	CY041600	CY041599	CY041598	CY041601
New_York/3324/2009	CY043202	CY043201	CY043200	CY043195	CY043198	CY043197	CY043196	CY043199
New_York/4735/2009	CY051670	CY051669	CY051668	CY051663	CY051666	CY051665	CY051664	CY051667
Omsk/02/2009	GU211242	GU211241	GU211240	GU211235	GU211238	GU211237	GU211236	GU211239
Sakai/1/2009	GQ267845	GQ267844	GQ267843	GQ267839	GQ267841	GQ261274	GQ267840	GQ267842
Shizuoka/759/2009	GQ334352	GQ334353	GQ334351	GQ334346	GQ334349	GQ334348	GQ334347	GQ334350
Sichuan/1/2009	GQ166228	GQ166227	GQ166226	GQ166223	GQ166225	GQ166224	GQ166229	GQ166230
Singapore/GP582/2011	CY124573	CY124574	CY124575	CY091696	CY124576	CY091697	CY124577	CY124578
South_Carolina/09/2009	GQ200222	GQ168854	GQ457472	GQ117056	GQ117052	GQ221795	GQ221796	GQ117054
Texas/42114261/2009	CY051894	CY051893	CY051892	CY051887	CY051890	CY051889	CY051888	CY051891
Tokushima/1/2009	GQ324577	GQ324576	GQ324575	GQ287625	GQ324573	GQ287626	GQ324572	GQ324574
Utsunomiya/1/2009	GQ334354	GQ334361	GQ334360	GQ334355	GQ334358	GQ334357	GQ334356	GQ334359
Yokohama/1/2009	GQ324583	GQ324582	GQ324581	GQ287627	GQ324579	GQ287628	GQ324578	GQ324580

Measure of antigenic distance

In this analysis, all HA sequences from pH1N1 strains isolated in RS, Brazil, from 2009 and 2011 deposited in the Influenza Research Database (<http://www.fludb.org/>) were utilized. The additional HA sequences included in the analysis are from the following strains (accession numbers are in parentheses): Rio Grande do Sul/4509/2009 (CY052048), Rio Grande do Sul/4772/2009 (CY052049), Rio Grande do Sul/4782/2009 (CY052050), Rio Grande do Sul/5395/2009 (CY052347), Rio Grande do Sul/7019/2009 (CY052348), Rio Grande do Sul/7108/2009 (CY054282), Rio Grande do Sul/277/2011 (CY099996), Rio Grande do

Sul/278/2011 (CY099997), Rio Grande do Sul/279/2011 (CY099998), Rio Grande do Sul/359/2011 (CY099999), Rio Grande do Sul/360/2011 (CY100001) and Rio Grande do Sul/361/2011 (CY100002).

The antigenic distances of the five epitopes of hemagglutinin of the samples in relation to those of the vaccine strain California/07/2009 were evaluated as described by Deem and Pan [17]. The p-distance is defined as the proportion of different amino acids for each epitope between two strains, and it was measured using MEGA software. The largest of the p-distance values is defined as p_{epitope} and can be used to estimate vaccine efficacy (E) by the equation $E = 0.47 - 2.47 \times p_{\text{epitope}}$ [17]. The difference of

Table 2 Amino acid substitutions in the HA protein from RS influenza A virus pH1N1 isolates

Strain	Amino acid position														
	2	47	56	100*	114	208*	214	220	222*	233*	240*	266	310	338	391*
California/04/2009	K	V	G	P	D	I	T	S	R	I	Q	V	Q	I	E
California/07/2009	.	.	.	.	.	L	A	.	.	.	R	.	.	.	.
Brazil/AVS03/2009	E	.	.	S	.	L	A	.	.	.	.	.	H	V	.
Brazil/AVS04/2009	E	.	.	S	.	L	A	.	.	.	.	.	H	V	.
Brazil/AVS06/2009	.	.	E	S	.	L	A	T	.	.	.	.	.	V	.
Brazil/AVS07/2009	.	.	.	S	.	L	A	T	.	.	.	.	.	V	.
Brazil/AVS08/2011	.	I	.	S	N	L	A	T	K	V	.	L	.	V	K
Brazil/AVS11/2011	.	.	.	S	N	L	A	T	K	V	.	L	.	V	K

Grey background, change in antigenic site in relation to the vaccine strain California/07/2009

* H1 antigenic sites (positions 100, 208, 222, 233 and 240 were described by Deem and Pan [17], and position 391 by Maurer-Stroh et al. [20])

• No amino acid change

the predicted vaccine efficacies between the pandemic (2009) and the post-pandemic (2011) periods in RS, Brazil, was evaluated by Mann-Whitney test using PAST software [18]. A P-value less than 0.05 was considered significant.

Results

Identification of amino acid substitutions

Six influenza A virus pH1N1 strains, four from the pandemic period (2009) and two from the post-pandemic period (2011), isolated in RS, Brazil were sequenced. Predicted proteins from the isolates were compared with those from the archetype strains California/07/2009 and California/04/2009, and the amino acid alterations are shown in Tables 2, 3, 4 and 5.

The 2011 isolates showed a higher number of amino acid substitutions than the 2009 isolates in relation to the archetype strains, notably in the proteins HA and NA (Tables 2 and 3 and Online Resource, Figures S1 and S2). The HA protein from the 2011 isolates had five modifications in antigenic sites, while that from the 2009 isolates had two modifications (Table 2). The amino acid substitution HA Q310H, which is associated with high mortality rates, was found in the RS samples AVS03 and AVS04 (2009) (Online Resource, Figure S2). However, the alteration D239N/G, which is associated with severe illness [19], was not observed in the isolates. The modification E391K, which is associated with changes in antigenic properties [20], is present in the 2011 isolates (Table 2 and Online Resource, Figure S2).

In the NA protein, there were two substitutions in antigenic sites in the post-pandemic isolates, and only one in the pandemic samples analyzed (Table 3). None of the NA proteins contained the modifications H275Y and S247N (Online Resource Figure S3), which are associated with oseltamivir resistance [21].

The 2011 isolates had more alterations in RNA polymerase complex proteins when compared to the 2009 isolates (Table 4).

Phylogenetic analysis

Preliminary phylogenetic analysis of each segment confirmed that all isolates were phylogenetically closer to pH1N1 than to other influenza A virus strains (Online Resource, Figure S3). Subsequently, whole-genome phylogeny was performed in order to determine the lineages of the isolates. The segments were aligned with those from representative strains of each of the seven clades determined by Nelson et al. [6]. All segment alignments were concatenated and subjected to phylogenetic analysis using maximum likelihood under the GTR model, which had the best performance in the test of nucleotide substitution models (Online Resource, Table S2).

As shown in Figure 1, the Brazilian isolates were distributed between clades 6 and 7, which had high aLRT values (96 and 99, respectively). These isolates exhibited the characteristic amino acid changes that define each clade [6]. Isolates from clade 6 had changes in HA (K2E and Q310H), NP (V100I) and NA (V106I and N248D), and those from clade 7 had substitutions in HA (S220T), NP (V100I), NA (V106I and N248D) and NS1 (I123V) (Tables 2, 3 and 5 and Online Resource, Figures S1 and S2).

The 2009 RS isolates from clade 6 were phylogenetically the closest to each other. In clade 7, the strain Brazil/AVS/06/2009 was closer to the strain Omsk/02/2009 than to the strain Brazil/AVS/07/2009.

The post-pandemic strains formed a monophyletic clade and shared a most recent common ancestor with clade 7 strains. Also, these strains accumulated more mutations than the pandemic strains, as shown by their longest branches in the phylogenetic tree.

Table 3 Amino acid substitutions in the NA proteins of RS influenza A virus pH1N1 isolates

Grey background, change in antigenic site in relation to the vaccine strain California/07/2009	California/07/2009	.	.	.	.	.	.	.	.	
	Brazil/AVS03/2009	.	.	I	.	.	D	.	.	
	Brazil/AVS04/2009	.	.	I	.	.	D	.	.	
	Brazil/AVS06/2009	.	.	I	.	.	D	.	.	
	Brazil/AVS07/2009	.	.	I	.	.	D	G	.	
* NA antigenic sites	Brazil/AVS08/2011	I	A	I	A	I	D	.	K	M
● No amino acid change	Brazil/AVS11/2011	.	A	I	A	I	D	.	K	M

* NA antigenic sites

• No amino acid change

Table 4 Amino acid substitutions in the PA, PB1 and PB2 proteins from RS influenza A virus pH1N1 isolates

Strain	Amino acid position																		
	PA								PB1						PB2				
	14	224	225	287	362	364	394	538	76	113	197	363	435	563	60	82	344	354	451
California/04/2009	V	P	S	A	R	S	D	E	D	V	K	K	I	R	D	N	V	I	I
California/07/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Brazil/AVS03/2009	.	S	.	.	.	N	.	.	.	.	.	.	.	.	N	.	M	.	.
Brazil/AVS04/2009	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Brazil/AVS06/2009	.	S	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	T
Brazil/AVS07/2009	.	S	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	V
Brazil/AVS08/2011	I	S	G	S	K	.	N	G	G	A	.	R	T	.	.	S	M	M	.
Brazil/AVS11/2011	I	S	G	.	K	.	N	G	.	A	R	R	T	.	.	S	M	M	.

