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Airborne SARS-CoV-2 – Indoor air contamination by SARS-CoV-2: characterization and development of a new detection tool

Priscilla Gomes da Silva

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

Priscilla Gomes da Silva

Airborne SARS-CoV-2 – Indoor air contamination by SARS-CoV-2: characterization and development of a new detection tool

Tese de Candidatura ao Grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador
Professor Doutor João Rodrigo Mesquita
Professor Auxiliar do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Co-orientadora
Doutora Sofia I. Vieira de Sousa
Investigadora Auxiliar da Faculdade de Engenharia da Universidade do Porto

Co-orientador
Doutor Ruben Soares
Investigador Pós-doutorado
Stockholm University

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Epidemiology of Communicable Diseases Laboratory, School of Medicine and Biomedical Sciences (ICBAS), Porto University

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“Ninguém educa ninguém, ninguém educa
a si mesmo, os homens se educam entre
si, mediatizados pelo mundo”.

Paulo Freire

Pedagogia do oprimido. Rio de Janeiro: Paz e Terra, 1987.

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List of abbreviations

ACE2 – Angiotensin-Converting Enzyme 2

ARDS – Acute Respiratory Distress Syndrome

BSL-3 – Biosafety Level 3

CDC – Centers for Disease Control and Prevention

CFR – Case-Fatality Ratio

CLIA – Chemiluminescence Immunoassay

COVID-19 – Coronavirus Disease 2019

C2CA – Circle-to-Circle Amplification

ddPCR – Droplet Digital Polymerase Chain Reaction

DPP4 – Dipeptidyl Peptidase 4

ECDC – European Centre for Disease Prevention and Control

ELISA – Enzyme-Linked Immunosorbent Assay

EPI – Equipamento de Proteção Individual (Personal Protective Equipment, PPE)

ExoN – Exoribonuclease

FFP2 – Filtering Face Piece 2 (Respirator Standard)

HAT – Human Airway Trypsin-Like Protease

HCoV – Human Coronavirus

ICU – Intensive Care Unit

IFN – Interferon

ISGs – Interferon-Stimulated Genes

LFIA – Lateral Flow Immunoassay

LFICS – Lateral Flow Immunochromatographic Strip

MERS-CoV – Middle East Respiratory Syndrome Coronavirus

NAAT – Nucleic Acid Amplification Test

NSPs – Non-Structural Proteins

ORF – Open Reading Frame

PMA – Propidium Monoazide

PLP – Padlock Probe

PoC – Point of Care

PP1a/PP1ab – Polyprotein 1a/Polyprotein 1ab

PPE – Personal Protective Equipment

RdRp – RNA-Dependent RNA Polymerase

RBD – Receptor-Binding Domain

RCA – Rolling Circle Amplification

RNA – Ribonucleic Acid

RNAse – Ribonuclease

RT-PCR – Reverse Transcription Polymerase Chain Reaction

SARS-CoV – Severe Acute Respiratory Syndrome Coronavirus

SARS-CoV-2 – Severe Acute Respiratory Syndrome Coronavirus 2

TMPRSS2 – Transmembrane Serine Protease 2

TRS – Transcriptional Regulatory Sequence

UCI – Unidade de Cuidados Intensivos (Intensive Care Unit, ICU)

UVC – Ultraviolet C

VOC – Variant of Concern

VOI – Variant of Interest

VUM – Variant Under Monitoring

WHO – World Health Organization

List of publications

In compliance with the *Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto*, the author of this thesis hereby declare that she participated in the Conception and execution of the experimental work, analysis and interpretation of the data leading to the results presented and writing of the respective manuscripts. The manuscripts provided in this thesis were not previously included in other theses and correspond to their integral versions. Copyright permissions can be found at the first page of each manuscript.

Under the scope of this thesis, the following manuscripts were published:

Research articles

1 da Silva, P.G., Gonçalves, J., Rodriguéz, E. et al. SARS-CoV-2 RNA Presence in Outdoor Air of Public Spaces in Valladolid During Winter, 2021. Food Environ Virol 17, 4 (2025). https://doi.org/10.1007/s12560-024-09615-1

2 da Silva PG, Hemnani M, Gonçalves J, Rodriguéz E, García-Encina PA, Nascimento MSJ, et al. Airborne SARS-CoV-2 is more frequently detected in environments related to children and elderly but likely non-infectious, Norway, 2022. Virol J 2023;20:1–8. https://doi.org/10.1186/s12985-023-02243-4

3 Silva PG da, Nascimento MSJ, Sousa SIV, Mesquita JR. SARS-CoV-2 in outdoor air following the third wave lockdown release, Portugal, 2021. J Med Microbiol 2023;72:1–6. https://doi.org/10.1099/jmm.0.001659

4 Silva PG da, Gonçalves J, Franco AT, Rodriguez E, Diaz I, Domingo AO, Pérez SG, Roselló GAM, Gutiérrez CJD, Nascimento MSJ, et al. Environmental Dissemination of SARS-CoV-2 in a University Hospital during the COVID-19 5th Wave Delta Variant Peak in Castile-León, Spain. Int J Environ Res Public Health. 2023;20:1574. https://doi.org/10.3390/ijerph20021574

5 Silva PG da, Gonçalves J, Lopes AIB, Esteves NA, Bamba GEE, Nascimento MSJ, et al. Evidence of Air and Surface Contamination with SARS-CoV-2 in a Major Hospital in Portugal. Int J Environ Res Public Health 2022;19:525. https://doi.org/10.3390/ijerph19010525

6 da Silva PG, Gonçalves J, Nascimento MS, Sousa SI V, Mesquita JR. Detection of SARS-CoV-2 in the Indoor and Outdoor Areas of Urban Public Transport

Systems of Three Major Cities of Portugal in 2021. Int J Environ Res Public Health 2022;19. https://doi.org/10.3390/ijerph19105955

Review articles

1 Silva PG, Branco PTBS, Soares RRG, Mesquita JR, Sousa SI V. SARS-CoV-2 air sampling: A systematic review on the methodologies for detection and infectivity. Indoor Air 2022;32:e13083. https://doi.org/https://doi.org/10.1111/ina.13083

2 da Silva PG, Nascimento MSJ, Soares RRG, Sousa SIV, Mesquita JR. Airborne spread of infectious SARS-CoV-2: Moving forward using lessons from SARS-CoV and MERS-CoV. Sci Total Environ 2021;764:142802. https://doi.org/10.1016/j.scitotenv.2020.142802

3 da Silva PG, Mesquita JR, de São José Nascimento M, Ferreira VAM. Viral, host and environmental factors that favor anthropozoonotic spillover of coronaviruses: An opinionated review, focusing on SARS-CoV, MERS-CoV and SARS-CoV-2. Sci Total Environ 2021;750:141483. https://doi.org/10.1016/j.scitotenv.2020.141483

Articles still to be submitted

1 Rolling circle amplification as a tool for direct viral RNA detection and variant profiling. To be submitted to the journal Accounts of Chemical Research.

Communications at Scientific Meetings

1 Priscilla Gomes da Silva, Maria São José Nascimento, Ruben Soares, Sofia I.V Sousa, João Rodrigo Mesquita. Assessment of SARS-CoV-2 presence and infectivity in air from indoor and outdoor environments from three European countries. Oral presentation at the 3rd Meeting PhD in Biomedical Sciences. 8th July, 2023. Porto, Portugal.

2 José Gonçalves, Priscilla Gomes da Silva, Andrés Torres Franco, Sofia I. V. Sousa, Elisa Rodriguez, Israel Diaz, João R. Mesquita, Pedro Garcia Encina. Assessment of air and surface contamination by SARS-CoV-2 in a hospital. Poster presentation at the 12th Micropol and Ecohazard Conference. 06-10

June, 2022. Santiago de Compostela, Spain.

3 Priscilla Gomes da Silva, Maria São José Nascimento, Sofia I. V. Sousa, João R. Mesquita. Assessing SARS-CoV-2 Presence in Outdoor Environments: A Winter Study in Valladolid, Spain. Poster presentation at the VII IBIMED Symposium – Sharing science, inspiring progresss. 23-24 May, 2024. Aveiro, Portugal.

4 Priscilla Gomes da Silva, João Carlos Varela, Mats Nilsson, Sofia I.V Sousa, João R. Mesquita, Ruben Soares. SARS-CoV-2 RNA Detection and Variant Profiling using Circle-to-circle Amplification (C2CA) and Padlock Probes (PLPs). Poster presentation at the One Health PhD Forum. 13th June, 2024. Porto, Portugal.

Abstract

The COVID-19 pandemic, caused by the novel coronavirus SARS-CoV-2, brought unprecedented challenges to global public health, highlighting the critical need to understand and mitigate airborne transmission of respiratory viruses. This thesis addresses this need by investigating the presence and dynamics of SARS-CoV-2 RNA in various environments, including hospitals, public transportation systems, and outdoor public spaces, across three European countries: Portugal, Spain, and Norway. The research conducted aimed to elucidate the factors influencing airborne transmission, evaluate the effectiveness of existing preventive measures, and develop a new diagnostic tool for viral detection and variant profiling towards enabling an expanded analytical toolbox for airborne transmission in future pandemics.

