UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

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Análise da influência de polimorfismos nos genes *ACE2, MTHFR, AnxA2, DDX58, RelA* e *IL-6* em desfechos clínicos da COVID-19 em uma população de Pernambuco

Recife
2023

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Tese apresentada ao Programa de Pós-Graduação em Genética da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de doutor(a) em Genética. Área de concentração: Genética.

Orientador (a): Dr. Lindomar José Pena

Coorientador (a): Dr. Ronaldo Celerino da Silva

Recife

2023

Catalogação na Fonte:
Bibliotecária Natália Nascimento, CRB4/1743

Reis, Wendell Palôma Maria dos Santos.
Análise da influência de polimorfismos nos genes ACE2, MTHFR, AnxA2, DDX58, ReIA e IL-6 em desfechos clínicos da COVID-19 em uma população de Pernambuco. / Wendell Palôma Maria dos Santos Reis. – 2023.

138 f. : il., fig.; tab.

Orientador: Lindomar José Pena.
Coorientador: Ronaldo Celerino da Silva.

Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Programa de Pósgraduação em Genética, 2023.
Inclui referências.

1. COVID 19. 2. Polimorfismos. 3. ANXA2 - via inflrmatória. I. Pena, Lindomar José. (Orient.). II. Silva, Ronaldo Celerino. (Coorient.). III. Título.

587 CDD (22.ed.) UFPE/CB – 2023-106

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Aprovado em: 28/02/2023.

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Dedico este trabalho ao meu filho, **Pedro**, luz da minha vida, razão de todas as coisas, que me dá forças para prosseguir.

Dedico também a todas as vítimas da COVID-19. Aos que se foram, aos familiares e aos profissionais de saúde.

Agradecimentos

Agradeço, primeiramente, a Deus por ter me dado forças para chegar até aqui. Foi um caminho longo e de muitas lutas, mas eu consegui.

À minha mãe, que sempre foi minha base e minha rede de apoio. Obrigada por sempre acreditar nos meus sonhos.

Às amigas da vida toda, Kênnia e Ingrid, que sempre se fizeram presentes, me encorajando e dando força.

Às amigas que a maternidade me presenteou, Catarina, Nathalia e Pamela, que são minha rede de apoio importantíssima, afinal só uma mãe entende outra.

Às amigas que a rotina no laboratório me trouxe, Willyene, Poliana e Renata. Obrigada por todo apoio e conhecimento dividido e, principalmente, pelo acolhimento e amizade genuínos.

Ao meu querido co-orientador, Ronaldo, que tem papel fundamental no êxito desse trabalho. Obrigada por toda paciência e sabedoria com que conduziu essa orientação e, mais do que tudo, pelas palavras de conforto que por vezes me disse.

A todos os colegas do grupo de virologia do Lavite, que são a força motriz para o sucesso coletivo. Obrigada pelo conhecimento compartilhado e pela amizade construída.

Ao LACEN-PE pelo fornecimento do material de estudo para este trabalho e, especialmente, ao Dr. Jurandy Magalhães por todo suporte e disponibilidade.

Ao meu orientador, Dr. Lindomar Pena, pela oportunidade de conhecer pessoas excepcionais e por todo aprendizado.

Por fim e mais importante, a todos aqueles que de alguma forma sofreram as consequências da pandemia. Todo o conhecimento produzido sempre será por vocês.

“Na vida nada deve ser temido, somente compreendido. Agora é hora de compreender mais, para temer menos.” (Marie Curie).

RESUMO

COVID-19 é uma doença respiratória com diferentes desfechos clínicos que podem estar relacionados à idade, o sexo, a presença de morbidades e a variabilidade genética viral e/ou hospedeira. Variações em genes de vias envolvidas na entrada viral, na coagulação e no processo inflamatório têm sido associadas aos desfechos graves da COVID-19. Neste sentido, investigamos a distribuição alélica e genotípica de SNPs nos genes ACE2(rs228566), MTHFR(rs1801133), ANXA2(rs7163836 e rs7170178), DDX58(rs10813831), RelA(rs7101916) e IL6(rs1800795) e suas possíveis relações com a gravidade da COVID-19. Para isso, foram utilizadas amostras de swab nasofaríngeo e dados clínico-epidemiológicos de 312 indivíduos diagnosticados com COVID-19. Destas, 100 amostras foram selecionadas aleatoriamente e submetidas a extração de DNA genômico por diferentes métodos (Chelex, Fenol/Clorofórmio e kit comercial), para selecionar o método mais adequado para as análises genéticas. Posteriormente, foi realizada a genotipagem através de PCR em tempo real usando sondas alelo-específicas, bem como a estratificação dos indivíduos quanto a gravidade em casos: leves(CL), graves(CG) e fatais(CF). A extração de DNA por chelex, mostrou-se mais barata, rápida e adequada para amostras nasofaríngeas. Entre os indivíduos com COVID-19, febre e tosse foram os sintomas mais frequentes (>76% em ambos) entre os CL, enquanto tosse (89,1%), baixa saturação (82,6%) e dispneia (78,3%) foram os mais comuns entre os CG. Nos CF, dispneia (85,7%), baixa saturação e tosse (ambos 75,7%) foram predominantes. Quanto as morbidades, doenças cardiovasculares (29,3% e 15,7% respectivamente) e diabetes mellitus (25% e 14,3% respectivamente) predominaram entre os CG e CF. Dentre as variantes genéticas, os genótipos A/G (rs7170178 em ANXA2) e G/G (rs10813831 em DDX58) foram associados a uma maior susceptibilidade à mortalidade por COVID-19 e os genótipos C/C e C/T (rs7101916 em RelA) foram associados à gravidade da doença. Novos estudos com abordagens genômicas e funcionais de variantes de ANXA2, DDX58 e RelA são necessárias para uma melhor compreensão do papel desses genes nos desfechos da COVID-19.

Palavras-chave: COVID-19; gravidade; polimorfismos; ANXA2, extração, via inflamatória.

ABSTRACT

COVID-19 is a respiratory disease with different clinical outcomes that may be related to age, gender, presence of morbidities and viral and/or host genetic variability. Variations in genes in pathways involved in viral entry, clotting, and the inflammatory process have been associated with severe outcomes from COVID-19. In this sense, we investigated the allelic and genotypic distribution of SNPs in the genes ACE2(rs228566), MTHFR(rs1801133), ANXA2(rs7163836 and rs7170178), DDX58(rs10813831), RelA(rs7101916) and IL6(rs1800795) and their possible relationships with the severity of COVID-19. For this, nasopharyngeal swab samples and clinical-epidemiological data from 312 individuals diagnosed with COVID-19 were used. Of these, 100 samples were randomly selected and submitted to genomic DNA extraction by different methods (Chelex, Phenol/Chloroform and commercial kit), to select the most adequate method for the genetic analyses. Subsequently, genotyping was performed through real-time PCR using allele-specific probes, as well as the stratification of individuals according to severity in cases: mild (CL), severe (CG) and fatal (CF). DNA extraction by chelex proved to be cheaper, faster and suitable for nasopharyngeal samples. Among individuals with COVID-19, fever and cough were the most frequent symptoms (>76% in both) among CL, while cough (89.1%), low saturation (82.6%) and dyspnoea (78.3 %) were the most common among GC. In CF, dyspnoea (85.7%), low saturation and cough (both 75.7%) were predominant. As for morbidities, cardiovascular diseases (29.3% and 15.7% respectively) and diabetes mellitus (25% and 14.3% respectively) predominated among the CG and CF. Among the genetic variants, the A/G (rs7170178 in ANXA2) and G/G (rs10813831 in DDX58) genotypes were associated with a higher susceptibility to COVID-19 mortality and the C/C and C/T genotypes (rs7101916 in RelA) were associated with disease severity. New studies with genomic and functional approaches of ANXA2, DDX58 and RelA variants are needed for a better understanding of the role of these genes in the outcomes of COVID-19.

Keywords: COVID-19; gravity; polymorphisms; ANXA2, extraction, inflammatory pathway.

LISTA DE ILUSTRAÇÕES

Capítulo I

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

Item	Definição
α-CoVs	Alfacoronavírus
β-CoVs	Betacoronavírus
δ-CoVs	Deltacoronavírus
γ-CoVs	Gamacoronavírus
ACE-1	Gene da enzima conversora de angiotensina 1
ACE-2	Gene da enzima conversora de angiotensina 2
Ang-1-7	Angiotensina 1-7
Ang-II	Angiotensina II
AnxA2	Gene da anexina A 2
ATP	Adenosina trifosfato
AVC	Acidente vascular cerebral
CIVD	Coagulopatia intravascular dissminada
COVID-19	*Coronavirus disease 19*
DM	Diabetes mellitus
DNA	Ácido desoxirribonucleico
dNTP	Desorribonucleotídeo trifosfatado

DPOC	Doença pulmonar obstrutiva crônica
ERGIC	Compartimento intermediário do complexo de Golgi
FT	Fator tecidual
HAS	Hipertensão arterial sistêmica
IFN	Interferon
IVS	Variação de sequência intrônica
IL	Interleucina
MAF	Frequência do alelo menor
MCP-1	proteína quimiotática de monócitos 1
MERS-CoV	Coronavírus da síndrome respiratória do Oriente Médio
MTHFR	Metileno tetrahidrofolato redutase
OMS	Organização Mundial de Saúde
ORFs	Matriz aberta de leitura
OR	*Oddis Ratio*
qPCR	Reação em cadeia da polimerase quantitativa em tempo real
RFLP	Polimorfismo por análise de fragmento de restrição
RNA	Ácido ribonucleico
SARS-CoV	Coronavírus da síndrome respiratória aguda grave

SNARE	*soluble N-ethylmaleimide-Sensitive Factor attachment protein receptor*
SNP	Polimorfismo de nucleotídeo único
TFBS	Sítio de ligação de fator de transcrição
TMPRSS	Serino protease transmembranar
TNF	Fator de necrose tumoral
WHO	*World Health Organization*

SUMÁRIO

1 INTRODUÇÃO

Em dezembro de 2019, o SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) foi identificado pela primeira vez em Wuhan na China, como causador de um quadro respiratório heterogêneo, denominado como COVID-19. O vírus rapidamente se disseminou ao redor do mundo, levando a um cenário de pandemia, confirmado em março de 2020 pela Organização Mundial de Saúde (OMS). Desde então, a OMS, já confirmou mais de 673.112.695 casos e 6.856.026 mortes em todo mundo, desde os primeiros casos até então.

A COVID-19 é uma doença altamente transmissível, sendo disseminada, principalmente, pelas vias aéreas. As pessoas infectadas podem ou não apresentar sintomas, os quais incluem febre, cansaço e tosse seca. Outras manifestações clínicas, como: mialgia, congestão nasal, faringite, cefaleia, anosmia, diarreia, dispneia grave, disgeusia, angina, paralisia, afasia, erupção cutânea ou descoloração dos dedos das mãos ou dos pés, também são comumente relatadas. Os pulmões se destacam entre os órgãos mais afetados, entretanto a infecção pode acometer: coração, rins, fígado e cérebro.

A necessidade de admissão hospitalar é comum em 20% dos casos e o risco de fatalidade é frequente em aproximadamente 14% para indivíduos maiores de 75 anos. O curso e desfecho clínico da doença dependem de uma série de fatores como: idade, sexo, presença de comorbidades e até mesmo variações genéticas do indivíduo. A maioria dos indivíduos que desenvolve as formas mais graves da doença são do sexo masculino, idosos e com comorbidades associadas. Doenças cardíacas e vasculares, diabetes mellitus e obesidade figuram entre as comorbidades mais frequentes entre estes indivíduos acometidos.

Nos quadros mais críticos da doença, distúrbios de coagulação têm sido frequentemente observados. A infecção pelo SARS-CoV-2 é capaz de induzir uma condição inflamatória aguda, levando a uma disfunção endotelial, ativação plaquetária e hipercoagulabilidade. O aumento excessivo na produção de coágulos induzido pela COVID-19 está associado a uma série de complicações, como: coágulos arteriais e venosos, sangramentos, trombose e embolia pulmonar.

A variabilidade genética humana apresenta considerável importância no contexto da doença. Polimorfismos genéticos podem influenciar desde a entrada do SARS-CoV-2, sua replicação e até mesmo, na evolução dos diferentes quadros da

doença. Genes relacionados à resposta imunológica e a predisposição à trombose, têm apresentado papéis de destaque, seja na susceptibilidade a infecção ou mesmo na gravidade da COVID-19.

Diante da grande heterogeneidade da COVID-19 e da variabilidade genética humana, a identificação de fatores genéticos de risco para a infecção e para a gravidade da doença, são essenciais na elucidação dos mecanismos moleculares envolvidos na infecção pelo SARS-CoV-2. Neste sentido, o presente trabalho visa relacionar polimorfismos de base única em genes relacionados a entrada viral e aos processos inflamatório e de coagulação, com os diferentes desfechos clínicos de COVID-19 em indivíduos do Estado de Pernambuco.

2 REVISÃO DA LITERATURA

2.1 Coronavirus e o SARS-CoV-2

Os coronavírus fazem parte da família Coronaviridae, são assim designados devido a sua estrutura circular contendo espículas, formando bulbos em sua região apical, assemelhando-se a uma coroa. Inicialmente, os coronavírus foram descobertos em aves domésticas podendo infectar também vários outros animais, causando doenças respiratórias, gastrointestinais, hepáticas e neurológicas (Woo et al. 2012).

De acordo com as características genéticas, os coronavírus são divididos em quatro gêneros: Alfacoronavirus (α-CoVs), Betacoronavirus (β-CoVs), Gamacoronavirus (γ-CoVs) e Deltacoronavirus (δ-CoVs). Os vírus que fazem parte dos grupos alfa e betacoronavírus são capazes de infectar humanos (Cui et al. 2019).

Dentre os betacoronavírus, três se destacam por estarem associados ao desenvolvimento de doenças respiratórias graves, são eles: Severe acute respiratory syndrome coronavirus (SARS-CoV) (Zhong et al. 2003), Middle East respiratory syndrome coronavirus (MERS-CoV) (Zaki et al. 2012) e Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Zhu et al. 2020a), sendo este último o agente causador da pandemia da COVID-19 (Zhong et al. 2003; Lu et al. 2020).

O SARS-CoV surgiu em 2002, na província de Guangdong na China, sendo responsável pela infecção de 8096 indivíduos e 774 mortes (taxa de mortalidade de 10%), mas foi controlado em 2003 (Zhong et al. 2003; WHO 2015; Su et al. 2016).

Em 2012, surgiu o MERS-CoV na península Arábica, ocasionando 2562 casos e 881 mortes, com uma taxa de mortalidade de 34%. Sua epidemia foi controlada em 2013, apesar de novos casos pontuais na península Arábica (Ramadan and Shaib 2017; WHO 2019).

Já o SARS-CoV-2, surgido em dezembro de 2019 em Wuhan na China, apesar de sua baixa mortalidade (2 a 3%) e brevidade do surgimento, apresenta-se como uma emergência sanitária global. O vírus apresenta altos índices de infectabilidade e transmissibilidade em comparação aos demais Betacoronavírus, provocando um maior número de exposições e o surgimento de quadros clínicos heterogêneos e desafiadores, dentro de um curto espaço de tempo (Hartley and Smith 2003; Bauch and Oraby 2013; Gao et al. 2020).

Semelhante ao MERS-CoV e ao SARS-CoV, o SARS-CoV-2 tem de 80-160 nM de diâmetro e seu genoma é composto por RNA de fita simples com polaridade positiva, contendo aproximadamente 30 kb de tamanho (Harcourt et al. 2020). A maior parte do genoma é constituída por ORFs (Open Reading frames – quadros abertos de leitura) denominadas Replicase 1a e 1b, que apresentam alto nível de conservação em todos os vírus da família Coronaviridae (Figura 1). Adicionalmente, codifica proteínas estruturais próximas à extremidade 3’: a glicoproteína S (Spike), as proteínas do envelope (E) viral, as proteínas da membrana (M) e a proteína nucleocapsídica (N); e proteínas não estruturais: RNA polimerase, helicase e a endoribonuclease (Wang et al. 2020).

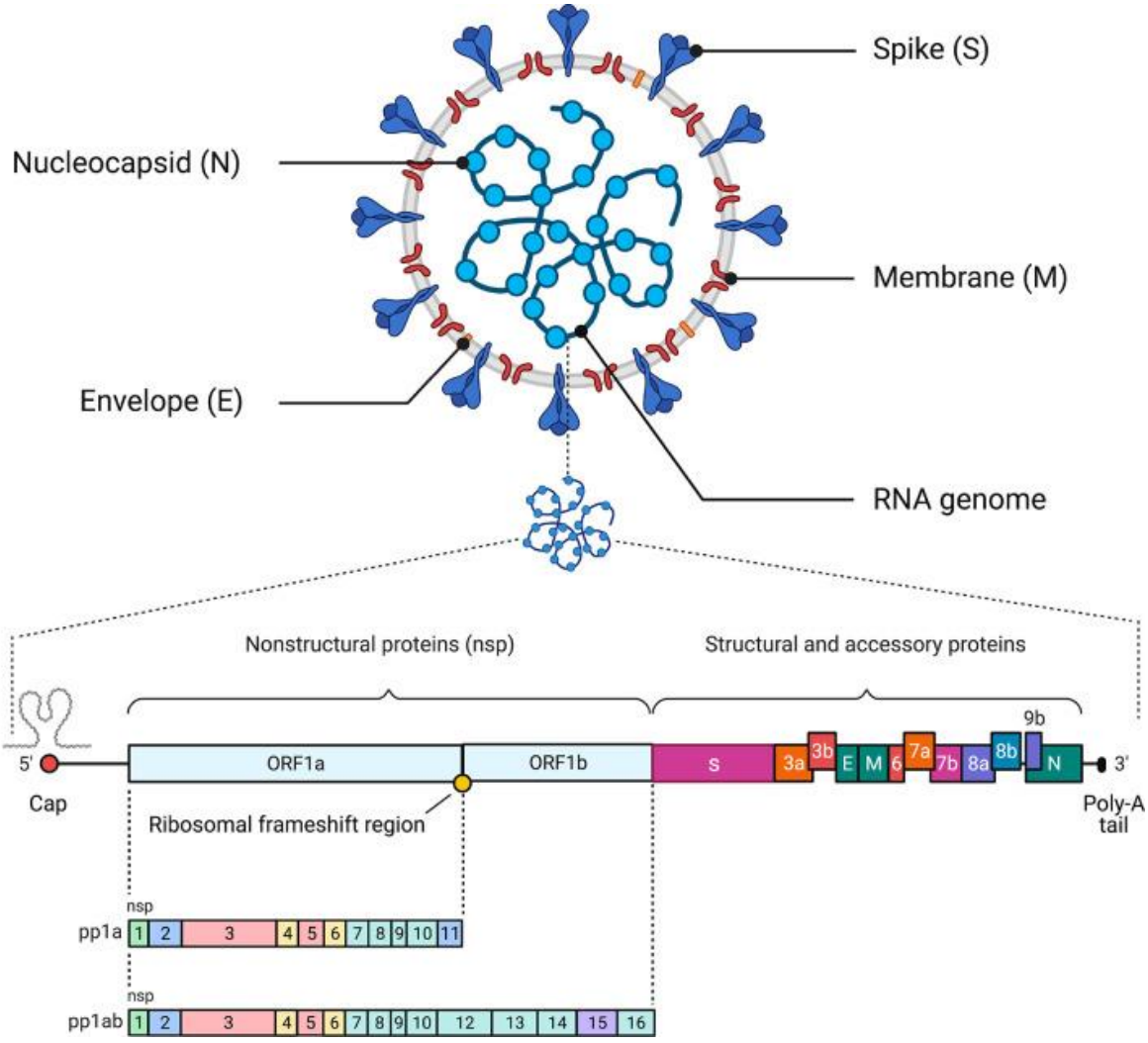

Figura 1. Estrutura do vírus SARS-CoV-2 e organização do genoma. Adaptado de (da Silva et al. 2022).

O gene N apresenta grande similaridade entre os vírus da mesma família, entretanto a região S1 da proteína Spike é bem variável (Yan et al. 2020; Walls et al. 2020). Variações na proteína Spike podem ser responsáveis pelo aumento da transmissibilidade e capacidade de infecção do vírus nas células do hospedeiro (Gupta et al. 2021).

2.1.1 Epidemiologia

Desde sua primeira descrição na China, o SARS-CoV-2 rapidamente se espalhou pelo planeta, ocasionando grande impacto nos sistemas de saúde e econômicos mundiais. Passados mais de três anos desde o seu surgimento, o vírus continua sendo uma ameaça à saúde pública, embora o número de casos e mortes tenha diminuído, graças à implantação em larga escala de vacinas eficazes (Dong et al. 2020).

Em todo o mundo já foram registrados 673.112.695 casos de COVID-19, sendo 6.856.026 casos fatais, segundo dados mais recentes (https://covid19.who.int, acesso 15/12/2023 as 10:45). A taxa global de mortalidade por COVID-19 tem sido estimada em aproximadamente 1,2% (Dong et al. 2020).

Dentre os países com maiores números de casos confirmados estão os Estados Unidos (102.626.386 casos), a Índia (44.685.601 casos), a França (38.527.780 casos), a Alemanha (37.949.446 casos) e o Brasil (36.949.318 casos), segundo estimativas da OMS (https://covid19.who.int , acesso 15/02/2023 as 10:00).

A Europa desponta com a região com o maior número de casos de COVID-19, seguido pelas Américas. Por outro lado, quase metade de todas as mortes por COVID-19 são provenientes das Américas. Essas disparidades se devem à escassez de testes, bem como a dificuldade de acesso aos serviços de saúde na maior parte dos países latino-americanos, o que pode ter impactado na verdadeira incidência da doença (https://www.worldometers.info/coronavirus/). De acordo com um estudo realizado no Brasil, no primeiro ano de pandemia cerca de 80% dos pacientes que precisaram de ventilação mecânica invasiva morreram. Este índice foi superior à mortalidade observada em indivíduos entubados na Europa (81,7% a 69%) (Ranzani et al. 2021).

O Brasil figura entre os países mais afetados pela COVID-19. Até o momento foram registrados mais de 36 milhões de casos confirmados e mais de 697 mil mortes, apresentando uma taxa de letalidade de 1.97% (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports). A doença abalou o sistema de saúde pública do país, exibindo as fragilidades sociais e econômicas das diferentes regiões brasileiras. Segundo dados do Ministério da Saúde, em número de casos as regiões brasileiras mais afetadas foram o Sudeste

(14.671.333 casos), Sul (7.849.101 casos) e Nordeste (7.296.837 casos). Já em relação ao número de óbitos, a Região Sudeste acumula a maior parte dos óbitos (335.585 óbitos), seguida por Nordeste (134.323 óbitos) e Sul (110.546 óbitos) (https://covid.saude.gov.br, acesso 15/02/2023 as 10:03).

No estado de Pernambuco, o primeiro caso de COVID-19 foi notificado em 12 de março de 2020. Atualmente, a taxa de incidência da doença no estado é de 12.040 por 100.000 habitantes e taxa de letalidade de 2%. Os casos confirmados da doença no estado estão distribuídos em 184 municípios, além do arquipélago de Fernando de Noronha (https://covid.saude.gov.br, acesso 15/02/2023 as 10:05).

2.1.2 Ciclo de replicação viral

A glicoproteína S do SARS-CoV-2 é um homotrímero formado pelos subdomínios S1 e S2 e é altamente glicosilado (Zhou et al. 2020a). O subdomínio de S1 é responsável pela ligação viral ao receptor da célula hospedeira, enquanto S2 está envolvido na fusão das membranas do vírus e da célula do hospedeiro (Figura 2) (Walls et al. 2020).

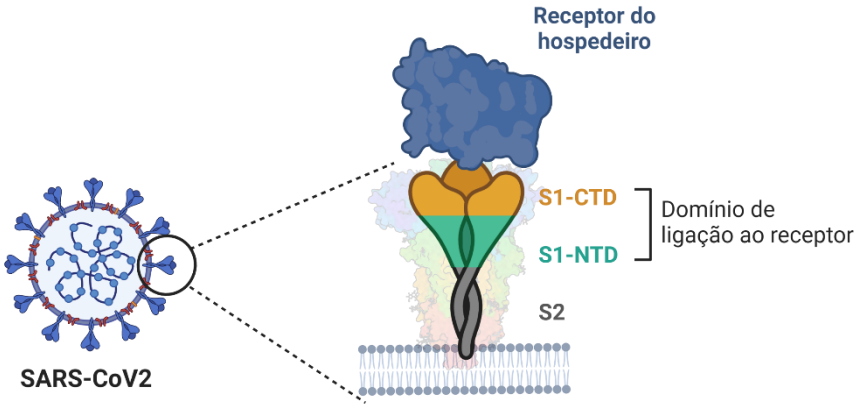

Figura 2. Mecanismo de ligação da proteína S ao receptor da célula hospedeira. S1-CTD – Domínio C-Terminal; S1-NTD – Domínio N-Terminal (Autor).

O processo de entrada do vírus na célula hospedeira se inicia pelo reconhecimento do receptor ACE2, expresso na superfície das células-alvo, pela

proteína S, a qual possui um sítio de clivagem de furina entre os domínios S1 e S2 (Hoffmann et al. 2020).

A clivagem é mediada através da serino protease transmembranar tipo II (TMPRSS2) no domínio S2 para ativar a fusão da membrana viral com a célula hospedeira (Figura 3). Após a clivagem da furina entre os domínios S1/S2, o vírus entra nas células através de endocitose mediada por clatrina. A fusão das membranas é semelhante à fusão da membrana celular mediada por SNARE (do inglês, Soluble N-ethylmaleimide-Sensitive Factor Attachment Protein Receptor) gerando um canal que permite a entrada das proteínas do nucleocapsídeo associadas ao RNA viral no citosol da célula hospedeira (Tang et al. 2020).

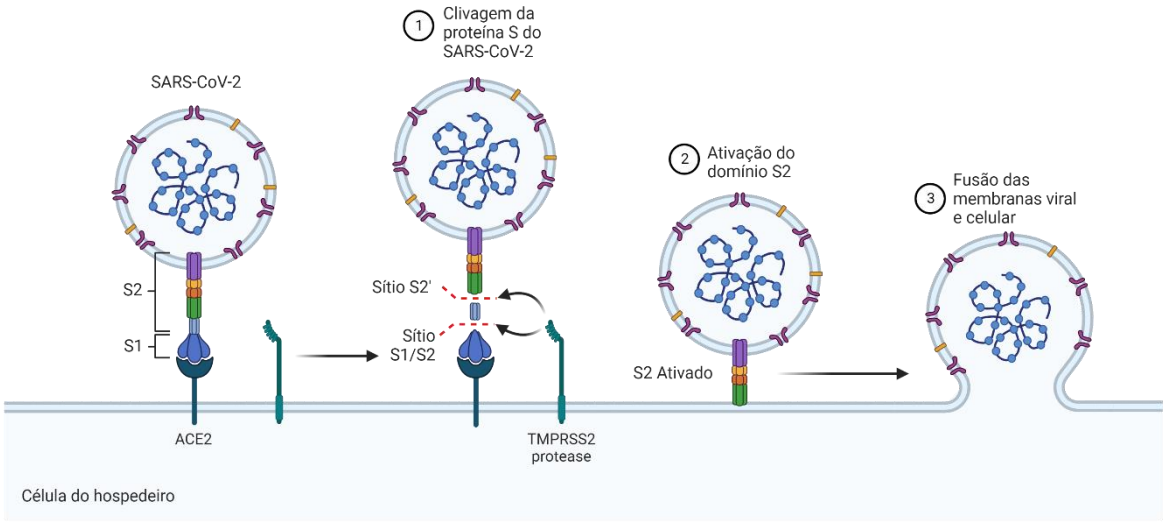

Figura 3. Mecanismo de reconhecimento e entrada do SARS-CoV-2 na célula do hospedeiro (Autor).

Adicionalmente, a proteína pleiotrópica anexina A2 (AnxA 2) tem demonstrado envolvimento nos processos de fixação, internalização, replicação e patogênese de vários vírus, incluindo o SARS-CoV. A AnxA2 interage com a RNA polimerase viral para replicação e ajuda no transporte, maturação e liberação dos novos vírus (Patil et al. 2021).