• No amino acid change

Table 5 Amino acid substitutions in the M1, M2, NEP, NP and NS1 proteins of RS influenza A virus pH1N1 isolates

Strain	Amino acid position										
	M1	M2	NEP			NP			NS1		
	150	21	22	29	115	100	101	483	98	122	123
California/04/2009	T	D	G	N	A	V	D	N	M	A	I
California/07/2009	.	.	.	.	.	.	G	.	.	.	.
Brazil/AVS03/2009	.	.	.	.	T	I	.	T	.	.	.
Brazil/AVS04/2009	.	G	E	.	.	I	.	.	.	.	.
Brazil/AVS06/2009	.	.	.	S	.	I	.	.	.	.	V
Brazil/AVS07/2009	I	.	.	.	.	I	.	.	I	.	V
Brazil/AVS08/2011	.	.	.	.	.	I	.	.	.	V	V
Brazil/AVS11/2011	.	.	.	.	.	I	.	.	.	V	V

• No amino acid change

Natural selection analysis

Genes from the pH1N1 isolates were tested for purifying and positive selection. No positive selection was detected in any of the genes of the isolates analyzed (Online Resource, Table S3). Results of the purifying selection test are listed in Table 6. Purifying selection was not found in the M2, NS1 and NEP genes of any of the isolates.

Notably, among the pandemic isolates, the polymerase genes PA, PB1 and PB2 were more frequently affected by purifying selection than other genes. Furthermore, the pattern of purifying selection was not found in any HA genes, while for the NA gene only one isolate (Brazil/AVS03/2009) presented such a pattern of natural selection.

Analysis of the post-pandemic isolates indicated that the HA, NA, M1, NP, PA, PB1 and PB2 genes were under purifying selection.

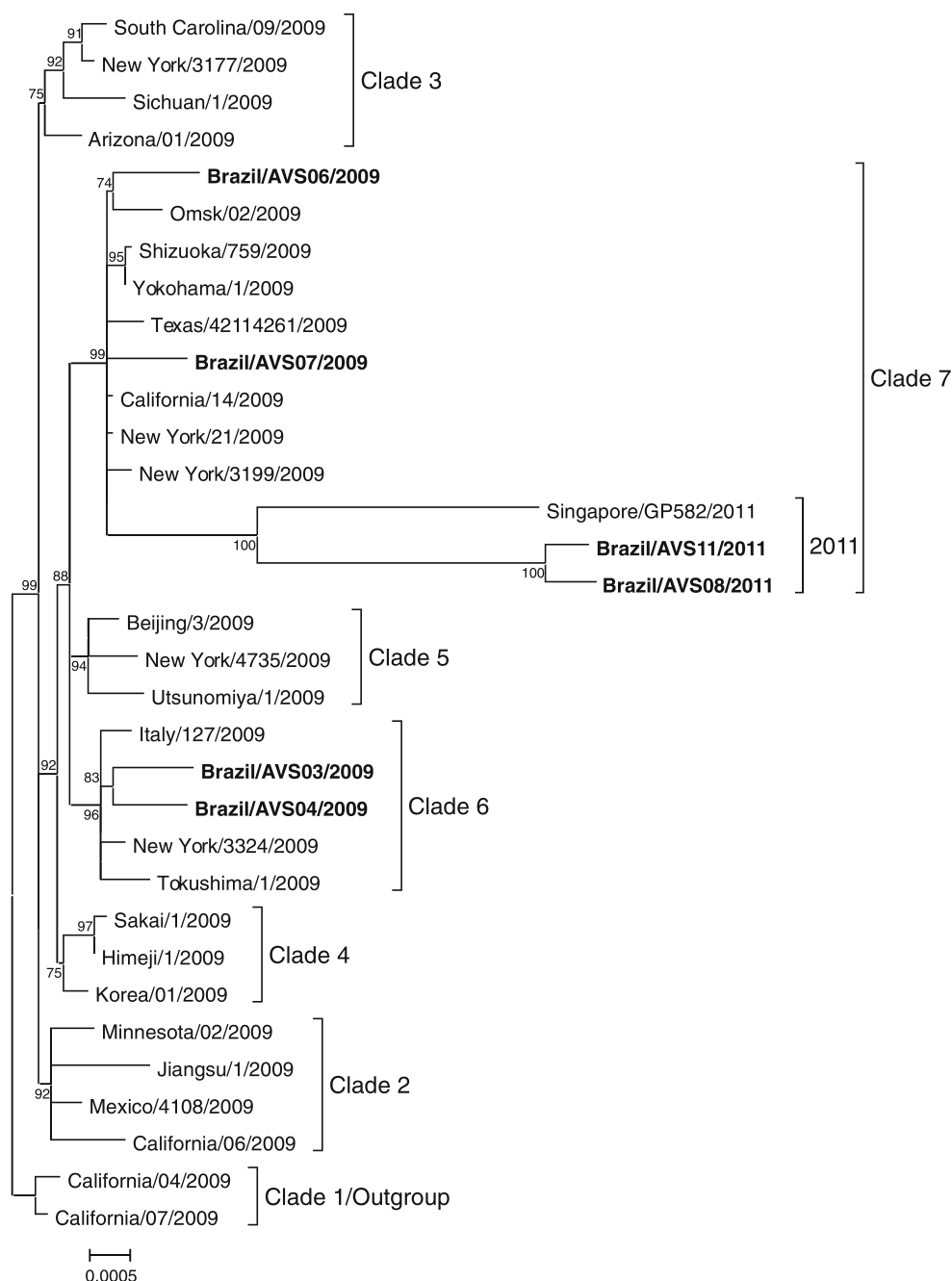


Fig. 1 Phylogenetic tree of concatenated genomic segments of RS influenza A pH1N1 viruses. The tree was constructed using the maximum-likelihood method. aLRT values greater than 50 % are

shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The RS H1N1 isolates are in bold

Vaccine efficacy prediction

The antigenic distances of five epitopes of HA from all isolates in relation to those from the vaccine strain California/07/2009 were computed in order to estimate the efficacy of the vaccine against these isolates using the p_{epitope} method [17], as shown in Table 7. The p_{epitope} of all pandemic strains was 0.029 (dominant epitope E),

culminating in a vaccine efficacy estimation of 39.735 %. The dominant epitope varied among the post-pandemic HA1 proteins, and the predicted vaccine efficacy values (median = 31.562 %) were lower than those from the pandemic strains (Mann-Whitney test P -value < 0.05) (Table 7). The relative vaccine efficacy medians of the pandemic and post-pandemic strains compared with a perfect-match virus ($p_{\text{epitope}} = 0$) were 84.543 % and 67.154 %, respectively.

Table 6 Purifying selection analysis of the genes from RS influenza A virus pH1N1 isolates

	HA		NA		M1		M2		NEP	
	dS - dN		dS - dN		dS - dN		dS - dN		dS - dN	
	1	2	1	2	1	2	1	2	1	2
1 California/04/2009										
2 California/07/2009	0.097		0.000		0.000		0.000		0.000	
3 Brazil/AVS03/2009	1.157	0.876	1.825	1.825	0.000	0.000	0.000	0.000	-1.008	-1.008
4 Brazil/AVS04/2009	0.702	0.336	1.061	1.061	1.042	1.042	-1.064	-1.064	-1.031	-1.031
5 Brazil/AVS06/2009	1.138	0.846	0.997	0.997	1.797	1.797	0.000	0.000	-1.038	-1.038
6 Brazil/AVS07/2009	0.871	0.537	1.297	1.297	1.194	1.194	0.000	0.000	0.000	0.000
7 Brazil/AVS08/2011	2.905	2.796	1.767	1.767	2.418	2.418	0.000	0.000	0.000	0.000
8 Brazil/AVS11/2011	2.642	2.534	1.667	1.667	2.418	2.418	0.000	0.000	0.000	0.000

	NP		NS1		PA		PB1		PB2	
	dS - dN		dS - dN		dS - dN		dS - dN		dS - dN	
	1	2	1	2	1	2	1	2	1	2
1 California/04/2009										
2 California/07/2009	-1.055		0.000		0.000		1.008		1.004	
3 Brazil/AVS03/2009	1.518	1.297	0.000	0.000	1.004	1.004	2.618	2.354	1.420	1.771
4 Brazil/AVS04/2009	1.241	0.992	1.071	1.071	1.823	1.823	2.963	2.679	2.294	2.496
5 Brazil/AVS06/2009	2.105	1.958	0.686	0.686	1.684	1.684	1.584	1.217	1.968	2.219
6 Brazil/AVS07/2009	1.592	1.391	-1.468	-1.468	1.964	1.964	1.220	0.704	1.897	2.151
7 Brazil/AVS08/2011	2.716	2.570	0.375	0.375	1.680	1.680	2.999	2.800	3.737	3.894
8 Brazil/AVS11/2011	2.716	2.570	0.375	0.375	2.362	2.362	3.428	3.237	3.737	3.894

dS and dN are the numbers of synonymous and nonsynonymous substitutions per site, respectively. Grey background indicates P-values less than 0.05. Genes with dS > dN and P-value < 0.05 were considered to be under purifying selection

Discussion

In this study, genomes of pH1N1 viruses from the pandemic (2009) and post-pandemic periods (2011) isolated in Rio Grande do Sul (RS), Southern Brazil, were investigated.