The first part of the study focuses on hospital environments, where the risk of nosocomial transmission is particularly high due to the concentration of COVID-19 patients and aerosol-generating medical procedures. Air and surface sampling were performed in hospitals in Portugal and Spain during different waves of the pandemic, dominated by the Alpha and Delta variants, respectively. Despite stringent infection control measures, including the use of personal protective equipment (PPE) and regular disinfection protocols, SARS-CoV-2 RNA was detected in several hospital areas. Notably, viral RNA was consistently found in the air of COVID-19 intensive care units (ICUs) and, in some cases, in non-COVID-19 zones, suggesting potential cross-contamination or the presence of asymptomatic carriers. Surface contamination was also observed in high-contact areas, indicating that while surface transmission may be less significant, it remains a potential route of infection in hospital settings.

In public transportation systems, which are vital for daily commuting and travel, the risk of airborne transmission was assessed through air and surface sampling in indoor and outdoor areas associated with public transport in Portugal. The study, conducted during the fifth wave of the pandemic, found that while the overall positivity rate for SARS-CoV-2 RNA was low, positive air samples were primarily collected in densely populated indoor environments, such as busy train stations during peak hours. These findings underscore the effectiveness of ventilation and mask-wearing in mitigating transmission risks but also highlight the residual risk present in crowded and poorly ventilated spaces. The lack of viral detection in surface samples suggests that rigorous disinfection protocols were effective, although continuous monitoring is necessary to ensure ongoing safety.

The third component of the research involved air sampling in outdoor public spaces across Portugal, Spain, and Norway during different seasons and stages of the

pandemic. The results revealed that outdoor environments generally pose a lower risk of SARS-CoV-2 transmission due to natural ventilation and environmental factors such as ultraviolet (UV) radiation. However, during periods of high crowd density or limited air movement, viral RNA was detected in outdoor air samples, particularly in settings like restaurant terraces where mask usage was inconsistent due to eating and drinking activities. Importantly, the study introduced RNAse pretreatment as a method to assess the viability of detected viral RNA, providing evidence that while RNA may be present, the virus is likely not infectious in well-ventilated outdoor environments.

In addition to environmental monitoring, this thesis presents the development of a novel detection tool based on padlock probes (PLP) and rolling circle amplification (RCA). This method was designed to address the challenges associated with the low concentration of viral RNA in air samples, which often hampers downstream molecular analysis, particularly when aiming at identifying specific mutations or viral variants. The PLP-based RCA method demonstrated the capability to detect as few as 10 copies/µL of SARS-CoV-2 RNA of specific viral variants using the C2CA method, making it a highly sensitive and specific tool for rapid diagnostic assessments.

Overall, the findings of this thesis emphasize the ongoing need for robust monitoring of airborne viruses and the enhancement of infection control measures in both healthcare and public settings. The research highlights the importance of acknowledging airborne transmission as a significant route for SARS-CoV-2 spread, particularly in enclosed or crowded environments. The novel detection method developed offers a promising approach for improving outbreak surveillance and rapid response capabilities, both in resource-rich and resource-limited settings. As new variants of the virus emerge, the strategies and tools outlined in this thesis will be vital for maintaining public health and preventing future pandemics.

Resumo

A pandemia de COVID-19, causada pelo novo coronavírus SARS-CoV-2, trouxe desafios sem precedentes à saúde pública global, destacando a necessidade crítica de compreender e mitigar a vírus respiratórios pelo ar. Esta tese aborda essa necessidade investigando a presença e a dinâmica do RNA do SARS-CoV-2 em vários ambientes, incluindo hospitais, sistemas de transporte público e espaços públicos ao ar livre, em três países europeus: Portugal, Espanha e Noruega. A pesquisa realizada visa elucidar os fatores que influenciam a transmissão pelo ar, avaliar a eficácia das medidas preventivas existentes e desenvolver um novo método de diagnóstico para deteção viral e identificação de variantes.

A primeira parte do estudo concentra-se nos ambientes hospitalares, onde o risco de transmissão nosocomial é particularmente elevado devido à concentração de pacientes com COVID-19 e ao uso extensivo de procedimentos médicos que geram aerossóis. Foram realizadas amostragens de ar e de superfícies em hospitais de Portugal e Espanha durante diferentes vagas da pandemia, dominadas pelas variantes Alfa e Delta, respetivamente. Apesar das rigorosas medidas de controlo de infeções, incluindo o uso de equipamento de proteção individual (EPI) e protocolos regulares de desinfeção, foi detetado RNA do SARS-CoV-2 em várias áreas hospitalares. Notavelmente, o RNA viral foi consistentemente encontrado no ar das unidades de cuidados intensivos (UCI) de COVID-19 e, em alguns casos, em zonas não COVID-19, sugerindo potencial contaminação cruzada ou a presença de portadores assintomáticos. A contaminação de superfícies também foi observada em áreas de alto contacto, indicando que, embora a transmissão por superfícies possa ser menos significativa, continua a ser uma via potencial de infeção em ambientes hospitalares.

Nos sistemas de transporte público, que são cruciais para o deslocamento diário e viagens, o risco de transmissão aérea foi avaliado através da amostragem de ar e de superfícies em áreas interiores e exteriores associadas ao transporte público em Portugal. O estudo, realizado durante a quinta vaga da pandemia, constatou que, embora a taxa de positividade geral para o RNA do SARS-CoV-2 fosse baixa, as amostras de ar positivas foram principalmente recolhidas em ambientes interiores densamente povoados, como estações de comboio movimentadas durante as horas de ponta. Estes resultados sublinham a eficácia da ventilação e do uso de máscaras na mitigação dos riscos de transmissão, mas também destacam o risco residual presente em espaços lotados e mal ventilados. A ausência de deteção viral em amostras de superfícies sugere que os protocolos rigorosos de desinfeção foram eficazes, embora

seja necessário um monitoramento contínuo para garantir a segurança em relação a superfícies.

A terceira componente da investigação envolveu a amostragem de ar em espaços públicos ao ar livre em Portugal, Espanha e Noruega, durante diferentes estações e fases da pandemia. Os resultados revelaram que os ambientes ao ar livre geralmente apresentam um risco menor de transmissão do SARS-CoV-2 devido à ventilação natural e a fatores ambientais como a radiação ultravioleta (UV). No entanto, durante períodos de alta densidade populacional ou movimento de ar limitado, foi detetado RNA viral em amostras de ar ao ar livre, particularmente em locais como esplanadas de restaurantes, onde o uso de máscaras era inconsistente devido às atividades de alimentação e bebida. Importa destacar que o estudo introduziu o pré-tratamento com RNAse como método para avaliar a viabilidade do RNA viral detetado, fornecendo evidências de que, embora o RNA possa estar presente, é provável que o vírus não seja infeccioso em ambientes ao ar livre bem ventilados.

Além do monitoramento ambiental, esta tese apresenta o desenvolvimento de um novo método de deteção baseado em sondas do tipo padlock (PLP) e amplificação por círculo rolante (RCA). Este método foi desenvolvido para enfrentar os desafios associados à baixa concentração de RNA viral em amostras de ar, o que muitas vezes dificulta a análise molecular subsequente. O método baseado em PLP-RCA demonstrou a capacidade de detetar até 10 cópias/µL de RNA do SARS-CoV-2, tornando-se uma ferramenta altamente sensível e específica para avaliações diagnósticas rápidas. Além disso, o método permite a identificação de variantes, fornecendo informações cruciais sobre as estirpes circulantes do vírus, essenciais para o rastreamento epidemiológico e a tomada de decisões em saúde pública.

Por fim, as conclusões desta tese enfatizam a necessidade contínua de um monitoramento robusto da qualidade do ar e do reforço das medidas de controlo de infeções em ambientes de saúde e públicos. A pesquisa destaca a importância de reconhecer a transmissão aérea como uma via significativa para a disseminação do SARS-CoV-2, particularmente em ambientes fechados ou lotados. O novo método de deteção desenvolvido oferece uma abordagem promissora para melhorar a vigilância de surtos e as capacidades de resposta rápida, tanto em ambientes com recursos abundantes quanto limitados. À medida que a pandemia evolui e novas variantes emergem, as estratégias e ferramentas delineadas nesta tese serão vitais para manter a saúde pública e prevenir futuras pandemias.

