

Universidade de Brasília
Instituto de Ciências Biológicas
Departamento de Biologia Celular
Programa de Pós-Graduação em Biologia
Microbiana

Rinovírus no Distrito Federal: caracterização epidemiológica e molecular por sequenciamento de alta performance

Larissa da Costa Souza

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Orientador: Prof. Dr. Tatsuya Nagata

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Resumo

Introdução: Infecções do trato respiratório estão associadas a um elevado índice de morbidade e mortalidade em todo o mundo, sendo os vírus os principais agentes etiológicos envolvidos. A implantação do Sistema Sentinela de Vigilância da Influenza e outros vírus teve início no ano 2000 no Brasil e é constituída por laboratórios centrais de saúde pública os quais são responsáveis pelo monitoramento pela identificação desses agentes virais. A metodologia utilizada para diagnóstico de vírus respiratórios é o RT-qPCR, o qual identifica apenas alvos específicos, resultando em diagnóstico inconclusivo para muitas amostras. Assim, o sequenciamento de alta performance (high-throughput sequencing, HTS) seria um método complementar na identificação de patógenos em amostras inconclusivas para RT-qPCR ou outros protocolos de detecção específicos. Vírus como os rinovírus humano (HRV) tinham sua incidência e relevância subestimadas pela ausência de alvos específicos. Estes vírus são patógenos virais humanos comuns relacionados a infecções do trato respiratório superior e inferior, que podem resultar em bronquiolite e pneumonia. Caracterizar aspectos epidemiológicos e moleculares dos rinovírus por HTS pode ser útil para compreender as formas de circulação e como ocorrem as interações entre eles e a população de Brasília, Distrito Federal, fortalecendo a rede de vigilância de vírus respiratórios (RVVR).

Objetivos: Este estudo teve como objetivo detectar vírus não identificados por RT-qPCR utilizando a abordagem HTS em amostras de nasofaringe / secreção traqueal coletadas no Distrito Federal, Brasil. A identificação dos rinovírus como principal agente etiológico por HTS nessas amostras permitiu analisar as características clínicas e os desfechos de pacientes HRV-positivos por RT-qPCR com primers desenvolvidos neste estudo, verificar o aprimoramento da definição de agentes etiológicos pela rede de vigilância, além de avaliar a ocorrência de rinovírus em determinados períodos.

Metodologia: Os ácidos nucleicos foram extraídos de amostras coletadas no período de inverno de 2016 e submetidas ao HTS. Os resultados foram confirmados pelo multiplex PR21 RT-qPCR, que identifica 21 patógenos respiratórios. Novos conjuntos de primers específicos foram desenhados e utilizados para detecção de rinovírus por RT-qPCR e sequenciamento Sanger de cDNA amplificado da região genômica 5', com posterior análise de filogenia de isolados representativos de HRV. RT-qPCR foi usado para monitorar a presença de vírus respiratórios, incluindo rinovírus no painel de vírus

respiratórios, em amostras de pacientes com Síndrome Gripal (SG) ou Síndrome Respiratória Aguda Grave (SARS). Perfis de amostra foram obtidos a fim de correlacionar com os resultados da detecção dos vírus avaliados. A análise estatística considerando o perfil dos pacientes e a correlação da incidência de rinovírus com os dados meteorológicos foram realizadas por meio do programa IBM SPSS com testes não paramétricos.

Resultados: Os principais vírus identificados pelo HTS foram das famílias *Herpesviridae*, *Coronaviridae*, *Parvoviridae* e *Picornaviridae*, com destaque para os rinovírus. A presença de vírus respiratórios nas amostras foi confirmada pelo multiplex RT-qPCR PR21. Coronavírus, enterovírus, bocavírus e rinovírus foram encontrados por multiplex RT-qPCR, bem como por análises HTS. O vírus mais prevalente, em amostras previamente negativas para vigilância da Influenza e outros vírus, foi o rinovírus (n = 40), incluindo as três espécies de rinovírus (rinovírus A, B e C). A razão de chance associada à infecção por HRV foi de 2,160 para pacientes com menos de 2 anos e de 4,367 para pessoas que vivem em áreas rurais. O principal sintoma associado à infecção pelo vírus foi a rinorreia. A análise múltipla mostrou associação também para menos casos de desconforto respiratório em pacientes HRV-positivos. A adição de primers específicos para rinovírus no painel de vírus respiratório aumentou significativamente a identificação de um agente etiológico viral. A prevalência de rinovírus (em relação aos demais vírus) apresentou correlação negativa significativa com as temperaturas mínimas, ou seja, o aumento da detecção de rinovírus é proporcional à diminuição das temperaturas mínimas registradas em Brasília, Brasil.

Conclusão: Grande diversidade de vírus foi encontrada por diferentes metodologias e alta frequência de ocorrência de rinovírus foi confirmada na população no inverno, mostrando sua relevância para a saúde pública. A presença de rinovírus em doenças respiratórias foi significativamente associada à idade menor que dois anos e à rinorreia. A incidência de rinovírus foi correlacionada com a queda da temperatura mínima, mas sem um padrão sazonal evidente para a população de Brasília no período estudado.

Palavras-chave: Vírus respiratório - saúde pública - sequenciamento de alta performance - RT-qPCR - rinovírus - epidemiologia – sazonalidade.

Abstract

Introduction: Respiratory tract infections are associated with a high rate of morbidity and mortality worldwide, with viruses being the main etiologic agents involved. The implantation of the Sentinel Surveillance System for Influenza and other viruses began in 2000 in Brazil and consists of central public health laboratories which are responsible for monitoring the identification of these viral agents. The methodology used for the diagnosis of respiratory viruses is the RT-qPCR, which identifies only specific targets, resulting in an inconclusive diagnosis for many samples. Thus, high-throughput sequencing (HTS) would be a complementary method for identifying pathogens in inconclusive samples for RT-qPCR or other specific detection protocols. Viruses such as human rhinoviruses (HRV) had their incidence and relevance underestimated due to the absence of specific targets. These viruses are common human viral pathogens related to infections of the upper and lower respiratory tract, which can result in bronchiolitis and pneumonia. Characterizing epidemiological and molecular aspects of rhinoviruses by HTS can be useful to understand the circulation forms and how interactions occur between them and the population of Brasília, Distrito Federal, strengthening the respiratory virus surveillance network (RVSN).

Objectives: This study aimed to detect viruses not identified by RT-qPCR using the HTS approach in nasopharyngeal / tracheal secretion samples collected in the Federal District, Brazil. The identification of rhinoviruses as the main etiological agent by HTS in these samples allowed to analyze the clinical characteristics and outcomes of HRV-positive patients by RT-qPCR using primers developed in this study, verify the improvement of the definition of etiological agents by the surveillance network, in addition to assessing the occurrence of rhinovirus in certain periods.

Methodology: Nucleic acids were extracted from samples collected in the winter period of 2016 and submitted to HTS. The results were confirmed by the PR21 RT-qPCR multiplex, which identifies 21 respiratory pathogens. New sets of specific primers were designed and used for the detection of rhinovirus by RT-qPCR and Sanger sequencing of amplified cDNA of the 5' genomic region, with subsequent phylogeny analysis of representative HRV isolates. RT-qPCR was used to monitor the presence of respiratory viruses, including rhinovirus in the panel of respiratory viruses, in samples from patients with Influenza-like Syndrome (ILS) or Severe Acute Respiratory Syndrome (SARS).

Sample profiles were obtained in order to correlate with the results of the detection of the evaluated viruses. Statistical analysis considering the profile of patients and the correlation of the incidence of rhinovirus with meteorological data were performed using the IBM SPSS program with non-parametric tests.

Results: The main viruses identified by the HTS were from the families *Herpesviridae*, *Coronaviridae*, *Parvoviridae* and *Picornaviridae*, with emphasis on rhinoviruses. The presence of respiratory viruses in the samples was confirmed by the RT-qPCR PR21 multiplex. Coronaviruses, enteroviruses, bocaviruses and rhinoviruses were found by multiplex RT-qPCR, as well as by HTS analyzes. The most prevalent virus, in samples previously negative for surveillance of Influenza and other viruses, was rhinovirus (n = 40), including three species of rhinovirus (rhinovirus A, B and C). The odds ratio associated with HRV infection was 2,160 for patients under 2 years of age and 4,367 for people living in rural areas. The main symptom associated with infection by the virus was rhinorrhea. The multiple analysis also showed an association for fewer cases of respiratory distress in HRV-positive patients. The addition of rhinovirus-specific primers to the respiratory virus panel significantly increased the identification of a viral etiologic agent. The prevalence of rhinovirus (in relation to other viruses) showed a significant negative correlation with minimum temperatures, that is, the increase in rhinovirus detection is proportional to the decrease in minimum temperatures recorded in Brasília, Brazil.

Conclusion: Great diversity of viruses was found by different methodologies and a high frequency of rhinovirus occurrence was confirmed in the population in winter, showing its relevance to public health. The presence of rhinovirus in respiratory diseases was significantly associated with age less than two years and with rhinorrhea. The incidence of rhinovirus was correlated with the drop in minimum temperature, but without an evident seasonal pattern for the population of Brasília in the studied period.

Keywords: Respiratory virus - public health - high throughput sequencing - RT-qPCR - rhinovirus - epidemiology - seasonality.

Capítulo 1

1.1 Introdução

1.1.1 Vírus respiratórios

Os vírus são os principais agentes etiológicos envolvidos em infecções do trato respiratório, com elevado índice de morbidade e mortalidade, estando entre as cinco principais causas de morte em todo mundo (WHO 2018). Complicações relacionadas a essas infecções respiratórias atingem principalmente os grupos considerados de risco: crianças menores de cinco anos, gestantes, idosos e pacientes imunossuprimidos (Zar, 2014; WHO 2015). Segundo a Organização Mundial de Saúde (OMS), as infecções respiratórias agudas (IRAs) são responsáveis por 40 a 60% de todos os atendimentos ambulatoriais em pediatria na América Latina. Cerca de 4 milhões de crianças menores de cinco anos morrem por infecção aguda das vias aéreas inferiores anualmente, principalmente em países em desenvolvimento (Salomão Júnior et al. 2011, WHO 2018). Além disso, essas doenças geram elevados custos diretos e indiretos com assistência à saúde (Girard et al. 2005) e representam a quarta causa de morte em crianças nos países de média renda, como o Brasil (Ferone et al. 2013).

Em pacientes adultos, os vírus respiratórios estão associados a pneumonias adquiridas na comunidade, além da exacerbação da doença pulmonar obstrutiva crônica e da asma, resultando em grande número de consultas e hospitalizações (Daubin et al. 2006). Os vírus mais prevalentes são vírus sincicial respiratório, rinovírus e vírus influenza, com positividade de 29,6% a 57,6% em pacientes com sintomatologia respiratória (Yu et al. 2012, Falsey et al. 2014, Stover & Litwin 2014, Nam & Ison 2019).

A nova pneumonia associada à doença COVID-19, causada pelo vírus SARS-CoV-2, é caracterizada por febre, fadiga, tosse seca e dispneia e acomete principalmente pacientes adultos e imunocomprometidos. A positividade de detecção desse vírus pandêmico varia de 29,0 a 66,6%, para síndrome gripal e síndrome respiratória aguda grave respectivamente (Brasil 2021).

Em crianças as infecções por vírus respiratórios são a principal causa de morbidade e de atendimentos médicos (Lambert et al. 2007; Quan et al. 2007). A positividade para vírus respiratórios nesse público varia de 35,3% a 84% com quadros de infecção do trato respiratório, com maior destaque para vírus sincicial respiratório e rinovírus (Jansen et al. 2011, Martin et al. 2013, Wang et al. 2016). Estes são responsáveis por 1 a 3% de mortalidade entre crianças menores de cinco anos de idade em países industrializados e por 10 a 15% dos óbitos em crianças de países em desenvolvimento (Quan et al. 2007). Os vírus respiratórios podem causar diversas complicações clínicas, como bronquiolite, pneumonias e crupe, o que geralmente envolve hospitalizações (Syrmis et al. 2004). Entre os vírus respiratórios, o vírus da influenza causa infecção respiratória que afeta 5-10% dos adultos e 20-30% das crianças (WHO 2012), atingindo 290.000-650.000 mortes por ano em todo o mundo (Iuliano et al. 2018).

O perfil de ocorrência das infecções virais respiratórias é variável e está relacionado com a região geográfica, clima e período do ano. Em locais de clima temperado, os vírus respiratórios normalmente seguem padrões sazonais de atividade, conforme fatores associados à temperatura (Monto 2004). Os vírus influenza, parainfluenza, metapneumovírus humano e vírus sincicial respiratório, por exemplo, possuem picos de incidência no inverno, enquanto enterovírus são comuns no outono e verão. Já infecções por rinovírus possuem maior incidência em estações frias, tanto para

países de clima temperado como para regiões tropicais, embora circulem durante o ano todo (Costa et al. 2006, du Prel et al. 2009).

Também em regiões de clima temperado, é comum a ocorrência de cocirculação de vários vírus em um dado período do ano, mesmo durante o pico de ocorrência de um determinado agente respiratório (Tamerius et al. 2013, Pica & Bouvier 2014). Um estudo na Inglaterra mostrou que a maioria das infecções por vírus respiratórios ocorreu na idade menor de cinco anos, com mais de 10% de infecções duplas ou múltiplas, sendo o rinovírus, o mais prevalente nessas coinfeções (Goka et al. 2015).

O Brasil, que inclui regiões temperadas e tropicais, exibe uma importante heterogeneidade regional da circulação desses vírus (Alonso et al. 2007, de Mello et al. 2009). As epidemias ou pandemias de gripe geralmente associam-se a climas frios e úmidos, mas também a fatores como idade, imunidade preexistente, polimorfismos genéticos e presença de comorbidades, influenciando na gravidade da epidemia e responsividade à vacina (Castrucci 2017). A vacinação é a principal medida de saúde pública utilizada para reduzir a frequência de casos graves de influenza, a qual é realizada na forma de campanhas anuais, em nosso país, desde 1999 (Domingues & Oliveira, 2012).

No Brasil, estudos mostram os agentes virais como a principal causa de infecção respiratória aguda, especialmente em crianças, como observado em estudos nas cidades de Fortaleza (de Arruda et al. 1991), Rio de Janeiro (Nascimento et al. 1991), São Paulo (Miyao et al. 1999), Curitiba (Tsuchiya et al. 2005), Porto Alegre (de-Paris et al. 2014) e Belo Horizonte (Monteiro et al. 2016).

1.1.2 Vigilância da influenza e de outros vírus respiratórios

A vigilância global epidemiológica para os vírus influenza foi iniciada em 1947, com o objetivo de monitorar os vírus circulantes e fornecer informações para subsidiar as recomendações da Organização Mundial da Saúde (OMS). Atualmente, a Rede Global de Vigilância de Influenza da OMS (GISN, do inglês WHO Global Influenza Surveillance Network) é constituída de mais de 140 laboratórios em diferentes países, denominados Centros Nacionais de Influenza (NIC – National), além de seis Centros Colaboradores (WHO CC). No Brasil, a implantação do Sistema de Vigilância Sentinela teve início em 2000 (Barros et al. 2004). O Sistema de Vigilância da Influenza e de outros vírus respiratórios está presente em todos os Estados brasileiros e existem três Centros Nacionais de Influenza (NICs). Os Lacens (Laboratórios Centrais de Saúde Pública) são responsáveis pela base da informação utilizada para vigilância a partir da identificação do agente etiológico, tipagem e subtipagem de vírus respiratórios circulantes. Um quantitativo das amostras processadas pelos Lacens é sistematicamente enviado para os Laboratórios de Referência Nacional para realização de análises complementares, como o sequenciamento genético (Brasil 2016). O objetivo da vigilância global da influenza é, portanto, identificar os vírus respiratórios circulantes, bem como sua sazonalidade e as populações mais susceptíveis.

A vigilância é realizada de forma sentinela para os casos de síndrome gripal (SG), definidos como pacientes apresentando febre e tosse ou dor de garganta além de um dos seguintes sintomas: cefaleia, mialgia ou artralgia. Em crianças menores de 2 anos, os casos de SG são definidos por: febre, tosse, coriza e obstrução nasal. A vigilância sentinela consiste em uma rede de unidades de saúde designadas (públicas ou privadas) distribuídas por todo o país, nas quais amostras aleatórias de casos respiratórios são

periodicamente coletadas para detecção e caracterização genética de vírus circulantes. Os casos de síndrome respiratória aguda grave (SRAG) são definidos com pacientes requerendo hospitalização e apresentando dispneia ou um dos seguintes sinais: saturação de oxigênio capilar periférico $< 95\%$, desconforto respiratório ou insuficiência respiratória aguda. A vigilância da SRAG é realizada de forma universal, onde todos os casos respiratórios graves internados em unidades de terapia intensiva (UTIs) e todas as mortes relacionadas à doença respiratória são investigadas em laboratório, quanto a suas causas (Barros et al. 2016).

1.1.3 Principais vírus respiratórios

Os agentes etiológicos virais mais frequentemente envolvidos com as infecções do trato respiratório (ITR) são os vírus influenza tipo A, B e C (*Influenza A virus*, *Influenza B virus*, *Influenza C virus*), vírus sincicial respiratório (VSR, *Human orthopneumovirus*), metapneumovirus humano (HMPV, *Human metapneumovirus*), vírus parainfluenza (PIV) tipos 1 (*Human respirovirus 1*), 2 (*Human orthorubulavirus 2*), 3 (*Human respirovirus 3*), 4A e 4B (*Human orthorubulavirus 4*), coronavírus humano (HCoV) 229E (*Human coronavirus 229E*), NL63 (*Human coronavirus NL63*), HKU1 (*Human coronavirus HKU1*), OC43 (*Betacoronavirus 1*), SARS-CoV (*Severe acute respiratory syndrome-related coronavirus*), MERS-CoV (*Middle East respiratory syndrome-related coronavirus*) e SARS-CoV-2, rinovirus (HRV) tipos A (*Rhinovirus A*), B (*Rhinovirus B*) e C (*Rhinovirus C*), enterovírus (EV, *Enterovirus C*), além dos bocavírus (HBoV, *Primate bocaparvovirus 1*) e adenovírus (AdV, *Human mastadenovirus C*) (van den Hoogen et al. 2001, Allander et al. 2005, Tregoning & Schwarze 2010, ICTV 2018, Abdelrahman et al. 2020), os quais são agrupados em sete famílias (quadro 1). Estes vírus são responsáveis por um espectro de manifestações clínicas que incluem o comprometimento alto e baixo do trato respiratório (Quan et al. 2007). Entretanto, os agentes causadores de uma parcela das infecções respiratórias ainda são desconhecidos.

Quadro 1. Características gerais dos principais vírus respiratórios.

Família	Vírus	Material Genético	Partícula Viral
<i>Orthomyxoviridae</i>	<i>Influenza A virus</i> <i>Influenza B virus</i> <i>Influenza C virus</i>	ssRNA (-) segmentado (10-14,6kb)	~100nm Envelopado Simetria helicoidal
<i>Pneumoviridae</i>	<i>Human orthopneumovirus</i> <i>Human metapneumovirus</i>	ssRNA (-) (13,2 – 17,4kb)	150 – 600nm Envelopado Simetria helicoidal
<i>Paramyxoviridae</i>	<i>Human respirovirus 1</i> <i>Human orthorubulavirus 2</i> <i>Human respirovirus 3</i> <i>Human orthorubulavirus 4</i>	ssRNA (-) (13,2 – 17,4kb)	150 – 600nm Envelopado Simetria helicoidal
<i>Coronaviridae</i>	<i>Human coronavirus HKU1</i> <i>Human coronavirus NL63</i> <i>Human coronavirus 229E</i> <i>Betacoronavirus 1</i> <i>Middle East respiratory syndrome-related coronavirus (MERS)</i> <i>Severe acute respiratory syndrome-related coronavirus (SARS)</i>	ssRNA (+) (27 – 32kb)	100-160nm Envelopado Simetria helicoidal
<i>Picornaviridae</i>	<i>Rhinovirus A</i> <i>Rhinovirus B</i> <i>Rhinovirus C</i> <i>Enterovirus C</i>	ssRNA (+) (7,2 – 8,5kb)	~30nm Desnudo Simetria icosaédrica
<i>Parvoviridae</i>	<i>Primate bocaparvovirus 1</i>	ssDNA (~5kb)	~25nm Desnudo Simetria icosaédrica
<i>Adenoviridae</i>	<i>Human mastadenovirus C</i>	dsDNA (~36kb)	70-90nm Desnudo Simetria icosaédrica

Fonte: Oliveira, 2016 (adaptado).

A influenza (gripe comum) é uma doença respiratória que ocorre no mundo todo causada pelo vírus influenza, pertencente à família *Orthomyxoviridae*, que inclui os vírus A, B e C em humanos. O genoma do vírus consiste em 8 segmentos de RNA (7 para Influenza C), onde cada um codifica pelo menos uma proteína com funções específicas (Vincent et al. 2014, Tian et al. 2012). Até o momento, foram identificados 18 subtipos antigênicos para a hemaglutinina (H1-18) e 11 subtipos diferentes da neuraminidase (N1-

11), sendo as principais cepas que circulam na população humana: H1N1, H1N2, H2N2 e H3N2 (WHO 2014).