Após a entrada na célula hospedeira, a replicação viral ocorre no citoplasma. O RNA viral utiliza a maquinaria enzimática da célula hospedeira para se replicar, expressar suas proteínas e montar novas partículas virais. A montagem dos vírions ocorre através da interação entre o RNA genômico viral, as proteínas estruturais localizadas no retículo endoplasmático (RE) e no compartimento intermediário do complexo de Golgi (ERGIC). Ao final, os vírions são liberados através de lisossomos

para a membrana plasmática e secretados pela célula via exocitose (Hoffmann et al. 2020).

2.2 A COVID-19

A doença respiratória causada pela infecção do SARS-CoV-2 foi chamada de COVID-19 (do inglês, Corona Virus Disease 2019), sendo caracterizada por manifestações clínicas que variam desde leves (80% dos casos) até graves (5 a 10% dos casos), existindo ainda, indivíduos que não apresentam nenhum sintoma, os assintomáticos (Leentjens et al. 2021).

Dentre os principais sintomas apresentados por indivíduos infectados estão: febre, tosse, mialgia, fadiga, sintomas respiratórios, dispneia, manifestações gastrointestinais etc. Além disso, os quadros mais graves podem apresentar insuficiência respiratória, cardíaca, sepse e falência de órgãos (Huang et al. 2020).
O curso clínico da infecção pode ser influenciado por diversos fatores, como: faixa etária, sexo, condições clínicas pré-existentes, fatores genéticos do vírus e do hospedeiro, o que confere a doença um caráter multifatorial.

Embora a COVID-19 apresente, principalmente, consequências relacionadas ao sistema respiratório, disfunções no processo de coagulação são comumente observadas entre os infectados (Wu et al. 2020).

A infecção pelo coronavírus ocasiona um aumento da produção de citocinas pró-inflamatórias e quimiocinas, como o fator de necrose tumoral alfa (TNF-α), interleucina 1 beta (IL-1β), e a proteína quimiotática de monócitos 1 (MCP-1), entre outros (Iba et al. 2020). Consequentemente, essas moléculas recrutam células do sistema imune que iniciam uma resposta imunológica hiper inflamatória sistêmica e lesões teciduais. Indivíduos com forma clínica grave de COVID-19 apresentam linfopenia e a tempestade de citocinas, como expressão máxima da desregulação da resposta imune (Zhou et al. 2020b; Giamarellos-Bourboulis et al. 2020).

A ativação imunológica exacerbada propicia a expressão do fator tecidual (FT) em células endoteliais e monócitos do sangue, ativando a cascata de coagulação (Mucha et al. 2020). Em aproximadamente 5% dos indivíduos sintomáticos, a

progressão do processo inflamatório irá deflagrar uma tempestade de citocinas, intensificando o quadro pró-coagulatório e ativando uma exacerbada cascata de coagulação. A coagulopatia associada a COVID-19 pode ser considerada uma endoteliopatia que causa o aumento da ativação plaquetária e hipercoagulabilidade, levando às manifestações clínicas pró-trombóticas associadas à infecção (Leentjens et al. 2021).

Uma vez que a infecção pelo SARS-CoV-2 acontece através da interação do vírus com o receptor da enzima conversora da angiotensina 2 (ACE-2), a COVID-19 está intimamente relacionada com a cascata de coagulação. A ACE2 faz parte do mecanismo fisiológico renina-angiotensina-aldosterona, o qual quando alterado pode ocasionar distúrbios de coagulação. Estudos avaliando alterações clínicas e laboratoriais nesse sistema têm demonstrado a presença de coagulopatias em até 50% dos indivíduos com formas graves de COVID-19 (Asakura and Ogawa 2021).

2.2.1 Fatores de Risco a COVID-19

Estudos epidemiológicos têm demonstrado que o sexo dos indivíduos é uma característica importante para o desenvolvimento da COVID-19 na população. As taxas de mortalidade e de gravidade da doença se encontram aumentadas com maior frequência em indivíduos do sexo masculino (Wang et al. 2020). Um estudo realizado na Itália, observou que os índices de morbidade e letalidade foram maiores em homens (59% e 17,7% respectivamente) (Foresta et al. 2021).

A presença de condições clínicas prévias e a idade avançada também se configura como fator de risco para a COVID-19 (Wu et al. 2020). Indivíduos com as formas mais graves, apresentam em média de idade superior aos 60 anos (Zhang et al. 2020b).

Doenças, como a hipertensão arterial sistêmica (HAS) e diabetes mellitus (DM), podem causar alterações na coagulação e no sistema imunológico, ocasionando uma superprodução de citocinas inflamatórias (Huang et al. 2020).

A HAS é a comorbidade mais prevalente na população que vai a óbito por conta da COVID-19 e possui expressiva prevalência entre os casos graves. Aproximadamente 43,9% dos indivíduos com quadros críticos de COVID-19 são hipertensos (Tiburi et al. 2021). Adicionalmente, portadores de DM apresentam um estado pró-trombótico induzido pela hiperglicemia, o que consequentemente leva a

um importante agravamento da coagulopatia induzida pela COVID-19 (Tiburi et al. 2021; Santos et al. 2021).

Indivíduos com coagulopatias pré-existentes (tromboembolismo venoso, trombose venosa profunda e embolia pulmonar) também possuem risco elevado para desenvolver a COVID-19 em suas formas mais graves (Wang et al. 2020).

Indivíduos portadores de comorbidades pré-existentes e infectados pelo SARS-CoV-2, no geral, apresentam contagem de dímero-D ainda mais elevada do que aqueles que não possuem nenhuma condição clínica prévia (Dobesh and Trujillo 2020).

Outros estudos indicam que os fatores genéticos também são determinantes para a susceptibilidade a infecção e para a evolução dos quadros clínicos mais graves da doença (2020; Debnath et al. 2020; Alshahawey et al. 2020). Semelhanças na susceptibilidade de COVID-19 foram observadas entre indivíduos geneticamente relacionados, sugerindo que pode haver uma seletividade do vírus por perfis genéticos específicos (Murray et al. 2020; Choudhary et al. 2021). Esses perfis genéticos somados aos demais fatores de risco podem ser cruciais para elucidar a progressão da gravidade e letalidade da doença.

2.2.2.1 Genes relacionados aos mecanismos de infecção viral, inflamação e coagulação

2.2.2.1.1 ACE2

O gene ACE2 está localizado no cromossomo Xp22 (Figura 4), é composto por 18 éxons e expresso em diversos tipos celulares em tecidos dos pulmões, rins, coração e trato gastrointestinal (Chappel and Ferrario 2006; Lambert et al. 2008). Codifica a enzima conversora de angiotensina 2, a qual atua como principal receptor para o vírus SARS-CoV-2 (Shang et al. 2020).

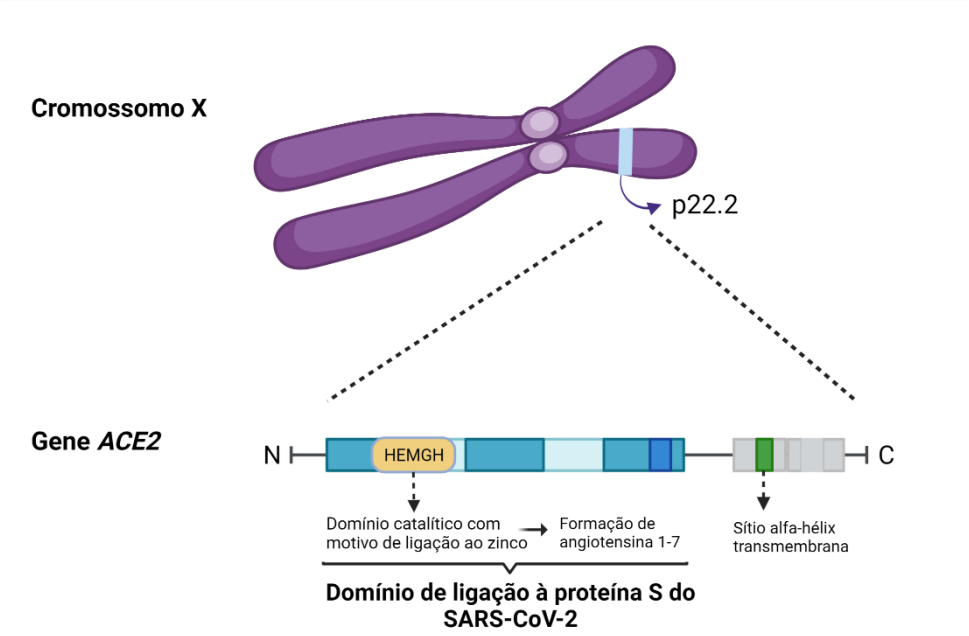

Figura 4. Localização e estrutura do gene ACE2. (Autor).

A proteína ACE2 também atua como regulador negativo no sistema renina-aldosterona pela hidrólise de angiotensina II (Ang II) em angiotensina 1-7 (Ang 1-7) (Lambert et al. 2008). A Ang II tem efeito profibrótico e vasoconstritor, mas ao ser hidrolisada, diminui o estresse oxidativo, uma vez que seu efeito é contrarregulado com ações antifibróticas e vasodilatadoras (Chappel and Ferrario 2006).

Alterações em ACE2 elevam os níveis de Ang II e reduzem a função cardíaca. Polimorfismos funcionais em ACE2, podem alterar a estrutura do receptor e/ou os níveis de expressão gênica. Tais variantes vêm sendo associadas ao risco de doenças cardiovasculares e pulmonares, consequentemente, podem contribuir para os diferentes desfechos da COVID-19 (Yan et al. 2020; Gemmati et al. 2020; Shang et al. 2020).

Os polimorfismos não-sinônimos (rs149039346 e rs147311723) apresentam uma forte influência na função e estabilidade de ACE (Paniri et al. 2021). O polimorfismo rs2285666 tem apresentado diferenças significativas entre populações europeias e asiáticas quanto a distribuição alélica aumentando a expressão gênica de ACE2 em até 50%, o que sugere um papel importante na susceptibilidade ao SARS-CoV-2 (AL-Eitan and Alahmad 2021).

2.2.2.1.2 MTHFR

O gene MTHFR, localizado no cromossomo 1 (1p36.3) e composto por 11 éxons, codifica a enzima metileno tetrahidrofolato redutase (MTHFR), a qual está envolvida no metabolismo do folato e na conversão da homocisteína em metionina (Ponti et al. 2021a).

A homocisteína tem sido alvo de muitas especulações desde a sua descoberta, exercendo papeis variados em diversos processos metabólicos e inflamatórios (Smith and Refsum 2021). Altos níveis plasmáticos de homocisteína aumentam significativamente a incidência de danos vasculares (Balint et al. 2020). Além disso, a hiper-homocisteinemia tem efeitos neurotóxicos, neuroinflamatórios, neurodegenerativos, pró-aterogênicos, pró-trombócitos e pró-oxidativos (Zoccolella et al. 2006).

Alguns estudos têm relacionado os níveis de homocisteína com a gravidade da COVID-19, o que possibilita o uso de homocisteína como um biomarcador para o prognóstico da doença (Yang et al. 2020).

A homocisteína pode ativar o receptor ACE2, através de um mecanismo regulatório chamado de ferroptose. A ferroptose é um tipo morte celular programada caracterizada pelo acúmulo de ferro e espécies reativas de oxigênio (Dixon et al. 2012). Tal mecanismo tem sido associado com distúrbios neurológicos comuns a COVID-19, como comprometimento cognitivo (Sun et al. 2020), ageusia e anosmia (perda de paladar e olfato) (Dinc et al. 2016).

Polimorfismos no gene MTHFR vêm sendo associados à gravidade da COVID-19 (Ponti et al. 2021b). Um dos polimorfismos mais frequentes estudado é o C677T (rs1801133), uma variação não-sinônima onde uma alanina é substituída por uma valina (Ala222Val) na posição 677. Os indivíduos homozigotos e heterozigotos para o polimorfismo apresentam níveis elevados e moderados de homocisteína, respectivamente (Liew and Gupta 2015).

Populações diferentes apresentam prevalências distintas para polimorfismos no gene MTHFR e seus efeitos na atividade da enzima (Yafei et al. 2012). Em populações americanas, europeias e asiáticas, foi observado que o genótipo homozigoto para MTHFR -677 estava relacionado a uma maior incidência e mortalidade por COVID-19 (Ponti et al. 2021b). Neste sentido, as informações

genéticas, juntamente com a dosagem de homocisteína podem ser relevantes na classificação de indivíduos acometidos com a doença.

Outro polimorfismo frequentemente estudado no gene MTHFR é a variante A1298C (rs1801131), uma variação do tipo não-sinônima na posição 1298, onde há uma troca de glutamato por uma alanina. Tal variante tem sido associada com aumento do risco de acidente vascular cerebral isquêmico (Zhang et al. 2014) e com uma aumentada gravidade e mortalidade em indivíduos com COVID-19 (Ponti et al. 2021b).

2.2.2.1.3 AnxA2

A anexina A2 (AnxA2) é uma proteína pleiotrópica dependente de cálcio que participa de diversas atividades biológicas relacionadas à membrana plasmática (Liu and Hajjar 2016). Possui um peso molecular de 36 kDa e é codificada pelo gene AnxA2, localizado no cromossomo 15q22.2 e constituído por 13 éxons. Pode se apresentar como monômero no citoplasma ou como heterotetrâmero na membrana extracelular. Esse tetrâmero (A2T) é gerado através da interação não covalente de duas moléculas de AnxA2 com duas moléculas S100A10 (p11) (Bharadwaj et al. 2013). Essa estrutura atua como um receptor para o ativador de plasminogênio tecidual (tPA), que converte o plasminogênio em plasmina e que participa do processo de fibrinólise (Madureira et al. 2011).

AnxA2 está envolvida em vários processos celulares, como: proliferação celular, metástase, angiogênese e inflamação. Adicionalmente, possui atividade de proteína de ligação ao RNA e como molécula sinalizadora para múltiplas vias de sinalização (Valapala and Vishwanatha 2011). Evidências também sugerem o envolvimento da anexina na fixação, internalização, replicação e patogênese de vários vírus, incluindo o SARS-CoV-2 (Fang et al. 2010; Taylor et al. 2018).

AnxA2 pode interagir com as proteínas S2 e RdRp do SARS-CoV-2, auxiliando na internalização e replicação viral. Depois que o vírus se liga à célula hospedeira, é internalizado através do auxílio da AnxA2 ligada à membrana. Este fato pode levar a uma diminuição da plasminólise da superfície celular mediada por A2T e ocasionar trombose intravascular em indivíduos com COVID-19. Uma vez dentro da célula, o

vírus irá se replicar com o auxílio da AnxA2 monomérica citoplasmática, o que pode resultar em uma onda inflamatória severa e tempestade de citocinas (Patil et al. 2021).

Estudos de variações no gene AnxA2 indicam uma associação do polimorfismo IVS-14-1046 (rs7163836; do inglês, intronic variation sequence) localizado no íntron 14 e na posição 1046, com o risco de acidente vascular cerebral (AVC) em indivíduos portadores de anemia falciforme (Baldwin et al. 2005; Baldwin et al. 2005). Outro polimorfismo bastante estudado é o rs7170178, caracterizado pela troca de uma guanina por uma alanina na posição 5681 e frequentemente associado com complicações clínicas específicas da anemia falciforme e com o desenvolvimento de osteonecrose (Pereira-Martins et al. 2020).

Até o momento, estudos de associação de polimorfismos em AnxA2 com os desfechos clínicos da COVID-19 ainda são escassos.

2.2.2.1.4 DDX58

O gene DDX58 está localizado no cromossomo 9 (9p.21.1) e codifica o receptor de resposta imune inata antiviral (RIG-I). Este receptor detecta ácidos nucléicos virais no citoplasma e ativa uma cascata de sinalização a jusante, produzindo interferons tipo I e citocinas pró-inflamatórias (Saito et al. 2007).

RIG-I é expressa em baixos níveis na maioria das células e sua abundância aumenta em resposta ao IFN (Saito et al. 2007). Estruturalmente é constituída por motivos RNA helicase-DEAD box e um domínio de recrutamento de caspases (CARD), importantes para interações com a proteína de sinalização antiviral mitocondrial (MAVS) (Kato et al. 2006).

Após o reconhecimento do RNA viral, RIG-I hidrolisa o ATP, sofre uma mudança conformacional e expõe um domínio de ligação para uma interação mais próxima com o RNA. A hidrólise do ATP também facilita a translocação de RIG-I ao longo do RNA, abrindo espaço para outras proteínas RIG-I oligomerizarem na molécula (Patel et al. 2013).

Os vírus desenvolveram mecanismos elaborados para favorecer sua replicação e escapar ou inativar a sinalização imune inata (Schindler et al. 2007). No caso do SARS-CoV, este apresenta um mecanismo antagônico para escapar das atividades antivirais do IFN (Zielecki et al. 2013). Entretanto, RIG-I reconhece a região 3' não

traduzida (3’-UTR) do RNA do SARS-CoV-2 por meio dos domínios da helicase. Esta interação não estimula a atividade ATPase e induz um efeito inibitório na replicação viral, sem ativar as vias convencionais de sinalização (Yamada et al. 2021), ajudando a explicar, em parte, porque muitos indivíduos infectados por SARS-CoV-2 tendem a apresentar doença assintomática ou leve. Tais fenótipos podem estar associados à falha na indução de uma imunidade adaptativa eficiente e duradoura (Ibarrondo et al. 2020).

Estudos observaram que células pulmonares de indivíduos com doença pulmonar obstrutiva crônica (DPOC) apresentaram níveis reduzidos de RIG-I e são mais suscetíveis à infecção por SARS-CoV-2. Neste sentido, os níveis de expressão de RIG-I podem ser determinantes intrínsecos para a defesa em células pulmonares humanas durante o processo inicial da infecção por SARS-CoV-2 (Yamada et al. 2021).

Polimorfismos no gene DDX58 são amplamente estudados. Um dos SNPs com maior destaque é o rs10813831, que causa uma alteração não sinônima de G por A com alto impacto funcional. Um estudo realizado na China sugere que indivíduos com HCV, portadores do alelo rs10813831-G, tiveram uma probabilidade significativamente menor de continuar uma infecção persistente do que os portadores do alelo A (Wu et al. 2019).

2.2.2.1.5 RelA

O gene RelA, localizado no cromossomo 11 (11q13.1), é composto por 11 éxons e codifica a proteína P65. Esta proteína é um fator de transcrição que, em conjunto com outros fatores de transcrição (RelB, c-Rel, P50 ou NF-KB1 e P52 ou NF-KB2) compõem o complexo NF-KB. NF-KB é um fator nuclear que se liga a um intensificador kappa do gene da cadeia leve de imunoglobulina kappa de células B ativadas (Baltimore 2009).

É expresso em diversos tipos celulares, sendo um importante regulador da resposta imune contra patógenos, da reação inflamatória e da proliferação e sobrevivência celular (Li and Verma 2002). É mantido no citoplasma em um estado inativo por inibidores de κB (IκBs), os quais quando degradados pela enzima IκB

quinase (IKK), leva à ativação do NF-κB, o qual se move para o núcleo e ativa a transcrição de genes específicos (Karin et al. 2004).

Os membros da família NF-κB formam homo e heterodímeros para induzir ou reprimir a transcrição, onde o heterodímero RelA/p50 normalmente regula a expressão gênica em resposta à IL-1 (Oeckinghaus and Ghosh 2009; Thomas-Jardin et al. 2020). A ativação excessiva de NF-KB pode desencadear a produção de citocinas pró-inflamatórias e uma tempestade de quimiocinas (O'Neill and Kaltschmidt 1997). RelA, RelB e c-Rel contêm domínios de ligação altamente conservados na região N-terminal chamada Domínio de homologia Rel (RHD). Essa região medeia a formação de hetero-dímeros, bem como a ligação e transativação do DNA (Oeckinghaus and Ghosh 2009).

O complexo homodimérico RelA-RelA NF-KB parece estar envolvido na ativação mediada por invasina da expressão de IL-8. Além disso, é considerado um fator de transcrição chave na regulação da resposta de IFN durante a infecção por SARS-CoV-2. Estudos demonstram que a proteína do nucleocapsídeo do SARS-CoV-2 pode ativar o NF-KB de maneira dose dependente. Esses achados fornecem informações sobre como o hospedeiro detecta o vírus e, consequentemente, desencadeia a resposta imune inata nas células infectadas (Yin et al. 2021).

Algumas variantes de RelA têm sido descritas. Dentre elas, o polimorfismo rs7101916 que está localizado próximo a extremidade 5' do gene. Esse polimorfismo foi previsto como um sítio de ligação do fator de transcrição (TFBS), podendo envolver a alteração da ligação do fator de transcrição e mediar a regulação da transcrição. O SNP rs7101916 pode influenciar a regulação transcricional do gene RelA e sua expressão, afetando subsequentemente a ativação da via NF-κB e a suscetibilidade à infecção viral (Yue et al. 2019).

2.2.2.1.6 IL6

O gene IL6 está localizado no cromossomo 7p15.3 e é composto por 5 éxons. Este gene codifica a glicoproteína interleucina 6 (IL6) de 21 KDa, uma citocina que atua na inflamação e na maturação de células B. A expressão deste gene está implicada em uma ampla variedade de patologias associadas à inflamação (Fei et al. 2015). A IL6 é produzida principalmente em locais de inflamação aguda e crônica,

sendo secretada no soro e induz resposta inflamatória transcricional através do receptor alfa da IL6. Níveis elevados de IL6 são comumente associados a infecções virais, incluindo o SARS-CoV-2 (Bhaskar et al. 2020).

A IL6 é considerada uma citocina inflamatória chave, é secretada por células imunes e não imunes no tecido pulmonar, como macrófagos, linfócitos T, células epiteliais alveolares tipo II (ECs) e fibroblastos pulmonares (Zhang et al. 2020a). A IL6 induz a apoptose linfocítica levando ao desenvolvimento de linfopenia em pacientes com COVID-19 (Tan et al. 2020; Abbasifard and Khorramdelazad 2020). Níveis elevados de IL6 prejudicam substancialmente a função dos linfócitos devido a diminuição significativa da expressão do antígeno leucocitário D humano (HLA-DR), juntamente com a diminuição de linfócitos CD4+, linfócitos CD19+ e células natural killer (NK). Além disso, IL6 tem um papel importante na gravidade dos pacientes com COVID-19, estando significativamente associada a resultados clínicos adversos (Liu et al. 2020; Chen et al. 2020; Gong et al. 2020).

Muitos polimorfismos já foram descritos nas regiões codificantes e não codificantes do gene IL6 (Fei et al. 2015). As diferenças na produção de citocinas entre indivíduos podem ser explicadas pela presença de SNPs nas regiões regulatórias, como promotores, íntrons e regiões 5' e 3'-UTR (Bidwell et al. 2001). Alguns estudos têm relatado que polimorfismos na região promotora do gene, estão associados ao risco e a gravidade de muitas doenças, como pneumonia, asma e doença obstrutiva pulmonar crônica (DPOC) (He et al. 2009; Chen et al. 2015; Jiménez-Sousa et al. 2017; Kirtipal et al. 2020). Dentre os SNPs mais estudados, estão rs1800795 (−174G>C), rs1800796 (−572G>C) e rs1800797 (−597G>A), localizados no promotor do gene IL6 e que podem estar correlacionados com a gravidade da COVID-19 (Verma et al. 2023).

O SNP rs1800795 é caracterizado pela troca de G por C na posição -174 do gene, localizado na região promotora, afetando os níveis de produção da citocina (Cussigh et al. 2011; Velazquez-Salinas et al. 2019). Estudos realizados na China indicaram que o aumento nos níveis séricos de IL6 é uma característica chave da gravidade da COVID-19 e pode servir como biomarcador para prever a progressão da doença (Zhu et al. 2020b; Liu et al. 2020).

3 OBJETIVOS

3.1 Objetivo geral

Verificar a relação entre polimorfismos de base única em genes relacionados à entrada viral, inflamação e coagulação e os diferentes desfechos clínicos de COVID-19 em indivíduos do Estado de Pernambuco.

3.2 Objetivos específicos

- ✓ Analisar qual o melhor método de extração de DNA genômico a partir de amostras de *swab* nasal de indivíduos positivos para COVID-19;
- ✓ Investigar as distribuições alélicas e genotípicas dos genes *ACE2, MTHFR, AnxA2, DDX58, RelA* e *IL-6* e sua possível relação com os diferentes desfechos clínicos da doença;
- ✓ Relacionar a distribuição genotípica destes genes com as morbidades e com a sintomatologia apresentadas por indivíduos com as formas graves e fatais de COVID-19.

4 ARTIGO 1 – COMPARISON OF DNA EXTRACTION METHODS FOR COVID-19 HOST GENETICS STUDIES.

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ABSTRACT

The coronavirus disease 2019 (COVID-19) pandemic has resulted in global shortages in supplies for diagnostic tests, especially in the developing world. Risk

factors for COVID-19 severity include pre-existing comorbidities, older age and male sex, but other variables are likely play a role in disease outcome. There is indeed increasing evidence that supports the role of host genetics in the predisposition to COVID-19 outcomes. The identification of genetic factors associated with the course of SARS-CoV-2 infections relies on DNA extraction methods. This study compared three DNA extraction methods (Chelex®100 resin, phenol-chloroform and the QIAamp DNA extraction kit) for COVID-19 host genetic studies using nasopharyngeal samples from patients. The methods were compared regarding number of required steps for execution, sample handling time, quality and quantity of the extracted material and application in genetic studies. The Chelex®100 method was found to be cheapest (33 and 13 times cheaper than the commercial kit and phenol-chloroform, respectively), give the highest DNA yield (306 and 69 times higher than the commercial kit and phenol-chloroform, respectively), with the least handling steps while providing adequate DNA quality for downstream applications. Together, our results show that the Chelex®100 resin is an inexpensive, safe, simple, fast, and suitable method for DNA extraction of nasopharyngeal samples from COVID-19 patients for genetics studies. This is particularly relevant in developing countries where cost and handling are critical steps in material processing.

Key words: Coronavirus disease, DNA extraction methods, Chelex®100, Phenol-Chloroform, QIAamp DNA extraction kit.

INTRODUCTION

The COVID-19 pandemic caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has posed an unprecedented challenge to humanity at many levels. Soon after SARS-CoV-2 emergence, the virus proved to have sustained human to human transmission and in specific settings, also high mortality (Ng *et al.*, 2021). Social distancing, mask wearing, hand hygiene and testing were the pillars of COVID-19 control prior to the advent and deployment of effective vaccines. The gold standard for COVID-19 diagnosis is viral detection through the amplification of nucleic acids in nasopharyngeal swab samples by real time polymerase chain reaction (RT-PCR) (Kevadiya *et al.*, 2021). As SARS-CoV-2 spread globally, a shortage in supplies for diagnostic tests and reagents for scientific research became an issue to many countries, especially in the developing world. This has had an impact in the diagnostics of COVID-19 and other diseases (Silva, Magalhães and Pena, 2021).

COVID-19 is a complex disease that present a broad spectrum of clinical manifestations ranging from an asymptomatic to a severe clinical course. Several risk factors have been associated with disease severity, including age, male sex and comorbidities. However, other variables such as host genetic background have been implicated in the outcome of this infection (Fricke-Galindo and Falfán-Valencia, 2021; Initiative, 2021). In addition to a well-designed and characterized study, the quality of extracted host DNA underpins any subsequent work.

A good extraction method needs to be safe, fast to perform and generate genomic DNA with good quality and in sufficient quantity for downstream analyses (Edwin Shiaw *et al.*, 2010; Popoiu G, Matei M, Azoicăi D, 2011; Gupta, 2019). The main DNA extraction techniques routinely used include organic extraction (phenol–chloroform method), nonorganic method (salting out and proteinase K treatment) and adsorption-based methods (silica–gel membrane). These techniques allow consistent DNA isolation from several biological specimens, but they differ in both the quality and the quantity of DNA yielded (Edwin Shiaw *et al.*, 2010; Gupta, 2019).

In-house methods usually have low cost, but they are oftentimes very laborious and time consuming, and requires many steps involving detergent-mediated lysis, proteinase treatment, extractions with hazardous organic solvents (phenol, chloroform and isoamylalcohol) and ethanol precipitation. Taken together, this increases the risk of DNA cross-contamination from sample to sample and poses chemical and ergonomic risks for the operator. Despite the existence of non-toxic extraction procedures, these require extensive dialysis or the use of filters, making their execution extremely laborious (Bailes *et al.*, 2007; Barnett and Larson, 2012). Commercial DNA isolation kits offer shorter extraction times, do not require specific training, have minimum equipment requirements and and result in a good DNA quality. However, the price per sample makes the process very expensive, and even prohibitive, for many laboratories in developing countries (Aidar and Line, 2007).