*CHAPTER 1**

General Introduction

Scientific Aims

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* Part of the content from this Chapter was reproduced or adapted from the following review papers:

1. *da Silva, P. G., Mesquita, J. R., de São José Nascimento, M., & Ferreira, V. A. M. (2021). Viral, host and environmental factors that favor anthropozoonotic spillover of coronaviruses: An opinionated review, focusing on SARS-CoV, MERS-CoV and SARS-CoV-2. The Science of the total environment, 750, 141483. https://doi.org/10.1016/j.scitotenv.2020.141483*
2. *da Silva, P. G., Nascimento, M. S. J., Soares, R. R. G., Sousa, S. I. V., & Mesquita, J. R. (2021). Airborne spread of infectious SARS-CoV-2: Moving forward using lessons from SARS-CoV and MERS-CoV. The Science of the total environment, 764, 142802. https://doi.org/10.1016/j.scitotenv.2020.142802*
3. *Silva, P. G., Branco, P. T. B. S., Soares, R. R. G., Mesquita, J. R., & Sousa, S. I. V. (2022). SARS-CoV-2 air sampling: A systematic review on the methodologies for detection and infectivity. Indoor air, 32(8), e13083. https://doi.org/10.1111/ina.13083*

1. General introduction

Twenty-one years ago, the emergence of the Severe Acute Respiratory Syndrome coronavirus (SARS-CoV-1) in southern China in February 2003 led to the SARS epidemic of 2003, affecting more than 24 countries in North America, Europe and Asia before being contained, and resulting in 8422 confirmed cases and 916 deaths [1]. As of the latest available information, there is no known transmission of SARS-CoV-1 globally, with the last reported human cases in China in April 2004 stemming from laboratory-acquired infections [2].

A decade later, the Middle East Respiratory Syndrome coronavirus (MERS-CoV) surfaced in the Arabian Peninsula in 2012 [3]. From April 2012 to date, a total of 2609 laboratory-confirmed cases of MERS were reported globally, with 939 associated deaths at a case-fatality ratio (CFR) of 36%. The majority of these cases were reported in Saudi Arabia, with 2200 cases and 858 related deaths (CFR: 39%) [4].

In December 2019, a novel coronavirus emerged, named SARS-CoV-2 by the International Committee for Taxonomy of Viruses, causing the disease known as COVID-19 [5,6]. Sequence and phylogenetic analysis indicated that SARS-CoV-2 shared 79–79.5% genomic homology with SARS-CoV-1 and 50% with MERS-CoV, classifying it as a new *Betacoronavirus* within the *Sarbecovirus* subgenus [7–10].

The emergence of SARS-CoV-2 and the COVID-19 pandemic marked a pivotal moment in global public health. The virus quickly gained international attention due to its association with a cluster of pneumonia cases linked to a seafood and live animals market [7]. Early investigations suggested a zoonotic origin, with the first cases primarily linked to animals from the market [11].

The rapid global spread of the virus was fueled by human-to-human transmission, prompting the World Health Organization (WHO) to declare the outbreak a Public Health Emergency of International Concern on January 30, 2020 [12], and subsequently labeling it a pandemic on March 11, 2020 [13]. The virus spread around the world quickly, affecting countries on every continent, with urban centers and densely populated areas experiencing particularly high transmission rates [14]. As of August 5th, 2024, there's been over 775 million cases of COVID-19 reported around the world, and over 7 million deaths [15].

SARS-CoV-2 primarily spreads through respiratory droplets produced when an infected person talks, coughs, or sneezes [16] (Fig 1). These droplets can reach the mucosa of the mouth or nose of nearby susceptible individuals, or be inhaled into their lungs, facilitating human-to-human transmission [17]. The virus ability to spread efficiently in this manner led to the implementation of various public health measures worldwide, including social distancing, mask-wearing, and lockdowns to curb its transmission [18].

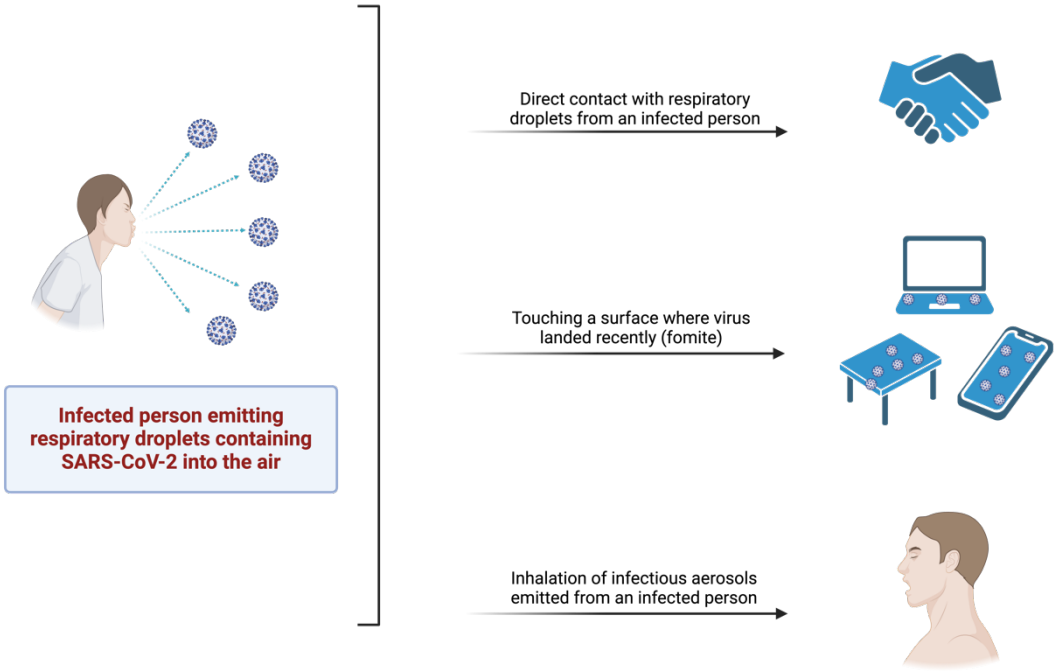

FIGURE 1: SARS-COV-2 TRANSMISSION MODES. CREATED WITH BIORENDER.COM.

The question of whether SARS-CoV-2 is airborne became a subject of significant debate and investigation [19–21]. While respiratory droplets were recognized as a primary mode of transmission, concerns were raised about the potential for airborne transmission in certain settings, particularly indoors or in enclosed spaces with poor ventilation [22]. The acknowledgment of airborne transmission by the WHO gradually evolved as more evidence emerged. The WHO as well as the Centers for Disease Control and Prevention (CDC) have updated their guidance to recognize that SARS-CoV-2 can spread through aerosols in addition to respiratory droplets at the end of April, 2021 [16,23].

The recognition of airborne transmission as a significant mode of SARS-CoV-2 spread prompted adjustments in the approach for control and mitigation measures. Once it became evident that the virus could be transmitted through aerosols, in addition to respiratory droplets, public health guidelines and strategies were adapted to address this mode of transmission [24].

According to the WHO, SARS-CoV-2 is primarily transmitted among individuals in close proximity (within 1 meter). Susceptible individuals can contract the virus by inhaling aerosols or droplets containing the virus, or through direct contact with the eyes, nose, or mouth. This transmission is believed to occur predominantly in inadequately ventilated and/or crowded indoor settings where individuals often spend extended periods [16]. Considering the consistent evidence of environmental contamination near infected individuals and the known transmission methods of other coronaviruses and respiratory viruses, surface (fomite) transmission is also considered to be a transmission mode for SARS-CoV-2, although now the risk is generally considered to be low [25,26].

1.1 Taxonomy of Coronaviruses

Coronaviruses are enveloped, positive-sense, single-stranded RNA viruses that have the ability to infect both animals and humans [27,28]. This viral group constitutes the largest category within the order *Nidovirales*, encompassing the families *Coronaviridae*, *Arteriviridae*, *Roniviridae*, and *Mesoviridae*. *Coronaviridae* family members have been categorized into three sub-families: *Orthocoronavirinae*, *Letovirinae*, and *Pitovirinae* [29]. The *Coronavirinae* subfamily is further divided into four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* [30,31]. *Alphacoronavirus* and *Betacoronavirus* infect mammals, particularly bats, while *Gammacoronavirus* and *Deltacoronavirus* mainly infect birds but can also infect mammals [32]. Amphibians are hosts for members of the subfamily *Letovirinae*, while bony fish are hosts for members of the subfamily *Pitovirinae* [33]. Within the *Betacoronavirus* genus, seventeen viruses are classified within four subgenera: *Embecovirus*, *Sarbecovirus*, *Merbecovirus*, and *Nobecovirus* [29]. SARS-CoV-1, MERS-CoV and SARS-CoV-2 are all classified within the subfamily *Orthocoronavirinae* and the genus *Betacoronavirus*. SARS-CoV-1 and SARS-CoV-2 fall into the *Sarbecovirus* subgenus, while MERS-CoV is part of the *Merbecovirus* subgenus [29].

1.1.1 Coronavirus genome and structure

Coronaviruses encode the largest single-stranded positive-sense RNA genomes among RNA viruses (27–32 kb) [34]. Their large genomes account for their diversity, leading to greater plasticity and greater chances to adapt to different hosts, being an advantage for host switching and interspecies infection [35].