A variabilidade genômica, que se traduz em diversidade antigênica, bem como as recombinações, compreendem importantes mecanismos de escape imunológico do vírus, os quais favorecem a infecção de novos hospedeiros suscetíveis, podendo diminuir também a eficácia das vacinas (Guarnaccia et al. 2013, Tewawong et al. 2015).

Esses fatores, juntamente com a alta transmissibilidade e o potencial zoonótico e pandêmico, tornam o desafio de prevenção e controle da influenza um problema crescente e de reconhecida importância na saúde pública (Zambon 2014, Jerigan & Cox 2013).

O vírus sincicial respiratório humano (VSR) é a causa mais comum de infecções do trato respiratório superior e inferior em crianças, de até dois anos de idade, seguido pelos vírus parainfluenza humanos (PIV) (Pecchini et al. 2015). O VSR acomete crianças principalmente nos primeiros meses de vida, podendo ainda infectar adultos e idosos, e estando associado à complicações como broncopneumonia, desnutrição e bronquiolite, mas também é comumente encontrado em coinfeção com outros vírus respiratórios (Raboni et al. 2015) e foi isolado pela primeira vez no Brasil por Candeias em 1964 (Candeias 1967). Divide-se em dois subgrupos antigênicos baseados em reações contra a glicoproteína G e posteriores análises genéticas: VSR-A, considerado mais virulento, e VSR-B, ainda subdividido em duas variantes, B1 e B2 (Mufson et al. 1985, Melero et al. 1997).

O metapneumovírus humano (HMPV) é um vírus da família *Pneumoviridae*, descoberto em 2001 na Holanda, caracterizado como agente causador de infecção respiratória aguda em todas as faixas etárias, mas principalmente em crianças até dois anos de idade (van den Hoogen et al. 2001, Panda et al. 2014). Esses vírus são classificados nos genótipos A e B (com posterior classificação em sub-linhagens A1, A2a,

A2b, B1 e B2) (van den Hoogen et al. 2004). Os principais sintomas são febre, tosse, congestão nasal, dispnéia, além de bronquiolite e pneumonia em casos mais graves (Kahn 2006, Broor et al. 2008, Feuillet et al. 2012). Em adultos, no geral a infecção é de leve a moderada; contudo, idosos e indivíduos imunocomprometidos podem apresentar complicações (Falsey et al. 2003). O primeiro genoma completo do HMPV sequenciado no Brasil foi relatado por Di Paola e colaboradores (2018).

Em relação aos PIVs, existem quatro espécies: PIV-1, -2, -3 e -4, sendo que PIV-4 está classificado em dois sorotipos, -4A e -4B. Estima-se que estes causem até 10% das infecções respiratórias agudas (IRA) na infância (Boivin et al. 2002). Os vírus PIV1 e PIV2 são a principal causa de crupe em crianças de 6 a 48 meses. O PIV3 e, em menor extensão, o PIV1, estão mais frequentemente associados a bronquiolite e pneumonia em crianças com menos de 1 ano. Os PIVs também causam doença grave, incluindo pneumonia e morte em pacientes transplantados, bem como infecções nosocomiais e surtos (Hasman et al. 2009). Estudo recente fez o primeiro relato de infecção por PIV-4 em crianças com IRA no Brasil, cuja prevalência ficou abaixo dos patógenos respiratórios mais comuns (VSR, adenovírus, influenza e HMPV), mas foi o segundo PIV mais prevalente, seguindo o PIV-3 (Thomazelli et al. 2018).

Os coronavírus humanos (HCoV) foram identificados pela primeira vez há mais de 50 anos. Esses vírus são classificados em ao menos 4 gêneros (delta, gama, beta e alfa), sendo sete destes vírus conhecidos por infectar humanos: NL63 e 229E do gênero alfa e OC43, HKU1, SARS-CoV, MERS-CoV e SARS-CoV-2 do gênero beta (Abdelrahman et al. 2020). Acredita-se que esses vírus tenham se originado de reservatórios animais, sendo os SARS-CoV e MERS-CoV exemplos mais recentes que emergiram de morcegos para civetas (gato-de-almofada) ou dromedários, respectivamente, e destes para humanos (van den Brand et al. 2015). Supõe-se que o SARS-CoV-2 emergiu de morcegos tendo

como possíveis hospedeiros intermediários os pangolins (Dimonaco et al. 2020). Estima-se que as taxas de fatalidade do SARS-CoV, MERS-CoV e SARS-CoV-2 sejam de 9,5%, 34,4% e 2,3%, respectivamente (Petrosillo et al. 2020).

Em relação às manifestações clínicas, os vírus 229E (*Alphacoronavirus*) e OC43 (*Betacoronavirus*) estão relacionadas com resfriado comum, enquanto NL63 (*Alphacoronavirus*) está associado com casos de crupe e bronquiolite. Os HCoV-HKU1 (*Betacoronavirus*) foram associados a sintomas como febre, rinorreia, sibilância e tosse (van der Hoek et al. 2005, Pyrc et al. 2007, Wu et al. 2008, Lee & Storch 2014).

Os vírus SARS-CoV (*Betacoronavirus*) causam pneumonia atípica, caracterizada por tosse, febre, mialgia, dores de cabeça, mal-estar, dispneia e menos comumente vômitos e diarreia (Hui & Chan 2010, van den Brand et al. 2014). As infecções por MERS-CoV (*Betacoronavirus*) possuem sintomatologia semelhante ao SARS, contudo consideradas mais graves e com maiores taxas de mortalidade. Ademais incluem aspectos clínicos como pneumonia grave, às vezes com lesão pulmonar aguda fatal ou síndrome respiratória aguda de angústia (Abdelrahman et al. 2020), além de retenção urinária e falência renal (Al-Tawfiq 2013, Groot et al. 2013, van den Brand et al. 2015).

Existe atualmente uma pandemia mundial de um novo vírus pertencente à família *Coronaviridae*, identificado pela primeira vez em dezembro de 2019 em Wuhan, China, cuja dispersão pelo mundo se deu de forma rápida. Este agente, foi designado como coronavírus relacionado à síndrome respiratória aguda grave 2 (SARS-CoV-2), cujo nome da doença associada é COVID-19 (CSGICTV 2020). O SARS-CoV-2 pode ser transmitido pela via respiratória, através de aerossóis, perdigotos e secreções respiratórias, ou por contato com fômites. A transmissão pela via ocular tem sido cogitada e o vírus pode também ser encontrado nas fezes de indivíduos infectados. A COVID-19 pode estar associada SRAG, pneumonia, além de quadros mais brandos ou subclínicos e

assintomáticos (Bchetnia et al. 2020). A infecção pelo SARS-CoV-2 já causou milhões de mortes, com sobrecarga dos sistemas de saúde e grande impacto econômico devido à política de isolamento social proposta para contenção da disseminação do vírus. Até o momento, não há terapia específica eficaz disponível, mas muitas vacinas para o vírus estão em desenvolvimento ou já foram aprovadas para aplicação na população adulta.

Os enterovírus humanos (EV) podem causar infecções respiratórias, mas também podem estar associados a diversas síndromes clínicas, incluindo infecções assintomáticas e subclínicas, doenças respiratórias, gastroenterites, miocardites e meningites. Os enterovírus pertencem à família *Picornaviridae* e estão divididos em doze espécies (EV A-L) com base em suas propriedades biológicas e moleculares. Foram descritas até o momento quatro espécies de EV A-D capazes de causar infecções em humanos, enquanto as outras espécies comportam vírus que infectam primatas, suínos e bovinos (Tapparel et al. 2013, ICTV 2018, Picornaviridae 2019). Além da transmissão por via respiratória, também são transmitidos por via fecal-oral. As infecções respiratórias por enterovírus podem variar desde assintomática até sintomáticas do trato respiratório superior (resfriado, faringite) ou inferior (pneumonia, bronquiolite ou exacerbação de asma na infância) (Chang 2008, Tapparel et al. 2013). No Brasil, Carney e colaboradores (2015) relataram pela primeira vez, dois casos de infecção pelo enterovírus EV-D68 em crianças com doença respiratória aguda, com sintomas como insuficiência respiratória, febre, diarreia e taquicardia. Outro enterovírus associado à infecção respiratória recentemente identificado no país foi o coxsackievírus B2. Ele foi identificado em uma paciente de um ano de idade, com sintomatologia leve de infecção respiratória (Lima 2017).

O bocavírus humano (HBoV) é classificado como genótipo 1 a 4 (Guido et al. 2016) e foi descrito pela primeira vez na Suécia em 2005, sendo identificado como agente de infecções respiratórias a partir de aspirados de nasofaringe de pacientes com infecções

do trato respiratório inferior (Allander et al. 2005, ICTV 2018). Estudos também descreveram a presença do bocavírus em amostras fecais provenientes de pacientes com gastroenterite, sugerindo tropismo do vírus pelo trato gastrointestinal para pelo menos algumas espécies (Kapoor et al. 2009, Kapoor et al. 2010).

Os adenovírus humanos (AdV) foram isolados primeiramente por Rowe e colegas, enquanto estudavam o cultivo de vírus em tecidos adenóides (Rowe et al. 1953). Os AdVs são divididos em sete espécies, AdV A-G, e ainda em mais de 100 genótipos (Robinson et al. 2011, Huang et al. 2013, Mao et al. 2019). Os adenovírus podem causar um amplo espectro de infecções em humanos, dentre elas, patologias respiratórias, gastroentéricas, hepáticas e oculares. A maioria dessas infecções ocorre em população pediátrica e, em indivíduos imunocompetentes é geralmente autolimitada. No caso das infecções do trato respiratório, os sintomas variam desde um quadro clínico de resfriado comum até casos mais graves de pneumonias e bronquiolites (Kunz & Ottolini 2010), sendo estes vírus responsáveis por 1 - 5% de todas as infecções respiratórias e por até 10% das pneumonias na infância (Ampuero et al. 2012).

1.1.4 Rinovírus

Os vírus mais frequentemente associados a infecções respiratórias são os Rinovírus humanos (HRV), tipicamente o resfriado comum, que inclui sintomas como espirros, obstrução nasal, coriza, dor de garganta, dor de cabeça, tosse e mal-estar.

Entretanto, com o avanço de técnicas de biologia molecular, esse vírus também vem sendo associado a infecções assintomáticas, disseminação sistêmica e do trato respiratório inferior, como pneumonia e bronquiolite, particularmente entre pacientes com asma, fibrose cística, doença pulmonar obstrutiva crônica (DPOC) e imunossupressão relacionada ao transplante (Peltola et al. 2009, Gern 2010, Tapparel et al. 2011).

Esses vírus são filogeneticamente classificados em três espécies, HRV-A, HRV-B e HRV-C (Choi et al. 2015, Martin et al. 2015), os quais compreendem uma grande diversidade genética, com mais de 160 sorotipos (Palmenberg et al. 2010). Os HRVs usam três diferentes glicoproteínas da membrana celular expressas no epitélio respiratório para entrar na célula hospedeira. Esses receptores virais são a molécula de adesão intercelular 1 (usada pela maioria dos tipos de RV-A e todos os tipos de RV-B), membros da família de receptores de lipoproteínas de baixa densidade (usados por 12 tipos de RV-A) e membros da família 3 relacionados à caderina (CDHR3; usado por RV-C) (Basnet et al. 2019).

Inicialmente a espécie C foi descrita como responsável por doença de maior severidade, porém estudos posteriores mostraram que essa característica é controversa (Pierangeli et al. 2013, Choi et al. 2015). Em muitos casos, os rinovírus são detectados juntamente com outros vírus respiratórios, principalmente associados ao VSR A e B em infecções de crianças e adultos (Brandão et al. 2016, Calvo et al. 2015).

Os rinovírus apresentam relevância mundial por circularem o ano todo em diferentes regiões do mundo, além de infectarem todas as faixas etárias; no entanto, bebês, crianças e idosos têm as taxas mais altas de infecção grave entre pacientes hospitalizados (Miller et al. 2013, Fry et al., 2011). A transmissão dos HRVs ocorre principalmente através de inalação de gotículas respiratórias, contato direto de pessoa a pessoa ou através de superfícies contaminadas ou inoculação direta do olho ou nariz mucosa com a ponta dos dedos (L'Huillier 2015).

O capsídeo do HRV é composto por quatro proteínas (VP1, VP2, VP3 e VP4), que completam o genoma de RNA. As três primeiras proteínas pertencem ao capsídeo e são responsáveis pela diversidade antigênica viral, enquanto a VP4 é internalizada, ancorando o RNA ao capsídeo (Jacobs et al. 2013). Os mecanismos de ação dos HRVs que envolvem o rompimento da barreira epitelial com vazamento e produção excessiva de muco, além da secreção de muco das células caliciformes em resposta a produtos de ativação de neutrófilos, estão provavelmente envolvidos na obstrução nasal, levando aos sintomas das vias aéreas (Papadopoulos et al. 2000).

1.1.5 Diagnóstico e caracterização molecular

Algumas técnicas tradicionais de diagnóstico são usadas há décadas na detecção de vírus respiratórios (como imunomicroscopia eletrônica, ensaio de imunoabsorção enzimática, imunofluorescência direta e indireta, imunocromatografia) (Murdoch 2009, Murdoch 2016a), entretanto técnicas específicas de diagnóstico molecular, têm permitido a identificação mais rápida de muitos patógenos que antes eram difíceis de detectar (Murdoch 2016b).

A técnica da reação em cadeia da polimerase (PCR) permite que um fragmento específico da molécula de DNA seja amplificado, de forma exponencial, em apenas algumas horas, sendo possível ainda a caracterização de patógenos, através da genotipagem. As vantagens da PCR são numerosas: velocidade, baixo custo, automação, sensibilidade e especificidade. Entretanto, o principal problema envolvendo o uso da PCR para diagnóstico é o fato de ela ser patógeno-específica, ou seja, só identifica alvos pré-definidos, o que supõe que o médico elaborou uma hipótese etiológica.

A utilização desta técnica está em constante atualização, como o uso cada vez mais disseminado da PCR em tempo real, a qual permite que a amplificação e detecção ocorram simultaneamente, com possibilidade de gerar resultados quantitativos com maior precisão, por meio de um sistema de monitoramento da emissão da fluorescência. Há atualmente diversos ensaios multiplex comerciais disponíveis em uma variedade de formatos. No contexto clínico, a detecção de um vírus respiratório em uma amostra respiratória é geralmente considerada suficiente para atribuir uma relação causal, embora seja importante avaliar a possibilidade de colonização ou até contaminação (Ruuskanen et al. 2011).

Essas novas metodologias, como as que envolvem sequenciamento genético, têm auxiliado na descoberta de novos vírus respiratórios, sendo que os últimos incluem o bocavírus humano, o metapneumovírus humano e uma variedade de coronavírus (SARS-CoV, CoV-NL63, CoV-HKU1 e MERS-CoV) (Berry et al. 2015, Lu et al. 2020).

De forma geral, as infecções do trato respiratório superior tendem a ser monomicrobianas e predominantemente causadas por vírus, com algumas exceções notáveis causadas por bactérias específicas (como por exemplo, faringite aguda causada por *Streptococcus pyogenes*). Infecções do trato respiratório inferior são causadas por uma ampla variedade de patógenos virais e bacterianos. Isso implica em complexidade, sendo que o papel de cada microorganismo nessas patologias ainda não é bem elucidado (El Kholi et al. 2016).

O sequenciamento de alto desempenho – do inglês *High Throughput Sequencing* (HTS), também chamado de sequenciamento de nova geração, é reconhecido como uma tecnologia poderosa para a detecção e identificação de microorganismos conhecidos ou não (Mallet & Gissoni-Lex 2014). Este sequenciamento amplifica fragmentos de DNA muitas vezes, gerando resultados com grande acurácia e aplicado ao diagnóstico microbiano permite, em teoria, a amplificação e análise de qualquer material genético presente na amostra clínica, mesmo em baixas concentrações e sem a necessidade de desenhar primers específicos para pré-amplificar as sequências-alvo (Reuter et al. 2015). Entretanto há entraves clínicos, computacionais e interpretativos, como por exemplo, a necessidade de enriquecimento das amostras a serem sequenciadas, visto que são provenientes de espécimes clínicos dos pacientes, extremamente contaminados com DNA humano e de outros microrganismos, os quais podem não causar sintomas (assintomáticos) ou estar presentes na microbiota normal e saudável (Xu et al. 2017, Taboada et al. 2014, Zhou et al. 2016).

A população humana está susceptível a um crescente número de doenças infecciosas, inclusive em razão da adaptação a humanos e emergência de vírus ainda não caracterizados. As mudanças climáticas, a globalização, assentamentos perto de habitats de animais silvestres e a convivência próxima de diferentes espécies, além do aumento do número de pessoas imunocomprometidas, provavelmente, contribuem para o surgimento e disseminação de novas infecções (Morens et al. 2004). Além disso, várias síndromes clínicas são suspeitas de ser de etiologia viral, mas o agente pode não ser isolado ou reconhecido por cultivo e ensaios moleculares tradicionais. Portanto, existe a necessidade de se aprimorar os métodos para a identificação de patógenos virais ou caracterização de novos agentes. Métodos de sequenciamento de alto desempenho tem permitido análises genômicas mais sensíveis e rápidas em relação às metodologias anteriores, utilizados por exemplo na descoberta do herpesvírus 8 humano (Chang et al. 1994), vírus GB humano (Simons et al. 1995), Torque teno virus (Nishizawa et al. 1997), bocavírus (Allander et al. 2005), parvovírus humano 4 (Jones et al. 2005), WU poliomavírus (Gaynor et al. 2007) e KI poliomavírus (Allander 2007).

Existem mais casos na literatura que mostram a utilização do sequenciamento de alta performance na descoberta de patógenos não identificados por métodos tradicionais, como ocorreu com a descoberta de um arenavírus em três pacientes que morreram de uma doença febril algumas semanas após transplante de órgãos sólidos a partir de um único doador (Palacios et al. 2008). A plataforma Illumina GA permitiu identificar um vírus influenza A a partir de esfregaços e montagem *de novo* de seu genoma (Yongfeng et al. 2011, Kuroda et al. 2010, Greninger 2010). A metodologia também permitiu a detecção de patógenos virais em amostras de aspirado nasofarínge de pacientes com infecções agudas do trato respiratório inferior (Yang et al. 2011), como um novo enterovírus, denominado enterovírus 109 (EV109) detectado em uma coorte de crianças nicaraguenses

com doença respiratória viral (Yozwiak et al. 2010). Foi também foi possível identificar um novo astrovírus HAstV-VA1 / HMO-C-UK1, associado a encefalite em pacientes imunossuprimidos (Brown et al. 2015).

O caso mais recente de utilização de sequenciamento de alto desempenho foi a descoberta do vírus SARS-CoV-2. O sequenciamento foi realizado a partir de amostras de fluido de lavagem broncoalveolar e isolados de nove pacientes internados, oito dos quais haviam visitado o mercado de frutos do mar Huanan em Wuhan, China. Sequências genômicas completas e parciais 2019-nCoV foram obtidas desses indivíduos, com mais de 99% de identidade de sequência. A determinação do genoma permitiu a identificação do domínio de ligação ao receptor e, posteriormente a identificação da enzima conversora da angiotensina 2 como receptor nas células do hospedeiro. (Lu et al. 2020).

1.2 Justificativa

A utilização de sequenciamento de alto desempenho (High-throughput sequencing: HTS) tem sido aplicada com sucesso em vários campos da virologia, incluindo descoberta de novos vírus, reconstrução de genoma completo e análise de variantes (Capobianchi et al. 2013, Lipkin 2010, Mokili et al. 2013, Smits & Osterhaus 2013, van Boheemen et al. 2012). A vantagem mais importante fornecida por estas plataformas é a determinação dos dados da sequência de fragmentos de DNA isolados de uma biblioteca que são separados em chips, evitando a necessidade de clonagem em vetores antes da aquisição de sequência (Barzon et al. 2011). Essas tecnologias são bem conhecidas por sua enorme produção de dados de sequência genética a um custo relativamente alto, mas decrescente. Outra vantagem é que este tipo de metodologia elimina a necessidade de projeto e validação de várias dezenas ou centenas de primers / sondas específicas para sequências virais, e não requer adaptação contínua desses iniciadores a cada nova variante ou espécie identificada.

Como o diagnóstico de vírus respiratórios é realizado no Lacen-DF (Laboratório Central de Saúde Pública do Distrito Federal) por meio de metodologia tradicional (RT-PCR em tempo real), agentes etiológicos em muitas amostras podem não estar sendo identificados, tendo em vista que essa metodologia é patógeno-específica. As amostras encaminhadas ao Lacen são oriundas de pacientes de hospitais da rede pública e privada de saúde com suspeita de infecção por vírus respiratórios, estando esses pacientes internados ou não. Os principais sintomas associados são: febre, tosse, dispneia, mialgia, dor de garganta e desconforto respiratório. Em torno de 50-60% das amostras encaminhadas são negativas para os vírus pesquisados na rotina (Influenza A e B, Vírus Respiratório Sincicial, Metapneumovírus humano, Adenovírus, Parainfluenza 1, 2 e 3).