Chelex®100 resin has emerged as a safe, economic, and sensitive method for nucleic acid extraction from many sample types, without DNA damage (Walsh, Metzger and Higuchi, 2013). Studies have shown that its efficiency in DNA extraction in several types of samples, including those comprising low numbers of cells (Suenaga and Nakamura, 2005; Nagdev *et al.*, 2010; Walsh, Metzger and Higuchi, 2013; Idris and Goodwin, 2015; Kallassy *et al.*, 2019; de la Cruz-de la Cruz *et al.*, 2020).

The method used for DNA extraction can have a significant impact on host genetic studies (Chacon-Cortes *et al.*, 2012). Given that nasopharyngeal samples are one of the most widely sample used for COVID-19 diagnostics, they can also reveal substantial information on host genetic markers involved in the susceptibility and resistance to SARS-CoV-2 infection (Philibert *et al.*, 2008; Grimaudo *et al.*, 2021). The present work compared three DNA extraction methods (Chelex®100, Phenol-chloroform and the QIAamp DNA Mini Kit) to isolate genomic DNA from nasopharyngeal swab samples and evaluated their performance and suitability for genotyping studies.

MATERIAL AND METHODS

Samples

A total of 100 nasopharyngeal swab samples were collected from individuals displaying respiratory symptoms and SARS-CoV-2 diagnosis was confirmed by qRT-PCR. The nasopharyngeal swab samples were collected using a cotton swab and placed in 3 mL virus transport medium (DEFINE), refrigerated, and shipped to the Pernambuco State Central Laboratory of Public Health "*Dr. Milton Bezerra Sobral (Lacen-PE)",* Brazil, for COVID-19 diagnosis. All samples were collected during the first wave of the COVID-19 pandemic in Pernambuco State in 2020. All samples were stored at -70ºC until use.

Ethical approval

This study was approved by the UFPE Institutional Review Board under protocol CAAE: 44390221.6.0000.5208 and was performed in accordance with relevant guidelines e regulations, including the Brazilian National Health Council (CNS) Resolution 466/2012. The requirement for informed consent study was waived because we used spent samples submitted to COVID-19 diagnosis and all patient identifying information was kept confidential.

DNA Extraction

The genomic DNA extraction was performed using three different protocols: Chelex®100 resin (Sigma-Aldrich), Phenol-chloroform (Sigma-Aldrich), and QIAamp DNA Mini Kit (Qiagen). A total of 250 microliters of nasopharyngeal swab sample was used for each protocol.

Extraction Protocols

a) Chelex®100 Resin

A 250 µL of nasopharyngeal swab sample aliquot was added to 100 µL of Chelex®100 resin (5g/mL). The mixture was vortexed and incubated at 56˚C for 1 hour in a 0.5 mL microtube in a dry bath. Next, it was heated to 96ºC for 30 minutes. After heating, the samples were centrifuged at 13000 rpm for 6 minutes. The supernatant was transferred to a clean microtube of 1.5 mL and 100 µL of ultrapure water were added for rehydration. The genomic DNA obtained was stored at 20°C.

b) Phenol-Chloroform

A volume of 250 µL of nasopharyngeal swab sample was added to 400 µL of TKM II buffer in a 2 mL tube. Then, 25 µL of 10% SDS and 5 µL of proteinase K were added and well mixed with a sterile tip. The sample was incubated in a dry bath at 55°C for 30 minutes. A 180 µL of 5M NaCl was added, mixed, and incubated at room temperature for 15 minutes. Centrifugation was performed at 13000 rpm for 10 minutes. The DNA containing supernatant was recovered and placed in a sterile tube (2 mL). 400 µL of chloroform/isoamyl alcohol (Sevag) and 400 µL of saturated phenol (pH 7.6-8) were added, then homogenized by vortexing. The proportion between chloroform/isoamyl alcohol and saturated phenol solutions should always be 1/1. Centrifugation was performed at 13000 rpm for 10 minutes. The DNA containing supernatant was transferred to a new sterile tube (2 mL) and 800 µL of chloroform/Isoamyl alcohol was added, vortexed, and centrifuged at 13000 RPM for 10 minutes. The supernatant was collected into a fresh tube where 10% of the supernatant volume (e.g. 80 µL) of 3M sodium acetate (pH 5.2) was added and 800 µL of ice-cold (-20°C) p.a. ethanol was added. The tube was shaken by inversion to precipitate the DNA and centrifuged at 13000 rpm for 10 minutes to aggregate the precipitated DNA on the tube wall. The supernatant was discarded and 500 µL of ice-cold 70% ethanol are added. The tube was mixed until the DNA detached from the tube wall, resuspended in ethanol and centrifuge at 13,000 RPM for 7 minutes. The supernatant was discarded, and the DNA was allowed to dry in a dry bath at 60°C with the tube open and resuspended in 50 µL of TE buffer or deionized water.

c) QIAamp DNA Mini Kit (Qiagen)

A total of 250µL of sample were used for DNA extraction using the QIAamp® DNA Mini and Blood Mini Handbook according to “DNA Purification from Buccal Swabs (Spin Protocol)” from the manufacturer (https://www.qiagen.com/us/resources/resourcedetail?id=62a200d6-faf4-469b-b50f-2b59cf738962&lang=en). In brief, 400 µl PBS were added to the sample, followed by the addition of QIAGEN protease stock solution and buffer AL. The mixture was incubated at 56°C for 10 minutes, washed with 100% ethanol and the provided buffers and then eluted in 150 µL Buffer AE.

Protocol runtime

To evaluate the average execution time of each protocol using a manageable number of samples (n=24) to be extracted in a single run, we measured the time spent by three different operators from beginning to end. We chose this sample size because it is the maximum capacity of tubes that can be processed in a regular benchtop centrifuge in a single run in our setting. The operators were equally trained and familiar with each protocol.

DNA concentration and purity

DNA concentration and quality were assessed by spectrophotometry using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) following the manufacturer´s instructions. DNA purity was estimated by determing 260/280 and 260/230 ratios.

DNA integrity on agarose gel

The integrity and presence of genomic DNA extracted by the three methods was analyzed on 1.5% agarose gel. DNA aliquots (3µL) were run at 100 V and 80 Amps for 30 minutes, stained with GelRed (Biotium) and visualized under an ultraviolet transilluminator (Loccus L-PIX).

DNA amplification

Genomic DNA was amplified by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and quantitative real time polymerase chain reaction (qRT-PCR) targeting the single nucleotide polymorphism (SNP) rs1801133 in *methylene tetrahydrofolate reductase* (*MTHFR*) gene as a representative marker for host genetic studies. The *MTHFR* gene is one of the regulatory enzymes involved in

folate metabolism and this SNP has been associated with various types of diseases, including hypertension (Mabhida *et al.*, 2022). In the *MTHFR* PCR-RFLP, the forward primer 5-TGAAG GAGAAGGTGTCTGCGG-3 and the reverse primer 5-AGGACGGTGCGGTGAGAGTG-3 were used. DNA amplification was performed using the commercial kit GoTaq G2 Flexi DNA Polymerase (Promega). Reactions were prepared in a final volume of 25µL: 5µL of GoTaq Flexi Buffer, 2.5 µL of 10mM dNTP mix, 1.5 µL of 25mM $MgCl_2$, 0.5 µL of forward primer, 0.5 µL of reverse primer, 0.2 µL of GoTaq G2 flexi DNA Polymerase (5u/µL), 12.8 µL ultrapure water and 2 µL of DNA sample. The cycling conditions used were 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, 62°C for 30 seconds, 72°C for 30 seconds and a final extension of 72°C for 7 minutes. The amplification success was verified by amplicon of 198 bp presence in a 1% agarose gel.

The amplicons were digested using the restriction enzyme *HinfI* (Thermo Fisher Scientific). The reactions were prepared for a final volume of 25µL, being 16.8µL of ultrapure water, 3 µL Buffer R, 0.3 of *HinfI* enzyme (10 U/µL) and 10µL of amplicon. The reactions were subjected to a temperature of 37ºC overnight (minimum of 12 hours), followed by inactivation at 85ºC for 5 minutes. The G/A transition creates a restriction site for the *HinfI*, generating fragments of varying sizes: the homoziygote for the wild-type allele (GG) produces a 198 bp fragment; the heterozygote (GA) produces 198, 175 and 23 bp fragments and the mutant homozygote (AA) produces 175 and 23 bp fragments. the analysis of the fragments was performed using a 3% agarose gel staing with GelRed® (Biotium).

For the qRT-PCR, the *MTHFR* rs1801133 SNP was genotyped using allelic specific probes (TaqMan SNP Genotyping Assays C_1202883_20) in an ABI7500 Real Time PCR machine (Applied Biosystems), according to manufacturer's recommendations.

Cost analysis of each DNA extraction method

To analyse the costs of the several consumables used in each DNA extraction method used, the value per one reaction was calculated based on Brazilian reagent prices at the time of the study (first semester of 2022) and were converted to US $.

Statistical analysis

The graphics and statistical analysis were done in GraphPad Prism 5 version 5.1 for Windows. DNA concentration, yield and purity values were evaluated by analysis of variance (ANOVA), and differences were compared with the post hoc Tukey test at a significance level of 0.05.

RESULTS AND DISCUSSION

DNA extraction is a cornerstone procedure for genetics and molecular biology studies. For best use in developing countries, methods should be fast, practical, affordable, free of contaminants and toxicity, and give DNA of high quantity and quality (Aidar and Line, 2007; Yang *et al.*, 2008; Gupta, 2019). We have looked at alternative solutions for DNA extraction for downstream applications, and compared three different DNA extraction protocols to choose the best extraction method for nasopharyngeal swab samples of COVID-19 patients.

Initially, each protocol was compared with respect to some technical-methodological criteria (Figure 1). In relation to steps number, the Chelex®100 protocol presented the shortest steps number (3 steps), followed by QIAamp DNA Mini Kit (10 steps) and phenol-chloroform (28 steps). Regarding protocol runtime, the QIAamp DNA mini kit showed the shortest time for extraction of 24 samples (1 hour and 44 minutes), followed by Chelex®100 (2 hours and 31 minutes) and phenol-chloroform (3 hours and 22 minutes). We chose this sample number because it allows comfortable manual DNA extraction using conventional tabletop centrifuges found in most labs. When compared to the total retrieved volume, the Chelex®100 protocol was able to recover the largest DNA volume (400µL) compared to QIAamp DNA mini kit (50µL) and phenol-chloroform (50µL).

We then compared the costs for consumables used in the different methods (Table 1). The Chelex®100 method gave the lowest cost (US$ 0.153), followed by phenol-chloroform (US$ 1.950) and the QIAamp DNA mini kit (US$ 5.066). Thus, the Chelex®100 was found to be 33 times cheaper than a commercial kit and almost 13 times cheaper than a classical phenol-chloroform method.

A greater number of steps implies greater human interference, including tube change/disposal, resulting in increased chances of DNA contamination- as well as increased amount of waste, higher costs, exposure to harmful chemicals (phenol, chloroform, isoamyl alcohol) and ergonomic risk (Aidar and Line, 2007; Edwin Shiaw *et al.*, 2010).

The concentration and yield of the DNA obtained by the three extraction methods using nasopharyngeal swab samples of COVID-19 individuals are shown in Figure 2. The DNA concentration was significantly higher (mean=191.8 ng/µl) for the Chelex®100 protocol than for the phenol-chloroform method (mean=22.1 ng/µl, $p<0.001$) and QIAamp DNA mini kit (mean=5.02 ng/µl, $p<0.001$) (Figure 2A). In relation to total DNA yield, samples extracted by Chelex®100 protocol produced a higher yield (mean=3836µg) than samples processed using phenol-chloroform (mean= 55.22µg, $p<0.001$) or QIAamp DNA mini kit (mean= 12.55µg, $p<0.001$) methods. The phenol-chloroform gave a higher DNA yield ($p<0.001$) than the QIAamp DNA mini kit (Figure 2B).

The DNA purities, as determined spectrophotometrically by the 260/280 and 260/230 ratios, for three extraction methods are shown in Figure 3. The Chelex®100 protocol showed the lowest 260/280 ratio (mean=1.284), differing significantly from phenol-chloroform (mean=1.861; $p<0.001$) and QIAamp DNA mini kit (mean=1.663; $p<0.001$) purifications. A statistically significantly difference was found between the phenol-chloroform and QIAamp DNA mini kit ($p<0.05$) (Figure 3A) with respect to this parameter. For the 260/230 ratio, the phenol-chloroform method gave the highest value (mean=1.207), significantly differing from the DNA extracted by Chelex®100 (mean=0.4107; $p<0.001$) and QIAamp DNA mini kit (mean=0.4167; $p<0.001$) (Figure 3B). The 260/230 ratio indicates the purity of the nucleic acid sample from salts and other contaminants which can absorb at 230 nm. Since proteins absorb light strongly at 280 nm wavelength, a low 260/280 ratio indicates the presence of high amounts of protein, relative to nucleic acids. The phenol-chloroform had the best DNA quality in comparison to Chelex®100 and QIAamp DNA mini kit as measured by the 260/280 and 260/230 ratios. However, the phenol-chloroform uses highly toxic reagents and is labour intense and time consuming (Silva *et al.*, 2014).

The literature shows a great deal of variability in DNA concentrations, yield and quality of the Chelex®100 method depending on the sample type used (Suenaga and

Nakamura, 2005; Walsh, Metzger and Higuchi, 2013; Silva *et al.*, 2014; Idris and Goodwin, 2015; Ip, Lin and Lai, 2015; Brito *et al.*, 2019). Some studies using blood samples (Idris and Goodwin, 2015), saliva placed onto cotton swabs and air-dried (Idris and Goodwin, 2015), semen (Silva *et al.*, 2014; Idris and Goodwin, 2015), human hair (Suenaga and Nakamura, 2005) and cigarette butts (Kallassy *et al.*, 2019). have reported higher quantity and yield of DNA extracted with Chelex®100, but lower quality in some cases as we observed in this study.

In our nasopharyngeal samples, the DNA integrity was also evaluated by running samples in 1.5% agarose gel and the results are shown in Figure 4. The agarose gel analysis revealed uniformity of the samples extracted by the Chelex®100 method (Figure 4A), phenol-chloroform (Figure 4B) and the QIAamp DNA mini kit (Figure 4C) in relation to the integrity of the genomic DNA, with no evident degradation. The differences of DNA quality seen in the different methods did not seem to interfere with the success of amplification using two different genotyping methodologies, RFLP and qRT-PCR (Figure 5). In the RFLP assays, samples extracted by three different protocols showed uniform amplification and were properly cut by the restriction enzyme used to detect the SNP (Figure 5 A-C). For qRT-PCR using allele-specific probes, the success of amplification was considered normal, allowing the correct genotyping of the tested SNP (Figure 5 D-F). In both genotyping methodologies, no divergence was found regardless of the DNA extraction method used, a fact that corroborates that DNA obtained with these methods can be successfully genotyped.

The success of DNA amplification by both PCR methodologies in our study differed from reports suggesting that samples extracted by Chelex®100 or phenol-chloroform may have PCR inhibitors (Walsh, Metzger and Higuchi, 2013). PCR inhibition in Chelex®100-extracted DNA seems to be associated with the type of biological sample used (Brito *et al.*, 2019). In blood samples, the removal of heme groups by Chelex®100 resin was reported to be inefficient, resulting in PCR inhibition (Brito *et al.*, 2019). In addition, some studies have suggested that the DNA extracted by Chelex®100 would degrade the DNA, which would make it unsuitable for RFLP analysis (Walsh, Metzger and Higuchi, 2013), which was also not supported by our results.

Taken together, our results suggest that in nasopharyngeal samples of COVID-19 patients, the use of QIAamp DNA mini kit is limited by its low DNA quantity and

quality, as well high cost per sample. On the other hand, the phenol-chloroform-based extraction is a laborious and potentially hazardous, despite the higher level of DNA purity observed. In contrast, Chelex®100 resin emerged as an affordable, effective, fast, and simple method, which can be carried out in few steps. The method does not require the use of organic solvents and manipulation steps, showing a low risk of sample contamination and offers lower hazard risk to the operator and the environment. Thus the Chelex®100 method is a cheap, safe, simple, fast, and effective method for the DNA extraction from nasopharyngeal samples of COVID-19 patients suitable for developing countries and minimal settings.

Acknowledgments

R.C.S is supported by a post-doctoral fellowship sponsored by the Fiocruz Inova Program, whereas S.C.L and W.P.M.S.R are supported by fellowships sponsored by the Foundation for Science and Technology of Pernambuco-Brazil and CAPES-Brazil, respectively. We would like to express our sincere gratitude and appreciation to the Pernambuco State Central Laboratory of Public Health Dr. Milton Bezerra Sobral (Lacen-PE) for providing us the samples used in this study. The work in Dr. Pena´s lab is supported by grants from the The International Development Research Centre (IDRC)-Canada (Project ID 109434), the Fiocruz Inova Program, and Brazilian National Council for Scientific and Technological Development (CNPq 441030-2020-3). A.K. is supported by the UK Medical Research Council (MC_UU_12014/8). BR acknowledges DST, Government of India for funding under BRICS Research Project (DST/INT/BRICS/COVID-19/2020-Part-1). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests

The authors declare no competing interests.

Table 1. Costs associated with the DNA genomic extraction by several methods, per one reaction (costs are based on Brazilian reagent prices at time of the study and are converted to US$).

Consumables	Costs per Sample Extracted		
	Chelex®100 resin	Phenol-chloroform	QIAamp DNA Mini Kit

Chelex®100 sodium form	0.001	-	-
QIAamp DNA mini kit (250 reations)	-	-	4.779
Phenol solution	-	0.171	
Pipet tips (200 µL)	0.039	0.097	0.039
Pipet Tips (1000 µL)	0.043	0.214	0.171
Proteinase K	-	0.970	-
Microcentrifuge tube (0.5 mL)	0.031	-	-
Microcentrifuge tube (2 mL)	0.039	0.155	0.077
Several chemical reagents*	-	0.343	
Price (US $)	**0.153**	**1.950**	**5.066**

*Reagents used in the phenol-chloroform extraction: magnesium chloride, sodium chloride, sodium acetate, chloroform, ethanol PA, amyl alcohol, tris(hydroxymethyl)aminomethane hydrochloride, sodium dodecyl sulfate, ethylenedinitrilotetraacetic acid.

Figure legends

Figure 1. Nasopharyngeal swab collection is the gold standard for diagnosis of SARS-CoV-2 and this sample can be used for DNA extraction for genetic analyses. DNA extraction can be performed by different methods such as Chelex®100, phenol-

chloroform, and commercial kits. Such methods differ in terms of principle, number of steps, time to perform the technique and yield of the extracted material.

Figure 2. Genomic DNA concentration and yield according to extraction methods. A) DNA concentration; B) DNA yield. DNA extraction methods were compared by one way analysis of variance by Tukey post-hoc test. Legend: ns = no significative value.

Figure 3. Genomic DNA purity measured by spectrophotometer. A) 260/280 ratio; B) 260/230 ratio. The DNA ratio obtained by different extraction methods were compared by one way analysis of variance by Tukey post-hoc test. Legend: ns= no significative value.

Figure 4. Genomic DNA quality according extraction methods measured by 1.5% agarose gel. A) Chelex®100; B) Phenol-Chloroform; C) QIAamp DNA mini kit. Legend: MW = molecular weight.

Figure 5. Applications of genomic DNA extracted by different methods in PCR RFLP and Real Time PCR with allelic specific probes (TaqMan) for SNP rs181133 in *MTHFR* gene. A, B, C – 3% Agarose gel of amplicons referent to PCR RFLP; D, E, F – Real time PCR curves. A – Genomic DNA extracted by chelex; B – Genomic DNA extracted by phenol-chloroform; C – Genomic DNA extracted by QIAamp DNA blood mini kit. Lane 1, 2, 4, 5, 6, 7, 8, 9, 11 and 12 – Genotype G/G; Lane 3 – Genotype A/A; Lane 10 – Genotype G/A; D – Curve of real time PCR referent to genotype A/A (sample 3); E – Curve of real time PCR referent to genotype G/A (sample 10); F – Curve of real time PCR referent to genotype G/G (Sample 1, 2, 4, 5, 6, 7, 8, 9, 11 and 12).

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Figure 1.

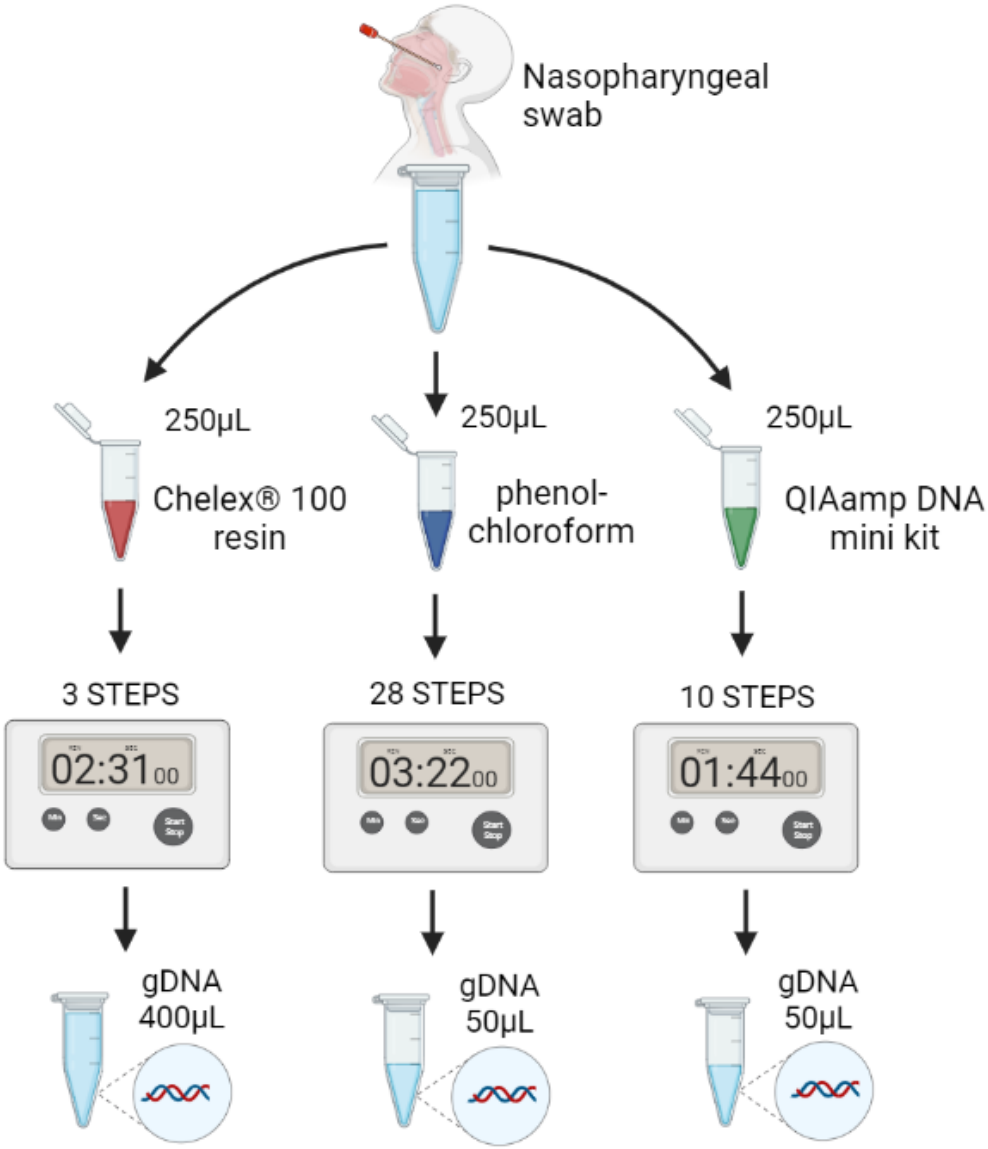

Nasopharyngeal swab
250µL
Chelex® 100 resin
250µL
phenol-chloroform
250µL
QIAamp DNA mini kit
3 STEPS
28 STEPS
10 STEPS
02:31 00
03:22 00
01:44 00
gDNA 400µL
gDNA 50µL
gDNA 50µL

Figure 2.

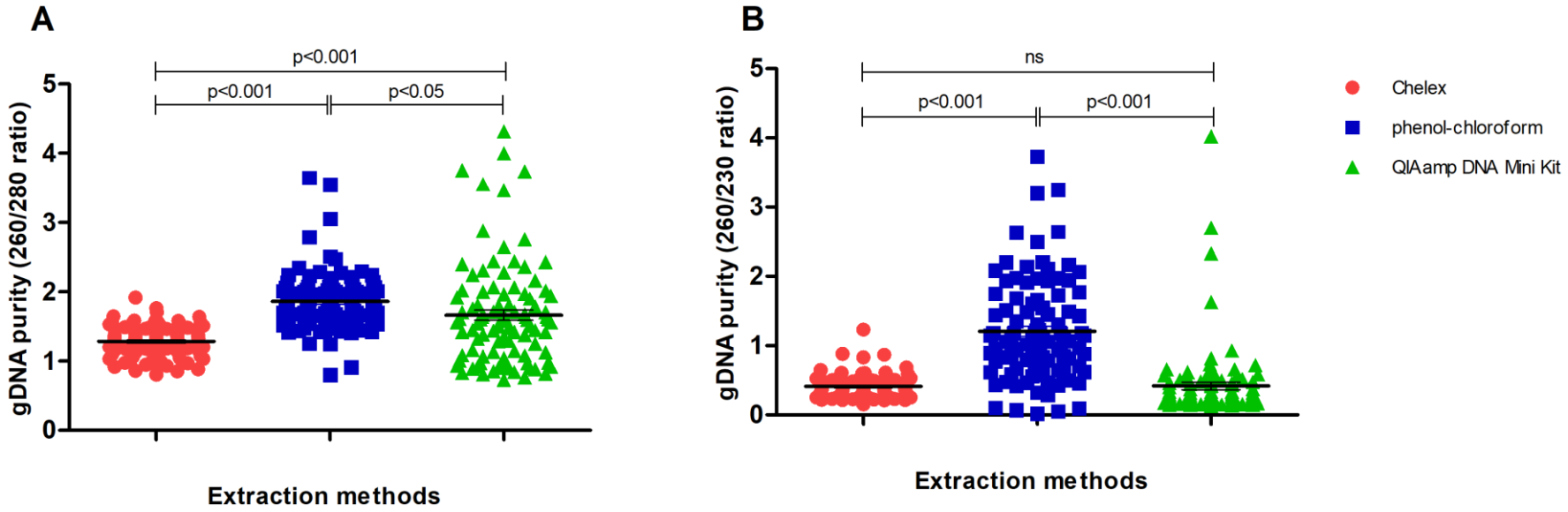
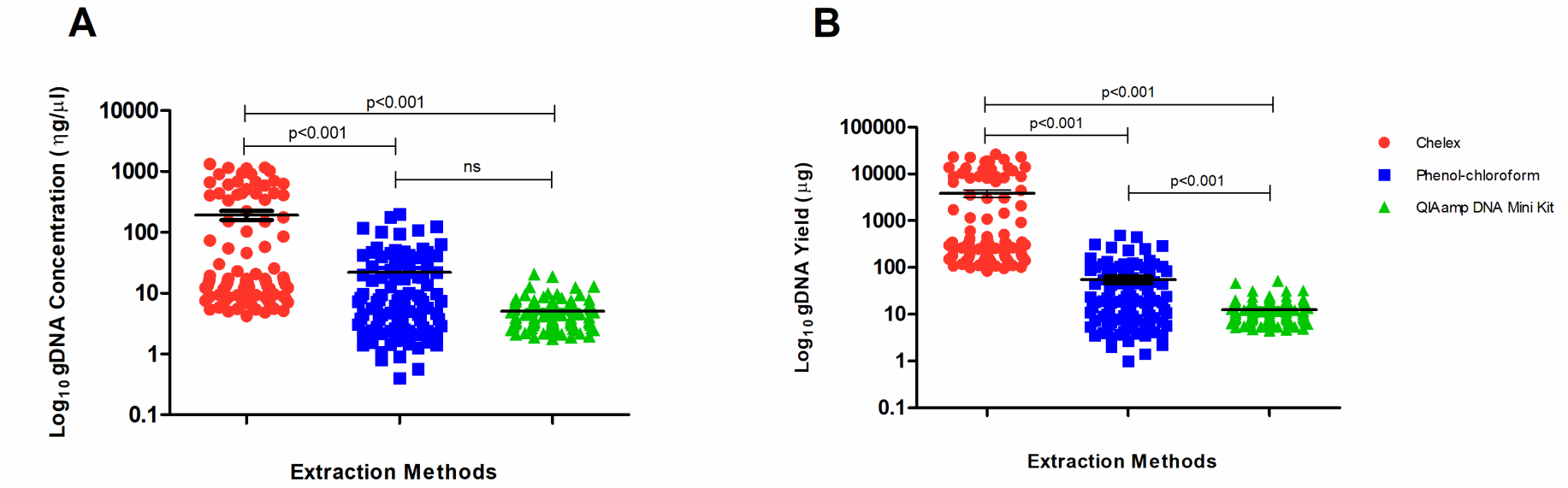


Figure 3.