All coronaviruses share a distinctive feature: the crown-like viral particle, from which they derive their name [36]. The virion is comprised of four essential structural proteins, namely spike (S), envelope (E), membrane (M), and nucleocapsid (N), which are essential for virion morphogenesis or infectivity or both [37,38] (Fig 2). S proteins, forming trimers, extend from the host-derived viral envelope and confer specificity for cellular entry receptors [39–41]. The S protein is comprised of two subunits, S1 and S2. While S1 facilitates the adhesion of the virus to the host cell, S2 plays a crucial role in facilitating the fusion of the virus with the host cell membrane [42].

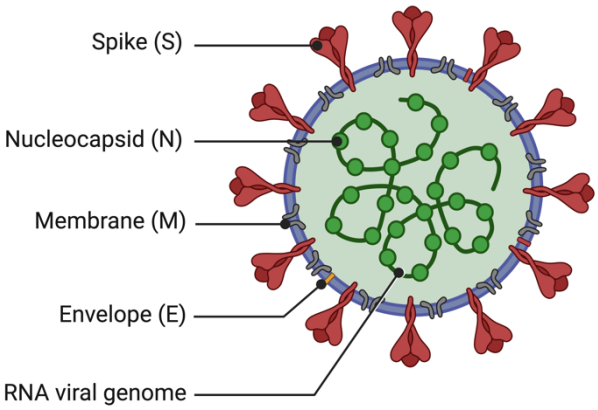

FIGURE 2: CORONAVIRUS PARTICLE STRUCTURE. CREATED WITH BIORENDER.COM

The S1 and S2 subunits are divided by the S1–S2 site, featuring a furin cleavage motif that undergoes cleavage within the virus-producing cell. Upon ACE2 binding on the target cell, the spike protein undergoes cleavage at the S2′ site by the transmembrane serine protease TMPRSS2 [43–45]. This cleavage activates the S2 subunit trimers, facilitating the fusion of viral and host lipid bilayers and the release of the viral ribonucleoprotein complex into the cell [46].

The genome of coronaviruses possesses at least six open reading frames (ORFs), namely ORF1a/b, S, E, M and N [29,47] (Fig. 3). The initial open reading frames, ORF1a/b, which encompasses about two-thirds of the entire genome at the 5' end, encodes the polyproteins pp1a and pp1ab [42,48]. Pp1a comprises NSP1 to NSP11, while pp1ab comprises NSP12 to NSP16. The ORFs located at the 3' end encode the four structural proteins: the S protein (responsible for recognizing host cell receptors) [42,49], the M protein (shaping virions and the envelope), the E protein (involved in virion assembly and release), and the N protein (responsible for packaging the RNA genome and influencing the virus pathogenicity as an interferon (IFN) inhibitor) [50]. Additionally, species-specific genes, namely ORF3a, ORF3d, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF14, and ORF10, encode nine accessory proteins [42,51] – which are now known to play significant roles in affecting immune escape and viral pathogenesis [50].

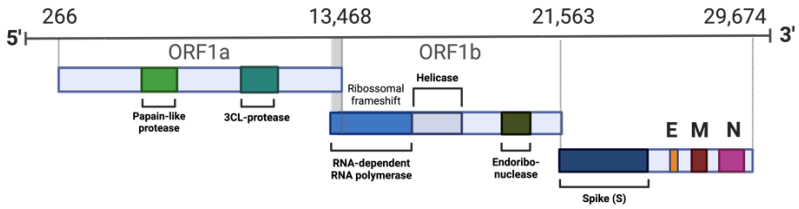

FIGURE 3: GENOMIC ORGANIZATION OF SARS-COV-2. CREATED WITH BIORENDER.COM.

Two distinct domains, the leader sequence, and the untranslated region (UTR) near the 5' end, form a multitude of stem-loop structures crucial for the replication and transcription of the viral genome. Each structural and accessory gene is preceded by transcriptional regulatory sequences (TRSs), essential for their functional expression. The structural component of 30 UTR is necessary for viral RNA replication [42,49]. Upon viral entry into the target cell cytoplasm, the RNA genome translates into pp1a and pp1b, further processing into 16 non-structural proteins (NSPs). This process forms a replication-transcription complex for genomic transcription and replication, leading to the synthesis of a nested set of sub-genomic RNAs through discontinuous transcription [42,47].

1.1.2 Infection cycle

SARS-CoV-2 exhibits a preference for invading mucus-producing goblet cells and ciliated cells within the respiratory tract [52]. This preference is indirectly inferred from the expression pattern of host entry factors, findings from in vitro and ex vivo tropism studies, and observations from post-mortem examinations [53–56]. Furthermore, in vivo studies have demonstrated the susceptibility of endothelial cells in the lung to SARS-CoV-2 infection [56,57].

Viral particles first bind with cellular attachment factors, and specific interactions of the S protein with cellular receptors (such as angiotensin-converting enzyme 2 - ACE2), coupled with host factors (such as the cell surface serine protease TMPRSS2), facilitate viral uptake and fusion at the cellular or endosomal membrane (Fig. 4). Upon entry, the genomic RNA undergoes release and uncoating, initiating the immediate translation of two extensive open reading frames, ORF1a and ORF1b. The resulting polyproteins, pp1a and pp1ab, undergo co-translational and post-translational processing, generating NSPs that constitute the viral replication and transcription complex [41,52,58].

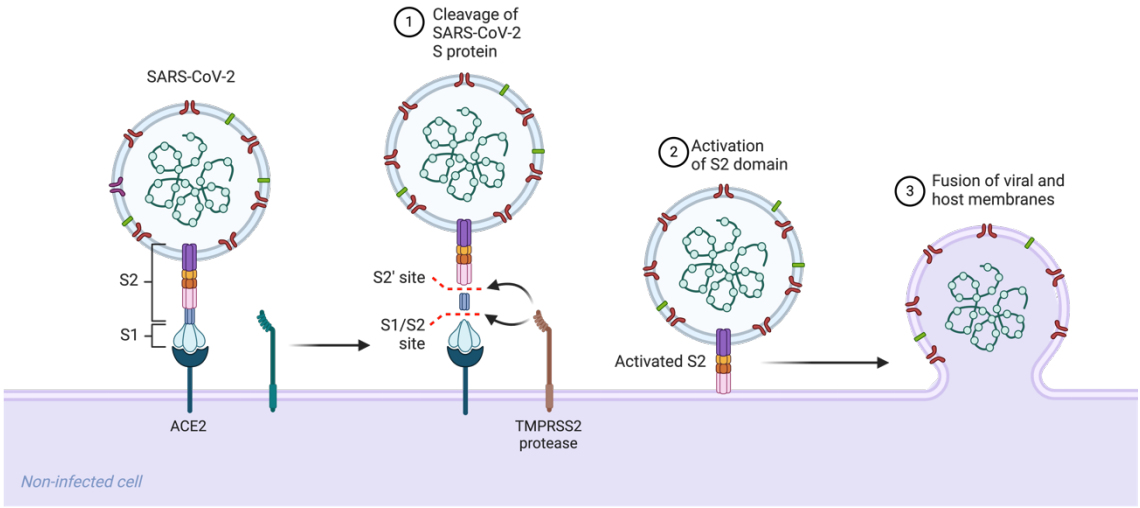

FIGURE 4: SARS-COV-2 VIRAL ENTRY. CREATED WITH BIORENDER.COM.

Concurrently with nsp expression, the formation of viral replication organelles occurs, characterized by perinuclear double-membrane vesicles, convoluted membranes, and small open double-membrane spherules. These structures create a protective microenvironment conducive to viral genomic RNA replication and the transcription of

subgenomic mRNAs, which constitute the distinctive nested set of coronavirus mRNAs [41,52,58].

Structural proteins translated during this process translocate into endoplasmic reticulum (ER) membranes, progressing through the ER-to-Golgi intermediate compartment. Interaction with N-encapsulated, newly produced genomic RNA results in budding into the lumen of secretory vesicular compartments. Ultimately, virions are released from the infected cell through exocytosis [41,52,58].

1.1.3 The importance of the Spike protein and ACE2

A crucial factor influencing interspecies transmission and host specificity of SARS-CoV-like viruses is the interaction between S protein and the host receptor ACE2 [59,60]. The S protein of SARS-CoV-1, MERS-CoV, and SARS-CoV-2 spans 1104 to 1273 amino acids, with an amino (N)-terminal S1 subunit and a carboxyl (C)-terminal S2 subunit [61,62]. Within the S1 subunit, the receptor-binding domain (RBD), covering about 200 residues, comprises two subdomains: the core and external subdomains [63,64]. The RBD core subdomain is pivotal for the formation of S trimer particles [37]. The external subdomain features two exposed loops on the surface that bind with ACE2 (Fig 5) [61,65].