Sendo assim, para fins de saúde pública, é fundamental conhecer quais possíveis patógenos estão acometendo os pacientes com sintomatologia típica de gripe ou resfriado, mas sem diagnóstico definido por metodologia tradicional.

Nesse contexto, identificar a alta prevalência de rinovírus, antes subestimado pela ausência de alvos no painel viral, por meio da avaliação de diferentes metodologias de diagnóstico é fundamental para fortalecer a pesquisa local e o laboratório de saúde pública.

Além disso, a caracterização molecular e de características clínico epidemiológicas dos rinovírus permitem compreender características filogenéticas e sintomatológicas dos vírus que acometem a população de Brasília. Isso se faz importante para melhorar o diagnóstico e conhecer a epidemiologia desses vírus e assim, aprimorar a atuação da rede de vigilância da influenza e de outros vírus respiratórios.

1.3 Objetivo Geral

Identificar possíveis vírus em amostras de secreções nasofaringe / traqueal de pacientes do Distrito Federal com sintomas de vírus respiratórios em amostras clínicas previamente negativas para os agentes etiológicos disponíveis no painel de vírus respiratórios.

1.3.1 Objetivos específicos

- 1) Realizar a caracterização molecular de vírus a partir de amostras clínicas (secreção da nasofaringe / aspirado traqueal), previamente negativas para o painel de vírus respiratórios do LACEN- DF e coletadas entre junho e agosto de 2016, por metagenômica, seguida de análise filogenética
- 2) Realizar o desenho de pares de iniciadores e sondas específicas para a detecção de rinovírus, a partir de resultados obtidos com a análise das amostras clínicas por Metagenômica, para serem utilizados em ensaio de rotina diagnóstica.
- 3) Avaliar a sintomatologia e dados sócio demográficos de pacientes em associação ao rinovírus, em amostras que haviam sido identificadas como negativas por métodos de diagnóstico de rotina do Lacen-DF.
- 4) Avaliar a positividade viral em relação aos meses do ano em associação a fatores climáticos da região.

1.4 Metodologia

Este projeto de pesquisa foi analisado e aprovado pelo comitê de ética da Faculdade de Ciências da Saúde (Universidade de Brasília), parecer de aprovação nº 3.052.443.

Primeiramente, amostras de secreção nasofaringe e traqueal recebidas no laboratório nos meses de junho, julho e agosto de 2016 foram testadas para o painel de vírus respiratórios realizado no Lacen-DF pelo RT-qPCR padrão (para Influenza A, Influenza B, Vírus sincicial respiratório humano, Metapneumovírus humano, Adenovírus C humano, Parainfluenza 1, Parainfluenza 2 e Parainfluenza 3). As que apresentaram resultados negativos tiveram o DNA / RNA extraídos na forma de pool (cinquenta e uma amostras de junho, 55 de julho e 39 de agosto de 2016, perfazendo uma amostra para cada mês). Foi realizada uma ultracentrifugação com colchão de sacarose a 20% e posterior extração de ácidos nucleicos seguindo as instruções do High Pure Viral Nucleic Acid Kit (Hoffmann-La Roche, Basel, Suíça), sem adição de RNA transportador. As três amostras de DNA / RNA foram submetidas ao sequenciamento de alta performance (high-throughput sequencing, HTS) usando Illumina HiSeq 2000 com escala de 3 G para 100 bases paired-end na Macrogen Inc. (Seul, Coréia do Sul). Em seguida, elas foram reunidas em uma amostra de RNA única e tratadas com DNase I (Promega, Madison, EUA) para diminuir o DNA humano ou bacteriano da amostra para enriquecimento do RNA viral e novamente submetidas ao HTS. A biblioteca de amostras de DNA / RNA ou RNA foi construída usando o kit TruSeq Standard total RNA.

As seqüências de baixa qualidade e os adaptadores dos dados brutos foram retirados usando a ferramenta Trimomatic v.036. As leituras metagenômicas de cada amostra foram avaliadas no site Kaiju (<http://kaiju.binf.ku.dk>) para traçar o perfil

taxonômico. As leituras de DNA foram filtradas com BWA v0.7.17 (17) e SAMtools v1.9 contra hg38 de referência (*Homo sapiens*) obtido do GenBank para remover sequências de DNA humano do conjunto de dados HTS *in silico*. As leituras obtidas por HTS foram montadas em sequências contíguas ("contigs") com os programas Velvet v.1.2.1 e SPAdes v.3.9. Para identificação da origem viral das sequências contig foi utilizado o tBlastx (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) contra o pacote de genoma viral (RefSeq Virus, NCBI, <https://www.ncbi.nlm.nih.gov/genome/viruses/>) implementado no programa Geneious R8.1 (Biomatters, Auckland, Nova Zelândia).

Parte dos achados na análise por HTS foi confirmada por meio de RT-qPCR utilizando o kit XGen PR21 (Biometrix, Curitiba, Brasil) para 21 patógenos respiratórios (Influenza A, Influenza B, Influenza A H1N1-sw1, Coronavírus NL63, Coronavírus 229E, Coronavírus OC43, Coronavírus HKU1, Parainfluenza 1, Parainfluenza 2, Parainfluenza 3, Parainfluenza 3, Parainfluenza 4, Parainfluenza Humana B, Vírus A e B sincicial respiratório humano, Rinovírus, Enterovírus, Parecovírus, Adenovírus humano, Bocavírus e *Mycoplasma pneumoniae*).

Os vírus identificados em maior prevalência nas amostras negativas estudadas por HTS foram os rinovírus, com isso, primers universais HRV direcionados a regiões conservadas em 5' UTR (região não traduzida) foram projetados para detecção de HRV usando o programa Geneious R8.1 (Biomatters, Auckland, Nova Zelândia). O RNA foi extraído das 145 amostras originais usando o kit Magna Pure LC de ácido nucléico total (Hoffmann-La Roche, Basel, Suíça), e depois amplificadas por RT-qPCR usando o sistema GoTaq® Probe 1-Step RT-qPCR (Promega, Madison, EUA). A positividade para rinovírus foi definida com limiar de ciclo (CT) menor ou igual a 40 (total de 45 ciclos na reação). O gene RNase P humano foi usado como controle endógeno.

Também foram desenhados primers HRV_Com_For e HRV_Com_Rev visando regiões conservadas com base em análises de viroma por HTS. Este par de iniciadores foi usado para amplificar um fragmento de aproximadamente 540 bp para sequenciamento de amplicon incluindo parte da 5' UTR e o gene da proteína VP4 / VP2 de rinovírus das espécies A, B e C. O cDNA das amostras selecionadas foi sintetizado usando a transcriptase MMLV (Thermo Fisher Scientific, Waltham, EUA) com primer hexâmero aleatório e, a seguir, amplificado com LongAmp Taq DNA Polymerase (New England BioLabs, Ipswich, EUA). O produto de PCR foi purificado e sequenciado pelo método Sanger na Macrogen Inc (Seul, Coreia do Sul).

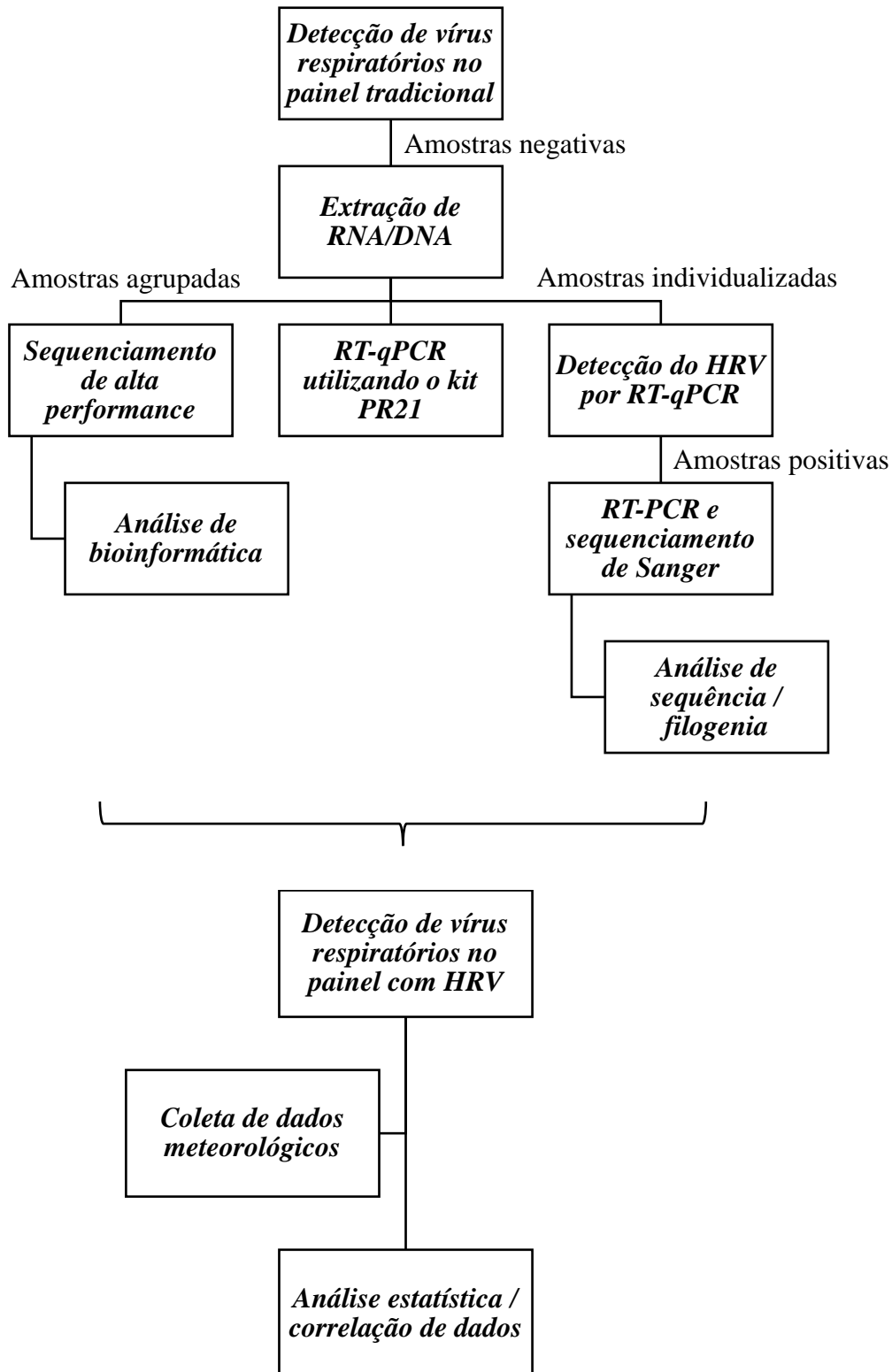
As sequências de nucleotídeos (nt) foram determinadas usando o programa Geneious R8.1 (Biomatters, Auckland, Nova Zelândia). A árvore filogenética baseada na 5' UTR e no gene da proteína VP4 / VP2 foi construída usando o software Mega X, com oito sequências de nucleotídeos obtidas neste estudo e outros quarenta isolados de rinovírus que estavam disponíveis no GenBank com sequências completas do genoma (para identificação do genótipo). A árvore filogenética foi inferida usando o método de máxima verossimilhança com o modelo Hasegawa-Kishino-Yano conforme indicado pela análise jModelTest.

Dados sócio demográficos e clínicos foram obtidos da ficha de notificação compulsória da síndrome gripal e síndrome respiratória aguda grave encaminhados com as amostras. Os dados foram analisados no software IBM SPSS (Chicago, EUA). Testes não paramétricos foram utilizados, conforme apropriado, para variáveis qualitativas e quantitativas.

A partir do desenho de primers universais, os rinovírus foram então inseridos no painel de vírus respiratórios do Lacen-DF a partir de agosto de 2019. Os resultados de

rinovírus e outros vírus respiratórios foram avaliados de agosto de 2019 a fevereiro de 2020. Dados dos 3 anos anteriores foram usados para comparar o percentual de identificação do agente etiológico. A partir de março de 2020, não foi possível dar continuidade à avaliação devido à pandemia de Coronavírus (SARS-CoV-2) que causou isolamento social e alteração da sazonalidade natural de outros vírus respiratórios, além de alterar os critérios de recebimento de amostra para diagnóstico. Dados meteorológicos da região de Brasília foram obtidas por meio de consultas diárias ao site oficial do Inmet (Instituto Nacional de Meteorologia) por meio de temperaturas e umidades relativas mínimas e máximas. A correlação não paramétrica de Spearman foi utilizada para avaliar a relação entre os dados meteorológicos e a incidência de rinovírus no período estudado. Todos os p-valores foram bicaudais ao nível de significância de 5%.

1.4.1 Fluxograma da metodologia



1.5 Referências Bibliográficas

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Capítulo 2

2.1 Human virome in nasopharynx and tracheal secretion samples

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Abstract

Background: In Brazil the implementation of the Sentinel Surveillance System of Influenza began in 2000. Central public health laboratories use RT-qPCR for diagnosis of respiratory viruses, but this protocol identifies only specific targets, resulted in inconclusive diagnosis for many samples. Thus, high-throughput sequencing (HTS) would be complementary method in the identification of pathogens in inconclusive samples for RT-qPCR or other specific detection protocols.

Objectives: This study aimed to detect unidentified viruses using HTS approach in negative samples of nasopharynx/tracheal secretions by the standard RT-qPCR collected in the Federal District, Brazil.

Methods: Nucleic acids were extracted from samples collected in winter period of 2016 and subjected to HTS. The results were confirmed by the multiplex PR21 RT-qPCR, which identifies 21 respiratory pathogens.

Findings: The main viruses identified by HTS were of families *Herpesviridae*, *Coronaviridae*, *Parvoviridae* and *Picornaviridae*, with the emphasis on rhinoviruses. The presence of respiratory viruses in the samples was confirmed by the PR21 multiplex RT-qPCR. Coronavirus, enterovirus, bocavirus and rhinovirus were found by multiplex RT-qPCR as well as by HTS analyses.

Main conclusions: Wide virus diversity was found by different methodologies and high frequency of rhinovirus occurrence was confirmed in population in winter, showing its relevance for public health.

Key words: Respiratory virus - public health - high-throughput sequencing - RT-qPCR

Introduction

Worldwide influenza surveillance was initiated in 1947 to monitor circulating viruses and provides information to support the vaccine-type recommendations by the World Health Organization (Geneva, Switzerland) and the Centers for Disease Control and Prevention (Atlanta, USA). Central laboratories (Lacens) in Brazil are responsible for the occurrence reports about the etiologic agents, types and subtypes of circulating respiratory viruses in patients with Influenza-like Syndrome (ILS) and Severe Acute Respiratory Syndrome (SARS).⁽¹⁾

The main technique for detecting respiratory viruses in Lacens is RT-qPCR (reverse transcription - quantitative polymerase chain reaction), which advantages are sensitivity, specificity, automation, relatively low cost and so on. However, this technique as well as the conventional RT-PCR are pathogen-specific, which only find pre-defined targets. Thus, a peculiar etiological agent or new genetic variants may not be detected, despite the use of a wide range of sensitive diagnostic tests.⁽²⁾

In this context, high-throughput sequencing (HTS) technologies without requiring any prior genomic information provide large volume of nucleotide sequences, resulting in elucidation of unidentified viruses (which were not detectable by the specific detection methods) and furthermore, allow the discovery of new viruses or new genomic variants using metagenomic approaches.

The human population is exposed to increasing burden of infectious diseases caused by the emergence of viruses not yet or rarely characterized. Globalization, climate change, settlements near wild animal habitats and increasing numbers of immunocompromised people are likely to contribute to the emergence and spread of new infections.⁽³⁾ HTS methods have allowed genomic analysis which is sensitive for

unknown or peculiar viruses over previous methodologies, such as the analysis of viral genomes used in the identification or discovery of human herpesvirus 8⁽⁴⁾, bocavirus⁽⁵⁾, human parvovirus 4⁽⁶⁾, human GB virus⁽⁷⁾, Torque Teno virus⁽⁸⁾, WU polyomavirus⁽⁹⁾ and KI polyomavirus.⁽¹⁰⁾

HTS has been used, for example, in the discovery of an arenavirus in three patients who died of a febrile illness a few weeks after transplant of solid organs from a single donor.⁽¹¹⁾ The Illumina platform allowed identifying hepatitis A virus among cases of acute febrile illnesses that occurred in Brazil which were suspected as yellow fever.⁽¹²⁾ The same platform was used to identify a new influenza virus from swabs samples and assembly of its genome.⁽¹³⁾ It also led to the detection of viral pathogens in nasopharyngeal aspirate samples from patients with acute lower respiratory tract infections, as a new enterovirus, termed enterovirus 109 (EV109) detected in a cohort of Nicaraguan children with viral respiratory disease.⁽¹⁴⁾

Thereby, this study aimed to detect unidentified viruses by HTS approach in negative samples of nasopharynx/tracheal secretions for diagnostics targeting several viruses collected from June to August 2016 in the Federal District (DF), Brazil.

Materials and methods

Detection of standard respiratory viruses - The nasopharyngeal and tracheal secretion samples received in the laboratory in June, July and August 2016 were tested for the respiratory virus diagnostic panel performed in Lacen-DF (Public health laboratory located in the Federal District, Brazil) by the standard RT-qPCR (for Influenza A, Influenza B, Human respiratory syncytial virus, Human metapneumovirus, Human adenovirus C, Parainfluenza 1, Parainfluenza 2 and Parainfluenza 3). Those that had

negative results were separated and stored in freezer -70°C for subsequent DNA/RNA extraction for HTS.

RNA/DNA extraction - Fifty one samples from June, 55 from July and 39 from August of 2016 were mixed, respectively (one for each month), and treated as three pooled samples. Individual sample had a volume of approximately 0.5 mL (total of 20-25 mL of each pooled sample). The volume was adjusted to 50 mL with 0.1 M sodium phosphate buffer (pH7.2) with Triton-X 100 at the final concentration of 1%. The solution in tubes were agitated for 1 h in a cold chamber, then, centrifuged at $4,800 \times g$ for 40 min. The supernatant was ultracentrifuged with 20% sucrose cushion at $140,000 \times g$ for 1 h using Ti45 rotor (Optima L-90K Ultracentrifuge, Beckman Coulter, Brea, USA). The pellet was resuspended in 0.1 M sodium phosphate buffer and DNA/RNA was extracted from the resuspended pellet following the instructions of the High Pure Viral Nucleic Acid Kit (Hoffmann-La Roche, Basel, Switzerland), without addition of carrier RNA in the lysis buffer. Three DNA/RNA samples were subjected to HTS. Next, part of all three samples were treated with DNase I (Promega, Madison, USA) in order to decrease the human or bacterial DNA in the sample for viral RNA enrichment. However, due to low amount of RNA after DNase I treatment, all three samples were mixed as one pooled sample for RNA HTS.

High-throughput sequencing - Approximately two μg of DNA/RNA and RNA samples in RNASTable tube (Biomatrica, San Diego, USA) were sent for HTS to Macrogen Inc. (Seoul, South Korea). The library of DNA/RNA or RNA samples was constructed using TruSeq Standard total RNA kit and sequenced using Illumina HiSeq 2000 with 3 G scale for each in 100 base paired-end.

Bioinformatics analysis - Low quality and adapter sequences of the raw data were trimmed using the Trimmomatic tool v.0.36.⁽¹⁵⁾ Metagenomic reads of each sample were then uploaded to Kaiju for profiling the reads in taxonomic assessment (<http://kaiju.binf.ku.dk>).⁽¹⁶⁾ DNA reads were filtered with BWA v0.7.17⁽¹⁷⁾ and SAMtools v1.9⁽¹⁸⁾ against reference hg38 (*Homo sapiens*) obtained from GenBank to remove human DNA sequences from HTS dataset *in silico*. Trimmed reads were assembled into contiguous sequences ('contigs') with Velvet v.1.2.1⁽¹⁹⁾ and SPAdes v.3.9⁽²⁰⁾ programs. The tBlastx search (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) against the viral genome package (RefSeq Virus, NCBI, <https://www.ncbi.nlm.nih.gov/genome/viruses/>) which is implemented in the Geneious R8.1 program (Biomatters, Auckland, New Zealand) was used to identify the contig sequences which were viral origins.

RT-qPCR using PR21 kit - A part of findings by HTS analysis was confirmed by means of RT-qPCR. The RNA was extracted from original 145 samples using the Magna Pure LC Total Nucleic Acid Kit (Hoffmann-La Roche, Basel, Switzerland), then a pool of five samples were projected to the multiplex RT-qPCR using the XGen PR21 kit (Biometrix, Curitiba, Brazil) for 21 respiratory pathogens (targeting Influenza A, Influenza B, Influenza A H1N1-sw1, Coronavirus NL63, Coronavirus 229E, Coronavirus OC43, Coronavirus HKU1, Parainfluenza 1, Parainfluenza 2, Parainfluenza 3, Parainfluenza 4, Human metapneumovirus A and B, Human respiratory syncytial virus A and B, Rhinovirus, Enterovirus, Parechovirus, Human adenovirus, Bocavirus and *Mycoplasma pneumoniae*).