A
Log10 gDNA Concentration (ηg/μl)
10000
1000
100
10
1
0.1
p<0.001
p<0.001
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Extraction Methods
B
Log10 gDNA Yield (μg)
100000
10000
1000
100
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Extraction Methods
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Figure 4.

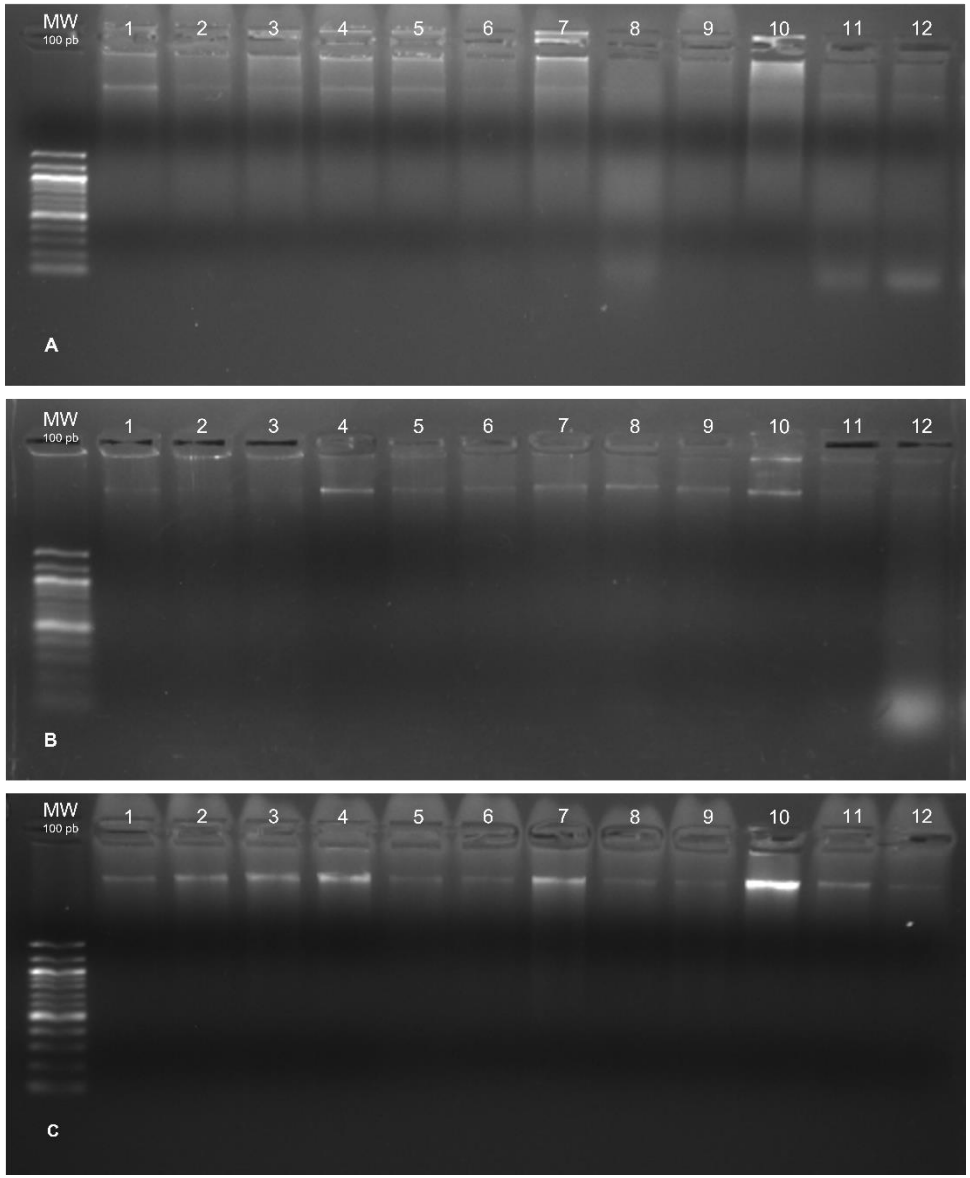
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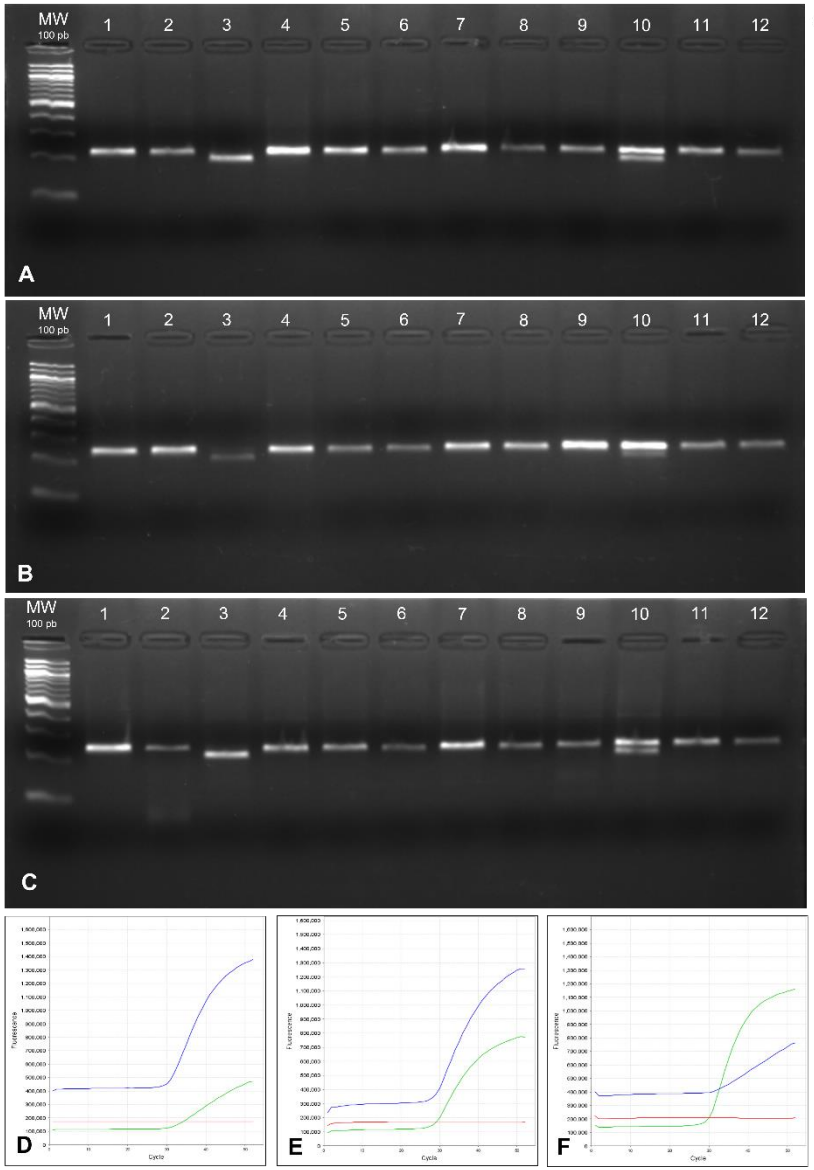
Figure 5.



5 ARTIGO 2 – ASSOCIATION OF SNP RS7170178 IN ANXA2 WITH THE COVID-19 MORTALITY IN A BRAZILIAN NORTHEASTERN POPULATION

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ABSTRACT

COVID-19 is a complex disease with a broad spectrum of clinical manifestations ranging from the absence of symptoms to severe systemic complications. The different disease outcomes can be related to factors such as: age, sex, presence of morbidities and the viral and host genetic variability. Polymorphisms in genes involved in viral entry and inflammation pathway have been linked to severe COVID-19 outcomes. We investigated the allelic and genotypic distribution of single nucleotide polymorphisms (SNPs) in the ACE2 (rs228566), *MTHFR* (rs1801133) and *ANXA2* (rs7163836 and rs7170178) genes and their possible relationship with the COVID-19 severity. For this, 312 nasopharyngeal swab samples of positive SARS-CoV-2 individuals were collected, and genomic DNA extracted. Based on clinical findings, individuals were stratified in mild, severe and fatal COVID-19. Genotyping was performed by real-time PCR, using allele-specific probes. Among individuals with mild COVID-19, fever and cough were the most frequent symptoms (>76% in both groups). On the other hand, cough (89.1%), low saturation (82.6%) and dyspnea (78.3) were the most common symptoms among severe cases. Already, among the fatal cases, the symptoms more common were dyspnea (85.7%), low oxygen saturation and cough (both with 75.7%). Regarding morbidities, cardiovascular diseases (29,3% and 15,7% respectively) and diabetes mellitus (25% and 14,3% respectively) were the most frequent among severe and fatal

individuals. Among the SNPs studies, only rs7170178 in *ANXA2* was associated with COVID-19 outcomes. The AG genotype was significantly more frequent among individuals with severe COVID-19 compared to those with mild forms, suggesting a greater susceptibility to disease severity. In this sense, new studies involving other variants in ANXA2 should be conducted in other populations to confirm the role of this gene in the different clinical outcomes of COVID-19.

Keywords: COVID-19; polymorphisms; ANXA-2, gravity.

INTRODUCTION

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), responsible for coronavirus disease (COVID-19), was identified for the first time in China in December 2019. With a high rate of transmissibility and infectivity, the virus quickly spread around the world, leading to a pandemic scenario announced in March 2020 by the World Health Organization (WHO) (Huang et al. 2020) .

COVID-19 is a highly contagious disease because it is spread mainly through the airways. Infected people may or no have symptoms, that include fever, tiredness, dry cough, myalgia, nasal congestion, pharyngitis, headache, anosmia, diarrhea, severe dyspnea, dysgeusia, angina, paralysis, aphasia, rash or cyanosis (Huang et al. 2020; Leentjens et al. 2021).

COVID-19 course and clinical outcomes depend on several factors such as age, sex, presence of comorbidities and even viral and host genetic variability/. Overall, individual who develop the most severe form of the disease are male, elderly and with associated comorbidities (Wu et al. 2020).

SARS-CoV-2 infection can induce an increase in pro-inflammatory cytokines and chemokines production, culminating in a systemic hyper-inflammatory immune response and tissue damage (Zhou et al. 2020b; Giamarellos-Bourboulis et al. 2020). 2020). Immune dysregulation induced by virus, lead to endothelial dysfunction, platelet activation and hypercoagulability (Mucha et al. 2020). Coagulation disorders have been frequently observed in the most critical stages of the coronavirus disease.

Genes associated with the immune response, clotting and inflammation, as *ACE2*, *MTHFR* and *ANXA2* have been related to COVID-19 outcomes (Shang et al. 2020; Ponti et al. 2021a).

The ACE2, the main receptor of SARS-CoV-2, downregulates the renin-aldosterone system by hydrolyzing angiotensin II (Lambert et al. 2008), which has a profibrotic and vasoconstrictor effect, but at the same time being hydrolyzed, it decreases oxidative stress (Chappel and Ferrario 2006). ACE2 alterations elevate Ang II levels and can reduce cardiac function. Already MTHFR is responsible by homocysteine conversion. High plasma levels of homocysteine, in addition to being related to increase of vascular damage and prothrombotic processes, have also been related to the severity of COVID-19 (Zoccolella et al. 2006; Balint et al. 2020). On the other hand, the annexin 2 interacts with viral proteins involved in the internalization and replication of SARS-CoV-2. On the cell surface, it can decrease plasminolysis and lead to intravascular thrombosis in individuals with COVID-19. Once inside the cell, the interaction of annexin 2 and viral proteins can result in a severe inflammatory wave and cytokine storm (Patil et al. 2021).

In this sense, it is necessary to carry out studies aimed at identifying genetic variants of susceptibility to unfavourable outcomes of COVID-19. In this study, we evaluated the allelic and genotypic distribution of single nucleotide polymorphisms in *ACE2* (rs228566), *MTHFR* (rs1801133) and *ANXA2* (rs7163836 and rs7170178) genes and their possible influence on the clinical outcome of COVID-19 in a Brazilian Northeastern population.

MATERIAL AND METHODS

Sample collection and stratification

Our study population was formed by 312 individuals of both sexes, with age from 18 to 60 years old and from the Pernambuco State – Brazil. Nasopharyngeal swabs samples were collected of individuals with positive diagnostic by PCR for SARS-CoV-2, through partnership with the Central Laboratory of Pernambuco (LACEN-PE), in period of March to December 2020.

According to the clinical record of everyone, forwarded at the time of diagnosis and World Health Organization (WHO) guidelines, the cases were stratified according to the severity of COVID-19 into mild, severe, and fatal.

The individuals without comorbidities and hospitalization by COVID-19 who presented mild symptoms such as cough, fever and absent of respiratory symptoms were classified as mild cases. On other hand, individuals with hospitalization by COVID-19, with or without comorbidities and presenting severe respiratory symptoms such as dyspnea, low oxygen saturation (< 95%) were classified severe cases. Lastly, individuals presenting severe respiratory symptoms, with or without comorbidities, hospitalized and who died as a result of COVID-19 complications were classified as fatal cases.

DNA isolation, SNPs selection and Genotyping

The genomic DNA extraction of nasopharyngeal swabs were performed using Chelex®100 resin (Sigma-Aldrich). DNA concentrations were evaluated by spectrophotometry using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). DNA purity and quality was estimated through the 260/280 and 260/230 ratios.

The SNPs selection was performed from public databases, such as: OMIM (https://www.omim.org), dbSNP (https://www.ncbi.nlm.nih.gov/snp/), SNPnexus (www.snp-nexus.org/v4/) and Ensembl (https://www.ensembl org/index.html); as well as based on the literature. The SNPs selection was based on the minimum allele frequency (MAF>0.1 in Caucasian and Yoruba populations), the functional role of the variant and previous associations with risk conditions for COVID-19.

The selected SNPs were the rs228566 C>T in *ACE2*, rs1801133 A>G in *MTHFR*, rs7163836 C>T and rs7170178 A>G in *ANXA2.* All variants were genotyped by qRT-PCR using allelic specific probes (TaqMan SNP Genotyping Assays) in an ABI7500 Real Time PCR machine (Applied Biosystems), according to manufacturer's recommendations.

Statistical analysis

Allelic and genotypic frequencies were estimated by direct counting among Genotype transposer (Cox and Canzian 2001). Hardy-Weinberg equilibrium (HWE) adherence and possible associations were determined through Chi-Square test (X^2) and Fisher's Exact Test, respectively. All tests were performed in R program version 2.11.1 and/or GraphPad Prism version 5.0, with a significance level of 0.05.

RESULTS

The study included 312 individuals SARS-CoV-2 positive, which 150 was classified as mild cases, 93 severe and 70 fatal cases. The clinical-epidemiological characteristics of these individuals were presented in Table 1.

Table 1. Clinical-epidemiological characterization of individuals with COVID-19.

Clinical/Epidemiological Characteristics	Mild Cases N (%)	Severe Cases N (%)	Fatal Cases N (%)
Sample Number			
	150	93	70
Sex			
Male	82 (54.7%)	53 (57.6%)	43 (61.4%)
Female	68 (45.3%)	39 (42.4%)	27 (38.6%)
Mean Age (years)			
	44.3	44.7	47.3
Symptomatology			
Saturation O2 < 95%	0	76 (82.6%)	53 (75,7%)
Dyspnea	0	72 (78.3%)	60 (85.7%)
Respiratory distress	0	43 (46.7%)	27 (38.6%)
Fever	115 (76.7%)	59 (64.1%)	49 (70%)
Cough	115 (76.7%)	82 (89.1%)	53 (75.7%)
Sore throat	64 (42.7%)	26 (28.3%)	6 (8.6%)
Headache	60 (40%)	21 (22.8%)	3 (4.3%)
Change/loss of smell or taste	41 (27.3%)	17 (18.3%)	12 (17.1%)
Myalgia	19 (12.7%)	13 (14.1%)	3 (4.3%)

Diarrhea	18 (12%)	15 (16.3%)	6 (8.6%)
Runny nose/Congestion	14 (9.3%)	8 (8.7%)	4 (5.7%)
Tiredness/Fatigue	4 (2.7%)	13 (14.1%)	2 (2.9%)
Nausea	4 (2.7%)	5 (5.4%)	3 (4.3%)
Asthenia	3 (2%)	2 (2.2%)	2 (2.9%)
Chills	2 (1.3%)	2 (2.2%)	0
Arthralgia	2 (1.3%)	0	1 (1.4%)
Puke	1 (0.7%)	12 (13%)	5 (7.1%)
Sneezing	1 (0.7%)	1 (1.1%)	0
Body pain	1 (0.7%)	1 (1.1%)	1 (1.4%)
Chest pain	1 (0.7%)	1 (1.1%)	0
Dizziness	1 (0.7%)	0	0
Other symptoms	0	14 (15%)	10 (14%)
Morbidities			
Heart and vascular diseases	0	27 (29.3%)	11 (15.7%)
Diabetes mellitus	0	23 (25%)	10 (14.3%)
Overweight/Obesity	0	14 (15.2%)	4 (5.7%)
Respiratory diseases	0	12 (13%)	4 (5.7%)
Hypertension	0	11 (12%)	4 (5.7%)
Chronic kidney diseases	0	4 (4.3%)	2 (2.9%)
Liver diseases	0	4 (4.3%)	2 (2.9%)
Immunosuppression	0	2 (2.2%)	2 (2.9%)
Chromosomal diseases	0	2 (2.2%)	0
Neoplasm	0	1 (1.1%)	0

Males were the most frequent (>54%) in the three studied groups. The mean age at diagnosis was 44.3 years old in mild cases, 44.7 years old in severe cases and 47.3 years old in fatal cases (Table 1).

In relation to symptomatology, fever (76.7%), cough (76.7%), sore throat (42.7%), headache (40.0%) and change/loss of smell or taste (27.3%) were the symptoms more frequent among the mild cases. Already, among sever cases, the symptoms more common were cough (89.1%), oxygen saturation < 95% (82.6%), dyspnea (78.3%), fever (64.1%) and respiratory distress (46.1%). On the other

hand, dyspnea (85.7%), oxygen saturation < 95% (75.7%), cough (75.7%), fever (70.0%) and respiratory distress (38.6%) were the main symptoms among fatal cases (Table 1).

Among severe and fatal cases, the morbidities more frequent were heart and vascular diseases (29.3% and 15.7%, respectively), diabetes mellitus (25% and 14.3% respectively), overweight/obesity (15.2% and 5.7%) respiratory diseases (13% and 5.7%, respectively) and hypertension (12% and 5.7% respectively) (Table 1).

Allelic and genotypic distribution of SNPs in *MTHFR* and *ANXA2* were presented in table 2. In relation to genotypic distribution, the Hardy-Weinberg adherence was observed for all studied variants.

Among the SNPs studied, only the rs7170178 in *ANXA2* showed significant differences between the groups (Table 2). The A allele was the most common among mild cases (70%) in relation to severe (66%) and fatal (61%) cases. Already, the G allele variant was more frequent in fatal cases (39%), followed to severe (34%) and mild (30%) cases. Similarly, the homozygous genotype AA was the predominant in all the groups, being more frequent among mild cases (52%) than in severe (46%) and fatal (36%) cases. The heterozygous genotype AG was significantly more frequent among individuals who died of COVID-19 (51%) than individuals with disease mild form (40%; OR=1.94, p=0.040), being associated to mortality susceptibility. On the other hand, the GG genotype was more frequent in severe (13%) and fatal (14%) cases than in mild cases (11%), but without difference significative (Table 2).

Other variants studied, despite the percentage differences between the different types of cases, no significant differences were observed (p>0.05) (table 2).

Allelic and genotypic distribution of SNPs in *ACE2* were presented in table 3. In relation to genotypic distribution, the Hardy-Weinberg adherence was observed for all studied variants.

The C allele was more frequent in male individuals who died of COVID-19 than in females from the same group. However, no statistically significant differences were observed.

The genotypic distribution was carried out according to the morbidities presented by the severe and fatal cases. The results were presented in tables 4 and 5. Despite the different genotypic distributions between mild, severe and fatal cases, no significant differences were observed ($p>0.05$).

Additionally, the genotypic distribution of SNPs in all genes studied according to the main symptoms of COVID-19 (low O_2 saturation, dyspnoea, respiratory discomfort, fever and cough) was also performed. The results were presented in tables 6 and 7. Only dyspnoea showed a significant association ($p=0.0002$) with the severity of COVID-19 in SNP rs228566 in *ACE2*. Regarding the other symptoms, despite the different genotypic distributions between severe and fatal cases, no significant differences were observed ($p>0.05$).

DISCUSSION

The different clinical manifestations of COVID-19 make the prognosis difficult and increase the chances of unfavorable outcomes on infected individuals (Leentjens et al. 2021). The comorbidities is an important factor involved in severe forms of the disease, due to the impairment of the immune response that facilitates viral replication on a larger scale (Wu et al. 2020).

Our findings corroborate previous studies, where cardiovascular diseases and diabetes are the most frequent among individuals with severe and fatal forms of COVID-19. Individuals with cardiovascular diseases can develop severe complications, due to changes in coagulation and the increase in the inflammatory state, induced by COVID-19 (Huang et al. 2020). Additionally, diabetes mellitus is also a risk morbidity for the most severe forms of COVID-19, since high blood glucose levels or non-treatment can lead to changes in coagulation and overproduction of inflammatory cytokines (Brito et al. 2020).

According to our findings, the rs7170718 polymorphism in the *AnxA2* gene was the only one that showed a significant association with the outcome of COVID-19. This polymorphism is a transition of T by G at position 5681, an intergenic region closes to the 3' end of the gene. This variant may be involved in the folding of chromatin loops, influencing the binding of enhancement factors in the *AnxA2*

promoter region and acting as a regulator of the genic expression levels (Baldwin et al. 2005). Thus, it is likely that individuals who have this polymorphism have molecular impairment in *AnxA2*.

Studies have identified an association of polymorphisms in the *AnxA2* with the development of osteonecrosis, as well as with an increased risk of stroke (Pereira-Martins et al. 2020). However, association studies of these polymorphisms with the different clinical outcomes of COVID-19 are still scarce. Our study was the first to make this relation. However, to confirm the real role of this gene in the clinical outcomes of COVID-19, studies including other functional variants in other populations and large number of samples are needed. Furthermore, it is important to evaluate *AnxA2* expression levels to elucidate the possible functional effect of the polymorphism in this gene.

Regarding the other genes included in the study, no statistically significant associations were observed. However, it is known that the determinants of different susceptibility to SARS-CoV-2 mainly involve genes related to the early stages of infection, the *ACE-1* and *ACE-2* angiotensin system played an important role in the pathogenesis of COVID-19 (Anastassopoulou et al. 2020). Functional polymorphisms of the *ACE1/ACE2* genes have been associated with the risk of cardiovascular and pulmonary diseases and therefore may also contribute to the outcome of COVID-19 (Gómez et al. 2020). Variations in the *ACE-2* gene can make it easier or harder for SARS-CoV-2 to invade cells, which is why cellular expression levels of *ACE-2* differ between individuals (Zhang et al. 2020b; Choudhary et al. 2021).

Evidence indicates that there is a racial difference between *ACE* gene polymorphisms, in which African Americans have higher rates of mortality from COVID-19 in the US. Likewise, Europeans also have high fatality rates. On the other hand, Asian populations have relatively lower mortality (Zheng and Cao 2020). Brazil has a highly mixed population, which may result in different levels of association between these polymorphisms.

Our results did not find association of the polymorphism in *MTHFR* gene with the outcome of COVID-19. The enzyme produced by *MTHFR* is responsible by homocysteine conversion and studies have suggested a relationship between

homocysteine levels and disease severity (Ponti et al. 2021b). Cohort analysis of 200 individuals with COVID-19 in Italy showed an association between elevated levels of homocysteine in the blood and the severe or lethal prognosis of disease (Ponti et al. 2021a).

Our findings showed that dyspnoea is a symptom significantly related to the severity of COVID-19. Individuals who have the most severe forms of the disease usually have important respiratory symptoms, which can lead to adverse outcomes of the disease (Wu et al. 2020).

In addition, although our studies did not find a significant association between the presence of morbidities and the severity of COVID-19, some studies suggest that individuals who have previous clinical conditions are more likely to develop the most severe forms of the disease (Wu et al. 2020; Tiburi et al. 2021).

Further studies including a greater number of variants of these genes, with a larger population should be conducted. As well as the functional analysis of these polymorphisms should be performed to determine the role of these variants with the clinical outcomes of COVID-19.

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of FEDERAL UNIVERSITY OF PERNAMBUCO (CAAE: 44390221.6.0000.5208).

Informed Consent Statement

Patient consent was waived due to fact that we used spent samples submitted to COVID-19 diagnosis.

Table 2. Allelic and genotypic distribution of single nucleotide polymorphisms in *MTHFR* and *ANXA2 genes in COVID-19* individuals of Pernambuco State.

Genes/ SNPs	Genetic Models	COVID-19 individuals			Fisher's Exact Test		
		Mild	Severe	Fatal	Mild vs Severe OR (95%CI), p-value	Mild vs. Fata OR (95%CI), p-value	Severe vs. Fatal OR (95%CI), p-value
MTHFR rs1801133	Allelic						
	A	204 (76.1))	152 (81.7)	104 (74.3)		Reference	
	G	64 (23.9)	34 (18.3)	36 (25.7)	0.71 (0.43-1.16), 0.165	1.10 (0.67-1.81), 0.717	1.54 (0.88-2.73), 0.134
	Codominant						
	AA	82 (61.2)	62 (66.7)	41 (58.6)		Reference	
	AG	40 (29.9)	28 (30.1)	22 (31.4)	0.93 (0.49-1.73), 0.882	1.10 (0.55-2.18), 0.870	1.19 (0.56-2.48), 0.726
	GG	12 (9.0)	3 (3.2)	7 (10.0)	0.33 (0.06-1.31), 0.102	1.16 (0.36-3.50), 0.797	3.49 (0.74-22.10), 0.094
	Dominant (AA vs AG+GG)				0.79 (0.43-1.42), 0.484	1.11 (0.59-2.09), 0.764	1.41 (0.71-2.82), 0.327
	Recessive (AA+AG vs GG)				0.34 (0.06-1.31), 0.107	1.13 (0.36-3.29), 0.804	3.31 (0.72-20.58), 0.101
	Overdominant (AA+GG vs AG)				0.99 (0.53-1.84), 1.000	0.93 (0.48-1.84), 0.873	0.94 (0.46-1.95), 0.865
ANXA2 rs7163836	Allelic						
	C	158 (52.7)	105 (56.5)	81 (57.9)		Reference	
	T	142 (47.3)	81 (43.5)	59 (42.1)	0.86 (0.58-1.26), 0.454	0.81 (0.53-1.24), 0.355	0.94 (0.59-1.51), 0.822
	Codominant						
	CC	46 (30.7)	32 (34.4)	23 (32.9)		Reference	
	CT	66 (44.0)	41 (44.1)	35 (50.0)	0.89 (0.47-1.70), 0.761	1.19 (0.56-2.54), 0.722	1.19 (0.56-2.54), 0.722
	TT	38 (25.3)	20 (21.5)	12 (17.1)	0.76 (0.35-1.62), 0.479	0.84 (0.31-2.22), 0.821	0.84 (0.31-2.22), 0.821
	Dominant (CC vs CT+TT)				0.84 (0.47-1.52), 0.573	0.90 (0.47-1.75), 0.757	1.07 (0.53-2.19), 0.868
	Recessive (CC+CT vs TT)				0.81 (0.41-1.55), 0.538	0.76 (0.31-1.78), 0.553	0.76 (0.31-1.78), 0.553
	Overdominant (CC+TT vs CT)				1.00 (0.57-1.74), 1.000	1.27 (0.65-2.48), 0.526	1.27 (0.65-2.48), 0.526
ANXA2 rs7170178	Allelic						
	A	199 (70.1)	123 (66.1)	82 (61.2)		Reference	
	G	85 (29.9)	63 (33.9)	52 (38.8)	1.20 (0.79-1.81), 0.417	1.48 (0.94-2.33), 0.075	1.24 (0.76-2.01), 0.409
	Codominan						
	AA	73 (51.4)	43 (46.2)	24 (35.8)		Reference	

	AG	53 (37.3)	37 (39.8)	34 (50.7)	1.18 (0.65-2.16), 0.568	1.94 (0.99-3.86), 0.040*	1.64 (0.79-3.45), 0.170
	GG	16 (11.3)	13 (14.0)	9 (13.4)	1.38 (0.55-3.39), 0.524	1.70 (0.58-4.75), 0.313	1.24 (0.40-3.68), 0.800
	Dominant (AA vs AG+GG)				0.81 (0.46-1.42), 0.505	0.53 (0.28-1.00, 0.038*	0.65 (0.32-1.30), 0.198
	Recessive (GG+AG vs AA)				1.28 (0.53-3.01), 0.549	1.22 (0.45-3.14), 0.653	0.95 (0.34-2.60), 1.000
	Overdominant (AA+GG vs AG)				1.11 (0.62-1.96), 0.784	1.72 (0.92-3.24), 0.073	1.55 (0.79-3.08), 0.198

* =significant p-value; OR = Odds ratio; 95% CI = 95% Confidence interval

Table 3. Allelic and genotype distribution of single nucleotide polymorphism (rs228566) in *ACE2* gene in COVID-19 individuals of Pernambuco State.