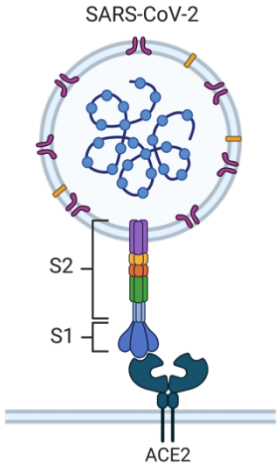

FIGURE 5: SARS-COV-2 BINDING TO ACE2 RECEPTOR. CREATED WITH BIORENDER.COM.

The S protein of SARS-CoV-2 contains a unique polybasic cleavage motif (RRAR) at the S1/S2 cleavage site that is absent in the S proteins of other related coronaviruses

[66], facilitating efficient cleavage by furin and other proteases, thereby influencing viral infectivity and host range [67]. Additionally, a leading proline (PRRA) is inserted at this site in SARS-CoV-2, creating a turn that is predicted to result in the addition of O-linked glycans to S673, T678, and S686, which flank the cleavage site and are specific to SARS-CoV-2 [68]. The functional implications of this polybasic cleavage site and the addition of O-linked glycans remain unknown. Previous studies with SARS-CoV-1 have demonstrated that the insertion of a furin cleavage site at the S1–S2 junction enhances cell–cell fusion without affecting viral entry [69], and the efficient cleavage of the MERS-CoV spike enabled MERS-like coronaviruses from bats to infect human cells [70]. Concerning the addition of O-linked glycans, it has been suggested that it might play a role in protein folding and immune evasion [68,71,72].

The infection of humans by SARS-CoV-like viruses relies on specific genetic changes in the RBD of the S1 subunit. These changes are believed to have evolved continuously, independent of recombination, with the bat-CoV ancestor acquiring or enhancing its ability to bind human ACE2 through point mutations when infecting intermediate hosts before efficiently infecting humans. This hypothesis is supported by the presence of at least three discontinuous, highly variable genomic regions between SARS-CoV-1 and SARS-CoV-like viruses [73]. The entry into host cells requires a defined RBD in the S1 subunit of the S protein, which must specifically recognize the host receptor ACE2 for docking and entry. Therefore, the RBD and ACE2 are crucial determinants for the cross-species and human-to-human transmission of SARS-CoV-like viruses [45,60,74].

The S protein of SARS-CoV-2 is believed to have undergone modification through homologous recombination, resulting from a combination of bat SARS-CoV-1 and an unidentified *Betacoronavirus* [75,76]. Studies have shown that SARS-CoV-2 utilizes the same ACE2 cell receptor and entry mechanism as observed in SARS-CoV-1 [75,77,78]. Notably, SARS-CoV-1 has demonstrated the ability to recognize and bind to ACE2 receptors in various animal species, including bats, palm civets, and raccoon dogs, providing insights into the possible events leading to interspecies transmission of SARS-CoV-2 [79].

Using human ACE2 as the benchmark molecule, specific residue changes in the ACE2 molecules of humans, mice, rats, and bats that hinder SARS-CoV-1 binding have been identified. Mice exhibit inefficient support for SARS-CoV-1 infections, rats show resistance to SARS-CoV-1 infections, and Daubentoni bats (*Myotis daubentonii*) support SARS-CoV-1 infections at low levels. These findings underscore critical residue

variations between human ACE2 and the ACE2 of mice, rats, or bats as major species barriers for SARS-CoV-1 infections [59].

As for MERS-CoV, it relies on the host cell entry facilitated by the S protein binding to the dipeptidyl peptidase 4 (DDP4) receptor. Variations in this receptor have been proven to prevent cell entry in host cells [80,81]. The adaptation of MERS-CoV to the DPP4 of the bat species *Desmodus rotundus* has been demonstrated, showcasing the virus's ability to swiftly adapt to novel species, underscoring the coronavirus capacity for host-switching [82].

Prior to fusion, host cellular proteases, including human airway trypsin-like protease (HAT), cathepsins, and transmembrane protease serine 2 (TMPRSS2), are essential for cleaving the S protein [31,76,83,84]. These cellular factors play a crucial role in determining host specificity and should be considered. Additionally, understanding other factors like the innate immune response, metabolic characteristics, and host cell restriction factors, which can either enhance or impede viral replication, is essential for a comprehensive understanding of SARS-CoV-2's infection mechanisms and pathogenicity. Investigating the divergence of these host factors between humans and the natural and intermediate hosts of coronaviruses may provide insights into cross-species infections and the potential barriers to cross-species transmission [11].

1.1.4 Genetic variability and the RNA-dependent RNA polymerase (RdRp)

Coronaviruses exhibit significant genetic variability primarily due to their high mutation rates stemming from the low fidelity of the RdRp. This characteristic allows these viruses to adapt to various hosts and undergo rapid evolution [85,86]. The RdRp's low fidelity results in unusually high mutation rates, estimated generally between 10^3 to 10^5, providing coronaviruses with the plasticity to respond to new environmental pressures and host changes [35,79,83].

However, in contrast to other positive-sense RNA viruses, coronaviruses replicate with higher fidelity. Larger members of the *Nidovirales* order, including coronaviruses, encode a 3′-to-5′ exoribonuclease (ExoN) domain crucial for replication fidelity and acting as a probable RNA-dependent RNA proofreading enzyme [87,88]. This feature may have facilitated genome expansion and contributed to the rapid evolution of the virus in response to changes in ecological settings while maintaining RNA transcription fidelity [85,87,89]. Inactivation of ExoN in engineered SARS-CoV-1 genomes results in a 10-fold or higher increase in mutation frequency, up to 18 times higher than the tolerance

for fidelity mutants of other RNA viruses. This suggests that ExoN likely functions as a proofreading enzyme, necessary for fidelity regulation and maintaining the integrity of these large genomes [27,85,90]. The identification of ExoN's role as a regulator of replication fidelity indicates the adaptability of RNA genome to diverse environmental conditions and selective pressures, enabling rapid virus evolution in changing ecological contexts [83,87,90].

Coronaviruses are suggested to employ a unique 'copy-choice' mechanism involving special random template switching during RNA replication, divided into two stages: genome replication and subgenomic RNA transcription [83,91–94]. In the first stage, plus-strand genome RNA is transcribed into a full-length minus-strand template RNA, generating more plus-strand genome RNAs. The second stage involves the transcription of 3′-nested subgenomic RNAs, serving as templates for viral protein translation [87]. Due to the RdRp's low capacity to copy the same template, the RNA replicase complex must pause and switch from the original to the acceptor template during synthesis [91,92]. For the switch to occur, the acceptor RNA must be in close proximity and exhibit sequence similarity to allow RdRp binding and continued RNA synthesis [86,88,95]. In coronaviruses, mRNA synthesis follows a discontinuous pattern, with the RNA replicase complex jumping between positions in the genome, leading to frequent template switching during replication and resulting in frequent recombination events [96].

The viral RdRp recognizes TRSs upstream of each subgenomic ORF during negative strand synthesis [87,97]. At these points, the polymerase faces two options: it can either read through to the next TRS or dissociate from the template strand and reassociate with the leader TRS in the 5′ UTR [87,90]. Subsequently, it completes the synthesis of a set of subgenomic negative strand RNAs containing an antileader RNA sequence identical in size to each viral mRNA. These subgenomic negative strand RNAs act as the primary templates for the production of subgenomic mRNAs that are 3′-coterminal with an ~70 nt 5′ leader sequence. Therefore, alterations in TRS sequences can impact viral replication efficiency [87]. Collectively, these factors provide coronaviruses with increased plasticity to adapt and modify genes [11], as evidenced by the numerous unique open reading frames (ORFs) and protein functions encoded toward the 3′ end of the genome [11,35,83,98].

1.1.5 SARS-CoV-2 variants

All viruses undergo evolutionary changes over time [99]. The majority of these changes have minimal to no impact on the virus's characteristics. Nevertheless, certain

alterations may influence factors like the virus transmissibility, disease severity, or the efficacy of vaccines, treatments, diagnostic tools, and other public health and social measures [100]. By late 2020, multiple variants of SARS-CoV-2 have surfaced, posing a significant risk to global public health [42]. The European Centre for Disease Prevention and Control (ECDC) and WHO employ three classification categories for SARS-CoV-2 variants to convey escalating levels of concern: variant under monitoring (VUM), variant of interest (VOI), and variant of concern (VOC) [101,102].

The VUMs are SARS-CoV-2 variants displaying genetic alterations that are believed to influence virus traits and potentially provide an early advantage in terms of growth compared to other variants present in circulation. This advantage may be observed either on a global scale or limited to a specific WHO region. However, the impact of these genetic changes on the virus's behavior and its effects on public health are still uncertain. Therefore, it is necessary to closely monitor and reevaluate this variant until further evidence becomes available. If a variant exhibits an abnormally high number of mutations in recognized antigenic regions, but has limited sequence data and cannot have its relative growth advantage estimated, it may be classified as a VUM, provided there is additional evidence of community transmission in at least two countries over a period of two to four weeks [102].