Ethics approval and consent to participate - This research project was reviewed and approved by ethics committee of the Faculty of Health Sciences (University of Brasília), approval letter No. 3.052.443 (in Appendix 1 of this thesis).

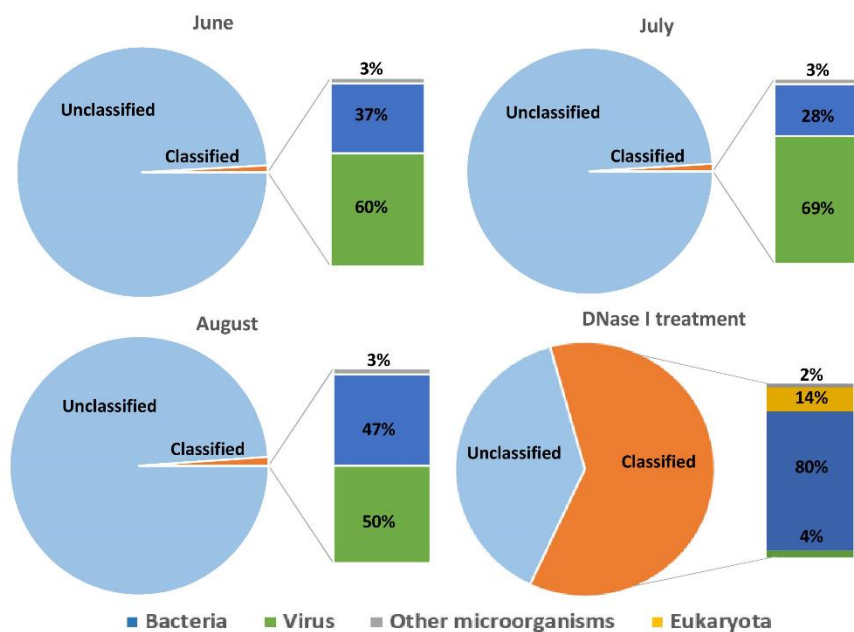
Results

Kaiju program is used for microbial and viral sequence profiling. For June samples a total of 87,317 (0.87%) out of 10,007,522 reads were classified as microbial sequences including virus; for July samples, 79,050 (0.96%) out of 8,215,186; for August samples 91,485 (1.11%) out of 8,271,632 and for the DNase I-treated RNA sample, 401,596 (61.26%) out of 655,558 reads were classified.

The analyses showed that nasopharyngeal secretion pooled samples of June, July and August of 2016 had similar metagenomic profiles in relation to the components of microorganisms classified found: predominance of viruses (supplementary Fig. 1), followed by bacteria and other microorganisms, such as Archaeas. However, the higher recovery of viral sequences in DNA/RNA samples was considered as misinterpretation by Kaiju program since the later fine analyses using BLASTn found that many of the human DNA sequences present in the samples were recognized as DNA viruses due to the similarity of some genomic regions. After *in silico* filtration of the reads by human DNA sequences, 77.26% of reads were classified as microbial sequences for June (274,269 out of 354,975), 81.22% for July (242,730 out of 298,846) and 82.50% for August (312,605 out of 378,895). This result showed that the contamination of human DNA was problematic for virus sequence search in these samples. As the human chromosomal and microbial DNA masked the presence of RNA viruses in the total DNA/RNA samples, the amount of DNA was reduced by DNase I treatment and the samples were subjected to the HTS again as one RNA sample.

After this treatment, the microorganism profile by Kaiju program had changed, where 4% of the sample was viruses unlike the DNase I-untreated samples (Supplementary Fig. 1). It is noted that the main microorganisms found in the RNA

sample after DNase I treatment were bacteria (80%) (Supplementary Fig. 1). The main bacterial family identified were *Burkholderiaceae*, *Streptococcaceae*, *Neisseriaceae*, *Veillonellaceae*, *Pseudomonadaceae*, *Pasteurellaceae*, *Veillonellaceae* and *Mycoplasmataceae*.



Supplementary Figure 1. Microorganism classified profiles found by Kaiju program in the DNA/RNA preparations of the nasopharyngeal and tracheal secretion samples of June, July and August (2016) and the RNA preparation treated with DNase I.

The RNA sample was the one that allowed the greater identification of RNA viruses. Therefore, the fine analyses of virus identification were performed using only the RNA sample, since most respiratory viruses possess RNA genomes. A total of 14,952

reads was classified as virus organisms. The main viruses identified using the Kaiju program were of the families *Picornaviridae*, *Herpesviridae*, *Parvoviridae* and *Coronaviridae*, with the emphasis on rhinoviruses (Table 1). The same viral families were also found in the DNA/RNA sample reads after the removal of human DNA sequences *in silico*, however, with less frequency. Only cytomegalovirus sequences were found more in DNA/RNA samples than RNA sample (Table 1), probably due to the negative effect on cytomegalovirus DNA genomes by DNaseI treatment in preparation of RNA sample.

Table 1. Human virus found in DNA/RNA and RNA sample (treated with DNase I) using Kaiju program.

Genetic material	Family	Subfamily	Genus	Specie	Total reads	
					DNA/RNA	RNA
*dsDNA	<i>Herpesviridae</i>	<i>Betaherpesvirinae</i>	<i>Cytomegalovirus</i>		387	40
ssDNA	<i>Parvoviridae</i>	<i>Parvovirinae</i>	<i>Bocaparvovirus</i>		6	18
ssRNA	<i>Coronaviridae</i>	<i>Orthocoronavirinae</i>	<i>Betacoronavirus</i>		-	16
ssRNA	<i>Picornaviridae</i>	-	<i>Enterovirus</i>	<i>Rhinovirus B</i>	26	1,087
ssRNA	<i>Picornaviridae</i>	-	<i>Enterovirus</i>	<i>Rhinovirus A</i>	19	995
ssRNA	<i>Picornaviridae</i>	-	<i>Enterovirus</i>	<i>Rhinovirus C</i>	90	2,721
ssRNA	<i>Picornaviridae</i>	-	<i>Enterovirus</i>	<i>Enterovirus J</i>	-	1

*ds = double strand, ss = single strand

Due to low accuracy of the short-read profiling by Kaiju program, the classification up to genus level was shown in this table, except for the *Enterovirus* genus, which was the one with the most reads founded, allowing the virus species definition.

To identify the contig sequences (1,621 contigs assembled by Velvet and 334 by SPAdes assemblers) of viral origin, tBlastx search plugged-in Geneious was used for the known virus genome sequences recorded in the RefSeq Virus (NCBI).

The analyses identified virtually the same viral families/genera found by the Kaiju program: *Picornaviridae*, *Herpesviridae* and *Parvoviridae* using contigs generated by both SPAdes (Table 2) and Velvet assemblers (Table 3). Only the *Coronaviridae* family was not identified by tBlastx in Geneious (but identified by Kaiju), probably due to limited sensitivity of *de novo* contig assembly, although we chose the most sensitive ones, Velvet and SPAdes.⁽²¹⁾

Table 2. Classification of human virus found in RNA sample (treated with DNase I) by tBlastx using SPAdes assembler.

Genetic material*	Family	Genus	Species	Contigs number	Alignment length	Query coverage (%)	Number of reads per contig	E-value
ssRNA	<i>Picornaviridae</i>	<i>Enterovirus</i>	<i>Rhinovirus B</i>	7	93 - 579	46.80 - 98.63	47 - 1,194	2.72×10^{-142} - 1.67×10^{-42}
ssRNA	<i>Picornaviridae</i>	<i>Enterovirus</i>	<i>Rhinovirus A</i>	12	177 - 1,158	53.96 - 99.48	11 - 254	3.81×10^{-76} - 1.99×10^{-11}
ssRNA	<i>Picornaviridae</i>	<i>Enterovirus</i>	<i>Rhinovirus C</i>	10	225 - 1,239	64.71 - 99.68	4 - 2,470	5.38×10^{-148} - 4.37×10^{-32}
ssRNA	<i>Picornaviridae</i>	<i>Enterovirus</i>	<i>Enterovirus B</i>	1	174	80.93	43	2.30×10^{-16}
ssRNA	<i>Picornaviridae</i>	<i>Enterovirus</i>	<i>Enterovirus C</i>	1	231	97.88	36	9.96×10^{-26}

*ss = single strand

Table 3. Classification of human virus found in RNA sample (treated with DNase I) by tBlastx using Velvet assembler.

Genetic material*	Family	Genus	Species	Contigs number	Alignment length	Query coverage (%)	Number of reads per contig	E-value
dsDNA	<i>Herpesviridae</i>	<i>Cytomegalovirus</i>	<i>Human betaherpesvirus 5</i>	3	96 - 153	95.05 - 96.84	23 - 24	3.15×10^{-32} - 4.76×10^{-16}
ssDNA	<i>Parvoviridae</i>	<i>Bocaparvovirus</i>	<i>Primate bocaparvovirus 1</i>	4	96 - 99	95.05 - 98.02	8 - 13	2.15×10^{-18} - 1.34×10^{-16}
ssRNA	<i>Picornaviridae</i>	<i>Enterovirus</i>	<i>Rhinovirus B</i>	29	93 - 570	63.00 - 100.00	3 - 668	2.01×10^{-117} - 3.20×10^{-11}
ssRNA	<i>Picornaviridae</i>	<i>Enterovirus</i>	<i>Rhinovirus A</i>	62	93 - 435	53.57 - 100.00	4 - 257	2.65×10^{-68} - 6.05×10^{-11}
ssRNA	<i>Picornaviridae</i>	<i>Enterovirus</i>	<i>Rhinovirus C</i>	54	87 - 885	64.15 - 100.00	11 - 962	1.23×10^{-152} - 7.86×10^{-11}
ssRNA	<i>Picornaviridae</i>	<i>Enterovirus</i>	<i>Enterovirus B</i>	1	174	80.93	43	2.30×10^{-16}
ssRNA	<i>Picornaviridae</i>	<i>Enterovirus</i>	<i>Enterovirus C</i>	1	117	77.48	72	1.05×10^{-13}
ssRNA	<i>Picornaviridae</i>	<i>Enterovirus</i>	<i>Enterovirus E</i>	1	99	98.02	9	2.33×10^{-11}

*ss = single strand, ds = double strand

It is observed in Table 2 (contigs assembled by SPAdes) that the number of contigs varied from 1 (*Enterovirus B* and *C*) to 12 (*Rhinovirus A*), with sizes of alignment between 93 and 1,239 nucleotides. The number of reads per contig also varied greatly, from 4 to 2,470 with the highest amount of reads per contig found in the *Rhinovirus C* species. The e-values from 5.38×10^{-148} to 1.99×10^{-11} indicates statistical significance in the alignments found using the SPAdes as assembler.

Table 3 shows the tBlastx results of contigs assembled by Velvet. The number of contigs varied between 1 (*Enterovirus B, C* and *E*) and 62 (*Rhinovirus A*) with sizes of alignment between 87 and 885 nucleotides. The number of reads per contig ranged from 3 to 962, with the highest number of reads found also for *Rhinovirus C*. E-values from 1.23×10^{-152} to 2.33×10^{-11} were also low, showing statistical significance of the alignment. The comparison of Tables 2 and 3 reveals that, when using Velvet as assembler, more contigs were formed, although with smaller length and fewer reads per contig. But this is important to identify more viruses in less abundance, as *Human betaherpesvirus 5*, Human bocavirus and *Enterovirus E* that have been identified using Velvet, but not when using SPAdes as assembler.

The Rhinovirus sequences showed greater abundance by the presence of reads in the sample, especially *Rhinovirus C*, that presented highest reads number (observed in Kaiju analysis), and the highest reads number per contig (in tBlastx analysis). This shows the relevance of this one virus in these negative samples by the standard RT-qPCR.

The presence of respiratory viruses in the samples was confirmed by the PR21 multiplex RT-qPCR kit, which identifies 21 respiratory pathogens. Coronavirus 63 (CT = 30), coronavirus 43 (CT = 22-31), coronavirus HKU 1 (CT = 20-33), enterovirus (CT = 33), bocavirus (CT = 19-35) and mainly rhinovirus (CT = 21-36) were found (Figure

1). The RT-qPCR assay showed the presence of coronaviruses which detection was failed by the HTS analyses.

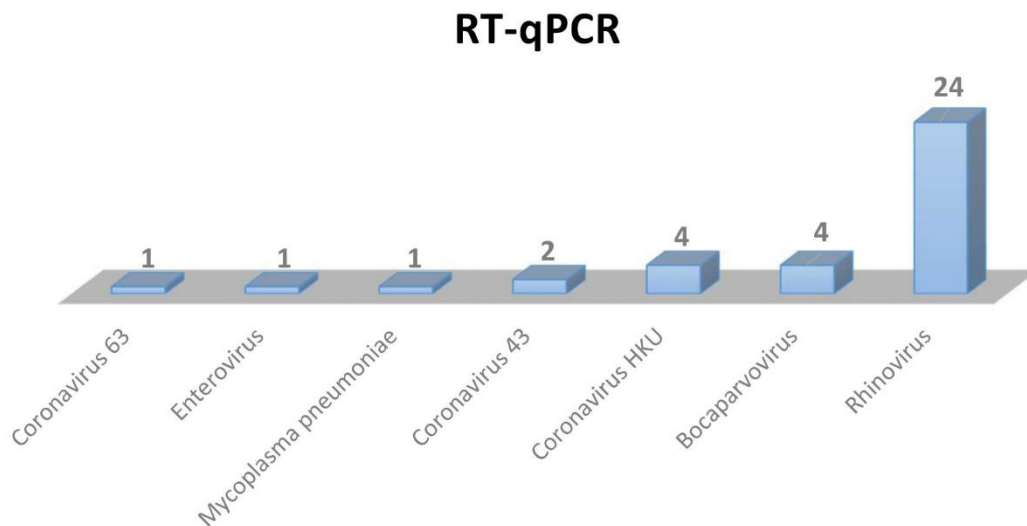


Figure 1. Respiratory pathogens found by PR21 multiplex RT-qPCR in nasopharyngeal and tracheal secretion samples which were negative by the standard RT-qPCR of influenza-like surveillance on June, July and August collected in Brasília, 2016.

Discussion

The HTS has been successfully applied in several fields of virology, including virus discovery, complete virus genome sequence determination, and genomic variant analyses.⁽²²⁾

Lacen-DF performs diagnosis of the respiratory virus by the standard RT-qPCR protocol with restricted targets, thus, many viruses may not be identified. The samples referred to Lacen are from patients of public and private hospitals with respiratory virus

infection suspected, whether hospitalized or not. Approximately 60% of the samples forwarded are negative for the virus detection.

In this study, *Human betaherpesvirus 5*, *Primate bocaparvovirus 1*, *Betacoronavirus*, *Enterovirus* and three species of rhinovirus were found by at least 2 different types of analyses in samples of patients from the DF, Brazil, with flu symptoms. *Human betaherpesvirus 5* (Cytomegalovirus) was identified by analyses with Kaiju and Geneious program, but not with PR21 RT-qPCR because specific primers for this pathogen were not included in the kit. DNA viruses as herpesvirus and parvovirus were also found in the sample treated with DNaseI. We assume that DNase I treatment doesn't exclude totally DNA in the sample, thus, herpesvirus and parvovirus reads were recovered. Coronavirus was identified with the Kaiju program but not by the contig analyses by tBlastx. However, its presence was confirmed by RT-qPCR of PR21 kit. Bocavirus (or *Primate bocaparvovirus 1*), and *Enterovirus* was also identified by PR21 RT-qPCR, Kaiju and Geneious programs only using Velvet, which is more sensitive method than SPAdes for contig assembly. *Mycoplasma pneumoniae* was found by PR21 RT-qPCR and many bacteria of the genus mycoplasma was also identified using the Kaiju program. Rhinovirus was identified using all methods used in this study.

These variations in results are explained by the differences in specificity and sensitivity in each method. For some cases, the limited sensitivity of HTS is explained by mixing some clinical samples forming one pooled sample for the cost reduction. Therefore, this sample preparation may mask the presence of viruses in low incidence or concentration. The limited capacity of *de novo* assembly programs is still a main problem in metagenomic analyses. Velvet and SPAdes *de novo* assemblers were used to build contigs in this study since these were reported as sensitive contig assembler for viral metagenomic study⁽²¹⁾, although there was no best *de novo* assembler concluded by the

same authors. HTS was reported to be less sensitive than RT-qPCR for some respiratory virus detection in such reasons.⁽²³⁾

Rhinovirus was the major viruses found in patients in the DF, Brazil with ILS or SARS samples which were negative by standard RT-qPCR of influenza-like surveillance (targeting Influenza A and B, Respiratory Syncytial Virus, Human Metapneumovirus, Adenovirus, Parainfluenza 1, 2 and 3). Several studies have shown the importance of rhinoviruses in acute respiratory infection in Brazil, especially in children.^(24, 25, 26) A study involving 120 children less than 12 years old in São Paulo, Brazil, found that human rhinoviruses (HRV) of species A, B, and C were the most frequent agents of acute respiratory infections (ARI). Moreover, these agents are also associated with up to 70% of virus-related wheezing exacerbations. The most recently identified *Rhinovirus C* has been detected in association with bronchiolitis, wheezing, and asthma exacerbations requiring hospitalization.⁽²⁷⁾

Other study made with hospitalized patients at an academic care center in Southern Brazil shows that HRV was usually detected in hospitalized children with respiratory infections and was often present in viral co-detections, mainly with enterovirus and respiratory syncytial virus. Comorbidities are closely associated with HRV infections, and this virus predominates during colder seasons,⁽²⁸⁾ period of which the samples used in this work were collected (winter). In investigations around the world, HRV, as well as agents causing acute respiratory infections, has its peak activity associated with the colder periods of the year.^(29, 30)

In this study, the relevance of HRV in public health in Brazil was again recognized and we suggest the inclusion of HRV for influenza-like surveillance.

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Capítulo 3

3.1 Molecular and clinical characteristics related to rhinovirus infection in Brasília, Brazil

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Abstract

Human rhinovirus (HRV) is one of the most common human viral pathogens related to infections of the upper and lower respiratory tract, which can result in bronchiolitis and pneumonia. However, the relevance of HRV in human health was under-estimated for long time due to the absence in targets for Influenza and Influenza-like syndrome surveillance in Brasilia, Brazil. In this study, the clinical characteristics and outcomes of HRV-positive patients, in comparison with patients without HRV and other common respiratory viruses, were analyzed. For this purpose, new specific primer sets were designed, in previous study, based on the high-throughput sequencing analysis and used for HRV detection by RT-qPCR and Sanger sequencing of amplified cDNA of 5' genomic region. The phylogeny analysis of representative HRV isolates was performed. Statistical analysis considering the patient profiles was also performed using IBM SPSS program with non-parametric tests. The most prevalent virus, in previously negative samples for the Influenza and Influenza-like Syndrome surveillance, was rhinovirus (n = 40), including three rhinovirus species (rhinovirus A, B and C). The odds ratio associated with HRV infection was 2.160 for patients younger than 2 years and 4.367 for people living in rural areas. The main symptom associated with virus infection was rhinorrhea. The multiple analysis showed association also for lower cases of HRV patients presenting respiratory distress. In this study, it was reported the predominance of rhinoviruses in cases of respiratory illness for negative patients for the Influenza and Influenza-like Syndrome surveillance, being rhinorrhea, the most significant symptom associated with the disease.

Key words: Rhinovirus – epidemiology - acute respiratory infections – symptoms

Introduction

Human rhinovirus (HRV) is one of the most common human viral pathogens. They are non-enveloped single-stranded RNA virus, member of *Enterovirus* genus, of *Picornaviridae* family [1, 2]. About half of common cold are caused by rhinoviruses, which have more than 160 serotypes described, in three species (rhinovirus A, B and C) [3]. In addition, cold leads to economic problems related clinical morbidity, overloading the health system, also causing absences at work and in schools [2, 4].

Rhinoviruses cause not only upper respiratory tract infections, but also lower respiratory tract infections, mainly in children and patients with chronic pulmonary disease or immunocompromised [5]. The most common symptoms of upper infection are sore throat, rhinorrhea, headache, cough, malaise and lower fever. However, in some cases, there may be aggravation for rhinosinusitis and otitis media [6, 7]. Infections of the lower respiratory tract are often associated with bronchiolitis and pneumonia especially in children [8-10].

In Brazil, the implementation of the sentinel surveillance system began in 2000 [11], so referral hospitals have been conducting active surveillance to detect respiratory viruses. Such surveillance includes notification and laboratory investigation of cases with the diagnostic criteria of Influenza-like Syndrome (ILS) and Severe Acute Respiratory Syndrome (SARS). This viral respiratory infection monitoring has resulted in important information about the circulation of community-acquired respiratory viruses. However, some viruses may not be identified by the absence of RT-qPCR target performed at central laboratories.

The present study reports the molecular and clinical characteristics, and outcomes of HRV infections in comparison with patients without HRV and other common respiratory viruses.