Sex	Genetics models	COVID-19 individuals			Fisher's Exact Test		
		Mild	Severe	Fatal	Mild vs Severe OR (95%CI), p-value	Mild vs. Fata OR (95%CI), p-value	Severe vs. Fatal OR (95%CI), p-value
Female	Allelic						
	C	95 (80.5)	55 (68.8)	41 (75.9)		Reference	
	T	23 (19.5)	25 (31.3)	13 (24.1)	1.87 (0.92-3.82), 0.065	1.31 (0.55-3.00), 0.546	0.70 (0.29-1.62), 0.436
	Codominant						
	CC	42 (71.2)	23 (57.5)	17 (63.0)			
	CT	11 (18.6)	9 (22.5)	7 (25.9)	1.49 (0.47-4.63), 0.443	1.56 (0.44-5.38), 0.562	1.05 (0.27-3.93), 1.000
	TT	6 (10.2)	8 (20.0)	3 (11.1)	2.41 (0.64-9.55), 0.145	1.23 (0.18-6.60), 1.000	0.51(0.08-2.57), 0.493
	Dominant (CC vs CT+TT)				0.55 (0.22-1.39), 0.197	0.69(0.24-2.05), 0.463	1.25 (0.41-3.89), 0.800
	Recessive (CC+CT vs TT)				2.19 (0.60-8.42), 0.240	1.10 (0.16-5.70), 1.000	0.50 (0.08-2.39), 0.504
	Overdominant (CC+TT vs CT)				1.26 (0.41-3.81), 0.799	1.52 (0.43-5.05), 0.569	1.20 (0.32-4.32), 0.777
Male	C	30 (71.4)	38 (71.7)	61 (83.6)			
	T	12 (28.6)	15 (28.3)	12 (16.4)	0.99 (0.37-2.69), 1.000	0.49 (0.18-1.36), 0.154	0.50 (0.19-1.28), 0.127

* =significant p-value; OR = Odds ratio; 95% CI = 95% Confidence interval;

Table 4. Genotype distribution of single nucleotide polymorphisms in *MTHFR* e *ANXA2* genes according to the morbidities

Morbidities/ Cases	*MTHFR rs1801133*		Fisher's Exact Test	*ANXA2 rs7163836*		Fisher's Exact Test	*ANXA2 rs7170178*		Fisher's Exact Test
	AA	AG+GG	OR (95%CI), p-value	CC	CT+TT	OR (95%CI), p-value	AA	AG+GG	OR (95%CI), p-value
Cardiovascular diseases									
Severe	19 (70.4)	8 (29.6)	2.77	11 (40.7)	16 (59.3)	1.80	15 (55.6)	12 (44.4)	5.38
Fatal	5 (45.5)	6 (54.5)	(0.53-15.43), 0.266	3 (27.3)	8 (72.7)	(0.33-12.95), 0.488	2 (18.2)	9 (81.8)	(0.87-60.48), 0.070
Diabetes Mellitus									
Severe	16 (69.6)	7 (30.4)	2.23	7 (30.4)	16 (69.6)	0.45	11 (47.8)	12 (52.2)	3.53
Fatal	5 (50.0)	5 (50.0)	(0.38-13.54), 0.433	5 (50.0)	5 (50.0)	(0.07-2.64), 0.433	2 (20.0)	8 (80.0)	(0.53-41.31), 0.245
Overweight/ Obesity									
Severe	10 (66.7)	5 (33.3)	5.42	6 (40.0)	9 (60.0)	0.68	5 (33.3)	10 (66.7)	nc
Fatal	1 (25.0)	3 (75.0)	(0.34-344.81), 0.262	2 (50.0)	2 (50.0)	(0.10-4.44), 0.697	0 (0.0)	4 (100.0)	
Respiratory diseases									
Severe	9 (69.2)	4 (30.8)	0.76	4 (30.8)	9 (69.2)	1.31	7 (53.8)	6 (46.1)	nc
Fatal	3 (75.0)	1 (25.0)	(0.01-13.68), 1.000	1 (25.0)	3 (75.0)	(0.07-87.21), 1.000	0 (0.0)	4 (100.0)	
Hypertension									
Severe	6 (54.5)	5 (45.5)	3.31	4 (36.4)	7(63.6)	0.59	4 (36.4)	7(63.6)	1.66
Fatal	1 (25.0)	3 (75.0)	(0.19-219.53), 0.569	2 (50.0)	2 (50.0)	(0.03-11.27), 1.000	1 (25.0)	3 (75.0)	(0.09-112.09), 1.000

nc = not calculated2

Table 5. Genotype distribution of single nucleotide polymorphisms in *ACE2* gene according to morbidities.

Morbidities/ cases	Female			Male		
	ACE2 rs228566		**Fisher's Exact Test**	***ACE2 rs228566***		**Fisher's Exact Test**
	CC	**CT+TT**	**OR (95%CI), p-value**	**C**	**T**	**OR (95%CI), p-value**
Cardiovascular diseases						
Severe	8 (72.7)	3 (27.3)	2.48	10 (62.5)	6 (37.5)	0.68
Fatal	2 (50.0)	2 (50.0)	(0.13-50.83), 0.560	5 (71.4)	2 (28.6)	(0.05-6.04), 1.000
Diabetes Mellitus						
Severe	3 (27.3)	8 (72.7)	0.21	8 (66.7)	4 (33.3)	0.81
Fatal	2 (66.7)	1 (33.3)	(0.003-5.54), 0.505	5 (71.4)	2 (28.6)	(0.05-8.56), 1.000
Overweight/ Obesity						
Severe	2 (100.0)	0 (0.0)		8 (66.7)	5 (33.3)	
Fatal	1 (33.3)	2 (66.7)	nc	1 (100.0)	0 (0.0)	nc
Respiratory diseases						
Severe	4 (57.1)	3 (42.8)		5 (83.3)	1 (16.7)	
Fatal	0 (0.0)	2 (100.0)	nc	0 (0.0)	2 (100.0)	nc
Hypertension						
Severe	2 (50.0)	2 (50.0)	1.00 (8.53e-3 –	6 (85.7)	1 (14.3)	
Fatal	1 (50.0)	1 (50.0)	1.17.e2), 1.000	2 (100.0)	0 (0.0)	nc

nc = not calculated

Table 6. Genotype distribution of single nucleotide polymorphisms in *MTHFR* e *ANXA2* genes according to the symptoms.

Symptoms/ Cases	*MTHFR rs1801133*		Fisher's Exact Test	*ANXA2 rs7163836*		Fisher's Exact Test	*ANXA2 rs7170178*		Fisher's Exact Test
	AA	AG+GG	OR (95%CI), p-value	CC	CT+TT	OR (95%CI), p-value	AA	AG+GG	OR (95%CI), p-value
O_2 saturation <95%									
Severe	53 (68.8)	29 (31.2)	1.82 (0.83-4.01),	25 (32.2)	17 (32.1)	1.02 (0.45-2.32),	37 (48.1)	40 (51.9)	1.60 (0.74-3.53),
Fatal	29 (54.7)	24 (45.3)	0.129	52 (67.5)	36 (67.9)	1.000	19 (36.5)	33 (63.5)	0.210
Dyspnoea									
Severe	47 (64.4)	26 (35.6)	1.29 (0.60-2.76),	25 (34.2)	48 (65.8)	1.21 (0.55-2.72),	36 (49.3)	37 (50.7)	1.84 (0.86-4.01),
Fatal	35 (58.3)	25 (41.7)	0.481	18 (30.0)	42 (70.0)	0.710	20 (34.5)	38 (65.5)	0.110
Respiratory discomfort									
Severe	28 (65.1)	15 (34.9)	1.48 (0.49-4.46),	13 (30.2)	30 (69.8)	0.63 (0.21-1.95),	18 (41.9)	25 (58.1)	1.27 (0.42-4.07),
Fatal	15 (55.6)	12 (44.4)	0.458	11 (40.7)	16 (59.3)	0.441	9 (36.0)	16 (64.0)	0.798
Fever									
Severe	37 (62.7)	22 (37.3)	1.74 (0.76-4.06),	22 (37.3)	37 (62.7)	1.22 (0.51-2.95),	27 (45.8)	32 (54.2)	1.63 (0.69-3.91),
Fatal	24 (49.0)	25 (51.0)	0.175	16 (32.7)	33 (67.3)	0.688	16 (34.0)	31 (66.0)	0.239
Cough									
Severe	55 (66.3)	28 (33.7)	1.62 (0.75-3.49),	27 (32.5)	56 (67.5)	1.11 (0.50-2.54),	37 (44.6)	46 (55.4)	1.43 (0.66-3.15),
Fatal	29(54.7)	24 (45.3)	0.207	16 (30.2)	37 (69.8)	0.851	18 (36.0)	32 (64.0)	0.367

nc = not calculated2

Table 7. Genotype distribution of single nucleotide polymorphisms in *ACE2* gene according to morbidities.

Symptoms/ cases	Female			Male		
	ACE2 rs228566		Fisher's Exact Test	*ACE2 rs228566*		Fisher's Exact Test
	CC	CT+TT	OR (95%CI), p-value	C	T	OR (95%CI), p-value
O_2 saturation <95%						
Severe	17 (56.7)	13 (43.3)	0.87 (0.23-3.17),	34 (72.3)	13 (27.7)	0.87 (0.23-3.17),
Fatal	12 (60.0)	8 (40.0)	1.000	24 (75.0)	8 (25.0)	1.000
Dyspnoea						
Severe	21 (61.8)	13 (38.2)	1.07 (0.29-3.82),	28 (71.8)	11 (28.2)	6.30 (2.19-19.61),
Fatal	12 (60.0)	8 (40.0)	1.000	11 (28.2)	28 (71.8)	0.0002*
Respiratory discomfort						
Severe	10 (62.5)	6 (37.5)	1.32 (0.18-9.29),	18 (66.7)	9 (33.3)	0.41 (0.06-2.04),
Fatal	5 (55.6)	4 (44.4)	1.000	15 (83.3)	3 (16.7)	0.308
Fever						
Severe	12 (54.5)	10 (45.5)	0.73 (0.16-3.20),	28 (75.7)	9 (24.3)	1.14 (0.43-4.68),
Fatal	10 (62.5)	6 (37.5)	0.744	22 (68.8)	10 (31.2)	0.207
Cough						
Severe	20 (60.6)	13 (39.4)	0.84 (0.20-3.27),	37 (74.0)	13 (26.0)	1.48 (0.52-4.21),
Fatal	11 (64.7)	6 (35.3)	1.000	23 (65.7)	12 (34.3)	0.472

nc = not calculated

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6 ARTIGO 3 – ASSOCIATION OF POLYMORPHISMS IN THE *DDX58* AND *RELA* GENES WITH THE COVID-19 SEVERITY IN A BRAZILIAN NORTHEASTERN POPULATION

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ABSTRACT

COVID-19 is a heterogeneous disease with diverse clinical manifestations. Different clinical outcomes can be related to factors such as age, sex, presence of morbidities and viral and host genetic variability. Polymorphisms in genes involved in inflammatory pathways have been linked to more severe outcomes of COVID-19. We investigated the allelic and genotypic distributions of single nucleotide polymorphisms (SNPs) in the *DDX58* (rs10813831), *RelA* (rs7101916) and *IL6* (rs1800795) genes and their possible relationship with the severity of COVID-19. For this, 312 nasopharyngeal swab samples of positive SARS-CoV-2 individuals were collected, and genomic DNA extracted. Based on clinical findings, individuals were stratified in mild, severe and fatal COVID-19. Genotyping was performed by real-time PCR, using allele-specific probes. Respiratory symptoms are more frequent in individuals who develop the most severe forms of the disease. In addition, the presence of morbidities such as cardiovascular

disease and diabetes mellitus are also more frequent among the most severe and fatal cases. Among the SNPs studies, rs10813831 in DDX58 gene and rs7101916 RelA gene was associated with COVID-19 outcomes. In the rs10813831 SNP, the G allele and GG genotype showed a significant difference when compared to the AA and AG genotypes with p=0,033 and 0,042, respectively, indicating a possible association with COVID-19 mortality. Regarding rs7101916 SNP, the T allele seems to be associated with the severity of COVID-19 (p=0.016). Furthermore, the CT and CC genotypes also seems to be associated with the severity of COVID-19 with p=0,001 and 0,003, respectively. New studies involving other populations should be conducted to confirm the role of these genes in the different clinical outcomes of COVID-19.

INTRODUCTION

COVID-19 is a highly contagious disease because it is spread mainly through the airways. Infected people may or no have symptoms, that include fever, tiredness, dry cough, myalgia, nasal congestion, pharyngitis, headache, anosmia, diarrhea, severe dyspnea, dysgeusia, angina, paralysis, aphasia, rash or cyanosis (Huang et al. 2020; Leentjens et al. 2021). Was identified for the first time in China in December 2019. With a high rate of transmissibility and infectivity, the virus quickly spread around the world, leading to a pandemic scenario announced in March 2020 by the World Health Organization (WHO) (Huang et al. 2020).

COVID-19 course and clinical outcomes depend on several factors such as age, sex, presence of comorbidities and even viral and host genetic variability. Overall, individual who develop the most severe form of the disease are male, elderly and with associated comorbidities (Wu et al. 2020). SARS-CoV-2 infection can induce an increase in pro-inflammatory cytokines and chemokines production, culminating in a

systemic hyper-inflammatory immune response and tissue damage (Zhou et al. 2020b; Giamarellos-Bourboulis et al. 2020).

Genes associated with the immune response and inflammation pathway as *DDX58*, *RelA* and *IL6* have been related to COVID-19 outcomes (Zhu et al. 2020b; Liu et al. 2020; Yamada et al. 2021).

The DDX58 gene encodes the antiviral innate immune response receptor (RIG-I). This receptor detects viral nucleic acids in the cytoplasm and activates a downstream signaling cascade, producing type I interferons and pro-inflammatory cytokines (Saito et al. 2007). RIG-I recognizes the 3' untranslated region (3'-UTR) of the SARS-CoV-2 RNA via helicase domains. In this sense, RIG-I expression levels may be intrinsic determinants of defense in human lung cells during the initial process of SARS-CoV-2 infection (Yamada et al. 2021).

The *RelA* gene encodes the P65 protein, which is a transcription factor that, together with other transcription factors (RelB, c-Rel, P50 or NF-KB1 and P52 or NF-KB2) make up the NF-KB complex. NF-KB is a nuclear factor that binds to a kappa enhancer of the kappa immunoglobulin light chain gene of activated B cells (Baltimore 2009). It is expressed in several cell types, being an important regulator of the immune response against pathogens, of the inflammatory reaction and of cell proliferation and survival (Li and Verma 2002). Excessive activation of NF-KB can trigger the production of pro-inflammatory cytokines and a chemokine storm. Furthermore, it is considered a key transcription factor in regulating the IFN response during SARS-CoV-2 infection (Yin et al. 2021).

Additionally, the IL6 gene encodes interleukin 6, a cytokine that acts on B cell maturation. IL6 is produced mainly in sites of acute and chronic inflammation, being secreted in serum and inducing a transcriptional inflammatory response (Fei et al.

2015). Elevated IL6 levels are commonly associated with viral infections, including SARS-CoV-2 (Bhaskar et al. 2020). IL6 is considered a key inflammatory cytokine, it is secreted by immune and non-immune cells in lung tissue, such as macrophages, T lymphocytes, type II alveolar epithelial cells (ECs) and lung fibroblasts (Zhang et al. 2020a). Furthermore, IL6 plays an important role in the severity of patients with COVID-19, being significantly associated with adverse clinical outcomes (Chen et al. 2015; Gong et al. 2020).

Thus, studies that can identify the association of genetic variants with susceptibility to unfavorable clinical outcomes of COVID-19 are needed. In this study, we evaluated the allelic and genotypic distribution of single nucleotide polymorphisms (SNPs) in *DDX58* (rs10813831), *RelA* (rs7101916) and *IL6* (rs1800795) genes and their possible influence on COVID-19 outcomes in a Brazilian Northeastern population.

MATERIAL AND METHODS

Sample collection and stratification

For this study, we selected 312 nasopharyngeal swabs samples collected of individuals of both sexes, with age from 18 to 60 years old from the Pernambuco State – Brazil with positive diagnostic by PCR for SARS-CoV-2, through partnership with the Central Laboratory of Pernambuco (LACEN-PE), in period of March to December 2020. The cases were stratified according to the severity of COVID-19 into mild, severe, and fatal according to their clinical record, forwarded at the time of diagnosis and World Health Organization (WHO) guidelines.

Were classified as mild cases the individuals without comorbidities and hospitalization by COVID-19 who presented mild symptoms such as cough, fever and absent of respiratory symptoms. In the severe cases group were included individuals

with hospitalization by COVID-19, with or without comorbidities and presenting severe respiratory symptoms such as dyspnea, low oxygen saturation (< 95%). Lastly, individuals presenting severe respiratory symptoms, with or without comorbidities, hospitalized and who died as a result of COVID-19 complications were classified as fatal cases.

DNA isolation, SNPs selection and Genotyping

The genomic DNA extraction of nasopharyngeal swabs were performed using Chelex®100 resin (Sigma-Aldrich). DNA concentrations were evaluated by spectrophotometry using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). DNA purity and quality was estimated through the 260/280 and 260/230 ratios. The SNPs selection was performed from public databases, such as: OMIM (https://www.omim.org), dbSNP (https://www.ncbi.nlm.nih.gov/snp/), SNPnexus (www.snp-nexus.org/v4/) and Ensembl (https://www.ensembl org/index.html); as well as based on the literature. The SNPs selection was based on the minimum allele frequency (MAF>0.1 in Caucasian and Yoruba populations), the functional role of the variant and previous associations with risk conditions for COVID-19.

The selected SNPs were the rs10813831 G>A in *DDX58*, rs7101916 C>T in *RelA*, rs1800795 G>C in *IL6.* All variants were genotyped by qRT-PCR using allelic specific probes (TaqMan SNP Genotyping Assays) in an ABI7500 Real Time PCR machine (Applied Biosystems), according to manufacturer's recommendations.

Statistical analysis

Allelic and genotypic frequencies were estimated by direct counting among Genotype transposer (Cox and Canzian 2001). Hardy-Weinberg equilibrium (HWE) adherence

and possible associations were determined through Chi-Square test (X^2) and Fisher's Exact Test, respectively. All tests were performed in R program version 2.11.1 and/or GraphPad Prism version 5.0, with a significance level of 0.05.

RESULTS

This study included 312 individuals SARS-CoV-2 positive, which 150 was classified as mild cases, 93 severe and 70 fatal cases.

In the three studied groups, males were the most frequent (>54%). The mean age at diagnosis was 44.3 years old in mild cases, 44.7 years old in severe cases and 47.3 years old in fatal cases.

Regarding the symptomatology, among severe cases cough (89.1%), oxygen saturation < 95% (82.6%), dyspnea (78.3%), fever (64.1%) and respiratory distress (46.1%) were more common. On the other hand, dyspnea (85.7%), oxygen saturation < 95% (75.7%), cough (75.7%), fever (70.0%) and respiratory distress (38.6%) were the main symptoms among fatal cases (data not published). Among severe and fatal cases, the morbidities more frequent were heart and vascular diseases 29.3% and 15.7%, respectively), diabetes mellitus (25% and 14.3% respectively), overweight/obesity (15.2% and 5.7%) respiratory diseases (13% and 5.7%, respectively) and hypertension (12% and 5.7% respectively) (data not published).

Allelic and genotypic distribution of SNPs in *DDX58*, *RelA* and *IL6* genes were presented in table 1. In relation to genotypic distribution, the Hardy-Weinberg adherence was observed for all studied variants.

Among the SNPs studied, only the rs10813831 in *DDX58* and rs7101916 in *RelA* showed significant differences between the groups (Table 1). Regarding the SNP rs10813831 in the DDX58 gene, the A allele was more frequent among mild cases

(65%) and less frequent in fatal cases (56%). The AG heterozygous genotype was the most frequent among fatal (63%) and mild (59%) cases, however no significant correlation was observed. When comparing mild and fatal cases, the homozygous GG genotype showed a significant difference when compared to the AA and AG genotypes (OR=3.35; p=0.042).

Regarding the SNP rs7101916 in *RelA*, the C allele was the most common among mild (86%) and fatal (83%) cases. The T allele was more frequent among severe (24%) and fatal (17%) cases. However, when we compared the frequency of the T allele between mild and severe cases, a significant correlation was observed with p=0.016, suggesting susceptibility to severity. The CC genotype was more common among mild (75%) cases, followed by fatal (72%) and severe (61%) cases. The CT genotype was more frequent among severe cases (21%) and fatal cases, 22%. However, when we compared mild and severe cases, a significant correlation was observed with a value of p=0.001. Furthermore, the CC genotype showed a significant difference in relation to the CT and TT genotypes (OR=2.46; p=0.003) between mild and severe cases. The CT genotype, on the other hand, showed a significant difference in relation to the homozygous CC and TT genotypes (OR=3.13; p=0.001).

In relation to the other variants studied, despite the percentage differences between the different types of cases, no significant differences were observed (p>0.05) (table 1).

The genotypic distribution was carried out according to the morbidities and symptomatology presented by the severe and fatal cases. The results were presented in tables 2 and 3. Regarding symptomatology, we observed a significant association only for the SNP rs10813831 in *DDX58*. Where, symptoms such as fever and cough (p=0,005 and p=0,001, respectively), as well as respiratory symptoms (low O2

saturation, p=0,0005 and dyspnoea, p=0,001) showed a dignified association between severe and fatal cases. For the other variants studied, as well as the presence of morbidities, despite the different genotypic distributions between mild, severe and fatal cases, no significant differences were observed (p>0.05).

DISCUSSION

COVID-19 is a highly heterogeneous disease that presents several clinical manifestations, making the prognosis difficult. Individuals with cardiovascular diseases can develop severe complications, due the increase in the inflammatory state induced by COVID-19 (Huang et al. 2020). Furthermore, diabetes mellitus is also a risk morbidity for the most severe forms of COVID-19, since high blood glucose levels or non-treatment can lead to changes in coagulation and overproduction of inflammatory cytokines (Brito et al. 2020).

According to our findings, only the SNPs rs10813831 in *DDX58* and rs7101916 in *RelA* showed a significant association with the outcome of COVID-19. The rs10813831 SNP in *DDX58* causes a non-synonymous change from G to A with high functional impact. A study conducted in China suggests that individuals with HCV carrying the rs10813831-G allele were significantly less likely to continue a persistent infection than those carrying the A allele (Wu et al. 2019). However, in our study population, we observed that the G allele showed a significant association when we compared the mild and fatal groups (p=0.033). Indicating that this allele seems to be associated with COVID-19 mortality in the study population. In addition, the GG genotype also showed a significant association with COVID-19 mortality. The divergence of results between populations can be explained by the high genetic

variability of the Brazilian population. However, studies that evaluate the functional effect of these polymorphisms must be conducted.

The rs7101916 C>T polymorphism in *RelA* gene is located near the 5' end of the gene. According to our findings, the T allele seems to be associated with the severity of COVID-19 (p=0.016). Furthermore, we also observed significant associations between CT and CC genotypes and COVID-19 severity with p=0.001 and 0.003, respectively. This polymorphism was predicted to be a transcription factor binding site (TFBS), which may involve altering transcription factor binding and mediate transcriptional regulation of the RelA gene and its expression, subsequently affecting the activation of the NF-κB pathway and susceptibility to viral infection (Yue et al. 2019). Studies performed in a Chinese population indicate that the T allele is associated with protection from HCV infection (Yue et al. 2019). So far, there are no studies relating the SNP rs7101916 C>T with COVID-19, our study is the first to find this association in a Brazilian population. However, studies that evaluate the functional effect of this polymorphism must be conducted.

Our results did not find association of the polymorphism in *IL6* gene with the outcome of COVID-19. The IL6 gene encodes the glycoprotein interleukin 6 (IL6), a cytokine that acts on inflammation and B cell maturation. IL6 is produced mainly in sites of acute and chronic inflammation, being secreted in serum and inducing a transcriptional inflammatory response. Elevated IL6 levels are commonly associated with viral infections, including SARS-CoV-2 (Bhaskar et al. 2020). Studies carried out in China indicated that the increase in serum IL6 levels is a key feature of the severity of COVID-19 and can serve as a biomarker to predict disease progression (Zhu et al. 2020b; Liu et al. 2020).

Although our studies did not find a significant association between the presence of morbidities and the severity of COVID-19, some studies suggest that individuals who have previous clinical conditions are more likely to develop the most severe forms of the disease (Wu et al. 2020; Tiburi et al. 2021).

Further studies including a larger population should be conducted. As well as the functional analysis of these polymorphisms should be performed to determine the role of these variants with the clinical outcomes of COVID-19.

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of FEDERAL UNIVERSITY OF PERNAMBUCO (CAAE: 44390221.6.0000.5208).

Informed Consent Statement

Patient consent was waived due to fact that we used spent samples submitted to COVID-19 diagnosis.

Table 1. Allelic and genotypic distribution of single nucleotide polymorphisms in *DDX58* and *RelA genes in COVID-19* individuals of Pernambuco State.