Whole-genome phylogenetic analysis of the pandemic isolates revealed that clade 6 and clade 7 pH1N1 strains co-circulated in RS in 2009. In a previous study using HA gene sequences, clade 6 strains were isolated in São Paulo (Southeast Brazil), and clade 7 strains in Mato Grosso and Distrito Federal (Center-West region, Brazil), and São Paulo [22]. However, these Brazilian strains were not the most similar to the RS isolates, considering all HA sequences deposited in the Influenza Research Database (<http://www.fludb.org/>) (data not shown). Therefore, it is likely that in 2009 multiple pH1N1 phylogenetic sublineages could have been introduced in Brazil. The assumption is also supported by the fact that pandemic RS isolates from clade 7 were not the most similar to each other.

On August 2010, the World Health Organization (WHO) declared that the pH1N1 pandemic had ceased and predicted that this virus type would circulate for the years to come, but causing minor problems [2]. In 2010, no cases of pH1N1 infections were detected in RS, Brazil, which was attributed to the intensive vaccination campaign adopted that year, when almost 45 % of the RS population was vaccinated [10]. However, in 2011, 103 cases (14 deaths) of pH1N1 were reported in RS [11].

Phylogenetic analysis of the post-pandemic 2011 RS isolates demonstrated that they belong to a monophyletic group that is nested in clade 7. During the 2009 pandemic, clade 7 strains were the most common in Latin America [22, 23] and in other countries such as the USA [24], India [25] and Canada [26]. The founder effect hypothesis would explain the global dominance of clade 7, since the initial foothold of this lineage occurred in New York State, which could have facilitated its rapid global spread via New York City's high international interconnectivity [24]. Further studies should be performed to evaluate if most of the currently circulating pH1N1 strains in Brazil were derived from the clade 7 lineage, since the WHO recommendation for influenza vaccine composition is to utilize the clade 1 strain California/07/2009, whose antigenic properties could be distinct from the former ones.

Evolution at high mutation rates is a genetic variation mechanism that ensures that RNA viruses survive [27].

As demonstrated previously, nonepitopic sites tend to accumulate fewer mutations than epitopic ones [28]. This phenomenon is also observed in internal viral proteins, such as matrix (M1), polymerase (PA, PB1, PB2), nucleoprotein (NP), and nonstructural proteins NS, since they are hidden from antibodies and are thus under less selective pressure to change. Furthermore, these proteins have a constrained structure, and mutations that impair their functionality are therefore promptly removed by purifying selection [29]. For instance, polymerase genes have the

Table 7 Predicted vaccine efficacy against RS influenza A virus pH1N1 isolates

	p-distance					Vaccine efficacy
	A	B	C	D	E	
Brazil/AVS03/2009	0	0	0	0.021	0.029	0.39735
Brazil/AVS04/2009	0	0	0	0.021	0.029	0.39735
Brazil/AVS06/2009	0	0	0	0.021	0.029	0.39735
Brazil/AVS07/2009	0	0	0	0.021	0.029	0.39735
Rio Grande do Sul/4509/2009	0	0	0	0.021	0.029	0.39735
Rio Grande do Sul/4772/2009	0	0	0	0.021	0.029	0.39735
Rio Grande do Sul/4782/2009	0	0	0	0.021	0.029	0.39735
Rio Grande do Sul/5395/2009	0	0	0	0.021	0.029	0.39735
Rio Grande do Sul/7019/2009	0	0	0	0.021	0.029	0.39735
Rio Grande do Sul/7108/2009	0	0	0	0.021	0.029	0.39735
Median						0.39735*
Brazil/AVS08/2011	0	0	0	0.063	0.029	0.31562
Brazil/AVS11/2011	0	0	0	0.063	0.029	0.31562
Rio Grande do Sul/277/2011	0	0	0	0.063	0.029	0.31562
Rio Grande do Sul/278/2011	0.042	0.045	0.031	0.042	0.059	0.3247
Rio Grande do Sul/279/2011	0	0.045	0	0.021	0.029	0.3577
Rio Grande do Sul/359/2011	0	0	0	0.063	0.029	0.31562
Rio Grande do Sul/360/2011	0	0	0	0.063	0.029	0.31562
Rio Grande do Sul/361/2011	0	0.045	0	0.021	0.029	0.35772
Median						0.31562*

p-distance - proportion of different amino acids for each HA1 epitope between the strain of interest and the vaccine strain California/07/2009

A, B, C, D and E - HA1 epitopes

Bold - p_{epitope} (largest p-distance)

Vaccine efficacy (E) is calculated by the equation $E = 0.47 - 2.47 \times p_{\text{epitope}}$

* Mann-Whitney test P -value = 2.285×10^{-5}

lowest dN/dS ratio among the virus genes, indicating that intense purifying selection acted on these genes [30].

Among the RS pH1N1 isolates, purifying selection was detected in most genes of the 2011 post-pandemic viruses. However, it was observed that some genes from 2009 pandemic isolates were already under purifying selection, even with the short time of divergence from the common ancestor shared with the archetype strains. Nevertheless, although purifying selection is an important driving force of pH1N1 virus evolution, it is worth noting that RS post-pandemic and pandemic isolates accumulated amino acid mutations relative to the vaccine strain. This observation is in agreement with the findings of Plotkin et al. [29], who suggested that influenza viruses are subject to an intragenomic conflict over the mutation rate: certain genes, and specific residues within those genes, experience frequency-dependent selection to change, while other genes experience purifying selection to remain fixed.

Studies have attempted to correlate the presence of the amino acid alterations in the major viral antigenic determinants HA and NA proteins with prognosis of illness,

pathogenicity, virulence, and resistance to antiviral drugs. In this respect, for instance, the substitution Q310H in the HA protein was detected in the isolates AVS03 and AVS04. Although a previous study pointed out the association of this mutation with fatal cases [8], this finding was recently challenged [9, 31].

Amino acid changes in NA associated with oseltamivir resistance were not found in the RS isolates. Assessments of oseltamivir resistance frequency among RS strains during the pandemics and the post-pandemic period (2010–2011) revealed that the H275Y mutation is extremely rare in RS [10, 32]. However, because there are limited options for antiviral treatment, the emergence of resistant strains is a public-health concern [33].

Some amino acid substitutions occurred in antigenic sites of the HA and NA, which could affect influenza vaccine efficacy. As expected, the post-pandemic strains had more amino acid changes in the antigenic sites of HA, culminating in a decrease in the predicted vaccine efficacy against the post-pandemic strains in relation to the pandemic ones. A study performed recently in Japan

demonstrated that, in post-pandemic strains containing multiple mutations in antigenic sites of HA (2 to 4 mutations), these mutations did not contribute to a change in antigenicity [34]. However, this probably depends on the site where the mutation occurred and also the type of amino acid substitution. For instance, viruses with the double mutation E391K and D142N have been associated with several breakthrough infections [35]. The D142N alteration was not observed in the 2011 isolates. Nevertheless, other changes in epitopic sites, such as R222K and I233V, which are both lacking in the archetype strains, must be evaluated.

It is worth noting that in 2011, there were 103 cases with 14 deaths due to influenza A virus pH1N1 in RS. In 2012, until October, 525 cases with 67 deaths were confirmed, an increase of 409.71 % and 378.57 %, respectively, in relation to 2011 [11]. Therefore, future studies are necessary to evaluate the efficacy of the current vaccine against the circulating pH1N1 strains in Brazil.