Materials and Methods

Detection of HRV by RT-qPCR - The nasopharyngeal and tracheal secretion samples received in the laboratory in June, July and August 2016 were tested for the respiratory virus diagnostic panel performed in Lacen-DF (Central Laboratory at Federal District, Brasilia, Brazil) by the standard RT-qPCR [12] (for influenza A virus, influenza B virus, human orthopneumovirus, human metapneumovirus, human mastadenovirus C, human respirovirus 1, human orthorubulavirus 2 and human respirovirus 3). The target genes of the standard RT-qPCR are shown in the Supplementary table 1. During transport, the samples were stored on ice and sent to the laboratory within 48 hours after collection. Those that had negative results were separated and stored in freezer at -70 °C for subsequent DNA/RNA extraction. The metagenomic analyses by high throughput sequencing (HTS) of those samples as one pooled virome was previous studied [13]. Based on this study, HRV universal primers targeting conserved regions in 5' UTR (untranslated region) were newly designed for HRV detection (Table 1) using the Geneious R8.1 program (Biomatters, Auckland, New Zealand). The RNA was extracted from 145 samples using the Magna Pure LC Total Nucleic Acid Kit (Hoffmann-La Roche, Basel, Switzerland), then all the 145 samples were projected to the RT-qPCR using GoTaq® Probe 1-Step RT-qPCR System (Promega, Madison, USA). Positivity for rhinovirus was defined with cycle threshold (CT) less than or equal to 40 (total of 45 cycles in the reaction). Human RNase P gene was used for endogenous control for the same sample set in separate form.

Supplementary table 1 The target genes of the standard RT-qPCR performed in Lacen-DF, Brasilia, Brazil

Name	Gene target
Influenza A virus	matrix gene (pos1) in segment 7
Influenza B virus	segment 8 NS1/NEP
Human orthopneumovirus	nucleocapsid protein (N) gene
Human metapneumovirus	fusion glycoprotein (F) gene
Human mastadenovirus C	hexon gene
Human respirovirus 1	hemagglutinin-neuraminidase (HN) mRNA
Human orthorubulavirus 2	hemagglutinin-neuraminidase (HN) mRNA
Human respirovirus 3	hemagglutinin-neuraminidase (HN) mRNA

Table 1 Rhinovirus primers and probe for RT-qPCR and amplicon sequencing

Name	Sequence	Use
HRV_Probe	5'- TCCGGCCCCTGAATGYGGCT - 3'(FAM/BHQ1)	RT-qPCR
HRV_For	5'- YCYAGCCTGCGTGGC - 3'	RT-qPCR
HRV_Rev	5'- ACACGGACACCCAAAGTAGT - 3'	RT-qPCR
HRV_Com_For	5'- ACTACTTTGGGTGTCCGTGT - 3	Amplicon sequencing
HRV_Com_Rev	5'-TCNGGHARYTTCCARCACC - 3	Amplicon sequencing

RT-PCR and Sanger sequencing - The HRV_Com_For and HRV_Com_Rev primers were newly designed (Table 1) targeting conserved regions based on virome analyses by HTS. This primer pair was used to amplify an approximately 540 bp fragment for amplicon sequencing including part of 5' UTR and the VP4 / VP2 protein gene of HRV A, B and C. cDNA of the selected samples were synthesized using MMLV transcriptase (Thermo Fisher Scientific, Waltham, USA) with random hexamer primer and, then, amplified with LongAmp Taq DNA Polymerase (New England BioLabs, Ipswich, USA). The PCR product was purified and sequenced by the Sanger method at Macrogen Inc (Seoul, South Korea).

Sequence analysis - The nucleotide (nt) sequences were determined using the Geneious R8.1 program (Biomatters, Auckland, New Zealand). Complete rhinovirus genome sequences were retrieved from Genbank as references and aligned with the sequences obtained in this study. This alignment was again trimmed to set the sequence size determined in this study (approximately 500 bases, the size has small variation due to indel mutation). The phylogenetic tree based on the 5' UTR and the VP4 / VP2 protein gene was constructed using the Mega X software [14], with eight nucleotide sequences obtained in this study and other forty rhinovirus isolates that complete genome sequences were available (for genotype identification). Phylogenetic tree was inferred using maximum likelihood method with Hasegawa-Kishino-Yano model as indicated by jModelTest analysis [15]. In order to obtain more precise nt identity in pairwise comparison, three more related virus sequences for each obtained sequence in this study were retrieved by BlastN search and the pairwise comparison was performed.

Statistical analysis – Medical records were obtained from compulsory notification sheet for ILS and SARS sent with the sample to the laboratory. Data were analyzed using the software IBM SPSS (Chicago, USA). Non-parametric tests were used

as appropriate, for qualitative and quantitative variables. All *p*-values were two-tailed and a value of < 0.05 was considered significant.

Ethics approval and consent to participate - This research project was reviewed and approved by ethics committee of the Faculty of Health Sciences (University of Brasília), approval letter No. 3.052.443.

Results

Rhinoviruses were identified as the main virus found in samples that had previously tested negative for the panel of respiratory virus survey used in Lacen-DF, including influenza A virus, influenza B virus, human orthopneumovirus, human metapneumovirus, human mastadenovirus C, human respirovirus 1, human orthorubulavirus 2 and human respirovirus 3, in a previously conducted study (REF). The RT-qPCR using newly designed primers and probe used in the present study (Table 1) detected HRV in 40 out of 145 samples analyzed. The mean CT scan was about 28 ranging from 17 to 39. These primers targeted the 5' UTR region (Table 1), which is most conserved among rhinoviruses.

To identify HRV at species level, amplicons obtained with primers targeting a region of approximately 540 bp of the 5' UTR-VP4 / VP2 protein gene region were sequenced. Five samples were concluded of HRV-A, one of HRV-B and two of HRV-C (Fig. 1), out of eight sequenced samples of the patients in the Federal District during the study period. The other samples were not of sufficient quality for sequencing. All sequences were deposited in the databank: rhinovirus A BSB_8 (Accession number LC549198), rhinovirus A BSB_16 (LC549199), rhinovirus A BSB_26 (LC549200), rhinovirus A BSB_33 (LC549201), rhinovirus A BSB_38 (LC549202), rhinovirus B

BSB_30 (LC549203), rhinovirus C BSB_2 (LC549204), rhinovirus C BSB_15 (LC549205). It is interestingly that though small number of samples sequenced in this study, the genetic variability of HRV-A was very wide, positioning in five different clusters in the phylogenetic tree (Fig. 1). Among them, only nt identities of 72.94-83.86% were observed (Table 2). On the other hand, two isolates of HRV-C were very similar, possessing 98.99% of nt identity.

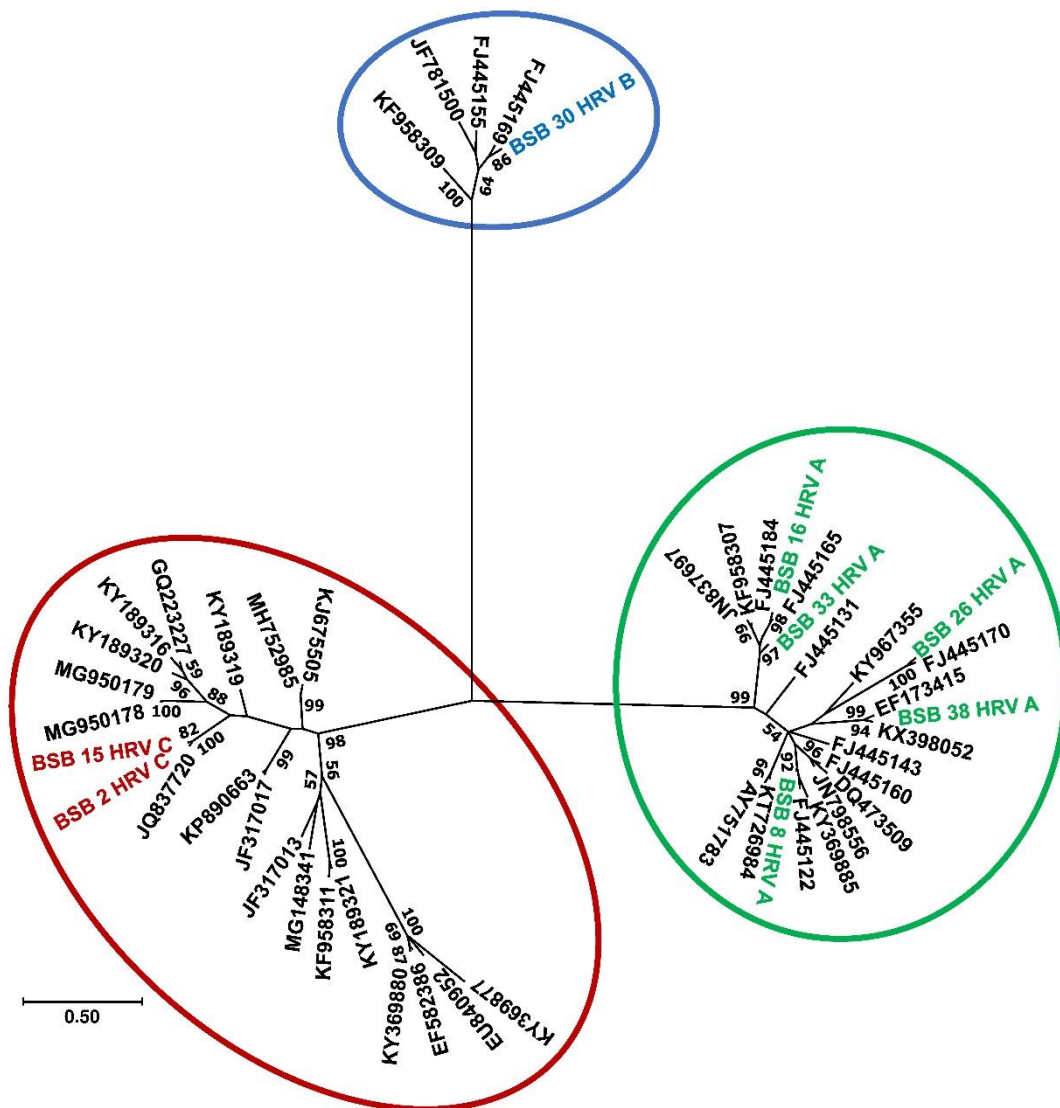


Figure 1 Phylogenetic tree of rhinoviruses based on 5' UTR and the VP4 / VP2 protein gene (approximately 500 bases). The tree was construct using Hasegawa-Kishino-Yano model of the maximum likelihood method using 48 isolates, which eight were from the Federal District, Brazil and the other 40 were genome sequences available on databases.

Bootstrap values above 50 (500 repetitions), and accession number of each sequence are indicated. Bar: number of substitutions per site. Green circle: *Rhinovirus A*, blue circle: *Rhinovirus B* and red circle: *Rhinovirus C*. The identification of isolate from the Federal District, Brazil are highlighted with colors used for species indication.

Table 2 Nucleotide identities (%) among HRV-A from Brasília, Brazil

	HRV-A				
	BSB_38	BSB_8	BSB_26	BSB_33	BSB_16
BSB_38		78.44	77.54	75.35	72.94
BSB_8	78.44		76.08	75.80	75.64
BSB_26	77.54	76.08		74.78	75.43
BSB_33	75.35	75.80	74.78		83.86
BSB_16	72.94	75.64	75.43	83.86	

By the identification of the country where the most related virus in the databases was isolated, we can observe that there is almost no relation in the geographical distribution (Table 3). The most related rhinovirus isolates came from several continents such as America, Asia, Europe and Oceania. This observation suggests that the dissemination of the rhinoviruses can happen worldwide in long distance and is different from the arbovirus geographical dissemination.

Table 3 The most related rhinovirus isolates with those from Brasília in this study

Isolate from Brasília	Best hit partner	Accession No. of reference	Origin	Pairwise comparison	Genotype
HRV-A BSB_8	Human rhinovirus A22 strain HRV-A22/Lancaster/2015	KY342346	United Kingdom	98.20%	A22
HRV-A BSB_16	Human rhinovirus A36 isolate A36/Singapore/1483/2010	MH648039	Singapore	96.36%	A36
HRV-A BSB_26	Human rhinovirus A isolate 12MYKLU0444 from Malaysia	KY094058	Malaysia	97.40%	nd*
HRV-A BSB_33	Human rhinovirus A isolate WM-09-156-1586	KF543936	Australia	98.80%	A58
HRV-A BSB_38	Rhinovirus A strain 20693_1_HRV-A	MK989737	Kenya	98.41%	A12
HRV-B BSB_30	Human rhinovirus B92 isolate B92/Singapore/1455/2010	MH648109	Singapore	97.00%	B92
HRV-C BSB_2	Human rhinovirus sp. strain C/Venezuela/IVE000070/2010	JX129430	Venezuela	95.75%	C17
HRV-C BSB_15	Human rhinovirus sp. strain C/Venezuela/IVE000070/2010	JX129430	Venezuela	95.55%	C17

*nd = not defined.

Patients positive (n = 40) and negative (n = 105) rhinovirus were compared in relation to socio-demographic and clinical parameters in order to evaluate possible risk factors associated with the involvement of these viruses. It can be observed in Table 4 that patients with rhinovirus were significantly different from patients negative for the main respiratory viruses in relation to age ($P = 0.042$) and to the area of residence ($P = 0.019$). Patients younger than 2 years presented 2.160 times more likely to be infected by rhinovirus than patients with 2 years old or more.

Table 4 Socio-demographic and clinical variables of patients with acute respiratory infection treated in the Federal District, Brazil, 2016

	Rhinovirus		<i>P</i> *	Odds ratio (IC 95%)
	<u>Yes</u> n (%)	<u>No</u> n (%)		
Month			0.505	-
June	15 (37.5)	36 (34.3)		
July	17 (42.5)	38 (36.2)		
August	8 (20.0)	31 (29.5)		
Diagnostic criteria			0.304	1.478 (0.701 – 3.119)
ILS	17 (42.5)	35 (33.3)		
SARS	23 (57.5)	70 (66.7)		
Sex			0.231	0.635 (0.301 - 1.339)
Male	15 (37.5)	51 (48.6)		
Female	25 (62.5)	54 (51.4)		
Categorized age			0.042	2.160 (1.021 - 4.567)
< 2 years	19 (47.5)	31 (29.5)		
≥ 2 years	21 (52.5)	74 (70.5)		
Breed			0.075	-
White	20 (54.1)	30 (33.7)		
Brown	14 (37.8)	53 (59.6)		
Black	3 (8.1)	6 (6.7)		
Zone			0.019	0.229 (0.061 - 0.860)
Urban	34 (85.0)	99 (96.1)		
Rural	6 (15.0)	4 (3.9)		
Risk factor			0.266	0.643 (0.294 - 1.404)
Yes	12 (30.0)	42 (40.0)		
No	28 (70.0)	63 (60.0)		
Vaccinated			0.625	1.222 (0.546 - 2.736)
Yes	12 (30.0)	27 (26.0)		
No	28 (70.0)	77 (74.0)		
Hospitalization			0.484	0.767 (0.365 - 1.612)
Yes	23 (57.5)	67 (63.8)		
No	17 (42.5)	38 (36.2)		
Medication			0.830	1.086 (0.510 - 2.313)
Oseltamivir	15 (37.5)	37 (35.6)		
No	25 (62.5)	67 (64.4)		
Use of ventilatory support			0.444	-
Yes, invasive	7 (17.5)	29 (27.6)		
Yes, no invasive	11 (27.5)	24 (22.9)		
No	22 (55.0)	52 (49.5)		

* Pearson's chi-squared test

In this study, patients with rhinovirus were significantly younger than patients without rhinovirus ($P = 0.042$) (Table 5). The median age for patients with the virus was 2.50 (interquartile range = 27.38) versus 26.00 (interquartile range = 50.13) years for patients without this virus. Thus, greater susceptibility of children to rhinovirus infection is reported. Figure 2 shows the difference between the groups with and without the virus in relation to the days of symptoms and age. It is observed that the days of symptoms did not differ significantly, but the age distribution of the patients was significantly lower in patients who presented rhinovirus respiratory symptoms ($P = 0.042$).

Table 5 Quantitative variables of patients with acute respiratory infection treated in the Federal District, Brazil, 2016

		Rhinovirus		<i>P</i> *
		<u>Yes</u>	<u>No</u>	
	Descriptive measures	n (%)	n (%)	
Age	n	40	105	0.042
	Mean	18.24	29.08	
	Median	2.50	26.00	
	Standard deviation	22.55	27.23	
	Minimum	0.05	0.02	
	Maximum	76.00	94.00	
	Interquartile range	27.38	50.13	
Days of symptoms	n	39	105	0.736
	Mean	5.36	5.38	
	Median	4.00	4.00	
	Standard deviation	4.56	5.25	
	Minimum	1.00	1.00	
	Maximum	19.00	41.00	
	Interquartile range	5.00	4.50	

* Mann-Whitney test

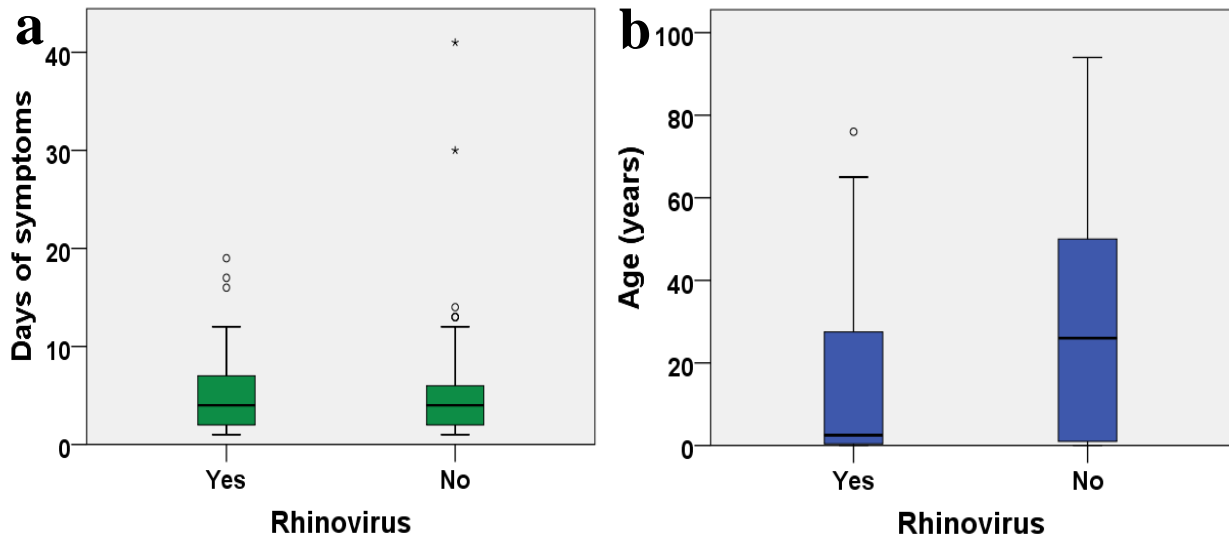


Figure 2 Box-plot of days of symptoms (a) and age (b) of patients with acute respiratory infection treated in the Federal District, Brazil, 2016. The comparison shows patients with and without rhinovirus infection, where only the age difference was statistically significant ($P = 0.042$). Extreme outliers are marked with an asterisk and moderate outliers with a dot.

Patients with rhinovirus were located significantly more in rural areas when compared to negative patients. The odds ratio for the rural area was 4.367 (1/0.229), which means that people resident in rural areas were 4.367 times more affected by rhinovirus when compared to urban dwellers.

Regarding the main symptoms of patients with acute respiratory infection, it can be observed in Table 6 that patients with rhinovirus presented significantly more rhinorrhea in relation to the patients without this virus detected ($P = 0.001$). Patients with rhinovirus presented 3.981 times more cases of presenting rhinorrhea in relation to negative patients. There was no statistically significant difference in relation to the other symptoms.

Table 6 Symptoms of patients with acute respiratory infection treated in the Federal District, Brazil, 2016

	Rhinovirus		<i>P</i> *	Odds ratio (IC 95%)
	<u>Yes</u> n (%)	<u>No</u> n (%)		
Fever			0.406	1.455 (0.599 - 3.532)
Yes	32 (80.0)	77 (73.3)		
No	8 (20.0)	28 (26.7)		
Cough			0.392	2.452 (0.524 - 11.479)
Yes	38 (95.0)	93 (88.6)		
No	2 (5.0)	12 (11.4)		
Sore throat			0.213	1.595 (0.763 - 3.334)
Yes	19 (47.5)	38 (36.2)		
No	21 (52.5)	67 (63.8)		
Dyspnea			0.157	0.585 (0.278 - 1.233)
Yes	22 (55.0)	71 (67.6)		
No	18 (45.0)	34 (32.4)		
Respiratory distress			0.133	0.567 (0.269 - 1.195)
Yes	15 (37.5)	54 (51.4)		
No	25 (62.5)	51 (48.6)		
Myalgia			1.000	1.000 (0.402 - 2.485)
Yes	8 (20.0)	21 (20.0)		
No	32 (80.0)	84 (80.0)		
O ₂ saturation < 95%			0.115	0.484 (0.194 - 1.209)
Yes	7 (17.5)	32 (30.5)		
No	33 (82.5)	73 (69.5)		
Rhinorrhea			0.001	3.981 (1.835 - 8.638)
Yes	27 (67.5)	36 (34.3)		
No	13 (32.5)	69 (65.7)		

* Pearson's chi-squared test

A multiple logistic regression with 20 explanatory variables (age, days of symptoms, month, diagnostic criteria, sex, breed, zone, risk factor, vaccinated, hospitalization, medication, use of ventilatory support, fever, cough, sore throat, dyspnea, respiratory distress, myalgia, O₂ saturation < 95% and rhinorrhea) was performed to analyze the variables associated with rhinovirus infection (response variable). In the

model containing only the constant, there was 71% agreement with the classification table. When entering the explanatory variables, the concordance increased to 79.8%.