Genes/ SNPs	Genetic Models	COVID-19 individuals			Fisher's Exact Test		
		Mild	Severe	Fatal	Mild vs Severe OR (95%CI), p-value	Mild vs. Fata OR (95%CI), p-value	Severe vs. Fatal OR (95%CI), p-value
DDX58 ***rs10813831***	Allelic						
	A	190 (66.4)	113 (60.8)	78 (55.7)		Reference	
	G	96 (33.6)	73 (39.2)	62 (44.3)	1.28 (0.85-1.91), 0.238	1.57 (1.02-2.43), 0.033*	1.23 (0.77-1.97), 0.366
	Codominant						
	AA	53 (37.1)	31 (33.3)	17 (24.3)		Reference	
	AG	84 (58.7)	51 (54.8)	44 (62.9)	1.04 (0.57-1.90), 1.000	1.63 (0.81-3.37), 0.151	1.57 (0.73-3.46), 0.283
	GG	6 (4.2)	11 (11.8)	9 (12.9)	3.10 (0.94-11.27), 0.057	4.58 (1.25-18.14), 0.012*	1.48 (0.45-4.88), 0.585
	Dominant (AA vs AG+GG)				1.18 (0.66-2.12), 0.581	1.83 (0.93-3.73), 0.065	1.55 (0.74-3.35), 0.229
	Recessive (AA+AG vs GG)				3.05 (0.99-10.43), 0.038*	3.35 (1.01-11.96), 0.042*	1.10 (0.38-3.12), 1.000
	Overdominant (AA+GG vs AG)				0.85 (0.49-1.50), 0.591	1.19 (0.63-2.24), 0.655	1.39 (0.70-2.77), 0.338
RELA ***rs7101916***	Allelic						
	C	233 (85.7)	133 (76.4)	108 (83.1)		Reference	
	T	39 (14.3)	41 (23.6)	22 (16.9)	1.84 (1.10-3.09), 0.016*	1.22 (0.65-2.22), 0.553	0.66 (0.35-1.22), 0.198
	Codominant						
	CC	108 (79.4)	53 (60.9)	47 (72.3)		Reference	
	CT	17 (12.5)	27 (31.0)	14 (21.5)	3.22 (1.54-6.89), 0.001*	1.88 (0.79-4.45), 0.142	0.59 (0.25-1.32), 0.192
	TT	11 (8.1)	7 (8.0)	4 (6.2)	1.29 (0.40-3.90), 0.607	0.84 (0.18-3.01), 1.000	0.65 (0.13-2.73), 0.544
	Dominant (CC vs CT+TT)				2.46 (1.30-4.71), 0.003*	1.47 (0.70-3.07), 0.284	0.60 (0.28-1.26), 0.168
	Recessive (CC+CT vs TT)				0.99 (0.31-2.94), 1.000	0.75 (0.17-2.65), 0.778	0.75 (0.15-3.11), 0.759
	Overdominant (CC+TT vs CT)				3.13 (1.51-6.65), 0.001*	1.91 (0.81-4.48), 0.142	0.61 (0.27-1.36), 0.203
IL6 ***rs1800795***	Allelic						
	A	182 (75.2)	140 (77.8)	103 (79.2)		Reference	
	C	60 (24.8)	40 (22.2)	27 (20.8)	0.87 (0.53-1.40), 0.564	0.80 (0.46-1.36), 0.441	0.92 (0.51-1.64), 0.782
	Codominan						
	AA	72 (59.5)	57 (63.3)	41 (63.1)		Reference	
	AC	38 (31.4)	26 (28.9)	21 (32.3)	0.86 (0.47-1.65), 0.647	0.97 (0.47-1.96), 1.000	1.12 (0.52-2.39), 0.858

CC	11 (9.1)	7 (7.8)	3 (4.9)	0.80 (0.25-2.44), 0.801	0.48 (0.08-1.97), 0.377	0.60 (0.09-2.82), 0.523
Dominant (AA vs AC+CC)				0.85 (0.47-1.55), 0.668	0.86 (0.44-1.67), 0.753	1.01 (0.49-2.06), 1.000
Recessive (CC+AC vs AA)				0.84 (0.26-2.50), 0.807	0.48 (0.08-1.93), 0.385	0.58 (0.09-2.65), 0.522
Overdominant (AA+CC vs AC)				0.89 (0.46-1.67), 0.763	1.04 (0.51-2.08), 1.000	1.17 (0.55-2.48), 0.724

* =significant p-value; OR = Odds ratio; 95%CI= 95% Confidence interval

Table 2. Genotype distribution of single nucleotide polymorphisms in *DDX58, RELA and IL6* genes according to the morbidities

Morbidities/ Cases	***DDX58* *rs1801133***		**Fisher's Exact Test**	***RELA* *rs7163836***		**Fisher's Exact Test**	***IL6* *rs7170178***		**Fisher's Exact Test**
	AA	**AG+GG**	**OR (95%CI), p-value**	**CC**	**CT+TT**	**OR (95%CI), p-value**	**AA**	**AG+GG**	**OR (95%CI), p-value**
Cardiovascular diseases									
Severe	8 (29.6)	19 (70.4)	4.08	19 (70.4)	8 (29.6)	0.54	13 (50.0)	13 (50.0)	1.19
Fatal	1 (9.1)	10 (90.9)	(0.43-204.99), 0.237	9 (81.8)	2 (18.2)	(0.05-3.54), 0.690	5 (45.5)	6 (54.5)	(0.23-6.34), 1.000
Diabetes Mellitus									
Severe	7 (30.4)	16 (69.6)		14 (60.9)	9 (39.1)	0.45	11 (47.8)	12 (52.2)	1.36
Fatal	0 (0.0)	10 (100.0)	nc	7 (70.0)	3 (30.0)	(0.04-3.19), 0.441	4 (40.0)	6 (60.0)	(0.24-8.46), 0.722
Overweight/ Obesity									
Severe	6 (40.0)	9 (60.0)	1.93	9 (64.3)	5 (35.7)	0.62	11 (73.3)	4 (26.7)	0.92
Fatal	1 (25.0)	3 (75.0)	(0.12-122.13), 1.000	3 (75.0)	1 (25.0)	(0.01-10.42), 1.000	3 (75.0)	1 (25.0)	(0.01-16.26), 1.000
Respiratory diseases									
Severe	5 (38.5)	8 (61.5)		6 (46.1)	7 (53.9)	0.31	10 (76.9)	3 (23.1)	1.61 (0.02-43.01),
Fatal	0 (0.0)	4 (100.0)	nc	3 (75.0)	1 (25.0)	(0.005-5.07), 0.576	2 (66.7)	1 (33.3)	1.000
Hypertension									
Severe	3 (27.3)	8 (72.7)	1.12	8 (72.7)	3 (27.3)		7 (63.6)	4 (36.4)	
Fatal	1 (25.0)	3 (75.0)	(0.05-78.45), 1.000	4 (100.0)	0 (0.0)	nc	0 (0.0)	3 (100.0)	nc

nc = not calculated

Table 3. Genotype distribution of single nucleotide polymorphisms in *DDX58, RELA and IL6* genes according to the major symptoms.

Symptoms/ Cases	*DDX58* *rs10813831*		Fisher's Exact Test	*RELA* *rs7101916*		Fisher's Exact Test	*IL6* *rs1800795*		Fisher's Exact Test
	AA	AG+GG	OR (95%CI), p-value	CC	CT+TT	OR (95%CI), p-value	GG	GC+CC	OR (95%CI), p-value
O_2 saturation <95%									
Severe	26 (33.8)	51 (66.2)	6.17 (1.93-26.09),	45 (59.2)	31 (40.8)	0.65 (0.28-1.44),	49 (64.5)	27 (35.5)	1.31 (0.59-2.91),
Fatal	4 (7.5)	49 (92.5)	0.0005*	36 (69.2)	16 (30.8)	0.268	29 (58.0)	21 (42.0)	0.574
Dyspnoea									
Severe	25 (34.2)	48 (65.8)	4.63 (1.67-15.02),	44 (60.3)	29 (39.7)	0.57 (0.25-1.26),	41 (57.7)	30 (42.3)	0.88 (0.41-1.91),
Fatal	6 (10.0)	54 (90.0)	0.001*	43 (72.9)	16 (27.1)	1.143	34 (60.7)	22 (39.3)	0.856
Respiratory discomfort									
Severe	12 (27.9)	31 (72.1)	1.69 (0.47-7.04),	25 (58.1)	18 (41.9)	0.42 (0.11-1.38),	28 (68.3)	13 (31.7)	1.57 (0.50-4.91),
Fatal	5 (18.5)	22 (81.5)	0.409	20 (76.9)	6 (23.1)	0.127	15 (57.7)	11 (42.3)	0.438
Fever									
Severe	20 (33.9)	39 (66.1)	4.45 (1.44-16.65),	35 (60.3)	23 (39.7)	0.76 (0.31-1.81),	36 (62.1)	22 (37.9)	1.26 (0.53-2.98),
Fatal	5 (10.2)	44 (89.8)	0.005*	32 (66.7)	16 (33.3)	0.548	26 (56.5)	20 (43.5)	0.688
Cough									
Severe	27 (32.5)	56 (67.5)	5.84 (1.85-24.57),	51 (62.2)	31 (37.8)	0.67 (0.29-1.49),	51 (62.2)	31 (37.8)	1.29 (0.59-2.80),
Fatal	4 (7.5)	49 (92.5)	0.001*	37 (71.2)	15 (28.8)	0.352	28 (56.0)	22 (44.0)	0.583

nc = not calculated

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7 DISCUSSÃO GERAL

A COVID-19 é uma doença altamente heterogênea que apresenta diversas manifestações clínicas, dificultando o prognóstico. Vários fatores podem influenciar o desfecho da doença, como idade, sexo, morbidades e fatores genéticos virais e do hospedeiro. Desta forma, a extração de DNA é um procedimento fundamental para análises genéticas e moleculares.

Para melhor uso em países em desenvolvimento, os métodos devem ser rápidos, práticos, acessíveis, livres de contaminantes e toxicidade e fornecer DNA de alta quantidade e qualidade (Aidar and Line 2007; Yang et al. 2008; Gupta 2019). Analisamos soluções alternativas para extração de DNA para aplicações posteriores e comparamos três protocolos diferentes de extração de DNA para escolher o melhor método de extração para amostras de swab nasofaríngeo de pacientes com COVID-19.

O presente trabalho comparou três métodos de extração de DNA (Chelex®100, Fenol-clorofórmio e QIAamp DNA Mini Kit) para isolar DNA genômico de amostras de swab nasofaríngeo e avaliou seu desempenho e adequação para estudos de genotipagem. Nossos resultados sugerem que em amostras nasofaríngeas de pacientes com COVID-19, o uso do mini kit QIAamp DNA é limitado por sua baixa quantidade e qualidade de DNA, bem como pelo alto custo por amostra. Por outro lado, a extração à base de fenol-clorofórmio é trabalhosa e potencialmente perigosa, apesar do maior grau de pureza do DNA observado. Em contraste, a resina Chelex®100 surgiu como um método acessível, eficaz, rápido e simples, que pode ser realizado em poucos passos. O método não requer o uso de solventes orgânicos e etapas de manipulação, apresentando baixo risco de contaminação da amostra e oferece menor risco de periculosidade ao operador e ao meio ambiente. Assim, o método Chelex®100 é um método barato, seguro,

simples, rápido e eficaz para a extração de DNA de amostras nasofaríngeas de pacientes com COVID-19, adequado para países em desenvolvimento e configurações mínimas.

As comorbidades são um importante fator envolvido nas formas graves da doença, devido ao comprometimento da resposta imune que facilita a replicação viral em maior escala (Wu et al. 2020).

Nossos achados corroboram estudos anteriores, onde doenças cardiovasculares e diabetes são as mais frequentes entre indivíduos com formas graves e fatais de COVID-19. Indivíduos com doenças cardiovasculares podem desenvolver complicações graves induzidas pela COVID-19 (Huang et al. 2020). Além disso, a diabetes mellitus também é uma morbidade de risco para as formas mais graves de COVID-19, pois níveis elevados de glicose no sangue ou o não tratamento podem levar a alterações na coagulação e superprodução de citocinas inflamatórias (Brito et al. 2020).

Além disso, genes relacionados a funções importantes da resposta imune, inflamação e coagulação também podem influenciar a progressão da gravidade e mortalidade por COVID-19 (Debnath et al. 2020; Choudhary et al. 2021). Em nosso estudo, os polimorfismos rs7170718 no gene *AnxA2*, rs10813831 em *DDX58* e rs7101916 em *RelA* apresentaram associação significativa com o desfecho de COVID-19. A variante rs7170718 no gene *AnxA2* pode estar envolvida no enovelamento das alças da cromatina, influenciando a ligação de fatores de intensificação na região promotora *AnxA2* e atuando como um regulador dos níveis de expressão gênica (Baldwin et al. 2005). Assim, é provável que indivíduos portadores desse polimorfismo apresentem comprometimento molecular em *AnxA2*.

Estudos identificaram uma associação de polimorfismos em *AnxA2* com o desenvolvimento de osteonecrose, bem como com um risco aumentado de acidente vascular cerebral (Pereira-Martins et al. 2020). Contudo, para confirmar o real papel desse gene nos desfechos clínicos da COVID-19, estudos incluindo outras variantes funcionais em outras populações e grande número de amostras são necessários. Além disso, é importante avaliar os níveis de expressão de AnxA2 para elucidar o possível efeito funcional do polimorfismo neste gene.

O SNP rs10813831 em *DDX58* causa uma mudança não sinônima de G para A com alto impacto funcional. Um estudo realizado na China sugere que indivíduos com HCV portadores do alelo rs10813831-G eram significativamente menos propensos a continuar uma infecção persistente do que aqueles portadores do alelo A (Wu et al. 2019). No entanto, em nossa população de estudo, observamos que o alelo G apresentou associação significativa quando comparamos os grupos leve e fatal (p=0,033). Indicando que esse alelo parece estar associado à mortalidade por COVID-19 na população do estudo. Além disso, o genótipo GG também mostrou associação significativa com a mortalidade por COVID-19. A divergência de resultados entre as populações pode ser explicada pela alta variabilidade genética da população brasileira.

O polimorfismo rs7101916 C>T no gene *RelA* está localizado próximo da extremidade 5' do gene. De acordo com nossos achados, o alelo T parece estar associado à gravidade da COVID-19 (p=0,016). Além disso, também observamos associações significativas entre os genótipos CT e CC e a gravidade da COVID-19 com p=0,001 e 0,003, respectivamente. Este polimorfismo parece ser um sítio de ligação do fator de transcrição (TFBS), que pode envolver a alteração da ligação do fator de transcrição e mediar a regulação transcricional do gene *RelA* e sua

expressão, afetando subsequentemente a ativação da via NF-κB e a suscetibilidade à infecção viral. Estudos realizados em uma população chinesa indicam que o alelo T está associado à proteção contra a infecção pelo HCV (Yue et al. 2019).

Com relação aos demais genes incluídos no estudo, não foram observadas associações estatisticamente significativas. No entanto, sabe-se que os determinantes de diferentes suscetibilidades ao SARS-CoV-2 envolvem principalmente genes relacionados aos estágios iniciais da infecção, o sistema angiotensina *ACE-1* e *ACE-2* desempenha um papel importante na patogênese de COVID-19 (Anastassopoulou et al. 2020). Os polimorfismos funcionais dos genes *ACE1/ACE2* têm sido associados ao risco de doenças cardiovasculares e pulmonares e, portanto, também podem contribuir para o desfecho da COVID-19 (Gómez et al. 2020). Variações no gene *ACE-2* podem facilitar ou dificultar a invasão de células por SARS-CoV-2, razão pela qual os níveis de expressão celular de ACE-2 diferem entre indivíduos (Zhang et al. 2020b; Choudhary et al. 2021).

Nossos resultados não encontraram associação do polimorfismo no gene *MTHFR* com o desfecho de COVID-19. A enzima produzida pelo *MTHFR* é responsável pela conversão da homocisteína e estudos têm sugerido uma relação entre os níveis de homocisteína e a gravidade da doença (Ponti et al. 2021a). A análise de coorte de 200 indivíduos com COVID-19 na Itália mostrou uma associação entre níveis elevados de homocisteína no sangue e o prognóstico grave ou letal da doença (Ponti et al. 2021b).

Por fim, nossos resultados não encontraram associação do polimorfismo no gene *IL6* com o desfecho de COVID-19. A IL6 é produzida principalmente em locais de inflamação aguda e crônica, sendo secretada no soro e induzindo uma resposta inflamatória transcricional. Níveis elevados de IL6 são comumente associados a

infecções virais, incluindo SARS-CoV-2 (Bhaskar et al. 2020). Estudos realizados na China indicaram que o aumento dos níveis séricos de IL6 é uma característica fundamental da gravidade da COVID-19 e pode servir como um biomarcador para prever a progressão da doença (Zhu et al. 2020b; Liu et al. 2020).

Nossos estudos não tenham encontrado uma associação significativa entre a presença de morbidades e a gravidade do COVID-19, alguns estudos sugerem que indivíduos que apresentam condições clínicas anteriores têm maior probabilidade de desenvolver as formas mais graves da doença (Wu et al. 2020; Tiburi et al. 2021).

Embora nosso estudo apresente limitações quanto ao baixo número de amostras entre os grupos e a ausência de análises funcionais dos polimorfismos estudados, trata-se do primeiro estudo a encontrar associações significativas entre polimorfismos nos genes *AnxA2* e *RelA* e os desfechos desfavoráveis da COVID-19. Esses achados possibilitam um direcionamento para estudos posteriores, com a finalidade de identificar possíveis marcadores genéticos para prever o prognóstico da COVID-19 e auxiliar nas condutas terapêuticas.

8 CONCLUSÕES

- A resina Chelex®100, mostrou-se ser um método barato, seguro, simples, rápido e adequado para extração de DNA genômico de amostras nasofaríngeas de pacientes com COVID-19 para estudos genéticos;
- Febre e tosse são os sintomas mais frequentes em indivíduos com as formas leves da COVID-19. Já aqueles com as formas graves e fatais, apresentam, além de tosse, sintomas respiratórios como baixa saturação de oxigênio e dispneia;
- Indivíduos que possuem morbidades como doenças cardiovasculares e diabetes mellitus, apresentam maior risco de desenvolver as formas graves e fatais da COVID-19;
- O polimorfismo rs7170178 no gene *AnxA2*, mostrou poder aumentar a susceptibilidade a morte por COVID-19. Enquanto outros polimorfismos estudados nos genes *MTHFR* (rs1801133), *AnxA2* (rs7163836) e *ACE-2* (rs228566) parece não estarem associados aos desfechos clínicos desfavoráveis da COVID-19;
- Os polimorfismos rs10813831 no gene *DDX58* e rs7101916 no gene *RelA* podem estar associados com a gravidade da COVID-19. Enquanto o polimorfismo rs1800795 no gene *IL6* parece não estar associado aos desfechos clínicos desfavoráveis da COVID-19 na população de Pernambuco;
- Os polimorfismos analisados não têm associação significativa com a presença de morbidades e os desfechos clínicos da COVID-19;

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ANEXO I

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: INFLUÊNCIA DE POLIMORFISMOS GENÉTICOS NAS COAGULOPATIAS ASSOCIADAS Á COVID-19

Pesquisador: Marcos André Cavalcanti Bezerra **Área Temática:**
Versão: 2

CAAE: 44390221.6.0000.5208

Instituição Proponente: Universidade Federal de Pernambuco - UFPE

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.638.742

Apresentação do Projeto:

O projeto de pesquisa intitulado “Influência de polimorfismos genéticos nas coagulopatias associadas á covid-19” do Laboratório de Hematologia do Centro de Biociências foi apresentado pelo Prof. Dr Marcos André Cavalcanti Bezerra (CB-UFPE) e os colaboradores Dr. Lindomar José Pena (Fiocruz-PE) e Dr Allyson Andrade Mendonça (Fiocruz-PE). Será realizado um estudo retrospectivo, descritivo quantitativo de pacientes positivos para COVID-19 por meio do teste de RT-qPCR realizado pelo LACEN/PE. Amostras de pacientes positivos para a infecção por SARS-CoV-2 serão obtidas a partir de amostras de rotina do LACEN/PE. Pretende-se testar amostras de pelo menos 600 pacientes no total, sendo 300 com a COVID-19 leve e 300 com as formas graves. As amostras foram coletadas nos hospitais e Unidades de Pronto Atendimento do Estado de Pernambuco integrantes do SUS. A coleta foi e será realizada pelos profissionais da rede de saúde como parte da demanda espontânea que busca o serviço laboratorial do LACEN/PE e não temos interferência neste processo.

Objetivo da

Pesquisa: a.

Objetivo

Geral

Determinar a associação de polimorfismos genéticos com as manifestações graves da COVID19.

b. Objetivos específicos

1. Identificar polimorfismos associados a mortalidade da COVID-19 em genes codificantes de componentesda resposta inflamatória.

2. Identificar polimorfismos associados a mortalidade da COVID-19 em genes codificantes de elementosenvolvidos na coagulação e fibrinólise.

Avaliação dos Riscos e Benefícios:

Riscos: O material coletado que será utilizado neste trabalho decorre da demanda espontânea do LACENPE para fins diagnóstico em âmbito externo as atividades deste projeto. Deste modo, a obtenção de amostras para presente pesquisa não contribui para exposição adicional de nenhuma forma de risco aos pacientes. Contudo, pode haver risco de exposição a dados dos pacientes. Este risco será limitado ao máximo com a seleção dos pacientes sendo realizada pelo laboratório colaborador e posterior encaminhamento das amostras codificadas para integrarem esta pesquisa.

Benefícios: Não há planejamento para concessão de nenhum benefício direto aos pacientes cujo material integrará essa pesquisa em virtude do seu anonimato. Contudo, os resultados deste trabalho podem contribuir com o entendimento da patogênese da Covid-19 e no desenvolvimento de melhores práticas clínicas que beneficiariam diretamente a sociedade.

Armazenamento dos dados coletados: O armazenamento das amostras de DNA obtidas, obedecerá aos requisitos estabelecidos na Resolução Nº 441 de 12 de maio de 2011 do Conselho Nacional de Saúde e Norma Operacional CNS Nº 001/2013, para o armazenamento de material biológico humano. Todo o material será mantido em ultrafreezers nas dependências do Laboratório Central, Av. Professor Moraes Rego, 1235 – Cidade Universitária – Recife/PE, sob a responsabilidade do Dr. Marcos André Cavalcanti Bezerra. Todas as amostras biológicas, e os resultados provenientes delas, serão usados apenas para as finalidades descritas no protocolo de pesquisa. Após a conclusão do estudo, qualquer material remanescente será destruído de acordo com as boas práticas clínicas.

Comentários e Considerações sobre a Pesquisa:

De acordo com a Resolucao 466/12 as pendências foram atendidas, conforme relato:

1. Solicitar ao Comite de Etica, atraves de documento, a dispensa do TCLE. Esse documento deve

ser anexado na Plataforma Brasil, contendo a justificativa pela qual a dispensa do TCLE esta sendo solicitada.
Resposta: Solicitação da dispensa do TCLE anexada como solicitado.

2. Descrever na metodologia qual sera a amostra utilizada nos experimentos.

Resposta:A descrição solicitada foi adicionada no item 3.1 da metodologia.

3. Corrigir no projeto as variascitacoes conflitantes sobre a coleta das amostras, conforme foi descrito noitem Comentarios e Consideracoes desse relatorio.

Resposta: O texto foi ajustado e os elementos conflitantes foram removidos. A alteração foi destacada com marca-texto amarelo, como indicado pelas recomendações da comissão.

4. Descrever como sera feito o uso de dados dos prontuarios e elencar esses dados, deixando claroquaisvariaveisserao estudadas nesta pesquisa.

Resposta: Não serão utilizados dados de prontuários, apenas parte dos dados encaminhados ao laboratório colaborador, juntamente com a amostra, durante sua rotina institucional. Os dados necessários para estratificação dos grupos analisados foram incluídos na metodologia no item 3.1.

5. Acrescentar o uso de dados secundarios na carta de anuencia do LACEN.

Resposta:O acesso aos dados secundários do paciente será realizado pelo próprio LACEN queencaminhará as amostras codificadas para utilização na pesquisadesde que cumpridos os requisitos para colaboração definidos por critérios institucionais e legais já presentes na carta de anuência apresentada.

6. Citar, no projeto e na Plataforma Brasil, os criterios de inclusao e exclusao.

Resposta:Os critérios de inclusão e exclusão foram adicionados ao texto do item 3.1 e no campo definido no site da plataforma Brasil.

7. Apresentar um esclarecimento sobre a fonte financeira do orçamento no valor de R$ 48.800,00 o qualsera da inteira responsabilidade do pesquisador principal.

Resposta:As atividades da pesquisa serão financiadas com recurso de projeto aprovado com colaboradores no edital Inova Covid-19 – Geração de conhecimento, FioCruz, descrito no item 4 do projeto de pesquisa.

8. Informar quais serao os riscos da pesquisa e as formas de minimiza-los.

Resposta:As informações solicitadas foram adicionadas no campo apropriado de descrição na plataforma Brasil.
9.Anexar Carta de Anuencia com a autorizacao para acessar os dados secundarios.

Resposta:O acesso aos dados dos pacientes será realizado pelo laboratório colaborador LACEN/PE e não pelos pesquisadores do projeto.

10. Ajustar o Cronograma de pesquisa, de maneira que a coleta de dados apenas seja realizada apos a aprovacao deste CEP, apos a correcao destas pendencias.
Resposta: O cronograma foi ajustado com início das atividades da pesquisa para o mês de maio de 2021, desde que seja obtido parecer favorável da comissão.
11.Corrigir no item "Riscos" que o uso dos dados do participante pode trazer riscos se houver quebra de sigilo. Assim esse risco sera minimizado a partir dos cuidados necessarios que serao tomados para evitar essa possibilidade.
Resposta:A possibilidade de risco de quebra de sigilo foi adicionada ao campo de “Riscos” no item 6, assim como as medidas adotadas para sua redução máxima.
12. Definir e explicar se o material utilizado sera de um biobanco e observar as resolucoes 196/96 e 441/96. OBS: De acordo com a resolucao 441/2011, no art. 1° Biobanco e uma colecao organizada de material biologico humano e informacoes associadas, coletado e armazenado para fins de pesquisa, conforme regulamento ou normas tecnicas, eticas e operacionais pre-definidas, sob responsabilidade e gerenciamento institucional. Ainda no artigo 1° (2.II) sera necessario o consentimento do sujeito da pesquisa, autorizando a coleta, o deposito, o armazenamento e a utilizacao do material biologico humano. Portanto, o pesquisador precisa esclarecer detalhadamente todas essas duvidas quanto ao material que sera utilizado.
Resposta:Foi adicionado um maior detalhamento da natureza do espécime clínico utilizado no trabalho tanto no item 3.1 quanto no item 3.3. De acordo com as definições na resolução N°441 de 12 de maio de 2011, a coleção de material biológico mantida apenasdurante a realização deste projeto se adequa a definição de biorrepositório. A informação foi corrigida na plataforma brasil.

Considerações sobre os Termos de apresentação obrigatória:

Os termos foram apresentados de acordo com a Resolução 466/12.

1. Folha de rosto – foi assinada pela Diretora do CB- UFPE.
2. Termo de Confidencialidade – foi apresentado.
3. Orçamento – o projeto demanda custos que estão discriminados no valor total de R$ 48.800,00 reais.
4. TCLE – Foi solicitado ao Comitê de Ética, através de documento, o pedido de dispensa do TCLE,conforme pendência da relatoria.
5. Currículos – apresentados.
6. Carta de Anuência – Foi a presentada corretamente conforme as pendência da relatoria.