Conclusion

Genome sequences provide fundamental data for the understanding of influenza virus evolution. In this study, we investigated whole-genomic sequences of Brazilian pH1N1 viruses and demonstrated that distinct lineages co-circulated in RS, Brazil. Moreover, we also showed that the pH1N1 isolates contained amino acid substitutions that could alter their biological and antigenic properties. At present, it is evident that the pH1N1 viruses persisted and are constantly evolving. Therefore, monitoring the circulating strains is crucial to ensure proper local prevention and control measures against these pathogens.

Acknowledgments This work was supported by grants from CNPq, CAPES (Auxpe- PNPD- 3047/2010) and JCVI/NIH. This project has been funded in whole or in part by federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services under contract number HHSN272200900007C.

References

1. CDC (2009) Swine influenza A (H1N1) infection in two children—Southern California, March–April 2009. *Morb. Mortal Wkly Rep* 58
2. Who (2010) Pandemic (H1N1) 2009—update 112. http://www.who.int/csr/don/2010_08_06/en/index.html
3. Ding N, Wu N, Xu Q, Chen K, Zhang C (2009) Molecular evolution of novel swine-origin A/H1N1 influenza viruses among and before human. *Virus Genes* 39:293–300. doi:10.1007/s11262-009-0393-7
4. Smith GJD, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M et al (2009) Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature* 459:1122–1125. doi:10.1038/nature08182
5. Medina RA, García-Sastre A (2011) Influenza A viruses: new research developments. *Nat Rev Microbiol* 9:590–603. doi:10.1038/nrmicro2613
6. Nelson M, Spiro D, Wentworth D, Beck E, Fan J et al. (2009) The early diversification of influenza A/H1N1pdm. *PLoS Curr* 1. doi:10.1371/currents.RRN1126
7. Deyde VM, Sheu TG, Trujillo AA, Okomo-Adhiambo M, Garten R et al (2010) Detection of molecular markers of drug resistance in 2009 pandemic influenza A (H1N1) viruses by pyrosequencing. *Antimicrob Agents Chemother* 54:1102–1110. doi:10.1128/AAC.01417-09
8. Glinsky GV (2010) Genomic analysis of pandemic (H1N1) 2009 reveals association of increasing disease severity with emergence of novel hemagglutinin mutations. *Cell cycle* 9:958–970
9. Lee R, Santos C, de Paiva TM, Cui L, Sirota F et al (2010) All that glitters is not gold - founder effects complicate associations of flu mutations to disease severity. *Virology* 7:297. doi:10.1186/1743-422X-7-297
10. Cevs (2011) Boletim Epidemiológico. http://www.saude.rs.gov.br/upload/1343328296_Boletim%20Epidemiologico%20Influenza.13,%20n.3,set.,%202011.pdf
11. Cevs (2012) Boletim epidemiológico preliminar da Influenza A (H1N1)2009 no RS até 10/10/12 (semana epidemiológica 41). http://www.saude.rs.gov.br/upload/1349960214_Avaliacao_Influenza_A_H1N1_10_10_12.pdf
12. Veiga ABG, Kretzmann NA, Correa LT, Goshiyama AM, Baccin T et al (2012) Viral load and epidemiological profile of patients infected by pandemic influenza a (H1N1) 2009 and seasonal influenza a virus in Southern Brazil. *J Med Virol* 84:371–379. doi:10.1002/jmv.23198
13. Zhou B, Donnelly ME, Scholes DT, St George K, Hatta M et al (2009) Single-reaction genomic amplification accelerates sequencing and vaccine production for classical and Swine origin human influenza a viruses. *J Virol* 83:10309–10313. doi:10.1128/JVI.01109-09
14. Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32:1792–1797. doi:10.1093/nar/gkh340
15. Tamura K, Peterson D, Peterson N, Stecher G, Nei M et al (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739. doi:10.1093/molbev/msr121
16. Dereeper A, Guignon V, Blanc G, Audic S, Buffet S et al (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res* 36:W465–W469. doi:10.1093/nar/gkn180
17. Deem MW, Pan K (2009) The epitope regions of H1-subtype influenza A, with application to vaccine efficacy. *Protein Eng Des Sel* 22:543–546. doi:10.1093/protein/gzp027
18. Hammer Ø, Harper DAT, Ryan P (2001) PAST: paleontological statistics software package for education and data analysis. *Palaeontol Electron* 4
19. Kilander A, Rykkvin R, Dudman SG, Hungnes O (2010) Observed association between the HA1 mutation D222G in the 2009 pandemic influenza A(H1N1) virus and severe clinical outcome, Norway 2009–2010. *Euro Surveill* 15
20. Maurer-Stroh S, Lee RTC, Eisenhaber F, Cui L, Phuah SP et al. (2010) A new common mutation in the hemagglutinin of the 2009 (H1N1) influenza A virus. *PLoS Curr* 2. doi:10.1371/currents.RRN1162
21. Hurt AC, Lee RT, Leang SK, Cui L, Deng YM et al. (2011) Increased detection in Australia and Singapore of a novel influenza A(H1N1)2009 variant with reduced oseltamivir and

- zanamivir sensitivity due to a S247N neuraminidase mutation. *Euro Surveill* 16
22. Goñi N, Moratorio G, Ramas V, Coppola L, Chiparelli H et al (2011) Phylogenetic analysis of pandemic 2009 influenza A virus circulating in the South American region: genetic relationships and vaccine strain match. *Arch virol* 156:87–94. doi:[10.1007/s00705-010-0825-7](https://doi.org/10.1007/s00705-010-0825-7)
 23. Barrero PR, Viegas M, Valinotto LE, Mistchenko AS (2011) Genetic and phylogenetic analyses of influenza A H1N1pdm virus in Buenos Aires, Argentina. *J Virol* 85:1058–1066. doi:[10.1128/JVI.00936-10](https://doi.org/10.1128/JVI.00936-10)
 24. Nelson MI, Tan Y, Ghedin E, Wentworth DE, St. George K et al (2011) Phylogeography of the spring and fall waves of the H1N1/09 pandemic influenza virus in the United States. *J Virol* 85:828–834. doi:[10.1128/JVI.01762-10](https://doi.org/10.1128/JVI.01762-10)
 25. Potdar VA, Chadha MS, Jadhav SM, Mullick J, Cherian SS et al (2010) Genetic characterization of the influenza A pandemic (H1N1) 2009 virus isolates from India. *PLoS ONE* 5:e9693. doi:[10.1371/journal.pone.0009693](https://doi.org/10.1371/journal.pone.0009693)
 26. Graham M, Liang B, Van Domselaar G, Bastien N, Beaudoin C et al (2011) Nationwide molecular surveillance of pandemic H1N1 influenza A virus genomes: Canada, 2009. *PLoS ONE* 6:e16087. doi:[10.1371/journal.pone.0016087](https://doi.org/10.1371/journal.pone.0016087)
 27. Domingo E, Holland JJ (1997) RNA virus mutations and fitness for survival. *Annu Rev Microbiol* 51:151–178. doi:[10.1146/annurev.micro.51.1.151](https://doi.org/10.1146/annurev.micro.51.1.151)
 28. Li W, Shi W, Qiao H, Ho S, Luo A et al (2011) Positive selection on hemagglutinin and neuraminidase genes of H1N1 influenza viruses. *Virol J* 8:183. doi:[10.1186/1743-422X-8-183](https://doi.org/10.1186/1743-422X-8-183)
 29. Plotkin JB, Dushoff J (2003) Codon bias and frequency-dependent selection on the hemagglutinin epitopes of influenza A virus. *Proc Natl Acad Sci USA* 100:7152–7157. doi:[10.1073/pnas.1132114100](https://doi.org/10.1073/pnas.1132114100)
 30. Sinha NK, Roy A, Das B, Das S, Basak S (2009) Evolutionary complexities of swine flu H1N1 gene sequences of 2009. *Biochem Biophys Res Commun* 390:349–351. doi:[10.1016/j.bbrc.2009.09.060](https://doi.org/10.1016/j.bbrc.2009.09.060)
 31. Ferreira JL, Borborema SE, Brígido LF, Oliveira MI, Paiva TM et al (2011) Sequence analysis of the 2009 pandemic influenza A H1N1 virus haemagglutinin gene from 2009–2010 Brazilian clinical samples. *Mem Inst Oswaldo Cruz* 106:613–616
 32. Souza TM, Mesquita M, Resende P, Machado V, Gregianini TS et al (2011) Antiviral resistance surveillance for influenza A virus in Brazil: investigation on 2009 pandemic influenza A (H1N1) resistance to oseltamivir. *Diag Microbiol Infect Dis* 71:98–99. doi:[10.1016/j.diagmicrobio.2011.05.006](https://doi.org/10.1016/j.diagmicrobio.2011.05.006)
 33. Sheu TG, Fry AM, Garten RJ, Deyde VM, Shwe T et al (2011) Dual resistance to adamantanes and oseltamivir among seasonal influenza A(H1N1) viruses: 2008–2010. *J Infect Dis* 203:13–17. doi:[10.1093/infdis/jiq005](https://doi.org/10.1093/infdis/jiq005)
 34. Dapat IC, Dapat C, Baranovich T, Suzuki Y, Kondo H et al (2012) Genetic characterization of human influenza viruses in the pandemic (2009–2010) and post-pandemic (2010–2011) periods in Japan. *PLoS ONE* 7:e36455. doi:[10.1371/journal.pone.0036455](https://doi.org/10.1371/journal.pone.0036455)
 35. Strengell M, Ikonen N, Ziegler T, Julkunen I (2011) Minor changes in the hemagglutinin of influenza A (H1N1) 2009 virus alter its antigenic properties. *PLoS ONE* 6:e25848. doi:[10.1371/journal.pone.0025848](https://doi.org/10.1371/journal.pone.0025848)

5.2. Anexo II

“Polymerase activity of influenza virus (H1N1)pdm09 ribonucleoprotein reassortment with H7N9 human influenza virus”

Luiz Gustavo dos Anjos Borges; Ana Beatriz Gorini da Veiga; Randy Albrecht; Adolfo García-Sastre; Shashank Tripathi.