The VOIs exhibit distinct genetic markers impacting viral transmissibility, virulence, reduced neutralization by antibodies from natural infection or vaccination, evasion of detection, or diminished effectiveness of therapeutics or vaccination. Notable VOIs include Epsilon (B.1.427 and B.1.429), Zeta (P.2), Eta (B.1.525), Theta (P.3), Iota (B.1.526), Kappa (B.1.617.1), Lambda (C.37), and Mu (B.1.621) [42].

The VOCs are variants that fits the criteria of a VOI as defined above and satisfies at least one of the following criteria when compared to other variants: the detrimental alteration in the severity of clinical disease; the alteration in the epidemiology of COVID-19 that has a substantial impact on the capacity of health systems to provide care to patients with COVID-19 or other illnesses, thereby requiring significant public health interventions; or the notable reduction in the efficacy of existing vaccines in safeguarding against severe disease [102,103]. Notable VOCs include Alpha (B.1.1.7 lineage), Beta (B.1.351 lineage or GH501Y·V2), Gamma (P.1 lineage or GR/501Y·V3), Delta (B.1.617.2 lineage) and Omicron (B.1.1.529) [42].

On 3 March 2023, the ECDC removed BA.2, BA.4, and BA.5 Omicron sub-variants from its list of SARS-CoV-2 VOC. This decision was made because these original

lineages were no longer in circulation. Currently, there are no variants of the SARS-CoV-2 virus that match the requirements to be classified as VOCs [101].

1.2 The zoonotic origins of SARS-CoV-2

There are four human seasonal coronaviruses (HCoV-NL63, HCoV-229E, HCoV-OC43, and HCoV-HKU1) distributed globally, generally causing mild respiratory infections with rounds of reinfection. While established in humans, their homologues are also found in various mammals, suggesting potential zoonotic origins. These mammals include cows (HCoV-OC43), camelids (HCoV-229E), and bats or rodents (HCoV-OC43 and HCoV-HKU1) [40,104,105].

The notion of a species barrier in infectious diseases arises from the genetic adaptations that limit the interaction between infectious agents, such as viruses, and their host species. This restriction develops through the co-evolution of the virus and its host species. In cases of viral spillover, where these viruses move from their natural hosts to new ones, severe diseases may arise due to the lack of adaptation of the new host [106]. The classification of coronaviruses reflects their host specificity: *Alphacoronaviruses* and *Betacoronaviruses* primarily infect mammals, while *Gammacoronaviruses* and *Deltacoronaviruses* predominantly infect birds [107,108].

Bats and birds serve as the primary reservoirs of coronaviruses (Woo et al., 2012). From these reservoirs, the viruses can be transmitted to other animals, including domestic pets or exotic animals, highlighting the potential for zoonotic spillover events [79,106].

Throughout these transmission events, viruses may undergo genetic mutations or acquire new genes through recombination mechanisms [109]. These genetic changes contribute to adaptations that facilitate the crossing of species barriers. Notably, alterations in the S protein, particularly in the RBD, have been crucial for the adaptation of coronaviruses like SARS-CoV-1 [110,111] and MERS-CoV [112,113] to human cells.

The primary candidate as an intermediate host for SARS-CoV-2 was initially thought to be the pangolin, supported by studies identifying SARS-CoV-2-related viruses in Malayan pangolins (*Manis javanica*) [114–118]. However, genetic analyses did not yield conclusive evidence [114,118,119]. Isolates of SARS-CoV-2 from infected individuals exhibited higher genetic similarity to the BatCov RaTG13 virus than those from pangolins [120].

Although Guangdong pangolin coronaviruses displayed a higher amino acid identity (97.4%) with SARS-CoV-2 than the bat coronavirus RaTG13 (89.2%) in the RBD [114,115,118,121], the rest of the genome showed a higher sequence identity between RaTG13 and SARS-CoV-2. Phylogenetic analysis based on synonymous sites of the RBD suggested a closer relationship between RaTG13 and SARS-CoV-2 than Guangdong's pangolin coronaviruses [114,115,118,121]. indicating that the virus might have originated through a natural spillover event from bats to humans in late 2019 [122].

Several animal species have tested positive for SARS-CoV-2. Infections have occurred due to close contact with humans or other animals infected with the virus, or during experimental infection studies conducted in laboratory settings. The list of animal species for which information on natural or experimental infection is available includes dogs, domestic cats, large cats, tigers, minks, ferrets, racoon dogs, rabbits, pigs, cattle, poultry, hamsters, Egyptian fruit bats, gorillas, deer, marmosets and macaques [123]. Reports from various countries, including Belgium, the Netherlands, France, Germany, Russia, and Spain, have documented sporadic cases in different domestic animals and mink farms [124]. Currently, the animal species identified as capable of transmitting SARS-CoV-2 include American mink, raccoon dog, cat, ferret, hamster, house mouse, Egyptian fruit bat, deer mouse, and white-tailed deer [124].

Studies have shown that while felines and dogs can be infected by SARS-CoV-2, only felines tend to develop symptoms [125]. The potential for domestic or livestock species to transmit the virus to humans remains uncertain [126]. Experimental infections have demonstrated the susceptibility of ferrets and other domestic animals to SARS-CoV-2, with efficient replication observed in ferrets and cats, and transmission occurring between cats via respiratory droplets [127,128]. However, SARS-CoV-2 replication is limited in dogs, pigs, chickens, and ducks [128]. Still, the origin of SARS-CoV-2 remains uncertain, as emphasized by the WHO [129]. Consequently, further investigations are required to determine the precise origin and identify potential intermediate hosts [52].

1.3 The disease COVID-19

1.3.1 Symptoms

While some cases of SARS-CoV-2 are asymptomatic, most infected individuals exhibit mild to moderate respiratory symptoms, including cough, fever, headache, myalgia, and diarrhea [130–134]. Severe illness typically emerges approximately 1 week

after symptom onset, with dyspnea (shortness of breath) being a common symptom resulting from hypoxemia [134,135]. Following the onset of dyspnea and hypoxemia, severe COVID-19 patients may develop progressive respiratory failure, meeting the criteria for acute respiratory distress syndrome (ARDS) [131,136]. ARDS is characterized by severe hypoxemia and bilateral radiographic opacities within 7 days of exposure to known predisposing factors, not fully explained by heart failure or fluid overload [137].

Individuals who have experienced COVID-19, regardless of hospitalization, may persistently suffer from symptoms referred to as long COVID or post-COVID-19 condition. Common manifestations of long COVID encompass fatigue, breathlessness, and cognitive dysfunction, involving issues like confusion, forgetfulness, or reduced mental focus and clarity. These enduring symptoms can impact an individual's capacity to carry out routine activities, including work and household chores [138].

1.3.2 Diagnosis

Existing diagnostic techniques involve the detection of viral RNA (molecular assays) and the antigen and antibodies linked to SARS-CoV-2 infection (serological assays). Currently, the US Food and Drug Administration and Emergency Use Authorizations (EUA) have approved more than 160 molecular diagnostic techniques for detecting SARS-CoV-2 [139]. The clinical diagnosis of COVID-19 primarily relies on the assessment of signs and symptoms and is further validated using nucleic acid amplification assays (NAAT), such as RT-PCR of nasopharyngeal or oropharyngeal swabs [140–142].

Although RT-PCR is the main NAAT used for COVID-19 diagnosis, it can take several days for a suspected COVID-19 patient to get the results, creating a period during which the infection could spread [143]. Additionally, individuals can transmit the virus before symptom onset, significantly contributing to its spread [144]. Given the significance of presymptomatic, asymptomatic and mild symptom transmission, reducing the time between testing and receiving results is crucial for preventing further transmission [143]. In this context, rapid antigen lateral flow tests were developed [145–149] as an alternative to achieve this reduction between testing and results, which would be difficult to achieve with large-scale PCR testing only [150].

These tests can detect a particular viral antigen, indicating an ongoing viral infection [154]. The majority of antigen tests can be administered at PoC or at home, and most

yield results within minutes. When a person exhibits COVID-19 symptoms, antigen tests are more effective in identifying a SARS-CoV-2 infection than when they are asymptomatic. Compared to RT-PCR and other NAATs, antigen tests for SARS-CoV-2 are typically less sensitive. Because of this, NAAT tests are recommended in scenarios when test sensitivity is crucial [151].

Serological tests can also be used for diagnosis, including lateral flow immunoaassay (LFIA), lateral flow immunochromatographic strip (LFICS), chemiluminescence immunoassay (CLIA), and enzyme-linked immunosorbent assay (ELISA), which are all based on the detection of antibodies [152,153]. However, it is important to note that despite being affordable, quick, and simple, serology tests have limited diagnostic value due to their low sensitivity and accuracy, high rates of false-positive and false-negative results, and inadequate levels of serum antibodies (including IgG and IgA) against SARS-CoV-2 spikes in blood samples taken at the onset of symptoms [153,154]. Thus, particularly during the second week of sickness, the serological assays are crucial supplementary tools for the diagnosis of COVID-19 in patients exhibiting persistent symptoms. During the first week, serological diagnostic tests use is limited [153].