Recomendações:

Nenhuma

Conclusões ou Pendências e Lista de Inadequações:

As pendências foram atendidas e o projeto encontra-se dentro das normas Resolução 466/12

Considerações Finais a critério do CEP:

As exigências foram atendidas e o protocolo está APROVADO, sendo liberado para o início da coleta de dados. Informamos que a APROVAÇÃO DEFINITIVA do projeto só será dada após o envio do Relatório Final da pesquisa. O pesquisador deverá fazer o download do modelo de Relatório Final para enviá-lo via "Notificação", pela Plataforma Brasil. Siga as instruções do link "Para enviar Relatório Final", disponível no site do CEP/CCS/UFPE. Após apreciação desse relatório, o CEP emitirá novo Parecer Consubstanciado definitivo pelo sistema Plataforma Brasil.
Informamos, ainda, que o (a) pesquisador (a) deve desenvolver a pesquisa conforme delineada neste protocolo aprovado, exceto quando perceber risco ou dano não previsto ao voluntário participante (item V.3., da Resolução CNS/MS Nº 466/12).
Eventuais modificações nesta pesquisa devem ser solicitadas através de EMENDA ao projeto, identificando a parte do protocolo a ser modificada e suas justificativas.
Para projetos com mais de um ano de execução, é obrigatório que o pesquisador responsável pelo Protocolo de Pesquisa apresente a este Comitê de Ética relatórios parciais das atividades desenvolvidas no período de 12 meses a contar da data de sua aprovação (item X.1.3.b., da Resolução CNS/MS Nº 466/12). O CEP/CCS/UFPE deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (item V.5., da Resolução CNS/MS Nº 466/12). É papel do/a pesquisador/a assegurar todas as medidas imediatas e adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e ainda, enviar notificação à ANVISA – Agência Nacional de Vigilância Sanitária, junto com seu posicionamento.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1713615.pdf	31/03/2021 11:00:03		Aceito
Outros	CartaRespostaPendencias.docx	31/03/2021	Marcos André	Aceito

Outros	CartaRespostaPendencias.docx	10:46:40	Cavalcanti Bezerra	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	DispensaTCLE.pdf	31/03/2021 10:46:13	Marcos André Cavalcanti Bezerra	Aceito

Projeto Detalhado / Brochura Investigador	ProjetoCovidDetalhado2.doc	31/03/2021 10:45:43	Marcos André Cavalcanti Bezerra	Aceito
Folha de Rosto	folhaDeRostoCovidassinada.pdf	10/03/2021 14:50:40	Marcos André Cavalcanti Bezerra	Aceito
Outros	CurriculoLattesAllysonAndradeMendonca.pdf	06/03/2021 12:50:43	Marcos André Cavalcanti Bezerra	Aceito
Outros	CurriculoLattesLindomarJosePena.pdf	06/03/2021 12:50:25	Marcos André Cavalcanti Bezerra	Aceito
Outros	CurriculoLattesMarcosAndreCavalcantiBezerra.pdf	06/03/2021 12:49:44	Marcos André Cavalcanti Bezerra	Aceito
Outros	CartaAnuenciaLacen.pdf	06/03/2021 12:48:36	Marcos André Cavalcanti Bezerra	Aceito
Outros	CartaAnuenciaCB.pdf	06/03/2021 12:48:17	Marcos André Cavalcanti Bezerra	Aceito
Outros	TermoConfidencialidade.pdf	06/03/2021 12:48:04	Marcos André Cavalcanti Bezerra	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

RECIFE, 08 de Abril de 2021

Assinado por:
LUCIANO TAVARES MONTENEGRO
(Coordenador(a))

APÊNDICE A – WIDESPREAD CONTAMINATION OF SARS-COV-2 ON HIGHLY TOUCHED SURFACES IN BRAZIL DURING THE SECOND WAVE OF THE COVID-19 PANDEMIC

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Summary

Although SARS-CoV-2 surface contamination has been investigated in health care settings, little is known about the SARS-CoV-2 surface contamination in public urban areas, particularly in tropical countries. Here, we investigated the presence of SARSCoV-2 on high-touch surfaces in a large city in Brazil, one of the most affected countries by the COVID-19 pandemic in the world. A total of 400 surface samples were collected in February 2021 in the City of Recife, Northeastern Brazil. A total of 97 samples (24.2%) tested positive for SARS-CoV-2 by RT-qPCR using the CDC-USA protocol. All the collection sites, except one (18/19, 94.7%) had at least one environmental surface sample contaminated. SARS-CoV-2 positivity was higher in public transport terminals (47/84, 55.9%), followed by health care units (26/84, 30.9%), beach areas (4/21, 19.0%), public parks (14/105, 13.3%), supply centre (2/21, 9.5%), and public markets (4/85, 4.7%). Toilets, ATMs, handrails, playgrounds and outdoor gyms were identified as fomites with the highest rates of SARS-CoV-2 detection. Taken together, our data provide a real-world picture of SARS-CoV-2 dispersion in highly populated tropical areas and identify critical control points that need to be targeted to break SARS-CoV-2 transmission chains.

Received 4 August, 2021; accepted 18 November, 2021.

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Introduction

Coronaviruses (CoVs) are members of the Coronaviridae family and represent a diverse group of viruses that cause respiratory and intestinal infections in animals and humans (Fehr and Perlman, 2015). The Orthocoronavirinae subfamily is divided into four genera – Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus. Alphacoronaviruses (HCoV-229E and HCoV-NL63) and betacoronaviruses (HCoV-OC43 and HCoV-HKU1) are commonly associated with mild respiratory disease in humans (Cui et al., 2019). However, in the last two decades, three highly pathogenic betacoronaviruses have emerged from animal sources to cause severe respiratory disease in humans: severe acute respiratory syndrome

coronavirus (SARS-CoV) (Zhong et al., 2003), Middle East respiratory syndrome coronavirus (Zaki et al., 2012), and more recently, the severe acute respiratory syndrome coronavirus 2 (SARSCoV-2) (Lu et al., 2020; Zhu et al., 2020; Zhou et al., 2020b).

SARS-CoV-2 first emerged in the city of Wuhan, Hubei province, China, in December 2019 causing an outbreak of a yet unknown acute pneumonia (Huang et al., 2020). The new coronavirus was found to be highly transmissible among humans and has spread rapidly around the globe prompting the World Health Organization (WHO) to declare a pandemic on March 11, 2020 (Petersen et al., 2020). As of September 30, 2021, there have been approximately 233.6 million confirmed cases of COVID19 across the world, with over 4.7 million deaths (Dong et al., 2020). Difficult to control viral transmission allied with the slow progress in the rollout of COVID-19 vaccines in most countries have contributed to the emergence of new variants of concern of SARS-CoV-2, which are more transmissible and can escape from natural and vaccine-acquired immunity (Abdool Karim and De Oliveira, 2021; Faria et al., 2021; Naveca et al., 2021; Peacock et al., 2021; Silva and Pena, 2021).

SARS-CoV-2 is spread from person to person mainly through exposure to respiratory fluids containing infectious virus. Virus exposure can occur in three main ways, which are not mutually exclusive: (i) inhalation of infectious virus present in very small fine droplets and aerosol particles; (ii) deposition of virus on exposed mucous membranes in the mouth, nose or eye by direct splashes and sprays, and (iii) touching mucous membranes with hands contaminated by exhaled respiratory fluids containing virus or from touching fomites containing the virus (Marquès and Domingo, 2021; CDC, 2021b). Notably, SARS-CoV-2 has been found to have high person-toperson transmission through direct contact with infected individuals (Hu et al., 2021), especially by coughing, sneezing and even breathing/talking by an infected person (Tang et al., 2013; Kutter et al., 2018; Leung et al., 2020; Stadnytskyi et al., 2020). SARS-CoV-2 enters the body through the mucous membranes of the eyes, mouth or nose and spreads to the nose line, sinus cavity and throat until deposition into the human respiratory tract (Kaur et al., 2020). Although transmission through direct contact, or airborne (respiratory droplets and/or aerosols) are considered the dominant routes for the spread of SARS-CoV-2 (Falahi and Kenarkoohi, 2020; Zhang et al., 2020), some epidemiological studies have found fomites as a possible source of infection (Brlek et al., 2020; Luo et al., 2020; Xie et al., 2020). The risk of infection by environmental surfaces is influenced by the distance from the source, the amount of virus to which a person is exposed and the length of time since the virus has been deposited on the surface, since SARS-CoV-2 viability over time is influenced by environmental factors such as type of surfaces, temperature, humidity and ultraviolet radiation (e.g. sunlight) (Chin et al., 2020; Zhang et al., 2020; Van Doremalen et al., 2020). Thus, understanding distribution and patterns of environmental contamination by SARSCoV-2 are relevant information for public health authorities. This knowledge allows the identification of critical points to establish effective control measures and may provide useful data to estimate silent circulation of SARS-CoV-2.

Several recent studies have investigated the presence of SARS-CoV-2 RNA in air and environmental surfaces, especially in health care settings (Chia et al., 2020; Colaneri et al., 2020; Liu et al., 2020; Mouchtouri et al., 2020; Santarpia et al., 2020; Tan et al., 2020; Ye et al., 2020; Zhou et al., 2020a; Dargahi et al., 2021). Previous studies under controlled laboratory conditions have

demonstrated the ability of SARS-CoV-2 to remain infectious on different types of common surfaces, such as stainless steel, glass and paper, for up to 28 days at 20C (Riddell et al., 2020), and it can also remain infectious in aerosols for up to 3 h (Van Doremalen et al., 2020). However, little is known about SARS-CoV-2 contamination of environmental surfaces in tropical public areas with a large flow and concentration of people. Therefore, studies investigating the presence of SARSCoV-2 RNA on surfaces, and the infectious potential of these particles are of paramount importance.

To address this gap of knowledge, we investigated the presence of SARS-CoV-2 RNA on highly touched surfaces in Recife, a large city in Pernambuco state with a tropical monsoon climate. Samples were collected during the second wave of the COVID-19 in Brazil, one of the most severely affected countries by the pandemic (Silva and Pena, 2021). Our findings showed widespread viral contamination across many urban public settings and poor adherence to COVID-19 mitigation measures. Taken together, our data provide a real-world picture of SARS-CoV-2 dispersion in public areas and identify critical control points that need to be targeted to halt SARSCoV-2 transmission.

Materials and methods

Study design and setting

This cross-sectional study was conducted in Recife, the capital of Pernambuco state, which is one of the most densely populated metropolitan regions in Brazil with 1 537 704 million people (https://cidades.ibge.gov.br/ brasil/pe/recife). The city is located on the coast of Northeast coast of Brazil and has a tropical monsoon climate under the Köppen climate classification, with warm to hot temperatures and high relative humidity throughout the year. To design the study, we selected the main public places of the city that have intense circulation and concentration of people.

Initially, we subdivided Recife's highly frequented areas into six categories, including: (i) transport terminals; (ii) health care units; (iii) beach areas; (iv) public parks; (v) supply centre; and (vi) public markets. A total of 400 environmental surface specimens were collected between February 2 and February 25, 2021 (Fig. 1). Samples were collected between 9:00 a.m. and 1:00 p.

m. During sample collection, the temperature was between 26C and 32C (average temperature 29C) and the average humidity was 72%. Environment data were obtained from the Time and Date AS website (http://

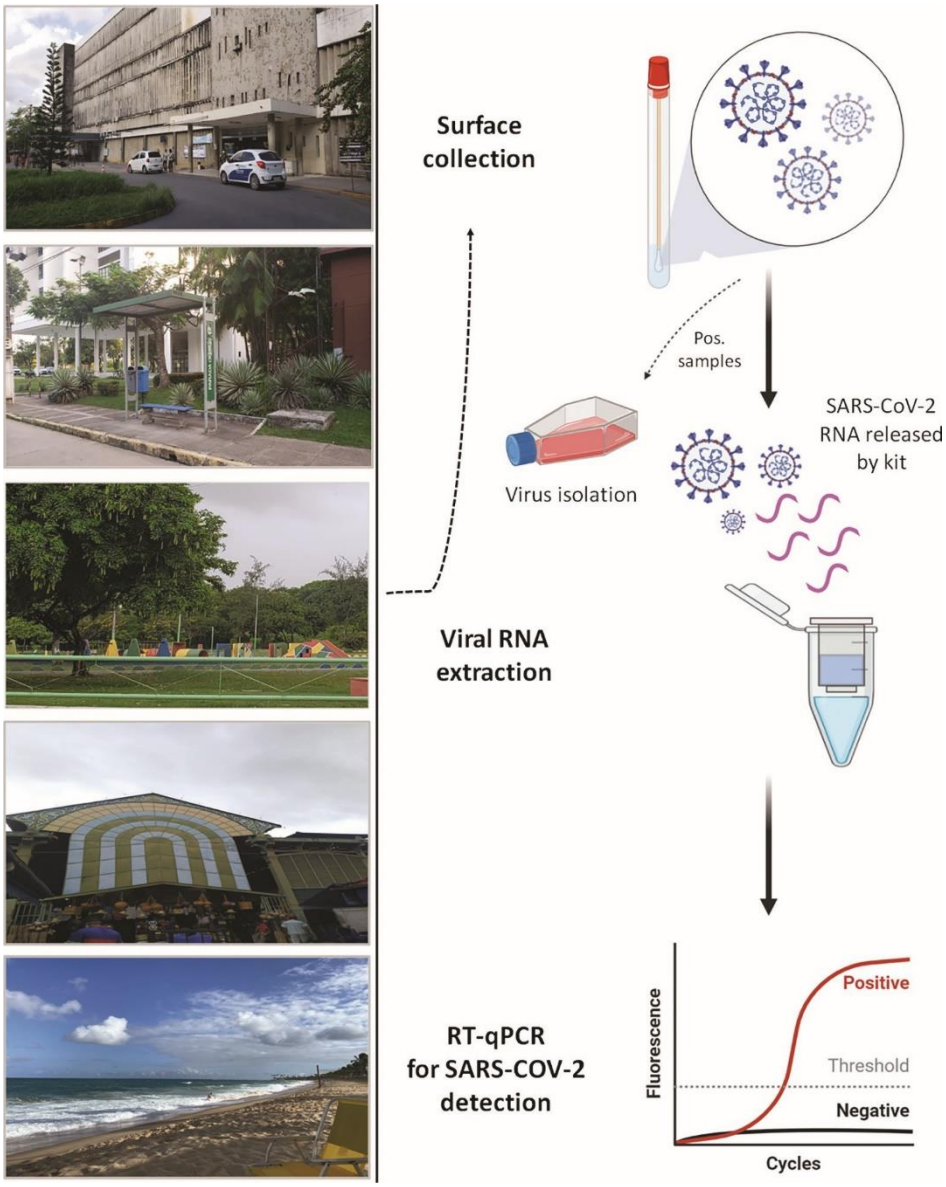

Fig. 1. Study design showing the collection points of surface samples and the graphical workflow used to test the swabs.

www.timeanddate.com/weather/brazil/recife/climate). This Sampling areas coincided with a period of progressive increase in the

Transport terminals. A total of 84 surface samples were

number of COVID-19 cases in Pernambuco state and collected from four public transport terminals with a large

Brazil, representing the ascendant phase of the second daily passenger flow and concentration. We strategically

wave during the COVID-19 pandemic course in this part selected transport terminals that connect several cities in

of the world and also the beginning of COVID-19 vaccithe metropolitan region of Recife. Twenty-one swabs

nation efforts in this state (Fig. 2). The ongoing pandemic were collected for each transport terminal. The collection

of COVID-19 in the Pernambuco state has resulted in points included the external area of the transport terminal

620 723 laboratory-confirmed cases and 19 740 deaths and neighbouring areas: (i) bus terminal entrance; (ii) bus

as of 30 September 2021. It is important to highlight that terminal exit; (iii) bus terminal access; (iv) subway station

Recife has a high concentration of specialized hospitals access; (v) ATM; (vi) toilet; (vii) handrail; (viii) bench;

and is considered a reference health centre for the North-

(ix) bus stop; (x) counter; (xi) faucet; (xii) ticket machine.

east region of Brazil.

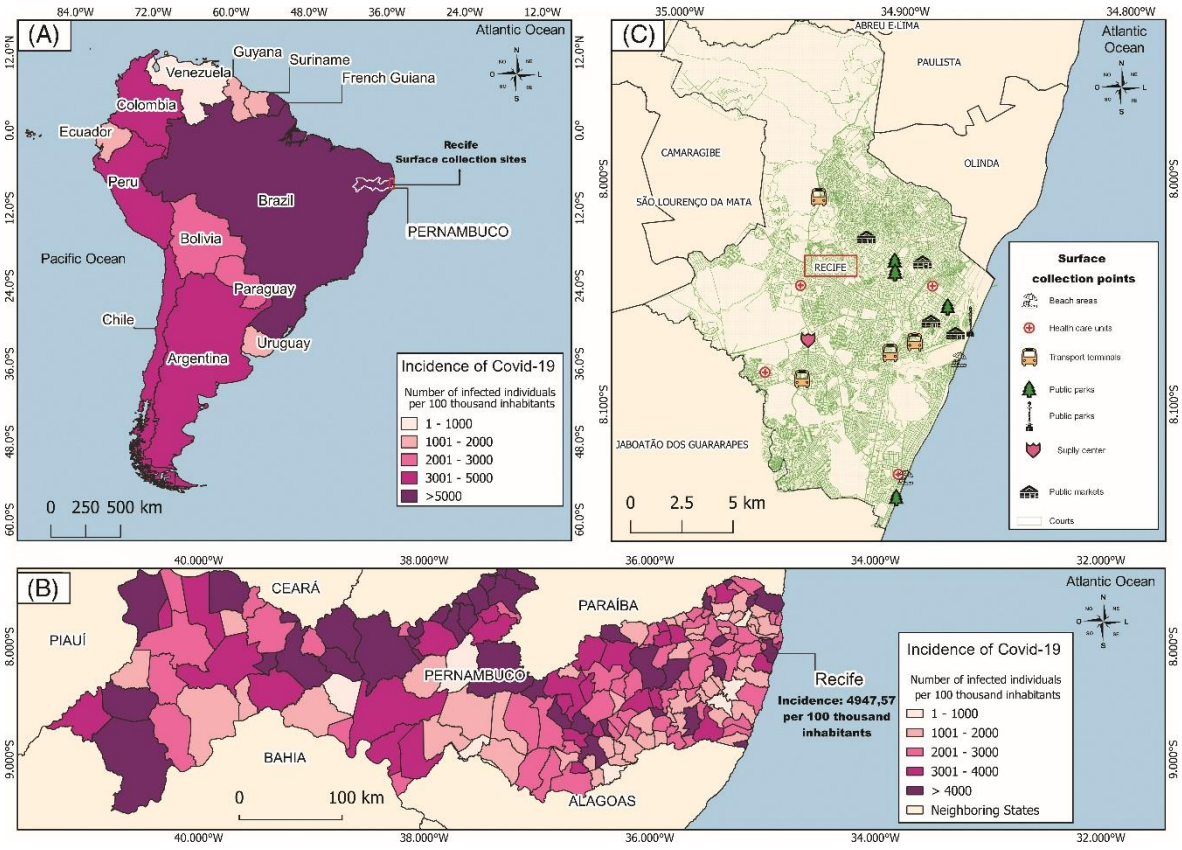

Fig. 2. Spatial distribution of surface collection points and incidence of COVID-19 in Latin America and Pernambuco state, Brazil. A. The incidence of COVID-19 per 100 000 inhabitants in Latin America.

B. The incidence of COVID-19 per 100 000 inhabitants in all cities in the state of Pernambuco, Northeast Brazil.

C. The spatial distribution of surface collection points (transport terminals, health care units, beach areas, public parks, supply centre and public markets) across Recife, Pernambuco state, Brazil.

Health care units. A total of 84 surface samples were collected from four reference hospitals for the treatment of COVID-19 patients in Recife, Brazil. Twenty-one swabs were collected for each hospital. The collection points included the external area of the hospital and neighbouring areas: (i) principal entrance; (ii) hospital access; (iii) ambulatory entrance; (iv) patient sample collection area; (v) toilet; (vi) traffic light button; (vii) coffee shop; (viii) public phone; (ix) bus stop; (x) resting area.

Beach areas. A total of 21 surface samples were collected from two beaches located in the coastal area of Recife, Brazil. Interestingly, the visited beaches had a high concentration of people during the time of surface collection and during all times of restrictive relaxation measures established by the state government during the COVID-19 pandemic. The collection points included: (i) toilets; (ii) benches; (iii) public bike station; (iv) outdoor gym; (v) fresh green coconut; (vi) handrails; (vii) faucet; (viii) traffic light button; (ix) bus stop; (x) resting area.

Public parks. A total of 105 surface samples were collected from five public parks. We strategically selected parks with high visitor flow, including children who access the playground. Twenty-one swabs were collected for each public park. The collection points included: (i) playground; (ii) recreation area; (iii) outdoor gym; (iv) toilet; (v) handrail; (vi) bus stop; (vii) public bike station; (viii) traffic light button; (ix) coffee shop; (x) faucet.

Supply centre. A total of 21 surface samples were collected from one food distribution centre located in Recife, Brazil. We selected this place as it is a place which serves as a gateway for people from all over the Brazilian territory, and acts as a source of food supply for the Northeast of Brazil. The collection points included: (i) toilet; (ii) restaurant; (iii) handrail; (iv) resting area.

Public markets. A total of 85 surface samples were collected from four public markets. Twenty-one swabs were collected for each public market with exception of one, where we collected 22 swabs. The collection points included: (i) principal entrance; (ii) side entrance; (iii) public market access; (iv) toilet; (v) kiosk; (vi) store; (vii) food hall; (viii) traffic light button; (ix) faucet; (x) resting area; (xi) outside area.

Surface sampling

Environmental samples were collected by qualified technicians who had received biosafety training and were equipped with personal protective equipment. For sample collection, sterile swabs (bioBoa Vista, Brazil) were used, which were put into a conical tube (15 ml) containing 2 ml of virus preservation solution (sterile phosphatebuffered saline, pH 7.2). Each swab was vigorously rubbed on the surface with a collection area of 25 cm^2. Samples were collected from distinct types of materials, including metal, plastic, wood, rock, concrete, and glass. The time of collection and climate conditions of the day were recorded during sampling. In addition, an environmental site assessment questionnaire was filled by technicians at each sample collection point with the aim to identify whether the collection environment and the population were following public health measures for preventing the rapid spread of SARS-CoV-2 and, subsequently, the COVID-19 transmission.

Sample transfer and processing

Surface samples were collected and immediately stored at 4C prior to transfer to the Biosafety Level 3 Laboratory (BSL-3) of Fiocruz Pernambuco, Brazil,

where all samples were processed until 72 h after collection with aim to preserve the infectivity or genome integrity of SARSCoV-2. After processing, each sample was taken directly tested according to the instructions described below.

Viral RNA extraction and RT-qPCR for SARS-CoV-2 detection

Viral RNA was extracted from surface samples (140 µl of transport solution) using the QIAamp Viral RNA Mini Kit (QIAGEN, Germany) following the manufacturer's protocol. RT-qPCR assay targeting the N protein according to protocols recommended by the Centres for Disease Control and Prevention – CDC USA was used to detect SARS-CoV-2 (Supplementary Table 1) (CDC, 2020). Samples were considered positive when they presented amplification for N1 target, considering the threshold for cycle quantification (Cq) value of 40 (CDC, 2020). Samples with Cq ≥40 were considered negative. Briefly, each reaction was prepared using the QuantiNova Probe RTPCR Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's protocol and the CDC-USA recommendations in a total volume of 10 µl. Negative (extraction control and non-template control) and positive controls (RNA extracted from SARS-CoV-2 cell supernatants) were included during all experiments. Primer and probe sequences were synthetized by IDT (Integrated DNA Technologies, Skokie, IL, USA). Thermal cycling was performed at 45C for 15 min for reverse transcription, followed by 95C for 5 min and then 45 cycles of 95C for 03 s and 55C for 30 s. All experiments were conducted using the Applied Biosystems QuantStudio 5 Real-Time PCR Systems (Applied Biosystems, USA). For data analysis, the QuantStudio software v1.5 was used with baseline and threshold automatic.

Cells

African monkey green kidney-derived cell line Vero CCL81 was used for virus isolation from positive environmental samples. Cells were cultured in Dulbecco's modified Eagle's medium, high glucose (Gibco, USA) supplemented with 10% heat-inactivated foetal bovine serum, 100 U ml^{1} penicillin and 100 µg ml^{1} streptomycin (Gibco); and maintained in a humidified atmosphere, at 37C and 5% CO_2.

SARS-CoV-2 isolation

Vero CCL-81 cells were cultured in 12-well plates at a density of 2 10^5 cells/well. After 24 h, the culture media was removed and cells were incubated with 300 µl of undiluted and filtered surface samples at 37C, 5% CO_2, for 1 h. Fresh media supplemented with 2% FBS (700 µl) was added to the cells and they were maintained at 37C, 5% CO^2. Cells were monitored daily for the visualization of virus-induced cytopathic effect (CPE). CPE images were acquired in Carl Zeiss Axio Observer 5 microscope coupled to a photographic camera. After 3 days post-infection supernatants were collected and 300 µl were transferred to a new 12-well plate. This procedure was repeated until completing three passages (P1, P2 and P3). Following this, cell culture supernatants were collected on t = 0 h and t = 72 h in each passage for viral RNA extraction and possible SARS-CoV-2 detection by RT-qPCR. All experiments were performed in a

BSL-3 facility.

Environmental site assessment questionnaire

Data regarding the social distancing, mask wearing, availability of hand sanitizers and COVID-19 control measures during sample collection in all locations were obtained using a structured questionnaire following the recommendations and guidelines established by WHO and CDC (CDC, 2021a). The questions aimed to identify the implementation and compliance with COVID-19

prevention measures, including social distancing, mask wearing, the availability of hand sanitizers, body temperature measurements for screening and the presence of informative charts for people education. The questionnaires were made with qualitative, with 'yes' or 'no' input, or quantitative inquiries.

Spatial location of collection surfaces

To georeference the locations where surface samples were obtained, we used the QGIS software (https://qgis. org/en/site/) to generate a map using the geographic coordinates of each publicly available location at https:// www.google.com.br/maps. First, we created a graduate map with information about the incidence of COVID-19 in the countries of Latin America (Fig. 2A) and all cities located in the State of Pernambuco, Brazil (Fig. 2B). The incidence per 100 thousand inhabitants was calculated using the database of the last Brazilian census available at http://censo2010.ibge.gov.br and epidemiological reports of COVID-19 cases from the Pernambuco State Health Department (SAÚDE, 2021) and the World Organization Health (WHO) (ORGANIZATION, 2021). Furthermore, we showed the spatial distribution of urban public places where the samples were collected including transport terminals, health care units, public parks, public markets, beach areas and food supply centre. We acquired the cartographic base in shapefile format through the Brazilian Institute of Geography and Statistics (IBGE) in the Geocentric Reference System for the Americas (SIRGAS) 2000 (Fig. 2C).

Data analysis

GraphPad Prism software version 5.01 for Windows (GraphPad Software, La Jolla, CA, USA) was used to plot most graphics. The association analysis between collection locations and type of materials was demonstrated based on the results from 97 positive surfaces collected in this study using the web-based Circos table viewer, version 0.63-9 (https:www.mkweb.bcgsc.ca/ tableviewer) (Krzywinski et al., 2009).

Ethics approval

This study was reviewed and approved under protocol number 03/2021 by the Fiocruz Pernambuco Internal Biosafety Commission, as part of quality assurance for working with highly pathogenic virus.

Results

Distribution of surface samples according to collection area and type of material

A total of 400 surface samples were collected in Recife, Pernambuco state in 19 sites divided into six subgroups (health care units, transport terminals, public parks, public markets, beach areas and a food supply centre). A total of 97 surface samples (24.2%) tested positive for SARS-CoV-2 RNA using the CDC-USA protocol by RTqPCR (Fig. 3A, Supplementary Fig. 1) in 18 out (94.7%) of 19 sites sampled (Table 1). The only site that tested negative was a public market. SARS-CoV-2 RNA was detected mainly around transport terminals (47/84, 55.9%), followed by health care units (26/84, 30.9%), beach areas (4/21, 19.0%), public parks (14/105, 13.3%), food supply centre (2/21, 9.5%) and public markets (4/85, 4.7%). (Fig. 3B, Supplementary Table 2). Regarding the type of material where environmental samples were collected, SARS-CoV-2 RNA was found most frequently on rock (10/22, 45.4%), followed by plastic (18/50, 36.0%), wood (12/47, 25.5%), metal (45/179, 25.1%), glass (2/10, 20.0%), concrete (8/55, 14.5%) and other (ceramic and rubber) (2/37, 5.4%) (Fig. 3C, Supplementary Table 2). Positive samples were predominantly found in toilets, ATMs, handrails, playground and outdoor gym; highlighting the importance of these fomites in SARS-CoV-2 surface contamination.

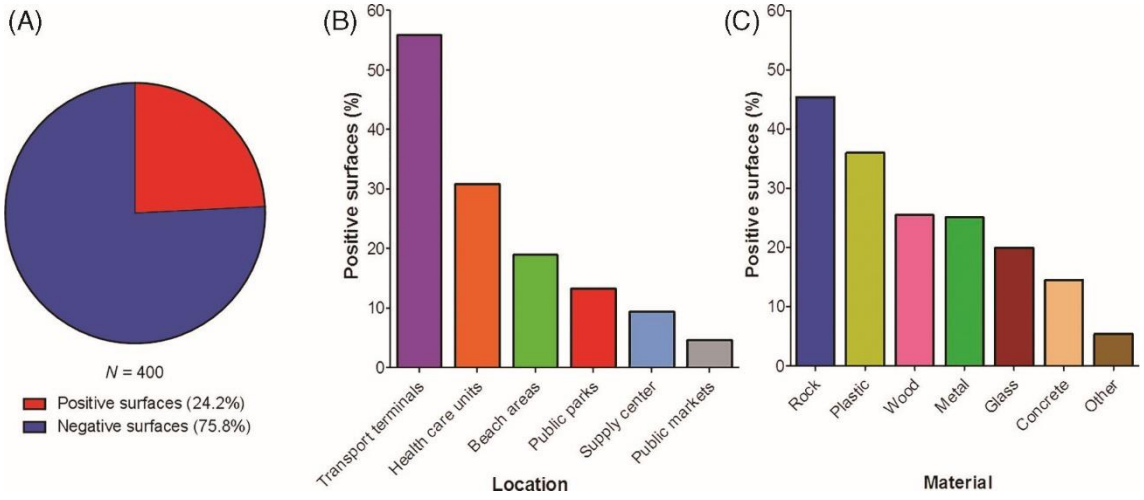

Fig. 3. Overall results for SARS-CoV-2 detection in surface samples.