- Short Communication – À ser submetido ao periódico Virus Research

**Polymerase activity of influenza A virus (H1N1)pdm09 ribonucleoprotein
reassortant with influenza A virus H7N9**

Luiz Gustavo dos Anjos Borges¹; Ana Beatriz Gorini da Veiga¹; Randy
Albrecht²; Adolfo García-Sastre^{2,3}; Shashank Tripathi²

Affiliations:

¹Laboratório de Biologia Molecular, Programa de Pós-Graduação em Patologia,
Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto
Alegre, RS, Brazil.

²Department of Microbiology, Icahn School of Medicine at Mount Sinai Hospital,
New York, NY, USA.

³Department of Medicine, Division of Infectious Diseases, Icahn School of
Medicine at Mount Sinai Hospital, New York, NY, USA.

Corresponding author: Shashank Tripathi

Microbiology Department, Icahn School of Medicine at Mount Sinai Hospital, 1
Gustave L. Levy Place, New York, NY 10029. Phone: (212) 241-6096 e-mail:
shashank.tripathi@mssm.edu

Keywords: avian influenza virus; NP; PB1; PB2; PA, viral fitness

Abstract

Introduction - The present century has reaffirmed the significance of infections from reassortant influenza A viruses. Although it was easily controlled after one year, a triple reassortant of the influenza virus A, (H1N1) pdm09, quickly spread globally and infected thousands of humans. A less transmittable human virus of avian-origin (H7N9) has caused aggressive disease in infected patients. A new reassortment from both viruses could be a threat for a novel pandemic virus.

Objective - The aim of this study was to investigate the polymerase activity of virus reassorted between influenza A (H1N1)pdm09 and 2013 H7N9 human influenza in human cells.

Methods - Sixteen different combinations of pDZ-PA, -PB1, -PB2, -NP of A/Anhui/1/2013 (H7N9) and A/Cal/04/2009 (H1N1) were prepared and transfected at 37°C on HEK293T cells using Lipofectamine2000 and pRL-TK and pPoll-FF/Luc. A second transfection was performed using three different incubation conditions (33/37/39°C) for combinations of pDZ-PA and -NP and -NP/-PA of H7N9. The polymerase activity was measured using the luciferase assay system. MTT assay was done using a gradient of concentration for pDZ-PA and -NP.

Results - The reporter gene assay showed that H7N9 NP as well as PA increased the activity of H1N1 polymerase up to 150% and 100%, respectively, when compared to H1N1 wild type. The higher polymerase activity was also confirmed at all temperatures used and an increase was observed mainly at 33°C for -PA/-NP combination and at 37°C and 39°C for NP combination.

Discussion - Most combinations in the reassortment that include NP or PA genes contribute to the activity of H1N1 polymerase on 293T cells *in vitro*. On

the other hand, PB1 and PB2 of H7N9 contribute low for the polymerase activity of the reassortant vRNPs.

Introduction

The last pandemic human influenza A virus (IAV), (H1N1)pdm09, demonstrated an exponential spread in 2009 (de Picoli Junior et al., 2011). Moreover, even absent of the hallmark of highly pathogenic strains, the pandemic of IAV (H1N1)pdm09 produced a more severe disease compared to previous seasonal H1N1 (Tscherne and García-Sastre, 2011). Even though the pandemic has been controlled, A(H1N1)pdm09 is still circulating in the population and also in swine poultry, being a potential candidate for novel recombination events (Liang et al., 2014).

Since isolated for the first time in 2013 from an elderly Chinese person, infection by H7N9 human IAV has been related to exposure to carrier poultry of the virus. Although the H7N9 subtype causes asymptomatic infection in chicken, the human strain of avian-origin virus has caused an aggressive disease in infected humans (Kageyama et al., 2013). Specific mutations on subunits of vRNP have been recently reported as responsible by the gain on polymerase activity of H7N9 (Zhu et al., 2015).

Despite being aggressive in human infection, H7N9 has not the fitness to spread in humans, so human-to-human infection is very rare. Nevertheless, some studies have reported that human H7N9 is capable of infecting pigs (Jones et al., 2013; Zhu et al., 2013). Considering the hypothesis that swine can serve as a mixing vessel (Tscherne and García-Sastre, 2011) of avian and human strains viruses, reassortment between an aggressive strain like human

H7N9 and a pandemic strain with fitness to spread like A(H1N1)pdm09 is unfortunately feasible.

The aim of this study was to investigate the polymerase activity of the reassortment between influenza virus A(H1N1)pdm09 and 2013 H7N9 human influenza viruses in human cells.

Material and methods

Minigenome assay

The human embryonic kidney-derived 293 cells, HEK-293T, were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were cultured at 37°C in 5% CO₂. We used an ambisense cloning vector pDZ for -NP, -PA, -PB1, and -PB2 from A/Anhui/1/2013 (H7N9), and A/Cal/04/2009 (H1N1) (Quinlivan et al., 2005). Also, we used a firefly luciferase vector (pPoll-FF/Luc) and a Renilla Luciferase control report vector with HSV-thymidine kinase promoter (pRL-TK) to be cotransfect the HEK-293T cells.

Fourteen different combinations of vRNP using the four plasmids containing one of each subunit (pDZ-PA, -PB1, -PB2, -NP) of A/Anhui/1/2013 (H7N9) and A/Cal/04/2009 (H1N1) strains were prepared. One combination for both vRNP wild type and one combination with absence of subunits (cell control) were included in the tests. We prepared the DNA solution with 40 ng of pPoll-FF/Luc, 10 ng of pRL-TK, 200 ng pDZ-NP, 50 ng of each pDZ-PA, pDZ-PB1, pDZ-PB2 in a total volume of 100 µL of OptiMEM (Gibco) for each combination. DNA solution was added to 100 µL of OptiMEM (Gibco) and 4 µL Lipofectamine 2000 mixture. The transfection was performed in four replicates on HEK293T cells seeded on 24 well tissue culture plates for 3 h at 37°C. After transfection

incubation OptiMEM was changed for DMEM with 10% FBS 1% penicillin/streptomycin (Gibco) and the cells were incubated for 24 h at 37°C, 5% CO₂. After incubation, polymerase activity was measured on 96 well plate using Dual-Luciferase Report 1000 Assay System (Promega) following instructions. We used Synergy-Hybrid Reader Biotek to read out plates.

A second transfection was performed using three different incubation conditions (33/37/39°C) for subunits presenting high active in all combinations. Following results of first transfection, we used pDZ-PA and -NP of A/Anhui/1/2013 on combinations. Moreover, a third transfection procedure was performed to check dose-response for NP and PA subunits. DNA solution with a range between 50-200 ng for pDZ-NP and pDZ-PA were prepared for the transfection. The toxicity for 293T cells on transfection assay protocol was measured using MTT assay. An alignment of NP and PA amino acids sequences was constructed using ClustalW (Thompson et al., 1994) in order to measure the similarity level of both sequences and the presence of hallmark for activities in mammalian cells or temperature sensitivity.

Statistical analysis

Statistical analysis was performed using SPSS version 20.0. The t-student test was applied for identified difference of vRNP activity. Values were considered statistically significant when $p < 0.05$.

Results and Discussion

The results show the vRNP activity of A/Anhui/1/2013 (H7N9), A/Cal/04/2009 (H1N1), and fourteen combinations expected from a reassortment of both vRNP

viral strains (Figure 1). The H7N9 strain (combination 16) had a higher polymerase activity than H1N1 strain (combination 1) ($p = 0.002$). There were eleven of fourteen combinations expressing significant higher polymerase activity than H1N1 wild type.