The regression model was statistically significant ($P = 0.005$), with adequate adjustments ($P = 0.862$ - Hosmer's test and Lemeshow) and $R^2 = 0.391$. To improve the model, a technique of variable selection was used by the backwise stepwise method. The best fit was obtained after 15 steps of exclusion of variables, remaining six explanatory variables. The new model was also statistically significant ($P < 0.001$), with optimal adjustment ($P = 0.923$ - Hosmer and Lemeshow test) and $R^2 = 0.319$. Three significant explanatory variables were obtained: respiratory distress, rhinorrhea and medication (Table 7).

Table 7 Logistic regression with variables selection of patients with acute respiratory infection treated in the Federal District, Brazil, 2016

	B	S.E	Wald	D.F	P	Exp(B)	95% C.I. for exp(B)	
							Inferior	Superior
Sex	0.89	0.48	3.41	1	0.065	2.446	0.946	6.326
Breed	-0.67	0.41	2.67	1	0.102	0.513	0.231	1.143
Zone	1.41	0.88	2.60	1	0.107	4.114	0.738	22.931
Respiratory distress	-1.09	0.56	3.86	1	0.049	0.335	0.113	0,997
Rhinorrhea	1.89	0.49	14.62	1	0.000	6.601	2.509	17.366
Medication	1.36	0.59	5.36	1	0.021	3.892	1.232	12.295
Constant	-3.74	1.38	7.30	1	0.007	0.024		

Abbreviations: B, [Beta coefficient]; S.E, [Standard Error]; Wald, [Wald test]; D.F, [Degree of freedom]; P, [p-value]; Exp(B), [Beta exponential]; C.I., [Confidence interval]

Patients with rhinovirus presented 2.94 (1/0.34) times less chance of experiencing respiratory distress, 6.60 and 3.89 times more chance of having rhinorrhea and using medication (Oseltamivir), respectively. Thus, it is observed that the main symptoms related to rhinovirus infection were the presence of rhinorrhea and absence of respiratory discomfort. The use of antiviral medication, Oseltamivir, was also significantly more observed in patients with rhinovirus in the multiple analysis.

To assess how respiratory symptoms were associated in patients with rhinovirus infection, a cluster analysis was performed with the 40 positive patients, using dendrogram with Ward method and measure of the quadratic Euclidean distance. It is observed that two groups of symptoms were mainly formed: fever, cough and rhinorrhea in the first group and myalgia, O₂ saturation < 95%, respiratory distress, dyspnea and sore throat in the second group) (Fig. 3). The symptoms of the first group may be associated with cases of common cold (upper airways infection), and those of the second group, with cases of complications of infection in the lower respiratory tract.

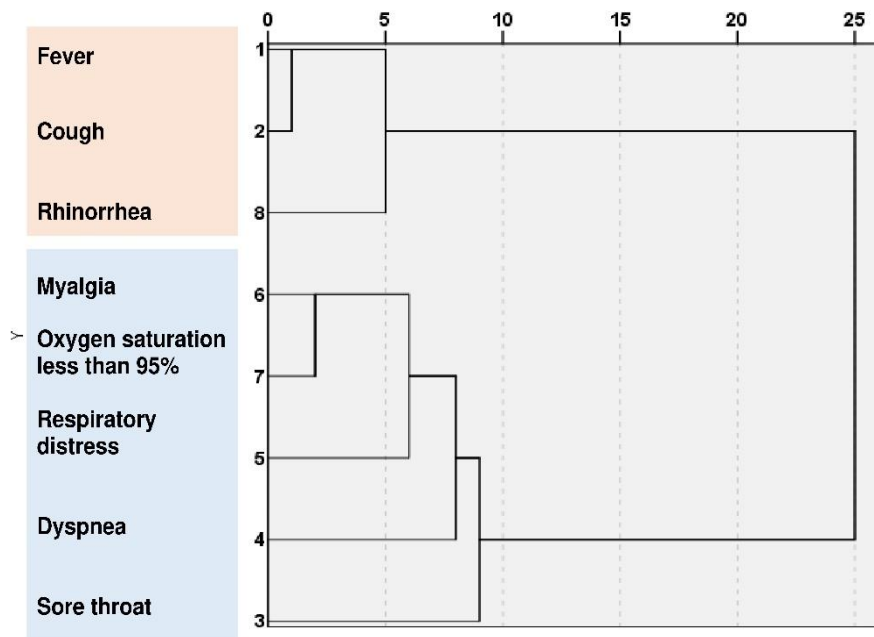


Figure 3 Cluster analysis of symptoms of rhinovirus patients treated in the Federal District, Brazil, 2016. The analysis shows the division into two groups that suggest infection in the upper (orange) and lower (blue) respiratory tract.

To investigate whether age was not a covariate of rhinorrhea, the Mantel-Haenszel test was performed, which evaluated the association between rhinorrhea symptom and age-corrected rhinovirus. It was observed that the association between rhinorrhea and rhinovirus occurred in the group of patients older than 2 years ($P = 0.001$), but not in the group younger than 2 years ($P = 1,000$) (Supplementary table 2), that is, children with and without rhinovirus presented rhinorrhea in a similar way. The Mantel-Haenszel test showed odds ratio = 3.865 (1.483 - 10.076; $P = 0.005$), which shows that even if adjusted for age, rhinorrhea was statistically significant to the rhinovirus, where rhinovirus patients had 3.865 times more chance of presenting this symptom compared with patients without the virus.

Supplementary table 2 Association between the rhinorrhea symptom and the presence of rhinovirus adjusted for age

Age		Rhinovirus			<i>P</i> *	
		Yes	No	Total		
			n (%)	n (%)	n (%)	
< 2 years	Rhinorrhea	Yes	17 (89.5)	27 (87.1)	44 (88.0)	1.000
		No	2 (10.5)	4 (12.9)	6 (12.0)	
	Total		19 (100.0)	31 (100.0)	50 (100.0)	
> ou = 2 years	Rhinorrhea	Yes	10 (47.6)	9 (12.2)	19 (20.0)	0.001
		No	11 (52.4)	65 (87.8)	76 (80.0)	
	Total		21 (100.0)	74 (100.0)	95 (100.0)	
Total	Rhinorrhea	Yes	27 (67.5)	36 (34.3)	63 (43.4)	0.001
		No	13 (32.5)	69 (65.7)	82 (56.6)	
	Total		40 (100.0)	105 (100.0)	145 (100.0)	

* Pearson's chi-squared test

Discussion

Since the beginning of the monitoring of respiratory virus circulation in the country, rhinovirus has been found with high frequency, either alone or co-detected with other respiratory viruses, mainly in the south and southeast regions [16, 17]. To date, however, the circulation of these viruses in the central region of the country has not been well studied. Therefore, the identification of these viruses is crucial to determine the etiologic agent of respiratory illness. In this case, the 5' UTR region is the most suitable to identify the virus by RT-qPCR, and this region has already been used by other laboratories with similar oligonucleotides [18, 19].

Rhinoviruses are reported to be the main cause of the common cold and spread from person to person via direct and indirect contact, mainly by infected respiratory secretions. The transmission by hands is important in this context [20]. The easy

contagion of rhinovirus can explain its rapid spread, over long distances, as shown in Table 3.

HRV infections were associated with diseases such as asthma, chronic pulmonary disease, bronchiolitis and pneumonia in children, the elderly and immunocompromised. Thus, proper clinical management, timely diagnosis and containment of outbreaks are becoming important [5, 16].

Rhinovirus infection was significantly associated with residency in rural areas. In this study, 7% came from rural areas, and among those infected by the virus the percentage was 15%. A study in Brazil has already reported that children from urban areas of school age are more susceptible to respiratory inflammatory problems compared to those in rural regions [21]. Thus, the negative cases for rhinovirus and other common respiratory viruses may be mainly associated with allergic inflammatory processes, which is more frequent in urban areas. With this, rural patients would present respiratory problems related mainly to viral infections, as observed in relation to HRV. In the multiple analysis, the relationship between rural residence and rhinovirus was not observed.

Patients younger than 2 years were more subject to HRV infection and had a significantly lower proportion of cases with fever, and more comorbidities such as asthma than patients without HRV [16, 22, 23]. In this study, it was observed that children younger than two years were more susceptible to rhinovirus infection, but there was no association with comorbidities. The main symptom associated with HRV was rhinorrhea rather than low frequency of fever. The HRV infections predominated during autumn and winter with significant negative correlation between the number of HRV cases and the average temperature [16]. So, the result obtained in this study, with rhinovirus dominance in negative samples for the other common respiratory viruses, was expected since the

samples were collected in late fall and winter (June, July and August), when the virus circulation is high.

The data here presented showed that patients with rhinovirus were significantly younger than patients without rhinovirus, consistent with other works that showed the detection rate of HRV infection in children was significantly higher than that in adults, mainly the HRV-C. Clinical manifestations associated with HRV-C appear to be more severe in children [24] and often associated with respiratory complications, like acute wheezing illness and bronchiolitis [25, 26]. In adults HRV-A is more common and is associated with cases of viral pneumonia [27].

Fever and nasal congestion were more frequently observed in pediatric group than the adults which were most diagnosed with severe respiratory disease entity [28]. Figure 3 presented two groups of symptoms. This division may be associated with the main affected area, upper or lower respiratory tract. Rhinorrhea was the clinical manifestation significantly associated with HRV infection in this study. Other studies also show rhinorrhea as the main symptom associated with rhinovirus infection [29, 30].

The use of oseltamivir was significantly associated with HRV infection in the multiple analysis (Table 7). In univariate analysis, this association was not verified (Table 4). However, a high percentage (37.5%) of patients with rhinoviruses who took the medication incorrectly were observed. Although rhinovirus and influenza symptoms can be similar and therefore confounded [31], the use of oseltamivir in patients with HRV has already been reported, but in a much smaller percentage (2.1 - 2.5%) [22, 31].

Conclusion

This study showed the predominance of rhinoviruses in cases of respiratory illness in clinical samples from negative patients by the Influenza and Influenza-like Syndrome surveillance. Rhinorrhea was the most significant symptom associated with the disease. All three virus species were identified in the Federal District, Brazil. A lower chance of presenting respiratory distress was also observed in the multiple analysis, although the symptoms of patients with HRV were associated with both upper airways infection and complications of infection in the lower respiratory tract (Fig. 3). In the patients with HRV, 55.0 and 37.5% presented exacerbations such as dyspnea and respiratory distress and 57.5% had to be hospitalized (Tables 4 and 6). As HRV is also identified in asymptomatic patients [32, 33], more detailed studies need to be made to better understand the prevalence, risk factors, and outcomes related to rhinovirus positivity in the population.

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Capítulo 4

4.1 Rhinovirus incidence in association with climate factors in Brasília, Brazil

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O manuscrito deste capítulo está em preparação.

Abstract

Background: Human rhinovirus (HRV) is the most common pathogen of acute respiratory infections. Evaluating the circulation of the virus throughout the months may be useful to delineate the circulation pattern of the agent and; therefore, previewing etiological agents for the respiratory virus surveillance network (RVSN).

Objective: The present study reports rhinovirus occurrence in relation to certain periods, as well as the definition improvement of an etiological agent for RVSN in Brasília, Brazil.

Methods: RT-qPCR was used to monitor the presence of respiratory viruses, including rhinoviruses from samples of patients with Influenza-like Syndrome (ILS) or Severe Acute Respiratory Syndrome (SARS). Sample profiles were obtained in order to correlate with the results of the detection of the evaluated viruses. The correlation of the incidence of rhinovirus with meteorological data was performed using non-parametric tests in the IBM SPSS software.

Results: The addition of rhinovirus-specific primers in the respiratory virus panel significantly increased the identification of a virus that is associated with respiratory symptoms. The prevalence of rhinovirus (in relation to the other viruses) showed a significant correlation with the minimum temperatures recorded, in other words, increasing detection of rhinovirus is proportional to the decrease in the minimum temperatures recorded in Brasília, Brazil.

Conclusion: The incidence of rhinovirus was correlated with the decrease in minimum temperature, but without an evident seasonal pattern for the population of Brasília in the studied period.

Key words: Rhinovirus – seasonality – RT-qPCR – respiratory virus surveillance

Introduction

The worldwide circulation of respiratory viruses is monitored through the global influenza surveillance since 1947. This network is also responsible for controlling possible outbreaks and epidemics, besides providing information to support the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) decide the type of vaccine recommendations. Central laboratories (Lacens) in Brazil are responsible for reporting the occurrence of etiologic agents, types and subtypes of circulating respiratory viruses in patients with Influenza-like Syndrome (ILS) and Severe Acute Respiratory Syndrome (SARS) (1).

Viral detection in acute respiratory infections (ARI) depends on several factors, such as sensitivity of the diagnostic methods, adequate patient selection influenced by the symptoms and severity of the disease, in addition to the climate (2).

Studies in Brazil have shown a higher incidence of respiratory viruses in moderately cold and dry months in regions with semitropical climate, in rainy seasons in tropical regions and during the colder months in subtropical areas (3-6).

Most respiratory viral infections happen in winter, but they are also common in other seasons. Environmental factors influence human airways physiology, immunity, virus stability and transmission rates (7). As a result, environmental changes are closely related to the incidence and frequency of infectious diseases.

Among the viruses that are associated with respiratory diseases, rhinoviruses (HRV) stand out for their high frequency, being the main etiological agent of "common colds". In most cases, it presents as a mild and self-limited disease (8), but it can also cause bronchiolitis and pneumonia especially in children (9). Thus, knowing the seasonality of infections by this virus could provide insights to determine the etiologic agent of the disease.

The present study reports the seasonality of rhinovirus infection, as well as the definition improvement of an etiological agent by RT-qPCR for the respiratory virus surveillance network in Brasília, Brazil.

Materials and Methods

Detection of HRV by RT-qPCR - The nasopharyngeal and tracheal secretion samples received in the laboratory since June 2016 were tested for the respiratory virus diagnostic panel performed in Lacen-DF (the Federal District, Brasilia, Brazil) by the standard RT-qPCR (for Influenza A virus, Influenza B virus, Human orthopneumovirus, Human metapneumovirus, Human mastadenovirus C, Human respirovirus 1, Human orthorubulavirus 2 and Human respirovirus 3). Those samples that were negative, and were collected between June and August 2016 were separated and stored in freezer at -70 °C for subsequent DNA/RNA extraction. The metagenomic analyses of those samples as one pooled virome was previous studied (10). Based on this study, rhinovirus was confirmed as the most prevalent etiologic agent in samples with negative result; therefore, HRV universal primers were designed for HRV detection (11).

Detection of standard respiratory viruses - The nasopharyngeal and tracheal secretion samples received in the laboratory from August 2019

were tested for the respiratory virus diagnostic panel performed in Lacen-DF (Public health laboratory located in the Federal District, Brazil) by the standard RT-qPCR plus Rhinovirs, after drawing universal primers.

Meteorological data – Brasília is the federal capital of Brazil and the seat of government of the Federal District. The capital is located in the Midwest region of the country, along the geographical region known as Planalto Central. It is located at 15°

50'16 "south latitude and 47°42'48" west longitude, with altitudes between 1,000 and 1,200 meters above sea level. Brasilia's climate is tropical with a dry and rainy season, with average monthly temperatures always above 18°C and annual rainfall of approximately 1,480 mm (mm), concentrated between the months from October to April. During the dry season, which starts in May and ends in September, the relative humidity can be below 30%. Information on minimum and maximum temperatures and relative humidities was obtained through daily consultations to the official website of Inmet (National institute of meteorology) (12).

Statistical analysis – Data on the results of rhinovirus and other respiratory viruses were computed from August 2019 to February 2020. Data from previous three years were used to compare the positive definition of the etiologic agent when performing the viral panel. From March 2020, it was not possible to continue the evaluation due to the Coronavirus pandemic (SARS-CoV-2) that caused social isolation and alteration of all natural seasonality of other respiratory viruses, in addition to changing the criteria for receiving a sample for diagnosis. Data were analyzed using the software IBM SPSS (Chicago, USA). Spearman's non-parametric correlation was used to assess the relationship between meteorological data and the incidence of rhinovirus in the studied period. All *p*-values were two-tailed and a value of < 0.05 was considered significant.

Ethics approval and consent to participate - This research project was reviewed and approved by ethics committee of the Faculty of Health Sciences (University of Brasília), approval letter No. 3.052.443.

Results

Samples from patients with Influenza-like Syndrome and Severe Acute Respiratory Syndrome are received by the Central Public Health Laboratory to assess a possible viral etiologic agent.

Data from August to February of 2016/2017, 2017/2018, 2018/2019 and 2019/2020 were used for comparison of diagnosis using the specific primers for the respiratory viruses panel including rhinovirus.

All positive results (for any respiratory virus) were divided by the number of samples from patients with suspected respiratory viral infection assessed on a monthly basis. Table 1 shows that there was a significant increase in the percentage of positive cases, that is, with the definition of an etiological agent from the insertion of rhinovirus in the panel of respiratory viruses. For the statistical analysis in question, the Kruskal-Wallis test was used, given the small number of data ($n = 7$ months) per group (evaluated period). The increase in the percentage of virus identification from the inclusion of rhinovirus can be better identified in figure 1.

Table 1 Comparison of the proportion of positive results of respiratory viruses in four periods. In the last period, the detection of rhinovirus in the viral panel was included, Brasília, Brazil.

Period	Samples	Proportion of positive results		<i>P</i> *
		Median	Interquartile range	
Aug/16 – Feb/17	362	0,368	0,085	0,009
Aug/17 – Feb/18	358	0,407	0,114	
Aug/18 – Feb/19	686	0,380	0,244	
Aug/19 – Feb/20	1297	0,587	0,185	

*Kruskal-Wallis test

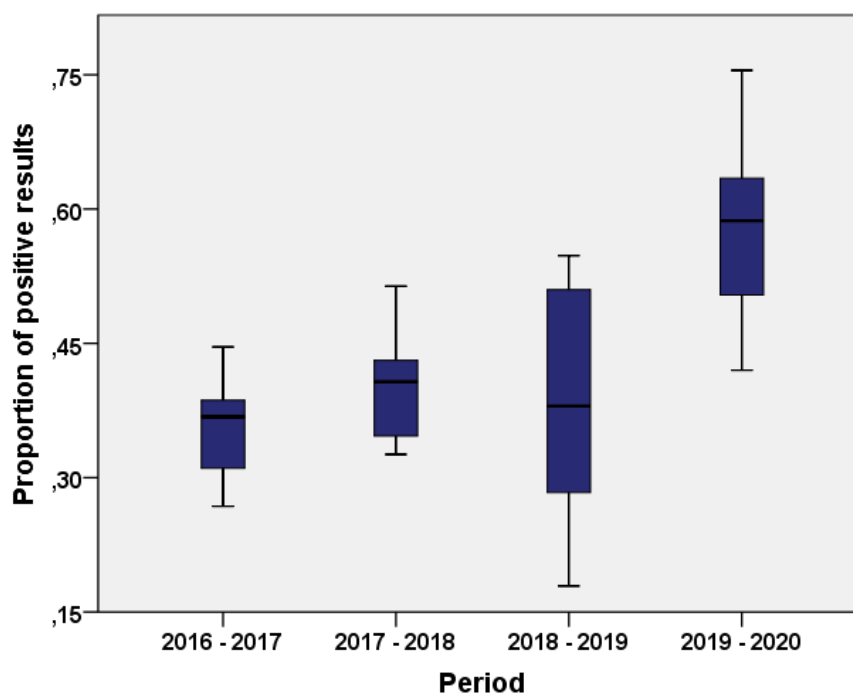


Figure 1 *Box-plot* of proportions of positive results of respiratory viruses in four periods of time. In the last period, the detection of rhinovirus in the viral panel was included, Brasília, Brazil.

Of the 1297 samples received from August 2019 to February 2020, 730 (56.28%) were positive for, at least, one of respiratory viruses. Among the total respiratory viruses identified, 37.26% were rhinovirus, 13.97% human respirovirus 3, 10.96% Influenza B virus, 10.82% Influenza A virus H1N1, 10.41% human metapneumovirus, 4.79% human mastadenovirus C, 3.97% non-subtypable Influenza A virus, 3.42% human orthopneumovirus, 2.74% Influenza A virus H3, 1.23% human respirovirus 1 and 0.41% were human orthorubulavirus 2. Coinfections were present in 62 samples (8.49% of positive samples), whose rhinovirus were the main viruses in co-detection (56.45%).

Minimum and maximum temperature and relative humidity data were obtained daily and the correlation of weekly averages and rhinovirus incidence was evaluated in relation to the other viruses identified in the corresponding week. Climatic data were

obtained from the week 31 of 2019 to the week 12 of 2020, totalizing 34 epidemiological weeks.