1.3.3 Host immune response

SARS-CoV-2 both activates and counteracts various facets of the immune response during infection. The virus triggers immune responses through components like dysfunctional mitochondria, ssRNA, dsRNA, cytosolic DNA, ORF8, E protein, S glycoprotein, and N protein. These viral elements prompt the production of type I and type III interferons and inflammasomes. The resulting cytokine responses, operating in autocrine and paracrine manners, activate IFN receptors (IFNAR, IFNLR), inducing interferon-stimulated genes (ISGs), IRF1, and IRF1 and NLRC5 lead to MHC class I expression. ISGs inhibit viral replication, activate RNase L, causing cellular and viral ssRNA cleavage, and induce cell death. These responses also recruit dendritic cells, CD8+ killer T cells, initiating the adaptive immune response involving CD8+ T cells, CD4+ T cells, and B cells (plasma cells) producing antibodies. Notably, at least 26 SARS-CoV-2 proteins antagonize specific aspects of the immune response [42,155].

1.3.4 Vaccines

There are different types of COVID-19 vaccines available, each employing a distinct methodology with the common goal of stimulating the immune system to recognize and combat the virus [156], namely:

1. Attenuated or Inactivated Viral Vaccines: These vaccines use a weakened or inactivated form of the SARS-CoV-2 virus. By utilizing a virus that is rendered non-pathogenic, these vaccines prevent illness while still eliciting an immune response.
2. Protein-Based Vaccines: Protein-based vaccines mimic the structure of the SARS-CoV-2 virus. They typically contain safe protein fragments or protein shells derived from the virus, which trigger an immune response without causing infection.
3. Viral Vector Vaccines: Viral vector vaccines utilize harmless viruses as carriers. These carrier viruses are incapable of causing disease but are engineered to deliver genetic material encoding coronavirus proteins into cells. This prompts the immune system to mount a response against the coronavirus.
4. RNA and DNA Vaccines: RNA and DNA vaccines are based on genetic engineering techniques. They involve introducing genetic material (either RNA or DNA) into the body, which instructs cells to produce coronavirus proteins. These proteins then trigger an immune response, priming the body to fight off future infections.

At the time of writing this thesis, 70.6% of the world population has received at least one dose of a COVID-19 vaccine, 13.53 billion doses have been administered globally, and 6,445 are now administered each day, with 32.9% of people in low-income countries having received at least one dose [157].

1.4 Airborne transmission of SARS-CoV-2

1.4.1 Definition and generation of aerosol

Airborne transmission and aerosol transmission are different terms that can be used to talk about the same thing: transmission of disease through small particles that are inhaled and that can remain in the air for hours [158]. In this context, infectious aerosols can be defined as suspensions of particles in the air containing infectious pathogens, with particle size being an essential factor of aerosol behavior [159]. Aerosol particles are defined by the Infectious Diseases Society of America as "respirable" particles (less than 10 μm, which can deposit in the upper or lower airways) and "inhalable" particles (between 10 and 100 μm, which primarily deposit in the upper airways) [160,161]. Even so, there is still a great deal of misunderstanding regarding the meaning and usage of pertinent terms, such as aerosols, particles, droplets, and droplet nuclei. This is mostly because different experts have different definitions for these terms.

To clarify the differences between airborne, droplet, and aerosol transmission, the WHO issued a technical report on April 18th, 2024, following over two years of consultations with more than 100 experts from various scientific fields. The report

removes the distinction between 'droplets' and 'aerosols,' which had been used during the pandemic to categorize particles larger or smaller than five micrometres, respectively. Instead, it introduces the term 'infectious respiratory particles' to refer to all such particles, irrespective of their size [158,162].

Viral stability in the environment is largely dependent on viral characteristics, such as whether the virus is enveloped or not [163]. The envelope normally contains the viral receptors needed for cell entry [164]. If the envelope is compromised, the virus will no longer be able to enter host cells. In contrast, non-enveloped viruses contain only the viral capsid, which is composed of structural proteins that self-assemble [165,166]. As a result, they are more resistant to environmental factors like heat, moisture, pH, UV light, and others, which suggests that they may continue to be infectious for extended periods of time [167].

The mechanism and rate at which the virus is expelled from the infected individual, the gravitational settling of respiratory droplets from the air onto surfaces, the concentration of viruses in respiratory secretions, the presence of particulates and organic matter, temperature and humidity, ventilation, heating, and air conditioning, and other factors all influence the actual risk of infection [168–170]. Additionally, the exposed person's health and immunization history must be considered, as well as variables like their distance from the sick person and if they are both wearing the proper masks. Overall, understanding the interplay between droplet dynamics, environmental conditions, and protective measures is crucial for effective management of aerosol-mediated transmission of SARS-CoV-2, both indoors and outdoors.

1.4.2 Evidence supporting airborne transmission of SARS-CoV-2

Our comprehension of the involvement of aerosols in SARS-CoV-2 transmission is still evolving. Initially, aerosols were largely disregarded as insignificant contributors to transmission dynamics [171,172]. However, in the last four years of the pandemic, we have seen a paradigm shift as emerging evidence and comprehensive analyses have challenged this perspective [20,21,173]. Now, it is widely acknowledged that aerosols indeed play a vital role in the transmission of SARS-CoV-2 [16,174].

Diseases such as measles and tuberculosis were conventionally believed to spread primarily through droplets or surface contact for many years before it became apparent that aerosols also played a significant role in transmission dynamics [175]. This historical

perspective underscores the evolving understanding of infectious disease transmission and the need for continual reassessment of prevailing assumptions.

Aerosols, emitted and inhaled in considerable quantities, can harbor infectious viruses for extended periods. In one study, laser light scattering observations were used to demonstrate that during loud speech, thousands of oral fluid droplets are emitted per second, affirming the potential for airborne virus transmission in confined environments [176]. This finding was corroborated by a study that demonstrated that aerosol release and superemission during human speech escalate with the volume of the voice [177]. By using a mathematical model, another study demonstrated the increased likelihood of inhaling aerosols compared to droplets [178]. Furthermore, one study conducted at a laboratory demonstrated the persistence of infectious SARS-CoV-2 viruses on aerosols for up to 3 hours [179].

Additionally, the infection risk associated with transmission via airborne droplet nuclei, following the evaporation of volatile components was quantified in one study [180]. In this study, it is suggested that in well-ventilated large rooms, the risk of aerosol transmission over large distances is significantly lower than that of close contact. However, in smaller spaces with poor ventilation, the risk escalates substantially. This underscores the importance of being cautious in environments characterized by prolonged exposure duration and a large, exposed population, as the potential for aerosol transmission remains considerable in these conditions.

Superspreading events may stem from various factors associated with the infected host, including the variability in individual emission rates of droplets and aerosols, as well as the viral load [181]. Specific behaviors, such as speaking, singing, shouting, and touching, can also promote the emission of aerosol containing virus [182]. Additionally, specific settings characterized by crowded environments and poor ventilation contribute to the occurrence of superspreading events [183].

The variability in individual emission rates of aerosols is a significant factor contributing to superspreading events. Moreover, a subset of individuals, termed "speech superemitters," consistently releases a substantially higher number of particles compared to others [177]. However, the factors determining why some individuals are speech superemitters remain unclear.

Early evidence indicates that aerosol-based transmission has played a significant role in many superspreader events [184]. In a hospital room study involving two patients,

aerosols containing infectious viruses were isolated up to 4.8 meters away from patients [185]. A simulation model of indoor scenarios demonstrated that very high emitters, particularly those not wearing masks, in typical settings like moderately ventilated offices or public transportation, could lead to elevated aerosol concentrations posing an increased risk of infection [186].

In a choir rehearsal in Washington, USA, one symptomatic individual infected 53 out of 61 attendees, leading to two fatalities [187]. The detailed reconstruction of the event suggested that aerosol transmission was likely, with fomite or ballistic droplet transmission considered unlikely to explain a substantial fraction of the cases. Similarly, carnival activities in a German town resulted in a superspreading event linked to virus emission during loud talking and singing [188].

Additionally, in a meat processing complex in Germany, an index case transmitted the virus to coworkers over a distance of more than 8 meters during three consecutive workdays [189]. Environmental conditions, including low temperature, poor air exchange rates, and close proximity between workers, were identified as factors facilitating efficient aerosol transmission of SARS-CoV-2 particles [190].

1.4.3 Air sampling for SARS-CoV-2

Airborne viruses can be recovered using a variety of sampling techniques, some of which are shown in Fig 6.