Analyzing the vRNP activity of combinations 2 to 5 only the combination 4 did not improve polymerase activity. In which PB1 of H1N1 strain was replaced by PB1 of H7N9 strain. The highest polymerase activities of H1N1 vRNP was observed when NP from H7N9 was present followed by recombination with PA and PB2 from H7N9 in that order.

The polymerase activities were significant different in all combinations with double substitutions (combinations 6 to 11). Interestingly, the polymerase activity had a significant decrease when PB1 and PB2 of H7N9 strain changed the respective vRNP subunits (combination 7) in comparison with wild type H1N1 polymerase.

Comparing with the polymerase activity of the H7N9 strain, six combinations had significant higher vRNP activities than H7N9 wild type (combinations 2, 5, 6, 9, 10, 13). The NP of H1N1 was shown to be able to improve the activity of vRNP H7N9. The high activity with NP from H1N1 has not observed when PA of H1N1 was present (in double or triple combination). These results show that PA of H1N1 is less effective than PA of H7N9 in mammalian cells.

Results suggest that the polymerase activity is not efficiently improved for combinations carrying at the same time the PA subunit from H1N1 and the PB1 subunit from H7N9 (combinations 4, 7, and 12). Perez and Donis (2001) have reported that PA might be responsible in mediating PB2-PB1-PA interaction and in to stimulating PB1 activity. Moreover, the PB2-PB1-PA interaction is essential

for virus viability in cell culture. Probably PA of H1N1 was not efficient to stimulate PB1 of H7N9 strain and consequently is not able to increase the polymerase activity of vRNP. However, the probable low affinity in the PA-PB1 interaction might not be the unique reason for these findings. The NP has been reported to be a major determinant in the regulation of the switch between transcription and replication activity that is performed by the PB1 subunit (Portela and Digard, 2002). This fact might explain the differences in vRNP activity between combination 4 (NP, PB2 and PA from H1N1, and PB1 from H7N9) and combination 8 (PB2 and PA from H1N1 NP, and PB1 from H7N9), the latter displaying significantly higher activity than the former in relation to wild-type vRNP of H1N1. Still, combination 8 had a lower activity compared to combinations such as 10 and 13, when both PB1 and PA from H7N9 were present.

Considering the roles of PA and NP in vRNP activity, we next analyzed the dose response (Figure 2) and the cytotoxicity (Figure 3) of these subunits. For dose response, the best concentrations were 50 ng and 200 ng for PA and NP, respectively. The MTT assay did not show evidence of cytotoxicity for PA and NP.

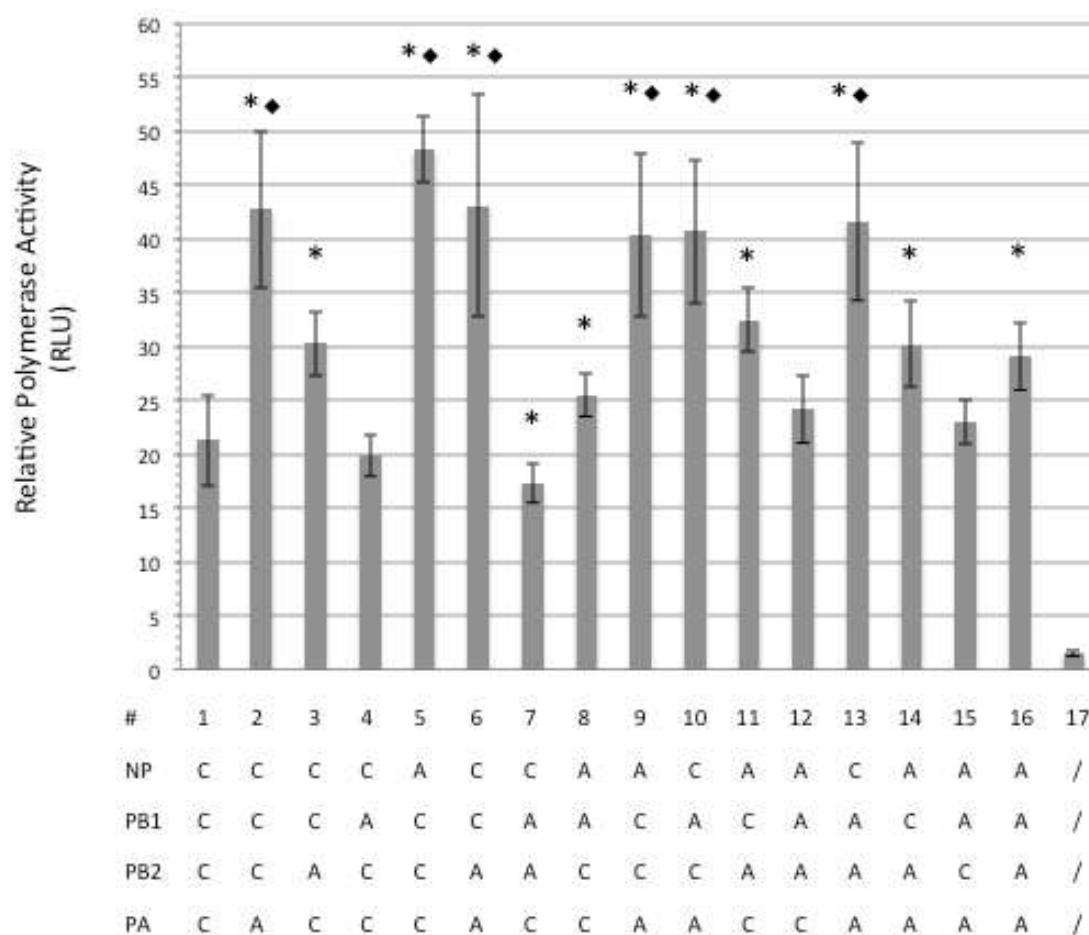


Figure 1 – Polymerase activity of 17 vRNP subunits combinations between A/California/04/2009 (C) and A/Anhui/01/2013 (A) strains. Results are shown as averages with standard deviation of four replicates. Symbol “/” indicates matches to non-subunit. * t-student Test – $p < 0.05$ comparing to A/California/04/2009; ♦ t-student Test – $p < 0.05$ comparing to A/Anhui/01/2013.

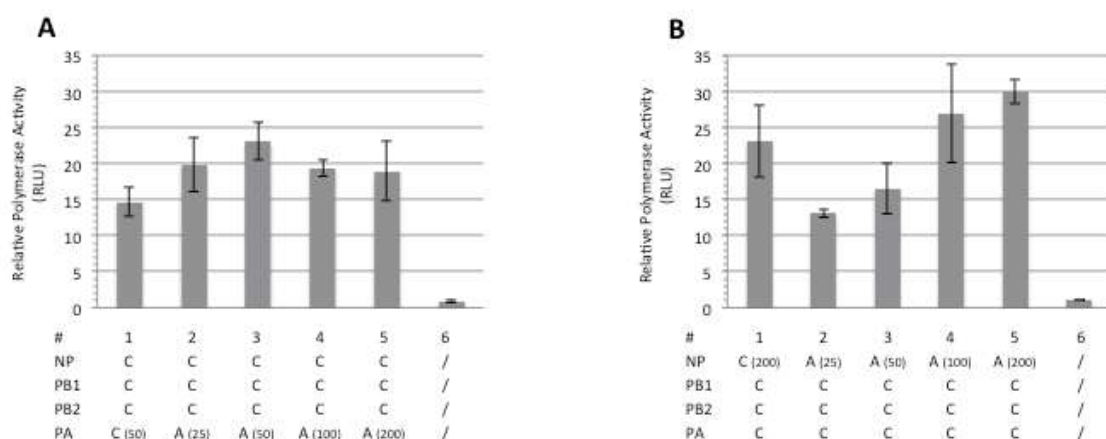


Figure 2 – Dose response of polymerase activity for H7N9 PA and NP subunits in PA dose response test (A) and NP dose response test (B). Subunit from A/California/04/2009 is represented by C; subunit from A/Anhui/01/2013 strain is represented by A. Absence of subunit is represented by symbol “/”. Numbers in parentheses indicate the concentration of subunit in nanogram. Results are shown as averages of relative luciferase unit. Standard deviation of four replicates represented by black bars.