For most parameters, the null hypothesis of normality of data was rejected by the Kolmogorov-Smirnov test. Then, Spearman's Rho non-parametric correlation test was chosen. There was statistically significant correlation between the ratio of rhinovirus / other viruses and minimum temperature (table 2). The correlation coefficient was negative, which indicates that the increase in the identification of rhinovirus in relation to the other respiratory viruses grows with the decrease in the minimum temperature. There was also a moderate negative correlation with the maximum temperature, although not statistically significant. There was no correlation between the humidity (maximum and minimum) and the detection of rhinovirus in relation to the other investigated viruses.

Table 2 Correlation between rhinovirus positivity in relation to other respiratory viruses and relative air temperature and humidity. August 2019 to February 2020, Brasília, Brazil.

		Rhinovirus / other viruses	
Spearman's Rho	Minimum temperature (°C)	Correlation Coefficient	-0,420
		<i>P</i>	0,013
		<i>n</i>	34
	Maximum temperature (°C)	Correlation Coefficient	-0,329
		<i>P</i>	0,057
		<i>n</i>	34
	Minimum humidity (%)	Correlation Coefficient	0,057
		<i>P</i>	0,751
		<i>n</i>	34
	Maximum humidity (%)	Correlation Coefficient	0,070
		<i>P</i>	0,696
		<i>n</i>	34

Figure 2 shows more clearly the downward trend in the rhinovirus / other viruses ratio with the higher minimum temperature in the studied period. There is also a tendency to the decrease of detection of rhinovirus with the increase in maximum temperature, although not significant. The detection of rhinovirus in relation to other viruses is not influenced by humidity.

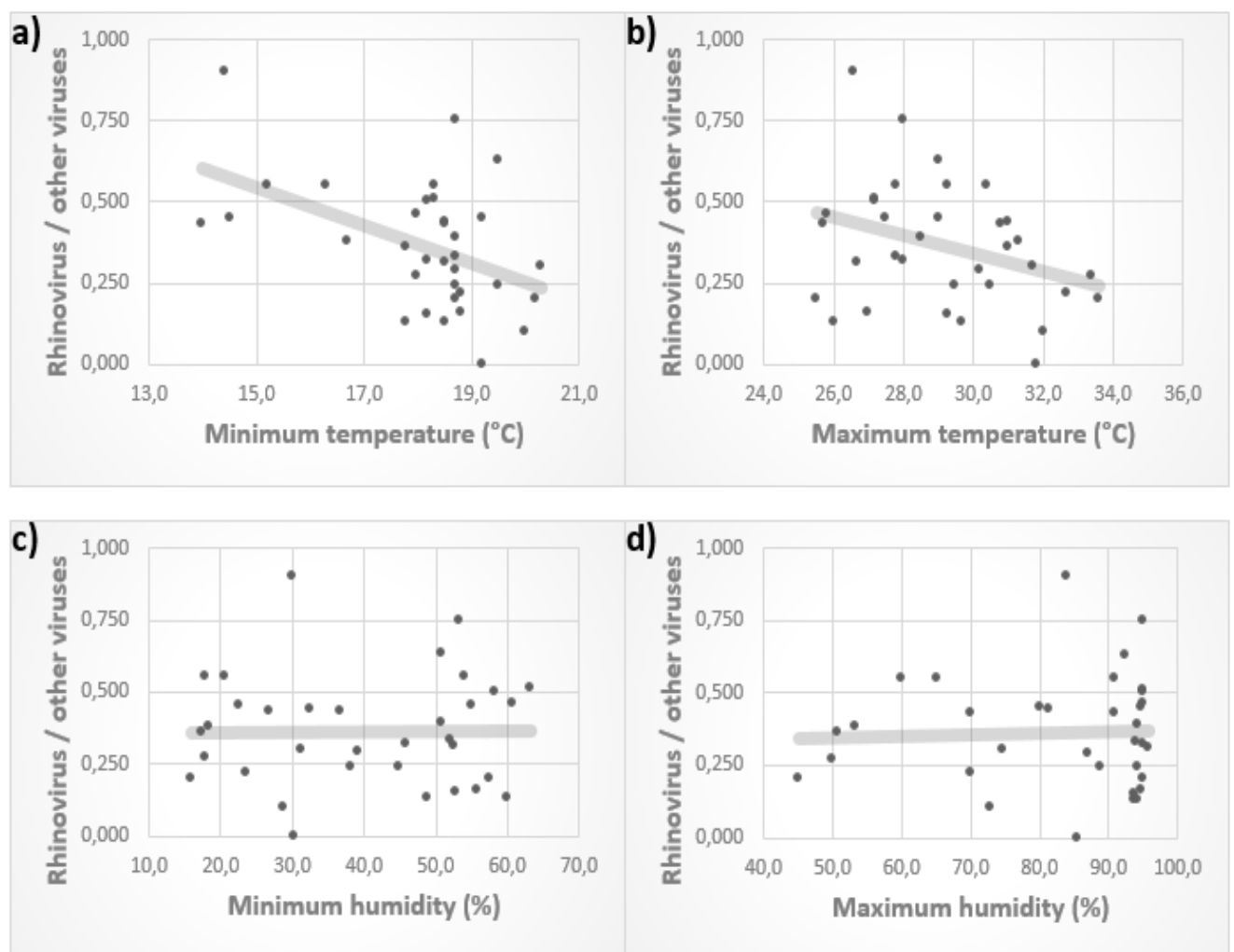


Figure 2 Dot plot with trend curve for the correlation between the rhinovirus / other viruses ratio and the minimum (a) and maximum (b) temperatures, in addition to the minimum (c) and maximum humidity (d). August 2019 to February 2020, Brasília, Brazil.

The seasonality of the circulation of rhinovirus and other respiratory viruses was assessed using the ratio of the total from the week 31 (2019) to week 26 (2020), with total of 48 weeks. As of week 13, it is already possible to observe the effects of social isolation (occurred in week 11) on the circulation of common respiratory viruses (excluding SARS-CoV-2) in Fig. 3. There was no clear pattern of positivity for respiratory viruses and rhinovirus in the population of Brasília in the period studied prior to the social isolation decreed in the region.

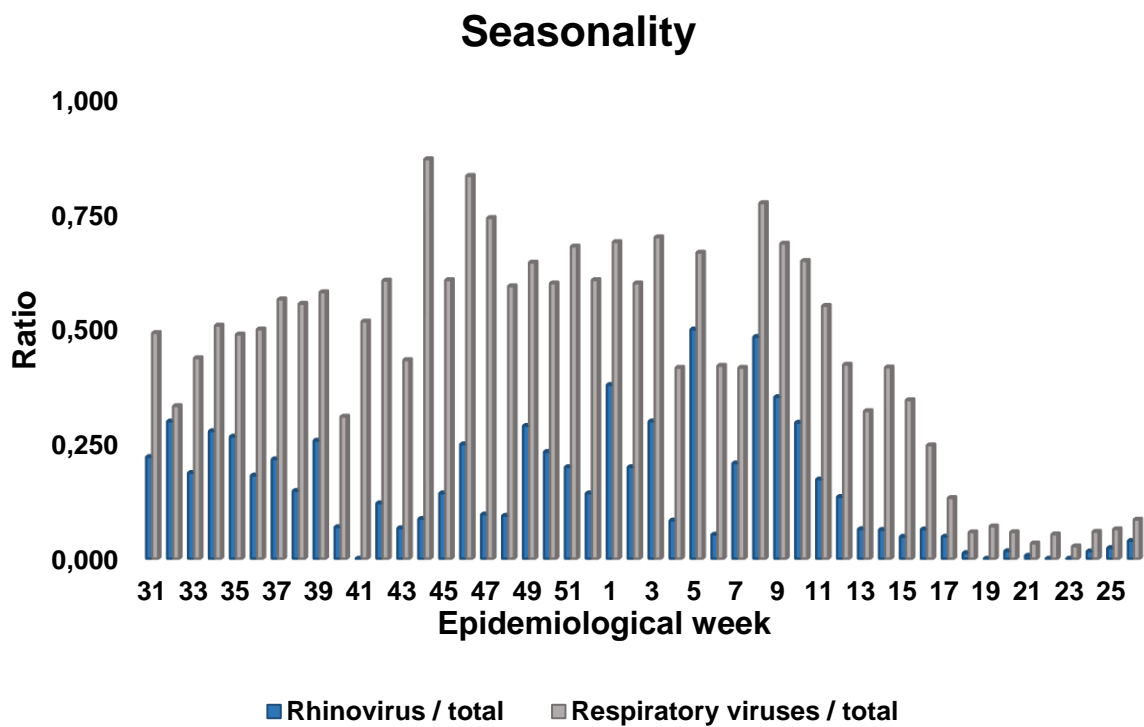


Figure 3 Weekly distribution of the ratio between the detection of rhinovirus or all respiratory viruses in relation to the total of tests carried out from August 2019 to June 2020. Brasília, Brazil.

Discussion

Since the beginning of the surveillance of respiratory viruses in the country, the improvement in the agility and sensitivity of detection of viral etiologic agents has been occurring in the Central Public Health Laboratories. Understanding seasonality patterns and climatic associations can help to suppose the target detection, which generates early diagnosis and consequently, agile surveillance action and clinical management associated with patient risk factors.

The significant increase in the percentage of positive cases, that motivated the inclusion of rhinovirus in the panel of respiratory viruses, will allow greater efficiency and agility in the definition of possible outbreaks and diagnosis for adequate treatment of patients with respiratory symptoms. More than a third of the viruses identified in the analyzed period were rhinoviruses, which shows the relevance of these viruses, previously unidentified, in the population of Brasília. Other studies have also shown a higher prevalence of rhinovirus as an etiologic agent of acute respiratory infection (13, 14).

Low air temperature and drier air are associated with an increased risk of viral infections (15-18). Some studies have shown that the decrease and dryness of the mucosa of the upper airways can increase the host's susceptibility to infections (19, 20).

Studies with Influenza viruses have shown that the decrease in temperature, as well as the decrease in absolute air humidity, appears to be important determinants of infectiousness and act as a trigger for flu seasons (21, 22). In southeastern Brazil, there was a significant trend towards an increase in viral respiratory infections as the temperature and relative humidity decrease (23).

In a study carried out in the south of the country, rhinovirus detection was more prevalent during the colder seasons (autumn and winter), with a moderate negative

correlation between the frequency of viral infection and temperature, but with no correlation with precipitation (13). The risk of rhinovirus infection was associated with a decrease in humidity and temperature during the three days prior to infection, showing a relationship between short-term variation in weather factors and HRV infections (24).

Studies on seasonality of influenza in Brazil have different patterns of viral circulation; in the Northeast region, influenza circulates in the first four months of the year, coinciding with the period of the highest humidity (25) unlike that found in other regions of the country. These different circulation profiles due to the climatic conditions of each region can impact the vaccine's effectiveness.

In our study, no seasonality was observed in the incidence of rhinovirus during the period from August 2019 to February 2020. This period coincides with the end of winter, spring and summer. During the fall and winter of 2020, social isolation decreased the circulation of non-SARS-CoV-2 respiratory viruses, which did not allow assessing whether in the country's capital this same pattern of increased incidence in cold seasons would be observed. In order to establish the seasonality of HRV infections more accurately, the analysis should include additional years, after pandemic control.

However, in this period it was already possible to verify a moderate negative association ($\rho = -0.420$, $p = 0,013$) with minimum temperatures and no correlation with humidity, which may lead to consider a probable association with the coldest seasons of the year.

The seasonality of rhinoviruses can also be associated with the species of circulating virus. One study showed that strains of HRV-A were identified throughout the year, the HRV-C strains appeared mainly in winter and HRV-B were found in low quantities, but mainly in the summer (26).

Conclusion

We confirmed an improvement in the detection of the etiologic agent for RVSN by including rhinovirus in the diagnosis. The incidence of rhinovirus was correlated with the decrease in minimum temperature, but without an evident seasonal pattern for the population of Brasília in the studied period.

Understanding the seasonal incidence of temperature-related rhinovirus infection can help with strategies to prevent and minimize transmission and provide the health network with more information for the clinical treatment of patients.

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Conclusão geral

Amostras clínicas negativas para o painel de vírus respiratórios do sistema de vigilância, por RT-qPCR, foram avaliadas por meio de sequenciamento de alto desempenho (HTS) a fim de identificar possíveis vírus em secreções da nasofaringe / aspirado traqueal de pacientes do Distrito Federal que apresentavam sintomas respiratórios.

Vírus pertencentes às famílias virais *Herpesviridae*, *Coronaviridae*, *Parvoviridae* e *Picornaviridae*, foram identificados com maior frequência, com destaque para os rinovírus. Houve variação nos resultados encontrados por diferentes metodologias de identificação, explicada pela diferença de especificidade e sensibilidade em cada método. Entretanto o sequenciamento genético foi capaz de identificar grande diversidade de vírus e alta frequência na ocorrência dos rinovírus, mostrando a relevância desses agentes etiológicos para a saúde pública do Distrito Federal.

Após identificação do rinovírus como o agente viral mais prevalente nas referidas amostras, foram desenhados primers em região consenso e primers na região VP2/VP4 para identificação de espécies. Grande parte das amostras analisadas foram positivas para rinovírus, sendo que todas as três espécies (rinovírus A, B e C) foram identificadas. A infecção por HRV foi associada à idade menor de 2 anos e à moradia em área rural, sendo a rinorreia o principal sintoma associado. A análise múltipla mostrou também menor chance de pacientes com HRV apresentarem desconforto respiratório em comparação aos pacientes com sintomas respiratórios por outros agentes.

A incorporação de primers específicos para rinovírus no painel de vírus respiratório aumentou significativamente a identificação do agente etiológico que causa infecção respiratória. Em relação à distribuição do vírus ao longo do ano, foi observada

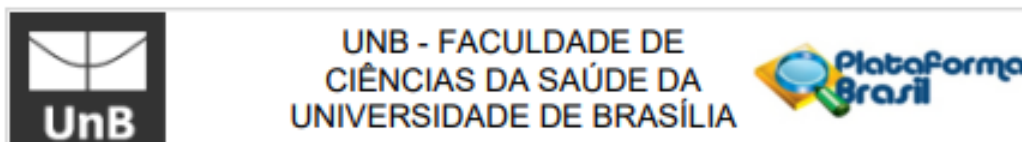
correlação significativa com as temperaturas mínimas, onde o aumento da detecção de rinovírus foi proporcional à diminuição das temperaturas registradas em Brasília, Brasil.

Estudos tem mostrado predominância de rinovírus no inverno, período de coleta das amostras utilizadas nesse trabalho. Porém, durante o outono e inverno de 2020, o isolamento social diminuiu a circulação de vírus respiratórios não SARS-CoV-2, o que não permitiu avaliar esse padrão de aumento da incidência nas estações frias. De forma geral, não foi observado um padrão sazonal evidente de rinovírus, mas há uma correlação da incidência com a queda da temperatura.

Compreender os agentes virais que acometem a população, identificar sintomas associados e a incidência sazonal desses vírus pode ajudar em estratégias para prevenir e minimizar a transmissão, além de fornecer à rede de saúde mais informações para o adequado manejo clínico e terapêutico das infecções respiratórias.

Apêndices

6.1 Apêndice 1 - Parecer do Comitê de Ética



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Análise do perfil viral de amostras de secreção nasofaringe/traqueal sem definição de diagnóstico por metodologia tradicional realizada no Lacen DF.

Pesquisador: Larissa da Costa Souza

Área Temática:

Versão: 3

CAAE: 90161518.8.0000.0030

Instituição Proponente: Instituto de Biologia da Universidade de Brasília

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.052.443

Apresentação do Projeto:

Resumo:

"Desde o ano 2000 a vigilância de influenza vem sendo feita no Brasil com o objetivo de monitorar os vírus circulantes e fornecer informações para subsidiar as recomendações da Organização Mundial da Saúde (OMS). Os Lacens são responsáveis pela base da informação utilizada para vigilância a partir da identificação do agente etiológico, tipagem e subtipagem de vírus respiratórios circulantes. No Lacen-DF o diagnóstico de influenza A e B por RT-PCR em tempo real começou em outubro de 2014, sendo esse painel ampliado para os principais vírus respiratórios em junho de 2016 (Vírus respiratórios sincicial, Adenovírus, Metapneumovírus, Parainfluenza 1, Parainfluenza 2 e Parainfluenza 3). A utilização do sequenciamento de última geração tem se mostrado fundamental na descoberta de patógenos não identificados por métodos tradicionais, como o RT-PCR em tempo real. Sendo assim, esse estudo tem por objetivo identificar possíveis vírus causadores de sintomas de vírus respiratórios de pacientes com diagnóstico negativo para os agentes etiológicos analisados no Lacen. Serão utilizadas aproximadamente 100 amostras por meio de um pool, com prévia purificação de material genético viral, para sequenciamento de nova geração utilizando o equipamento Illumina 2000 HiSeq 100 par-end. Depois, por meio de banco de dados, as amostras sequenciadas serão alinhadas com sequências conhecidas para identificação de possíveis vírus.*

Hipótese:

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Alguns vírus respiratórios podem não estar sendo identificados por RT-qPCR em tempo real, pois esta técnica pressupõe que o agente etiológico seja conhecido. Embora seja a técnica padrão ouro para a identificação de vírus respiratórios, o sequenciamento genético de vírus permite a identificação de todos os vírus presentes na amostra, o que permite a identificação de qualquer vírus, e não dos 8 preestabelecidos na metodologia tradicional.

Metodologia:

Serão obtidas aproximadamente 100 amostras de secreção nasofaringe negativas nos 3 meses analisados (junho a agosto de 2018). Os pacientes com SG e SRAG terão suas amostras colhidas nos hospitais sentinela e ao realizar o preenchimento da ficha epidemiológica responderão ao Termo de Consentimento Livre e Esclarecido, concordando ou não em participar do estudo. Procedimento: Semi-purificação de vírus para Sequenciamento de próxima Geração Material: Processador de alimentos, Beckers. Solução: Tampão fosfato 0.1 M (Misturar 1M de fosfato monobásico e dibásico) pH 7.8, EDTA 1mM pH 8.0, B-Mercaptoetanol 0.2%, Sacarose 20% no tampão fosfato para atenuar. Lise das células de secreção nasofaringe/traqueal com Triton X-100 a 1%. Agitação em temperatura ambiente por 1 hora. Centrifugação a baixa velocidade em tubos Falcon de 50 mL (5000 x g) durante 30 min num rotor Swingle. Ultracentrifugação com 17 mL de amortecedor a 20% em 35000 rpm (rotor Ti 45) (142032 x g) durante 1 h 15 min. Suspensão de Pellet com tampão de extração de RNA para posterior extração de RNA com Kit da Zymo®. Tratamento de no mínimo de 2 ug de RNA seco com estabilizador para sequenciamento. Condição para o sequenciamento: • RNA -> controle de qualidade (MacroGen, Bioanalyzer) • Construção de biblioteca usando TruSeq para kit RNA-Seq para Illumina 2000 HiSeq 100 par-end • NGS 4-5g escala e receber dados em bruto Processo de bioinformática: (1) Aparar dados brutos por Galaxy (ou Geneious) (2) Alinhamento por Velvet (Galaxy) (3) Utilizar Blast X com referência genômica Viral (RefSeq Viro) (4) Acerto do alinhamento é estendido para arranjar leituras (montagem para referência). (5) Montar o genoma completo, se possível.

Objetivo da Pesquisa:

Considerando que os métodos tradicionais não são capazes de identificar novos patógenos, objetiva-se com este estudo identificar possíveis vírus de amostras de secreções nasofaringe/traqueal de pacientes do Distrito Federal com sintomas de vírus respiratórios, mas com resultado negativo para os agentes etiológicos conhecidos, nos meses de junho a agosto de 2018.

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Continuação do Parecer: 3.052.443

Objetivo Secundário:

"Caracterizar os possíveis vírus causadores de sintomas respiratórios, com desenho de primers e sondas específicos para confirmar utilizando a técnica de diagnóstico tradicional (RT-PCR em tempo real). Avaliar a sintomatologia específica dos pacientes que possa estar associada à infecção por esses patógenos não utilizados no diagnóstico rotineiro do Lacen."

Avaliação dos Riscos e Benefícios:

De acordo com a pesquisadora:

"Riscos: Os riscos decorrentes de sua participação na pesquisa são mínimos, pois não há risco imediato ou tardio no estudo para os participantes, tendo em vista, ser apenas um exame laboratorial para identificação de Vírus Respiratórios.

Benefícios: Este estudo vai contribuir na orientação de Políticas de Saúde Pública no Distrito Federal, afim de aprimorar o diagnóstico de Vírus respiratórios realizado no Lacen-DF"

Comentários e Considerações sobre a Pesquisa:

Trata-se de um projeto de pesquisa de doutorado do Programa de Pós-graduação de Biologia Microbiana da UnB, de responsabilidade de Larissa da Costa Souza sob orientação do Prof. Dr. Tatsuya Nagata. O estudo visa o sequenciamento genético de próxima geração para a identificação de possíveis vírus em amostras de secreção nasofaríngea/traqueal de aproximadamente 100 pacientes com Síndrome Gripal e Síndrome Respiratória Aguda Grave, com resultado negativo para os Vírus Respiratórios realizados por metodologia tradicional no Lacen-DF. As amostras são colhidas nos hospitais sentinela durante os meses junho-agosto de 2018. O cronograma de pesquisa prevê início das coletas de dados em junho 2018 e finaliza em julho 2019. O orçamento financeiro que consta na Plataforma Brasil apresenta valor de R\$ 5.000,00 - referente a primers e sondas. Será custeado por recursos próprios do pesquisador responsável.