The distribution of positive and negative samples using a total of 400 environmental samples.
The distribution of positive samples according to the collection areas, including transport terminals, health care units, beach areas, publicparks, supply centre and public markets.
The distribution of positive samples according to the type of material including metal, plastic, wood, rock, concrete, glass and others.

Distribution of positive surface samples according to point of collection

Transport terminals. Forty-seven (48.4%) surface samples were positive for SARS-CoV-2 RNA around public transport terminals with Cq values ranging from 31.1 to 38.7 by RT-qPCR (Supplementary Table 2). Positive samples were distributed particularly in 11 different locations, including ATM (9/47, 19.1%), handrails (9/47,

19.1%), bus terminal access (7/47, 14.8%), bench (6/47, 12.7%), toilet (5/47, 10.6%), ticket machine (3/47, 6.3%), bus stop (2/47, 4.2%), subway station access (2/47, 4.2%), faucet (2/47, 4.2%), bus terminal exit (1/47, 2.1%) and ticket counter (1/47, 2.1%) (Fig. 4A, Supplementary Table 2).

Health care units. Twenty-six (26.8%) surface samples were positive for SARS-CoV-2 RNA in the surroundings of health care units with Cq values ranging from 31.1 to 38.7 by RT-qPCR (Supplementary Table 2). Positive samples were found in nine different locations from four reference hospitals for COVID-19 treatment. The areas with highest number of positive samples were hospital access (10/26, 38.4%), bus stop (4/26, 15.3%), traffic light button (4/26, 15.3%), principal entrance (2/26,

7.6%), resting area (2/26, 7.6%), toilet (1/26, 3.8%), ambulatory entrance (1/26, 3.8%), coffee shop (1/26, 3.8%) and public phone (1/26, 3.8%) (Fig. 4B, Supplementary Table 2).

Beach areas. Four (4.1%) surface samples were positive for SARS-CoV-2 RNA in beach areas with Cq values ranging from 36.1 to 37.9 by RT-qPCR (Supplementary Table 2). Positive samples were collected from three different locations, including toilets (2/4, 50.0%), bench (1/4, 25.0%) and resting area (1/4, 25.0%) (Fig. 4C, Supplementary Table 2). No positive samples were detected from the outdoor gym, public bike station, bus stop, fresh coconut, handrail, faucet, or traffic light button.

Public parks. Fourteen (14.4%) surface samples were positive for SARS-CoV-2 RNA around public parks, with Cq values ranging from 36.2 to 39.7 by RT-qPCR (Supplementary Table 2). Positive samples were collected from five different locations, including playground (5/14, 35.7%), recreation area (4/14, 28.5%), outdoor gym (2/14, 14.2%), toilet (2/14, 14.2%) and handrails (1/14,

7.1%) (Fig. 4D, Supplementary Table 2). There were no positive samples from the public bike station, bus stop, coffee shop, traffic light button, or faucet.

Table 1. Positivity of SARS-CoV-2 RNA on public touched surfaces at different locations in Recife, Pernambuco state, Brazil.

Location	Site #	Number of samples collected	Number of positive samples (%)
Transport terminals	01	21	20 (95.2%)
	02	21	16 (76.1%)
	03	21	8 (38.0%)
	04	21	3 (14.2%)
Health care units	05	21	4 (19.0%)
	06	21	3 (14.2%)
	07	21	18 (85.7%)
	08	21	1 (4.76%)
Public parks	09	21	3 (14.2%)
	10	21	4 (19.0%)
	11	21	3 (14.2%)
	12	21	2 (9.52%)
	13	21	2 (9.52%)
Public markets	14	21	2 (9.52%)
	15	21	0 (0%)
	16	21	1 (4.76%)
	17	22	1 (4.54%)
Beach areas	18	21	4 (19.0%)
Supply centre	19	21	2 (9.52%)

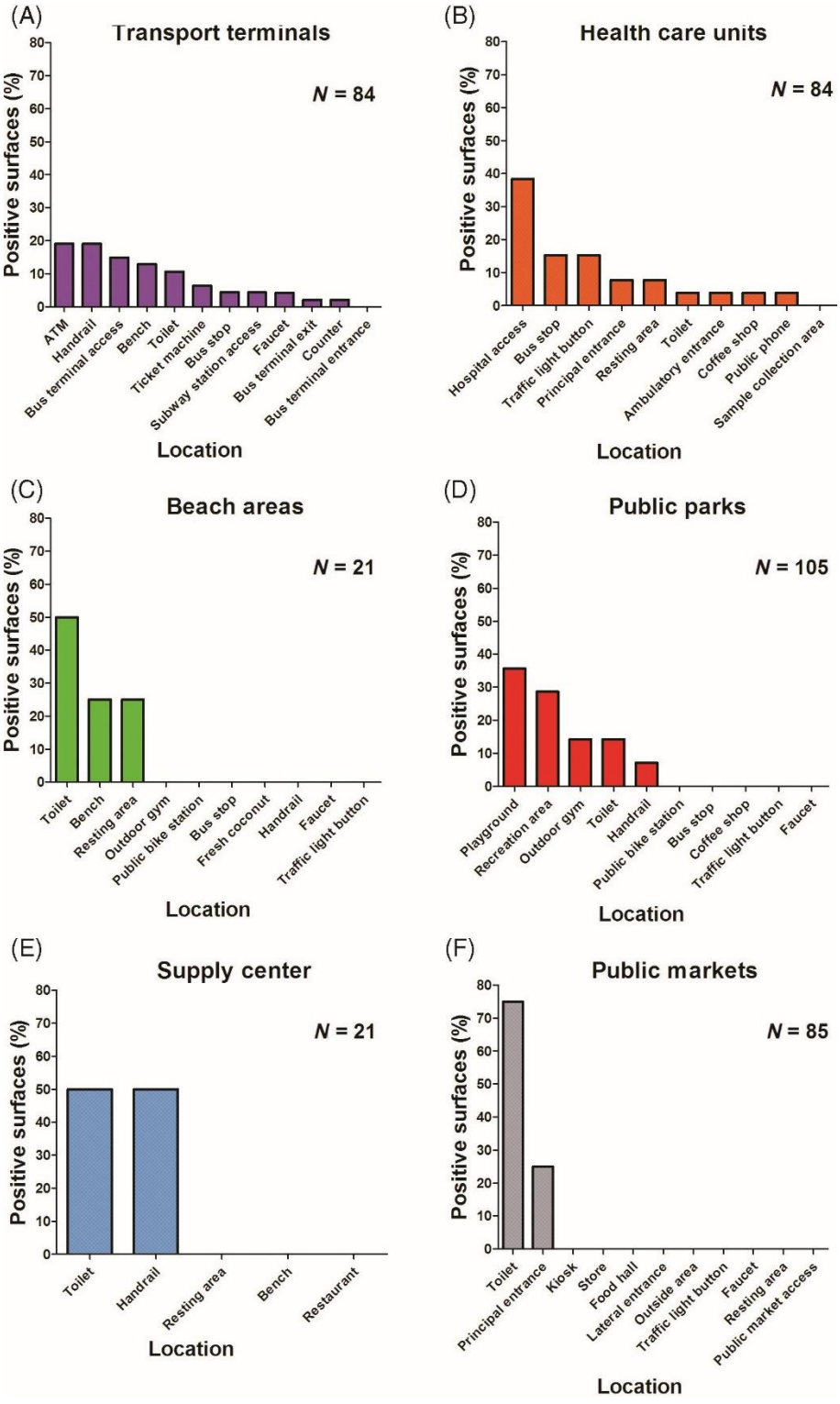

Fig. 4. Distribution of positive surface samples according to collection areas. A. The distribution of positive samples in transport terminals.

B. The distribution of positive samplesaround health care units.
C. The distribution of positive samplesin beach areas.
D. The distribution of positive samplesin public markets.
E. The distribution of positive samplesin a supply centre.
F. The distribution of positive samplesin public markets.

Supply centre. Two (2.0%) surface samples were positive for SARS-CoV-2 RNA in a food distribution centre with Cq values ranging from 38.0 to 38.7 by RT-qPCR (Supplementary Table 2). Positive samples were collected from two different locations, including toilet (1/2, 50.0%) and handrails (1/2, 50.0%) (Fig. 4E, Supplementary Table 2). No positive samples were found in restaurants or resting benches.

Public markets. Three out of four public markets sampled returned at least one positive sample. Four (4.1%) surface samples were positive for SARS-CoV-2 RNA in public markets with Cq values ranging from 36.9 to 38.1 by RT-qPCR (Supplementary Table 2). Positive samples were collected from two different locations, including toilets (3/4, 75.0%) and principal entrance (1/4, 25.0%) (Fig. 4F, Supplementary Table 2). No positive samples were found at the kiosk, store, lateral entrance, outside area, food hall, public market access, traffic light button, faucet, or resting area.

Types of surface materials positive for SARSCoV-2 RNA

From the 47 positive samples in transport terminals, 21 (44.6%) samples were identified mainly on metal surfaces, especially from handrails at bus terminals, ATM buttons, protection grids, and faucets. Nineteen (19.1%) samples were recovered from plastic surfaces, especially around biometrics sensors in ATMs and faucets in the toilet. Five (10.6%) samples were found in concrete surfaces, most being found in pillars near the bus stop and one sampled from a bench. Four (8.5%) samples were collected on rock surfaces, with virus being detected on walls in the toilet and bus terminal, and one sample was collected at the terminal service desk. Four (8.1%) samples were identified on wood surfaces, all being from benches near the bus stop of transport terminals. Two (4.2%) samples were detected on glass surfaces, mainly on the ticket machine screens. In addition, one (2.1%) sample was collected on a toilet seat (porcelain) and one (2.1%) was detected on the ticket machine (rubber) (Fig. 5, Supplementary Table 2).

From the 26 positive samples found in health care units and neighbouring areas, 12 (46.1%) samples were recovered from metal surfaces mostly, located at the entrance to hospitals and near bus stops. Seven (26.9%) samples were identified in plastic surfaces, especially from traffic light buttons, near bus stops and in the toilets. Four (15.3%) samples were detected in rock surfaces found at the entrance to hospitals. Two (7.6%) samples were identified in wood surfaces at the entrance to hospitals. One (3.8%) sample was detected on the concrete surface from a nearby bus stop (Fig. 5, Supplementary Table 2).

From the 14 positive samples found in public parks, seven (50.0%) samples were identified on the metal surfaces of handrails in the playground and outdoor gym. Four (28.5%) samples were recovered from wood surfaces in the playground, and one tourist attraction point. Two (14.2%) samples were detected in concrete surfaces of the playground. One (7.1%) sample was identified in the plastic surface from a faucet in the toilet (Fig. 5, Supplementary Table 2).

From the four positive samples in beach areas, two (50.0%) were detected in rock surfaces, one from toilet wall and one from a bench. One (25.0%) sample was identified in a metal surface from a faucet in the toilet, and a further one (25.0%) was detected in a wood surface on a handrail that gives access to the beach (Fig. 5, Supplementary Table 2).

From the four positive samples in public markets, three (75.0%) samples were detected on metal surfaces at the entrance to public markets, and from a toilet faucet. One (25.0%) sample was identified in wood surfaces from a door in the toilet (Fig. 5, Supplementary Table 2).

Lastly, from the two positive samples from one food distribution centre, one (50.0%) sample was detected on a plastic surface from a faucet in the toilet and one (50.0%) was identified on a metal handrail surface at the entrance of a bank (Fig. 5, Supplementary Table 2).

Viability of SARS-CoV-2 from positive surfaces samples

To assess infectivity of samples that tested positive by RT-qPCR, nine samples with Cq value <34 (Cq ranging from 31.0 to 33.7) were inoculated into 12-well plates seeded with Vero CCL-81 cells. Samples were considered negative after three blind passages of the supernatant. Under these conditions it was not possible to isolate the virus, as determined by the absence of CPE and negative RT-qPCR results from the third passage supernatant (Supplementary Fig. 2, Supplementary Table 3). The risk of infection from these contaminated surfaces is therefore not clear.

Poor adherence of COVID-19 mitigation measures by society

Data regarding the adoption of public health measures and community perception of COVID-19 disease were collected during surface collection in all locations by using a structured environmental site assessment questionnaire. In the 19 collection points, 70% alcohol-based hand sanitizer was available at the entrance in 26.3% (5/19) of the locations, whereas 42.1% (8/19) had a sink with soap and water for hand hygiene. Temperature measurements at the entrance were carried out in 15.8% (3/19) of the sites, and information material on preventive measures to prevent SARS-CoV-2 transmission was found in 42.1% (8/19) of the sites. High mask wear adherence was seen [94.7% (18/19)], although only 57.3% of people (average calculated for every 10 people per collection point) were wearing masks in a proper way. Regarding social distancing, only 26.3% (5/19) of the people present at collection points were maintaining

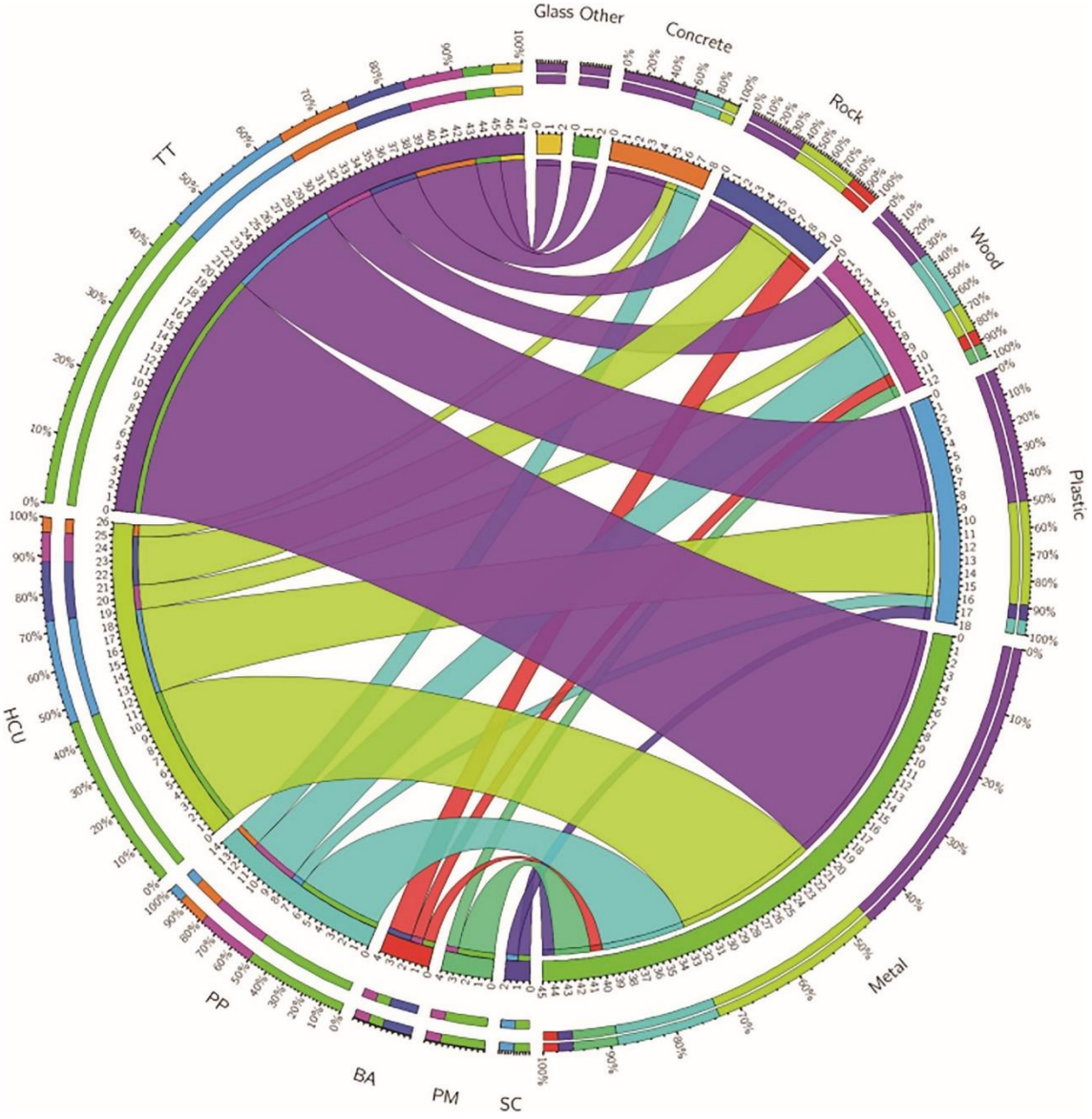

Fig. 5. Association between the surface collection areas and type of material where SARS-CoV-2 RNA was detected. TT: transport terminals; HCU: health care units; PP: public parks; BA: beach areas; PM: public markets; SC: supply centre.

the recommended social distance of 2 m. Furthermore, only 5.3% (1/19) of collection sites were limiting the number of people who accessed the location point

(Supplementary Table S4). We found no positive correlation between adherence of COVID-19 mitigation measures and SARS-CoV-2 positivity (data not shown). Overall, our findings indicated poor adherence of COVID19 mitigation measures in our study areas.

Discussion

Since the emergence of SARS-CoV-2, first identified in China, the highly pathogenic coronavirus has spread rapidly around the world causing an unprecedented health security crisis and drastically affecting the global economic stability. Thus, understanding the modes of transmission of SARS-CoV-2 among humans is a critical step to establish effective prevention policies and prioritize resources to break the chain of SARS-CoV-2 transmission. The transmission through direct contact and via airborne (respiratory droplets and/or aerosols) are pointed as the dominant routes for the transmission of SARS-CoV-2 in humans (Falahi and Kenarkoohi, 2020; Zhang et al., 2020; Greenhalgh

et al., 2021) and animal models, like ferrets (Richard et al., 2020), golden hamsters (Sia et al., 2020), and mice (Bao et al., 2020).

However, some epidemiological reports have found fomites as a possible source of infection (Brlek et al., 2020; Luo et al., 2020; Xie et al., 2020). The transmission dynamics of SARS-CoV-2 by environmental surfaces and their role in the transmission chain remain unclear and may be multifactorial, especially in urban areas with a large flow of people with real-life challenges. Here, we investigated the presence of SARS-CoV-2 RNA on public high-touch surfaces in a large metropolitan city during a relevant section of the second wave of the COVID-19 pandemic course in Brazil. Our findings represent specific collection points of the city and its specific conditions at the time of sampling.

A recent study investigated the presence of SARSCoV-2 RNA on public surfaces in Belo Horizonte, a large city with a tropical savanna climate in Southeast Brazil using a total of 933 surface specimens from different locations (health care units, public squares, bus terminals, public markets and other public places) were collected between April and June 2020 (Abrahao~ et al., 2021). The results showed that 49 (5.25%) of surface samples were tested positive for SARS-CoV-2 RNA, although the infectious potential of positive samples was not investigated. Considering the proportion of positivity in the different places, the authors pointed out that bus terminals exhibited the highest positivity rate, followed by public markets, public squares and health care units (Abrahao~ et al., 2021). In our study, we found higher positivity of SARS-CoV-2 RNA (97/400, 24.2%) when compared to the Belo Horizonte survey. Moreover, most of the positive samples in our study were detected in the surroundings of public transport terminals (47/84, 55.9%), followed by health care units (26/84, 30.9%), beach areas (4/21, 19.0%), public parks (14/105, 13.3%), other places (2/21, 9.5%) and public markets (4/85, 4.7%). The difference in the positivity rate of both cities cannot be explained by climate differences as Recife is hotter and more humid than Belo Horizonte, conditions that decrease the stability of SARS-CoV-2 in the environment (Biryukov et al., 2020) and its transmissibility (Qi et al., 2020). A more plausible explanation for this disparity is the number of confirmed COVID-19 cases in these cities by the time of sample collection. Although Belo Horizonte reported 400–5000 (https://ciis.fmrp.usp. br/covid19/bh-mg/) daily cases between April and June 2020, Recife had 60 000–70 000 (https://ciis.fmrp.usp.br/ covid19/recife-pe/) in February 2021.

Regarding the distribution of positive samples according to the type of material, we found the SARSCoV-2 RNA mainly on rock, followed by plastic, wood, metal, glass and concrete. Previous studies performed under controlled laboratory conditions have shown that SARS-CoV-2 remains infectious on different types of surfaces, such as stainless steel, glass and paper, for up to 28 days at 20C (Riddell et al., 2020), depending on the type of environmental surface; and can remain viable in aerosols for up to 3 h (Van Doremalen et al., 2020). Notably, the viral load decreases over time and depends on the length of time since the virus has been deposited on the surface, which may be reflected in the presence of infectious or non-infectious viral particles and, consequently, infection risk in humans in field settings (Chin et al., 2020; Zhang et al., 2020; Van Doremalen et al., 2020). Other studies have suggested that several environmental stressors can compromise and damage the integrity of SARS-CoV-2 viral particles, including temperature and relative humidity (Biryukov et al., 2020; Riddell et al., 2020). Additionally, our data demonstrated that the positive samples for SARS-CoV-2 RNA were mainly collected in toilets. This finding also corroborates with outcomes obtained by other research groups (Chia et al., 2020; Liu et al., 2020; Luo et al., 2020), which pointed toilets as an area of high positivity rate for SARSCoV-2 RNA. Taken together, our findings revealed other specific locations with high rates of positivity: ATMs, handrails, playgrounds and outdoor gyms.

In order to elucidate the transmission dynamics of SARS-CoV-2 by environmental surfaces in real-life conditions, several studies have investigated the presence of SARS-CoV-2 in air and environmental surfaces/areas, including health care settings (Chia et al., 2020; Colaneri et al., 2020; Liu et al., 2020; Mouchtouri et al., 2020; Santarpia et al., 2020; Tan et al., 2020; Zhou et al., 2020a; Dargahi et al., 2021) and urban settings (Cai et al., 2020; Di Carlo et al., 2020; Harvey et al., 2020; Luo et al., 2020; Abrahao~ et al., 2021). In general, these studies have found varying levels of environmental contamination, ranging from extensive (Chia et al., 2020; Zhou et al., 2020a) to low contamination (Colaneri et al., 2020; Abrahao~ et al., 2021), or even no contamination of SARS-CoV-2 RNA. However, many of these studies did not determine the ability of SARSCoV-2 to be cultured from such environmental swabs, which would help to understand the implications of SARS-CoV-2 RNA positive environmental samples in terms of infectious potential for the human population (Santarpia et al., 2020; Zhou et al., 2020a; Rocha et al., 2021). Another important factor that must be considered is the minimal infectious dose of SARS-CoV-2 to start an effective infection in humans. A recent study estimated to be 100 viral particles, although further studies are required to confirm this infective dose (Karimzadeh et al., 2021).

In the current analysis, we evaluated the infectious potential of positive surface samples (Cq value <34) in Vero CCL-81 cells, but SARS-CoV-2 could not be cultured. This finding is supported by recent studies, which have demonstrated the low isolation rate of infectious virus in environmental swabs (Colaneri et al., 2020; Mondelli et al., 2020; Zhou et al., 2020a). This may explain the lack of success in virus isolation given the short half-life of SARS-CoV-2 in the environment. Serial sampling of highly touched surfaces in places with large people flow might produce culturable SARS-CoV-2. Nevertheless, our findings identify the locations and objects that pose the highest risk of contamination through fomites and should be considered as COVID-19 critical control points. The difficulty in culturing viruses from environmental samples arises from low viral load concentrations and instability of SARS-CoV-2 outside the human host. Recent studies aggregated environmental sampling has shown high RT-qPCR Cq values (>30) for most of the positive samples, which may explain the difficulty of SARS-CoV-2 to be cultured from the environmental specimens (Zhou et al., 2020a; Abrahao~ et al., 2021; Dargahi et al., 2021).

SARS-CoV-2 contamination of public surfaces suggests the circulation of infected people and the risk of infection in these locations either by direct or indirect contact with infected patients. Direct contact with an infectious source is important for the establishment of COVID19 clinical features and this has been established using animal models. Transmission studies in the ferret SARSCoV-2 model have demonstrated that airborne transmission is likely but is considerably less efficient than direct contact transmission, whereby direct contacting animals are exposed to infected ferrets and share with them the same food, water, bedding and breathe the same air (Kim et al., 2020; Richard et al., 2020).

Regarding the adherence of COVID-19 mitigation measures by society, a number of studies have been performed in order to evaluate the adoption of measures to prevent the SARS-CoV-2 transmission (Azene et al., 2020; Azlan et al., 2020; Shewasinad Yehualashet et al., 2021). To assess the community's adherence to mitigation measures to combat the rapid spread of SARS-CoV-2, a recent cross-sectional study conducted in Malaysia employed 4850 Malaysian residents, between 27th March and 3rd April 2020 (Azlan et al., 2020). The findings revealed that most participants (83.1%) held positive attitudes toward the successful control of COVID-19 (Azlan et al., 2020). Interestingly, the number of COVID-19 cases in Malaysia remained stable, with a progressive increase observed only between September and November 2020 (https:// ourworldindata.org/covid-cases). Here, although the number of places evaluated was limited (19) and the method of answering the

questionnaires was different, it is important to highlight that are places with high circulation and concentration of individuals. Our data demonstrated low adherence of COVID-19 mitigation measures by society regarding the social distancing, precaution measures adoption and community's perception about the COVID19 disease. Therefore, these results highlight the importance of consistent messaging from government and health authorities to improve levels the adoption of measures to prevent and contain the spread of SARS-CoV-2.

In summary, our data demonstrated the extensive viral RNA contamination of surfaces in a range of public urban settings in the absence of viral isolation, which suggests low potential risk from environmental contamination for the human population. However, we identified poor adherence to COVID-19 mitigation policies by wider society regarding the adoption of control measures, and this may be reflected in the frequent detection of the viral RNA. Studies such as these can contribute to assess the prevalence of SARS-CoV-2 in specific settings. Finally, we suggest that further studies are urgently performed to elucidate the relative contribution of various modes of transmission for SARS-CoV-2 in both healthcare and urban settings.

Funding

The work in Dr. Pena's lab is funded by the Fiocruz Inova Program, IDRC-Canada and the Foundation for Science and Technology of Pernambuco – FACEPE, Brazil (grant number APQ-0560-2.12/19). S.J.R.d.S. is supported by a PhD fellowship sponsored by the Foundation for Science and Technology of Pernambuco (FACEPE), reference number IBPG-1321-2.12/18. A.K. is funded by the UK Medical Research Council (MC_UU_12014/8). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We are grateful to MSc. Larissa Krokovsky for her support and critical analysis for the design of the work. We thank Josenildo do Sobral for helping to collect surface samples. We would like to express our sincere gratitude and appreciation to the Fiocruz Pernambuco team for their support. Figure 1 was created with Biorender.com under academic license.

Author Contribution Statement

L.P., A.K. and S.J.R.d.S. conceived the work. S.J.R.d.S.,

J.C.F.d.N., W.P.M.d.S.R., C.T.A.d.S., P.G.d.S., R.P.G.M., A.A.M., B.N.R.S. and J.J.F.d.M. performed the experiments. S.J.R.d.S., J.C.F.d.N., W.P.M.d.S.R., P.G.

d.S. A.A.M., J.J.F.d.M., A.K. and L.P. performed data analysis and interpretation. S.J.R.d.S., J.C.F.d.N.,

W.P.M.d.S.R., A.A.M. and J.J.F.d.M. wrote the original draft. S.J.R.d.S., A.K. and L.P. wrote the final manuscript. L.P. supervised the work. All authors critically revised the manuscript and approved the final version of the submitted manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site: Appendix S1: Supplementary Information