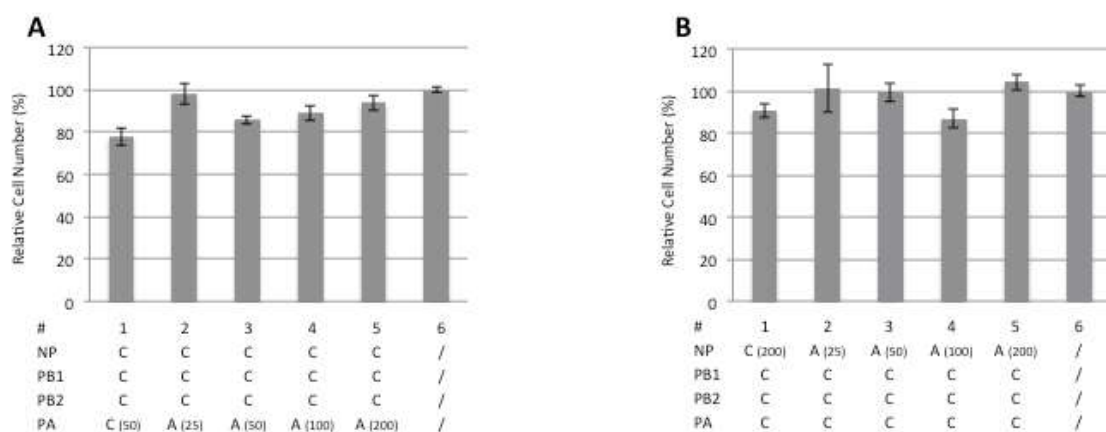


Figure 3 – Cytotoxicity test of PA and NP subunits in PA MTT test (A) and NP MTT test (B). Subunit from A/California/04/2009 strain is represented by C;

subunit from A/Anhui/01/2013 strain is represented by A. Absence of subunit is represented by symbol “/”. Numbers in the parenthesis indicate the concentration of subunit in nanogram. Results are shown as relative average of viable cells in comparison to results obtained for combination number 6 (absence of subunits transfected).

The vRNP reassortment for combinations of H1N1 subunits using NP and PA subunits from H7N9 were performed under 33°C, 37°C, and 39°C (Figure 4). The activity was higher for all combinations in comparison with the wild type H1N1 at different temperatures. Bussey et al. (2011) reported that PA of A/California/04/2009 has three specific residues, 85, 186, and 336 that might contribute for enhancing avian polymerase activity in mammalian cells. Observing the H1N1 and H7N9 alignment of PA subunit we found difference in amino acids in the residues mentioned above between the two strains (data not shown). Despite this PA of H7N9 confirmed to be the fittest in all tested temperatures when compared to wild type H1N1 that presents those specific residues.

The vRNP with both NP and PA of H7N9 showed to be more active at 33°C than other combinations. Even though the present study had showed that PA of H7N9 might improve the polymerase activity of H1N1 polymerase, results also show that, at higher temperatures, NP bursts the vRNP. A study by Jin et al. (2003) reported that residue 34D on NP is a major factor for temperature sensitivity and might result in a reduction of virus titer at 39°C. The strain of human H7N9 as well as of H1N1 used in the present study do not have the

amino acid reported on residue 34 of NP, therefore they were able to be active also at high temperatures like 39°C.

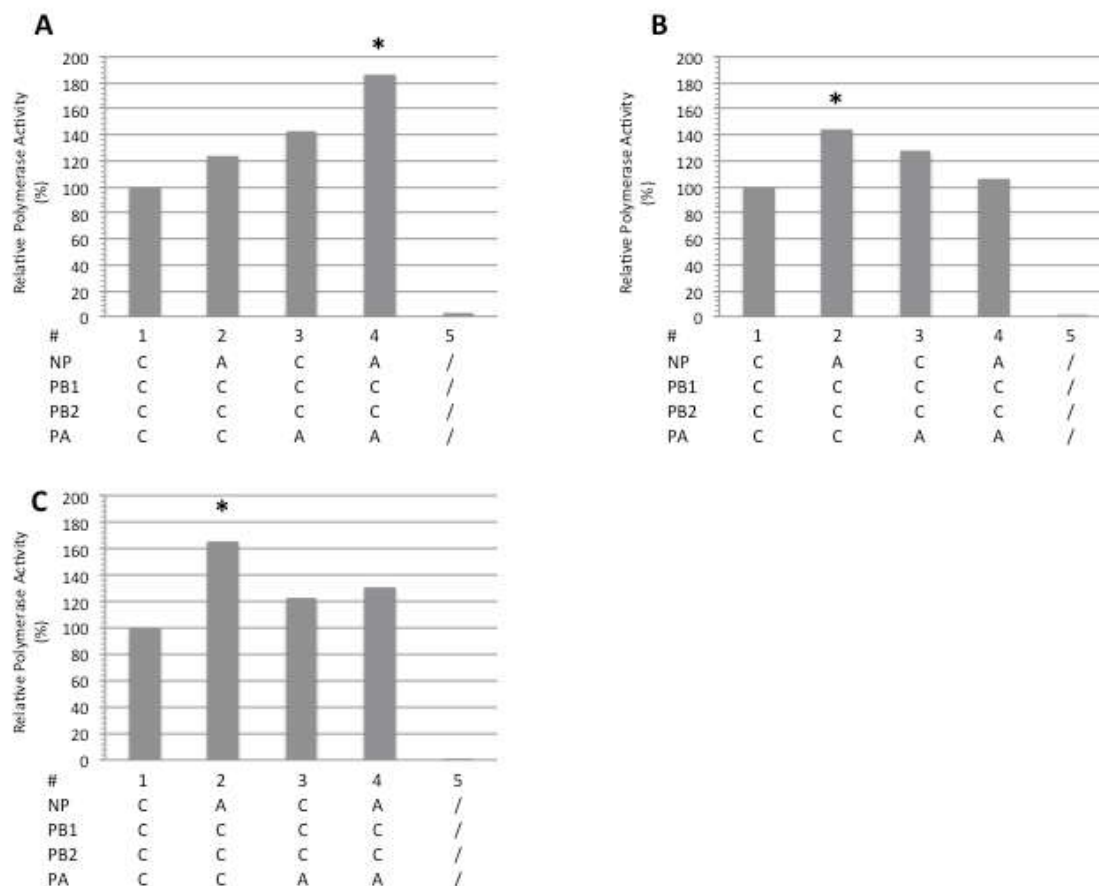


Figure 4 – Polymerase activity of PA and NP subunits combinations at different temperatures, 33°C (A), 37°C (B) and 39°C (C). Subunits from A/California/04/2009 strain are represented by C and subunits from A/Anhui/01/2013 strain are represented by A. Absence of subunits are represented by symbol “/”. Results are shown as the percentage of polymerase activity average in comparison to polymerase activity of H1N1 wild type, * t-student Test – $p < 0.05$ comparing to A/California/04/2009.

Conclusion

The reporter gene assay used in this study showed that H7N9 NP as well as PA increase the activity of H1N1 polymerase when compared to H1N1 wild type up to 150% and 100%, respectively. Also, while the polymerase activity is not efficiently improved for combinations carrying at the same time PA subunit from H1N1 and the PB1 subunit from H7N9, but it can be improved when the NP subunit of H7N9 is present. The present study showed that a novel recombinant of H7N9 and H1N1 influenza A virus might be aggressive and powerful if infect humans. More studies need to be done to have insight about control and prevention against the a future H7N9 recombinant virus.

Acknowledgments

This research was supported by grants and fellowships of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Ministry of Education, Brazil) and FAPERGS (PqG Grant 2014).

References

- Bussey, K. A, Desmet, E. A, Mattiaccio, J.L., Hamilton, A., Bradel-Tretheway, B., Bussey, H.E., Kim, B., Dewhurst, S., Takimoto, T., 2011. PA residues in the 2009 H1N1 pandemic influenza virus enhance avian influenza virus polymerase activity in mammalian cells. *J. Virol.* 85, 7020–8. doi:10.1128/JVI.00522-11
- de Picoli Junior, S., Teixeira, J.J.V., Ribeiro, H.V., Malacarne, L.C., dos Santos, R.P.B., dos Santos Mendes, R., 2011. Spreading patterns of the influenza

- A (H1N1) pandemic. PLoS One 6, e17823. doi:10.1371/journal.pone.0017823
- Jin, H., Lu, B., Zhou, H., Ma, C., Zhao, J., Yang, C.F., Kemble, G., Greenberg, H., 2003. Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains (FluMist) derived from cold-adapted A/Ann Arbor/6/60. Virology 306, 18–24. doi:10.1016/S0042-6822(02)00035-1
- Jones, J.C., Baranovich, T., Zaraket, H., Guan, Y., Shu, Y., Webby, R.J., Webster, R.G., 2013. Human H7N9 influenza A viruses replicate in swine respiratory tissue explants. J. Virol. 87, 12496–8. doi:10.1128/JVI.02499-13
- Kageyama, T., Fujisaki, S., Takashita, E., Xu, H., Yamada, S., Uchida, Y., Neumann, G., Saito, T., Kawaoka, Y., Tashiro, M., 2013. Genetic analysis of novel avian A(H7N9) influenza viruses isolated from patients in China, February to April 2013. Euro Surveill. 18, 20453. doi:10.1093/cid/cit294
- Liang, H., Lam, T.T.-Y., Fan, X., Chen, X., Zeng, Y., Zhou, J., Duan, L., Tse, M., Chan, C.-H., Li, L., Leung, T.-Y., Yip, C.-H., Cheung, C.-L., Zhou, B., Smith, D.K., Poon, L.L.-M., Peiris, M., Guan, Y., Zhu, H., 2014. Expansion of Genotypic Diversity and Establishment of 2009 H1N1 Pandemic-Origin Internal Genes in Pigs in China. J. Virol. 88, 10864–10874. doi:10.1128/JVI.01327-14
- Perez, D.R., Donis, R.O., 2001. Functional analysis of PA binding by influenza A virus PB1: effects on polymerase activity and viral infectivity. J. Virol. 75, 8127–36. doi:10.1128/JVI.75.17.8127-8136.2001
- Portela, A., Digard, P., 2002. The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. J. Gen.