Considerações sobre os Termos de apresentação obrigatória:

Documentos analisados para emissão desse parecer:

Informações Básicas do Projeto: "PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1128273.pdf" postado em 19/10/2018;

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Carta Resposta ao CEP: documento editável "Carta_Resposta2.docx" e "Carta_Resposta2.pdf" postadas em 19/10/2018, contendo as respostas aos questionamentos desse CEP.

Temo de Dispensa de TCLE: nova versão documento "DispensaTCLE3.doc" e "DispensaTCLE3.pdf" postados em 19/10/2018 reitera a justificativa para dispensa de aplicação do TCLE e acrescenta novos esclarecimentos.

Recomendações:

Não se aplicam.

Conclusões ou Pendências e Lista de Inadequações:

Análise das respostas às pendências emitidas pelo parecer consubstanciado no. 2.807.548 e 2.959.104:

1. Nos documentos "PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1128273.pdf" e "Projeto_de_pesquisa.docx", não foram apresentados os critérios de inclusão e exclusão dos participantes da pesquisa, bem como, a forma de seleção dos participantes de pesquisa. Solicita-se incluir essas informações nos documentos acima mencionados.

RESPOSTA/ANÁLISE: "Os critérios de inclusão e exclusão foram incluídos nas metodologias dos documentos "PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1128273.pdf" e "Projeto_de_pesquisa2.docx" na página 5." Copio o parágrafo que consta nos documentos referente aos itens citados: "Critério de inclusão: os critérios de inclusão do estudo são: todas as amostras devidamente coletadas e encaminhadas ao Lacen-DF para realização do diagnóstico painel de vírus respiratório de pacientes que apresentem características de síndrome gripal (febre, tosse e/ou dor de garganta e pelo menos um dos sintomas: mialgia, cefaléia, artralgia, dispnéia conjuntivite, mal estar geral e perda do apetite). As amostras são encaminhadas em conjunto com a ficha epidemiológica preenchida com os dados epidemiológicos do paciente para fins de controle e monitorização das ações de vigilância." e, "Os critérios de exclusão são: amostras colhidas inadequadamente (ex: em meio de transporte inadequado ou com swab de algodão ao invés do swab de rayon) ou com algum dano no frasco que contamine o seu interior." PENDÊNCIA ATENDIDA.

2. A pesquisadora apresenta o Termo de concordância da Coparticipante, assinado pelo diretor do LacenDF autorizando a realização pesquisa, porém o CNPJ da instituição FEPECS não foi incluído no

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documento "PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1128273.pdf". Solicita-se a inserção da SES-DF como instituição coparticipante na Plataforma Brasil, por meio do CNPJ, confirmando vínculo com o "5553 - Comitê de Ética em Pesquisa - FEPECS/SES-DF", para que, uma vez aprovado pelo CEP/FS, possa ser automaticamente replicado nesse CEP para apreciação.

RESPOSTA/ANALISE: "O CNPJ da instituição coparticipante SES-DF foi inserido na Plataforma Brasil". Copio o parágrafo que consta no documento referido acima: "CNPJ Nome da Instituição Co-participante Nome do Responsável Comitê de Ética Instituição Selecionada Via Plataforma Brasil 00.394.700/0023-13 DISTRITO FEDERAL Jorge Antônio Chamon Júnior SECRETARIA DE SAUDE Fundação de Ensino e Pesquisa em Ciências da Saúde/ FEPECS/ SES/ DF." PENDÊNCIA ATENDIDA.

3. No arquivo "DispensaTCLE.pdf", a pesquisadora requer a dispensa do TCLE devido aos seguintes fatores, a saber: "o objetivo do estudo ser a caracterização dos possíveis vírus causadores de sintomas respiratórios utilizando técnicas de diagnóstico tradicionais ..."; "as amostras que serão utilizadas no estudo são normalmente enviadas ao LACEN para realizar o painel viral de Vírus Respiratórios por se enquadrarem SRAG e SG" e "... não haverá através da amostra identificação do provedor. No entanto, a Resolução CNS N° 441 de 2011, artigos 9º e 10 orienta que "o material biológico pertence ao participante de pesquisa, o qual tem o direito de retirar, a qualquer momento, o consentimento de guarda e utilização do material biológico armazenado em biobanco ou biorrepositório. Esse manifesto deve ser realizado por escrito pelo participante ou seu responsável legal." Assim, solicita-se anexar o documento TCLE, esse deve conter informações sobre a coleta (natureza do material biológico e os riscos da coleta), quantidade a ser coletada, propósito da coleta, local e tempo de armazenamento, bem como, se há intenção de usar o material biológico em futuras pesquisas, isso deve estar contido no mesmo. Esclarecer se a coleta é rotina do serviço e se há alguma autorização ou conhecimento do voluntário.

RESPOSTA/ANALISE: "No novo documento "DispensaTCLE2.docx" reitera-se o pedido de dispensa de TCLE, com as devidas explicações e justificativas que atendem à Resolução CNS N° 441 de 2011, ao informar que a coleta de amostra é rotina de diagnóstico de Vírus Respiratório realizada sob orientação do grupo GT Influenza do Ministério da Saúde em parceria com a Vigilância epidemiológica local. Assim, sendo o Lacen-DF não se enquadra como biobanco ou biorrepositório e o projeto de pesquisa em questão visa avaliar quais os possíveis vírus contidos nas amostras com resultados negativos, para melhorar o diagnóstico do agravo em questão, sem contato ou risco ao paciente". PENDÊNCIA ATENDIDA.

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4. No arquivo "PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1128273.pdf" no item Riscos, página 3 de 7, lê-se: "Os riscos decorrentes de sua participação na pesquisa são mínimos, pois não há risco imediato ou tardio no estudo para os participantes, tendo em vista, ser apenas um exame laboratorial para identificação de Vírus Respiratórios." Conforme item V, Res. CNS 466/2012, risco da pesquisa é a possibilidade de danos à dimensão física, psíquica, moral, intelectual, social, cultural ou espiritual do ser humano, em qualquer pesquisa e ainda sendo necessário estratégias de cuidados para minimizá-los. Solicita-se descrever, os meios de minimizar os possíveis riscos e desconfortos que os participantes serão submetidos para a coleta do material biológico nos arquivos "PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1128273.pdf"; o "Projeto_de_pesquisa.docx" e no TCLE a ser anexado na plataforma Brasil.

RESPOSTA/ANALISE: "Os riscos e benefícios diretos ao paciente foram melhor esclarecidos nos documentos "PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1128273.pdf" e "Projeto_de_pesquisa2.docx" na página 5. Copio o parágrafo sobre riscos que consta no documento "Projeto_de_pesquisa2.docx": "Assim sendo, neste projeto será apenas utilizado material genético purificado para identificação de etiológica viral e não serão utilizados os dados referentes aos pacientes, não acarretando riscos diretos aos mesmos. Os riscos relacionados à utilização dessas amostras armazenadas podem ser considerados baixos, pois garante-se que a identidade dos pacientes que tiverem suas secreções incluídas na pesquisa permanecerá sempre em sigilo (ou seja, não será revelada), assim como os dados que permitam de algum modo sua identificação. PENDÊNCIA ATENDIDA.

5. No arquivo "Projeto_de_pesquisa.docx", solicita-se inserir a planilha orçamentária detalhada e esclarecer a fonte de financiamento da pesquisa.

RESPOSTA/ANALISE: "A planilha orçamentária detalhada foi inserida e a fonte de financiamento da pesquisa foi esclarecida no documento "Projeto_de_pesquisa2.docx" na página 8.". PENDÊNCIA ATENDIDA.

6. Nos arquivos "Projeto_de_pesquisa.docx" e "PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1128273.pdf" solicita-se atualização do cronograma de pesquisa para início de coleta após aprovação desse CEP.

RESPOSTA/ANALISE: "O cronograma desta pesquisa foi atualizado nos arquivos "PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1128273.pdf" e "Projeto_de_pesquisa2.docx" na página 7.". PENDÊNCIA ATENDIDA.

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7. No documento "DispensaTCLE2.doc", postado em 17/08/2018, a pesquisadora reitera a dispensa do TCLE, porém as justificativas e explicações apresentadas pela pesquisadora, não esclarecem os seguintes aspectos da pesquisa:

7.1 Caso a pesquisadora comprove que a nova técnica é melhor em comparação a técnica tradicional, como será incorporado a nova metodologia nas rotinas de análises do LACEN? Solicita-se esclarecimentos.

RESPOSTA/ANÁLISE: "Os esclarecimentos referentes à incorporação da nova metodologia estão no documento "DispensaTCLE3.doc", postado em 22/10/2018, na página 3"

Copio o paragrafo sobre a incorporação da nova técnica no LACEN: "A técnica de sequenciamento de próxima geração (alvo-inespecífico) ainda é muito cara e não disponível em laboratórios de análises clínicas, sendo restrita à pesquisa científica. O alto custo inviabiliza o uso dessa tecnologia para cada amostra individualizada, por isso foram realizados pools de amostras neste trabalho. Mesmo não sendo possível incorporar a tecnologia na rotina de análise do Lacen, ela é fundamental para identificar possíveis novos vírus respiratórios que circulem na população do Distrito Federal e com essa identificação é possível desenhar primers e sonda específicos para incorporar esse(s) novo(s) vírus na rotina diagnóstica do Lacen DF por meio de RT-qPCR (alvo-específico)."

PENDÊNCIA ATENDIDA.

7.2 Se caso amostras com o diagnostico negativo pela técnica tradicional apresentar alteração positiva com utilização da nova metodologia, qual será ação tomada pela pesquisadora? Solicita-se esclarecimentos.

RESPOSTA/ANÁLISE: "Os esclarecimentos referentes à ação tomada caso o resultado seja positivo para outros vírus respiratórios estão no documento "DispensaTCLE3.doc", postado em 22/10/2018, na página 3."

Copio o trecho "Como este trabalho tem por objetivo identificar outros possíveis vírus que estejam causando sintomas respiratórios, podem ser identificados um ou mais vírus respiratórios não utilizados na rotina de diagnóstico do Lacen-DF. Reitero que caso sejam identificados outros vírus respiratórios, não haverá contato com o paciente (por ligação ou qualquer outro meio), tendo em vista que nem seria possível identificar a amostra de qual paciente o vírus pertencia, pois, a análise de sequenciamento genético de próxima geração é feita em um pool (conjunto de várias amostras)". PENDÊNCIA ATENDIDA.

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Conclusão: Todas as pendências foram atendidas. Não há óbices éticos para a realização deste projeto. Protocolo de pesquisa está em conformidade com a Resolução CNS 466/2012 e Complementares.

Considerações Finais a critério do CEP:

De acordo com a Resolução 466/12 CNS, itens X.1.- 3.b. e XI.2.d, os pesquisadores responsáveis deverão apresentar relatórios parcial semestral e final do projeto de pesquisa, contados a partir da data de aprovação do protocolo de pesquisa. O início das atividades de coleta dos dados do projeto devem aguardar a aprovação do projeto pelo CEP da instituição coparticipante, se for o caso.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1128273.pdf	19/10/2018 18:21:21		Aceito
Outros	Carta_Resposta2.pdf	19/10/2018 18:19:50	Larissa da Costa Souza	Aceito
Outros	Carta_Resposta2.docx	19/10/2018 18:19:37	Larissa da Costa Souza	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	DispensaTCLE3.pdf	19/10/2018 18:19:03	Larissa da Costa Souza	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	DispensaTCLE3.doc	19/10/2018 18:18:45	Larissa da Costa Souza	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_de_pesquisa2.docx	17/08/2018 11:24:27	Larissa da Costa Souza	Aceito
Folha de Rosto	Folha_Rosto.pdf	22/05/2018 12:18:59	Larissa da Costa Souza	Aceito
Outros	cartaencaminhprojeto_ao_CEPFS.pdf	21/05/2018 09:54:34	Larissa da Costa Souza	Aceito
Outros	cartaencaminhprojeto_ao_CEPFS_.docx	17/05/2018 12:28:12	Larissa da Costa Souza	Aceito
Declaração de Pesquisadores	CV_Tatsuya.pdf	17/05/2018 12:26:35	Larissa da Costa Souza	Aceito
Declaração de Pesquisadores	CV_Tatsuya.doc	17/05/2018 12:26:20	Larissa da Costa Souza	Aceito
Declaração de	CV_Larissa.pdf	17/05/2018	Larissa da Costa Souza	Aceito

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Pesquisadores	CV_Larissa.pdf	12:26:10	Souza	Aceito
Declaração de Pesquisadores	CV_Larissa.doc	17/05/2018 12:26:02	Larissa da Costa Souza	Aceito
Declaração de Pesquisadores	TermoRespCompromPesq_CEPFS.pdf	17/05/2018 12:25:17	Larissa da Costa Souza	Aceito
Declaração de Pesquisadores	TermoRespCompromPesq_CEPFS_documento	17/05/2018 12:25:03	Larissa da Costa Souza	Aceito
Declaração de Instituição e Infraestrutura	TermoConcordancia_Participante.pdf	17/05/2018 12:24:39	Larissa da Costa Souza	Aceito
Declaração de Instituição e Infraestrutura	TermoConcordCoPartic.doc	17/05/2018 12:24:19	Larissa da Costa Souza	Aceito
Declaração de Instituição e Infraestrutura	TermoConcord_CEPFS_documento	17/05/2018 12:22:00	Larissa da Costa Souza	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

BRASILIA, 03 de Dezembro de 2018

Assinado por:
Keila Elizabeth Fontana
(Coordenador(a))

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6.2 Apêndice 2 - Página de rosto da publicação 1

ORIGINAL ARTICLE

Mem Inst Oswaldo Cruz, Rio de Janeiro, Vol. 114: e190198, 2019 1 | 6

Human virome in nasopharynx and tracheal secretion samples

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BACKGROUND In Brazil the implementation of the Sentinel Surveillance System of Influenza began in 2000. Central public health laboratories use reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for diagnosis of respiratory viruses, but this protocol identifies only specific targets, resulted in inconclusive diagnosis for many samples. Thus, high-throughput sequencing (HTS) would be complementary method in the identification of pathogens in inconclusive samples for RT-qPCR or other specific detection protocols.

OBJECTIVES This study aimed to detect unidentified viruses using HTS approach in negative samples of nasopharynx/tracheal secretions by the standard RT-qPCR collected in the Federal District, Brazil.

METHODS Nucleic acids were extracted from samples collected in winter period of 2016 and subjected to HTS. The results were confirmed by the multiplex PR21 RT-qPCR, which identifies 21 respiratory pathogens.

FINDINGS The main viruses identified by HTS were of families *Herpesviridae*, *Coronaviridae*, *Parvoviridae* and *Picornaviridae*, with the emphasis on rhinoviruses. The presence of respiratory viruses in the samples was confirmed by the PR21 multiplex RT-qPCR. Coronavirus, enterovirus, bocavirus and rhinovirus were found by multiplex RT-qPCR as well as by HTS analyses.

MAIN CONCLUSIONS Wide virus diversity was found by different methodologies and high frequency of rhinovirus occurrence was confirmed in population in winter, showing its relevance for public health.

Key words: respiratory virus - public health - high-throughput sequencing - RT-qPCR

Worldwide influenza surveillance was initiated in 1947 to monitor circulating viruses and provides information to support the vaccine-type recommendations by the World Health Organization (Geneva, Switzerland) and the Centers for Disease Control and Prevention (Atlanta, USA). Central laboratories (Lacens) in Brazil are responsible for the occurrence reports about the etiologic agents, types and subtypes of circulating respiratory viruses in patients with influenza-like syndrome (ILS) and severe acute respiratory syndrome (SARS).⁽¹⁾

The main technique for detecting respiratory viruses in Lacens is reverse transcription-quantitative polymerase chain reaction (RT-qPCR), which advantages are sensitivity, specificity, automation, relatively low cost and so on. However, this technique as well as the conventional RT-PCR are pathogen-specific, which only find pre-defined targets. Thus, a peculiar etiological agent or new genetic variants may not be detected, despite the use of a wide range of sensitive diagnostic tests.⁽²⁾

In this context, high-throughput sequencing (HTS) technologies without requiring any prior genomic information provide large volume of nucleotide sequences, resulting in elucidation of unidentified viruses (which were not detectable by the specific detection methods) and furthermore, allow the discovery of new viruses or new genomic variants using metagenomic approaches.

The human population is exposed to increasing burden of infectious diseases caused by the emergence of viruses not yet or rarely characterised. Globalisation, climate change, settlements near wild animal habitats, and increasing numbers of immunocompromised people are likely to contribute to the emergence and spread of new infections.⁽³⁾ HTS methods have allowed genomic analysis which is sensitive for unknown or peculiar viruses over previous methodologies, such as the analysis of viral genomes used in the identification or discovery of human herpesvirus 8,⁽⁴⁾ bocavirus,⁽⁵⁾ human parvovirus 4,⁽⁶⁾ human GB virus,⁽⁷⁾ Torque Teno virus,⁽⁸⁾ WU polyomavirus⁽⁹⁾ and KI polyomavirus.⁽¹⁰⁾

HTS has been used, for example, in the discovery of an arenavirus in three patients who died of a febrile illness a few weeks after transplant of solid organs from a single donor.⁽¹¹⁾ The Illumina platform allowed identifying hepatitis A virus among cases of acute febrile illnesses that occurred in Brazil which were suspected as yellow fever.⁽¹²⁾ The same platform was used to identify a new influenza virus from swabs samples and assembly of its genome.⁽¹³⁾ It also led to the detection of viral pathogens in nasopharyngeal aspirate samples from patients with acute lower respiratory tract infections, as a new enterovirus, termed enterovirus 109 (EV109) detected in a cohort of Nicaraguan children with viral respiratory disease.⁽¹⁴⁾

Thereby, this study aimed to detect unidentified viruses by HTS approach in negative samples of nasopharynx/tracheal secretions for diagnostics targeting several viruses collected from June to August 2016 in the Federal District (DF), Brazil.

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6.3 Apêndice 3 – Página de rosto da publicação 2



Molecular and clinical characteristics related to rhinovirus infection in Brasília, Brazil

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Abstract

Introduction Human rhinovirus (HRV) is one of the most common human viral pathogens related to infections of the upper and lower respiratory tract, which can result in bronchiolitis and pneumonia. However, the relevance of HRV in human health was under-estimated for long time due to the absence of molecular targets for influenza and influenza-like syndrome surveillance in Brasília, Brazil.

Objectives The main objective of this study was analyze the clinical characteristics and outcomes of HRV infections in comparison with patients without HRV and other common respiratory viruses.

Materials and Methods For this purpose, new specific primer sets were designed based on the high throughput sequencing analysis in previous study. These primers were used for HRV detection by RT-qPCR and Sanger sequencing of amplified cDNA of 5' genomic region. The phylogenetic tree with representative HRV isolates was constructed using the Mega X software. Statistical analysis considering the patient profiles were performed using IBM SPSS program with non-parametric tests.

Results The most prevalent virus in negative samples was rhinovirus ($n = 40$), including three rhinovirus species (rhinovirus A, B, and C). The odds ratio associated with HRV infection was 2.160 for patients younger than 2 years and 4.367 for people living in rural areas. The multiple analysis showed lower chance of patients with HRV presenting respiratory distress.

Conclusion In this study, it was reported the predominance of rhinoviruses in cases of respiratory illness for negative patients for the influenza and influenza-like syndrome surveillance, being rhinorrhea, the most significant symptom associated with the disease.

Keywords Rhinovirus · Epidemiology · Acute respiratory infections · Symptoms

Introduction

Human rhinovirus (HRV) is one of the most common human viral pathogens. They are non-enveloped single-stranded RNA virus, member of *Enterovirus* genus, of *Picornaviridae* family [1, 2]. About half of common cold are caused by rhinoviruses, which have more than 160 serotypes described, in three species (rhinovirus A, B, and C) [3]. In addition, cold leads to economic problems related clinical

morbidity, overloading the health system, also causing absences at work and in schools [2, 4].

Rhinoviruses cause not only upper respiratory tract infections but also lower respiratory tract infections, mainly in children and patients with chronic pulmonary disease or immunocompromised [5]. The most common symptoms of upper infection are sore throat, rhinorrhea, headache, cough, malaise, and lower fever. However, in some cases, there may be aggravation for rhinosinusitis and otitis media [6, 7]. Infections of the lower respiratory tract are often associated with bronchiolitis and pneumonia especially in children [8–10].

In Brazil, the implementation of the sentinel surveillance system began in 2000 [11], so referral hospitals have been conducting active surveillance to detect respiratory viruses. Such surveillance includes notification and laboratory investigation of cases with the diagnostic criteria of influenza-like syndrome (ILS) and severe acute respiratory syndrome (SARS). This viral respiratory infection monitoring has resulted in important information about the circulation of

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