- Viol. 83, 723–734. doi:10.1099/0022-1317-83-4-723
- Quinlivan, M., Zamarin, D., Garcia-Sastre, A., Cullinane, A., Chambers, T., Palese, P., 2005. Attenuation of equine influenza viruses through truncations of the NS1 protein. *J. Virol.* 79, 8431–8439. doi:10.1128/JVI.79.13.8431-8439.2005
- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680. doi:10.1093/nar/22.22.4673
- Tscherne, D.M., García-Sastre, A., 2011. Virulence determinants of pandemic influenza viruses. *J. Clin. Invest.* 121, 6–13. doi:10.1172/JCI44947.genicity
- Zhu, H., Wang, D., Kelvin, D.J., Li, L., Zheng, Z., Yoon, S.-W., Wong, S.-S., Farooqui, A., Wang, J., Banner, D., Chen, R., Zheng, R., Zhou, J., Zhang, Y., Hong, W., Dong, W., Cai, Q., Roehrl, M.H.A., Huang, S.S.H., Kelvin, A.A., Yao, T., Zhou, B., Chen, X., Leung, G.M., Poon, L.L.M., Webster, R.G., Webby, R.J., Peiris, J.S.M., Guan, Y., Shu, Y., 2013. Infectivity, transmission, and pathology of human-isolated H7N9 influenza virus in Ferrets and Pigs. *Science.* 341, 183–186. doi:10.1126/science.1239844
- Zhu, W., Zou, X., Zhou, J., Tang, J., Shu, Y., 2015. Residues 41V and/or 210D in the NP protein enhance polymerase activities and potential replication of novel influenza (H7N9) viruses at low temperature. *Virol. J.* 12, 71. doi:10.1186/s12985-015-0304-6

5.3. Anexo III

Parecer do Comitê de Ética em Pesquisa

Parecer Consubstanciado de Projeto de Pesquisa

Título do Projeto: Co-infecção do vírus influenza e bactérias produtoras de proteases envolvidas no processo de infecção viral e nos mecanismos de defesa do hospedeiro.

Pesquisador Responsável : Ana Beatriz Gorini da Veiga

Parecer 1774/12

Data da Versão

Cadastro 928/12

Data do Parecer 11/06/2012

Grupo e Área Temática

Classificação utilizada pela CONEP

Objetivos do Projeto

Geral:: Identificar o aumento do poder de infectividade dos vírus Influenza prevalentes no RGS baseado na ocorrência de co-infecção e estrutura do gene que codifica a glicoproteína HA.

Sumário do Projeto

Itens Metodológicos e Éticos	Situação
Título	Adequado
Autores	Adequados
Local de Origem na Instituição	Adequado
Projeto elaborado por patrocinador	Não
Aprovação no país de origem	Não necessita
Local de Realização	Outro (citar no comentário)
Outras instituições envolvidas	Sim
Condições para realização	Adequadas

Comentários sobre os itens de Identificação

Envolvidos na pesquisa: Ufcs e Lacen

Introdução	Adequada
-------------------	----------

Comentários sobre a Introdução

Objetivos	Adequados
------------------	-----------

Comentários sobre os Objetivos

Pacientes e Métodos	
Delineamento	Adequado
Tamanho de amostra	Total Local
Cálculo do tamanho da amostra	Adequado
Participantes pertencentes a grupos especiais	Não
Seleção equitativa dos indivíduos participantes	Não se aplica
Critérios de inclusão e exclusão	Adequados
Relação risco- benefício	Adequada
Uso de placebo	Não utiliza
Período de suspensão de uso de drogas (wash out)	Não utiliza
Monitoramento da segurança e dados	Adequado
Avaliação dos dados	Adequada - quantitativa
Privacidade e confidencialidade	Adequada
Termo de Consentimento	Adequado
Adequação às Normas e Diretrizes	Sim

Comentários sobre os itens de Pacientes e Métodos

Cronograma	Ausente
Data de início prevista	
Data de término prevista	
Orçamento	Adequado
Fonte de financiamento externa	Não Informado

Comentários sobre o Cronograma e o Orçamento

Referências Bibliográficas	Adequadas
-----------------------------------	-----------

Comentários sobre as Referências Bibliográficas

Recomendação

Aprovar

Comentários Gerais sobre o Projeto

Após reanálise do projeto decide-se pela aprovação.

Os demais itens da pesquisa atendem as exigências, em seus aspectos éticos e metodológicos, das Diretrizes e Normas, especialmente as Resoluções 196/96, 251/97, 292/99, 346/05, 347/05 e complementares do Conselho Nacional de Saúde.

5.4 Anexo IV

Ficha de Investigação

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 $\geq 38^{\circ}\text{C}$ E tosse OU dor de garganta OU dispnéia.

Dados Gerais	1	Tipo de Notificação		2 - Individual																	
	2	Agravado/doença		Código (CID)	3	Data da Notificação															
	INFLUENZA HUMANA POR NOVO SUBTIPO (PANDÊMICO)		J11																		
	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-E ensino fundamental completo (antigo ginásio ou 1º grau) 5-E ensino médio incompleto (antigo colegial ou 2º grau) 6-E 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 05 - Posto de Saúde/Hospital 09 - Ignorado 02 - Vizinhança 06 - Outro Estado/Município 10 - Meio de Transporte 03 - Trabalho 07 - Sem História de Contato 11 - Outro 04 - Creche/Escola 08 - Outro País														
38		Informações sobre Deslocamento (datas e locais freqüentados no período de até 10 dias antes do início dos sinais e sintomas)																			
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																		
44		Comorbidade				Cardiopatia crônica				Renal Crônico				Imunodeprimido				Doença Metabólica Crônica			
		1 - Sim 2 - Não 9 - Ignorado						Pneumopatia crônica				Hemoglobinopatia				Tabagismo				Outros	

Atendimento	45 Ocorreu Hospitalização ☐ 1 - Sim 2 - Não 9 - Ignorado			46 Data da Internação			47 UF	
	48 Município do Hospital			Código (IBGE)		49 Nome do Hospital		
Dados Laboratoriais	PCR			51 Tipo de Amostra			52 Resultado	
	50 Data da Coleta			1 - Secreção de Nasofaringe 4 - Tecido pós-morte 9 - Ignorado 2 - Lavado Bronco-alveolar 5 - Soro 3 - Fezes 6 - Outro			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			56 Tipo de Amostra			57 Resultado	
	55 Data da Coleta			1 - Secreção de Nasofaringe 4 - Tecido pós-morte 9 - Ignorado 2 - Lavado Bronco-alveolar 5 - Soro 3 - Fezes 6 - Outro			1 - Positivo 3 - Não realizado 2 - Negativo	
	INIBIÇÃO DA HEMAGLUTINAÇÃO			59 Resultado			☐	
	58 Data da Coleta			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			63 Se sim, resultado			☐	
	62 Data da Realização			1 - Normal 2 - Infiltrado Intersticial 3 - Consolidação 4 - Misto 5 - Outros				
Conclusão	64 Classificação Final			65 Critério de Confirmação			☐	
	1 - Influenza por Novo Subtipo Viral 3 - Descartado 2 - Outro agente infeccioso			1 - Laboratorial 2 - Clínico-Epidemiológico				
	Local Provável de Fonte de Infecção			66 O caso é autóctone do município de residência?			67 UF 68 País	
				1-Sim 2-Não 3-Indeterminado				
	69 Município			Código (IBGE)		70 Distrito		71 Bairro
	72 Doença Relacionada ao Trabalho			73 Evolução do Caso			☐	
	1 - Sim 2 - Não 9 - Ignorado			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	