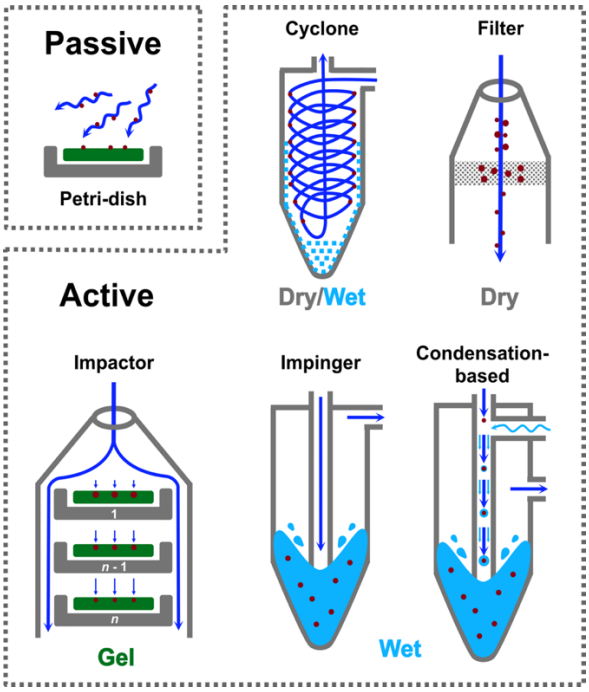

FIGURE 6: SCHEMATIC REPRESENTATION OF EACH AIR SAMPLING METHOD APPLIED FOR THE DETECTION OF SARS- COV- 2 PARTICLES.

Different types of air samplers have been employed in studies focused on SARS-CoV-2 air sampling, each utilizing distinct methodologies with specific advantages and limitations [191–193]. Filter-based samplers are commonly used, involving the passage of air through a filter to capture particles, including viral material. However, it's important to note that this method may desiccate collected material, potentially impacting the viability of the captured viruses [194]. Impinger samplers, on the other hand, draw air through a liquid medium, allowing for the collection of aerosols. While effective for larger particles, impingers may be less efficient for smaller fractions, and their impact on virus viability remains an area of consideration [195].

Cyclone samplers, utilizing centrifugal force to separate particles based on size, have shown promise for SARS-CoV-2 RNA collection [196–198]. However, questions persist about how this collection method might affect the virus's infectivity. Water-based condensation methods have demonstrated the ability to detect viable viruses in air samples [185,199]. This method mimics the human lung by gently depositing airborne particles into a liquid, maintaining their infectivity for analysis [200].

The selection of an appropriate air sampler for SARS-CoV-2 sampling depends on various factors, including the specific goals of the study, cost considerations, and the

need to preserve viral infectivity during the collection process. However, sampling of SARS-CoV-2 in air lacks a universally accepted protocol or consensus. Varied parameters, including airflow rate, air volume collected, sampler placement within the sampling area, sampler type, and collection media, have been observed across most studies to date that have identified SARS-CoV-2 RNA. In addition, crucial parameters influencing virus recovery and infectivity, such as the number of air changes, relative humidity, temperature, and lighting conditions in the chambers where air collection occurred, are frequently omitted or not mentioned in the publications [201].

Because of the absence of standardized air sampling methodologies, it is challenging to ascertain the relative efficiency of different methods. Understanding the distinction between each sampler and the air collection methodology employed can assist in identifying the optimal parameters for a particular experimental environment. Factors such as the time of collection, level of activity, and amount of foot traffic in the area can impact the sampling findings, hence it is important to clearly define these factors for each specific setting [201].

1.4.4 Conditions that affect infectivity of airborne particles

Physical characteristics of the environment such as ultraviolet light (UV), temperature, relative humidity, as well as wind currents and ventilation systems, are critical environmental factors that will determine the settling time of airborne particles [202], highlighting that persistence and exposure risk to airborne viruses might vary between indoor and outdoor environments [203,204].

There are three types of UV light: UVA (320–400 nm), UVB (280–320 nm), and UVC (200–280 nm). UVC is known to be absorbed by RNA and DNA bases, resulting in the photochemical fusion of two adjacent pyrimidines into covalently linked dimers, which in turn lose the ability to pair with each other [205]. Previous studies have shown that UVC is able to inactivate aerosolized coronaviruses [206,207], with more recent studies on the subject reporting that simulated sunlight is also able to inactivate airborne SARS-CoV-2. UVC has also been suggested as non-contact disinfection method to inactivate SARS-CoV-2 and other viruses [203,204].

Temperature is another significant factor for virus survival because it can affect the state of viral proteins and the virus genome [208]. Temperatures above 60 °C for more than 60 min are thought to be sufficient to inactivate most enveloped viruses and depending on the presence of any surrounding organic material such as saliva, the virus

might be insulated against extreme environmental changes [209]. In a study by Pan et al. (2019), it was reported that artificial saliva could better protect infectious viruses from deactivation by preventing viruses of reaching the air-water-interface, possibly due to the complex structure of the mucin component [210]. In a similar study, Woo et al. (2012) reported that the inactivation efficiency of droplet and aerosolized viruses under different humidity levels and UV irradiation at a constant intensity were low in artificial saliva, indicating that solids present in it might exhibit a protective effect [211].

The relative humidity is also significant for virus survival and stability because phospholipid–protein complexes in enveloped viruses are usually more likely to denature in the air at medium to high relative humidity. In contrast, the protein coats of non-enveloped viruses denature easier at low relative humidity [212], which explains why most enveloped viruses tend to survive longer at a lower relative humidity [209]. In addition to that, when faced with high humidity, such as in tropical regions as the Amazon rainforest where relative humidity values can get close to 100% during the rainy season, viruses are associated with larger droplets that settle down much faster, which can be a limiting factor to transmission [213].

In an attempt to study the effects of temperature and relative humidity on the viability of the SARS-CoV, a study found that low temperature and low humidity were able to prolong survival of virus on contaminated surfaces [214]. The same was found to be true for MERS-CoV. A study reported the stability of MERS-CoV at 20 °C and 40% relative humidity; 30 °C and 30% relative humidity; and 30 °C and 80% relative humidity and concluded that MERS-CoV was more stable at lower temperature and lower humidity conditions [215]. In another study, two sets of climatic conditions were used in order to establish the inactivation of MERS-CoV: one represented the common indoor office environment (25 °C and 79% relative humidity) and the other represented the climatic conditions of the Middle Eastern region where the virus outbreak started (38 °C and 24% relative humidity) [216]. Authors found that the virus had a better survival rate at a lower temperature, with virus decay being higher in hot and dry air.

In one study, atomic force microscopy was applied to investigate the topographical changes of SARS-CoV-2 virions exposed to high-temperature treatments, reporting that after the treatment, the virus had much fewer less distinct spikes, with their trigonal shape not being able to be resolved, suggesting heat-induced inactivation of SARS-CoV-2 [217]. Another study reported that the virus was stable at 4 °C in virus transport medium, but sensitive to heat, and that at 22 °C and 65% relative humidity had a negative effect on viral survival on smooth surfaces [218]. Other studies have reported the effects of

humidity and temperature on SARS-CoV-2 transmission based on meteorological data and statistical analysis [219–228]. Although all of them have reported a correlation between temperature and relative humidity and the number of new COVID-19 cases, there is still some controversy regarding whether one or both variables have a positive, negative or no effect on the number of new cases.

1.4.5 SARS-CoV-2 infectivity in air samples

It has been highlighted that air sampling technologies do not accurately replicate the actual mechanisms involved in human respiratory infection through inhalation, as natural human exhalation and inhalation flow velocities differ from the parameters involved in air sampling with current available air-sampling techniques, making them less likely to cause shear stress damage to the viral structure [229]. Because of that, lack of viable viruses in air samples does not necessarily correlate to the absence of viable virus in RNA positive samples, and the presence of RNA should be interpreted as the probable presence of a viable virus, especially considering that viral culture is very difficult as it requires a week or more for completion and specialized laboratory equipment and skills, therefore being much less sensitive than detection by molecular methods [230,231].

Nonetheless, alternative methods for assessing viral infectivity, such as sample pre-treatment before nucleic acid extraction with propidium monoazide (PMA) should be explored and validated. [232–234]. This method assumes that virus inactivation is associated with the loss of integrity of viral outer structures such as the envelope and capsid [235]. As PMA is a photoreactive intercalating dye with a high affinity for DNA and RNA, it forms a covalent linkage upon exposure to intense visible light. This reaction inhibits RT-qPCR amplification of modified DNA templates by removing modified DNA during purification and inhibition of template amplification by DNA polymerases. Because the dyes cannot penetrate the intact viral capsid, when a sample containing both live and dead viruses is treated with it, only dead viruses with compromised capsids are susceptible to DNA modification [232], which in turn allows for assessment of viable virus, expanding the applications of PCR-based detection, and eliminating the need of a BSL-3 laboratory facility to assess cell infectivity through the culture method.

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2. Scientific aims

General and specific objectives

The general objective of this PhD thesis was to strengthen the evidence base for the transmission of SARS-CoV-2 in indoor and outdoor environments considering that, when this work started, airborne transmission still was not acknowledged by public health authorities as one of the transmission modes. Additionally, this thesis aimed to develop and evaluate a novel detection tool for the virus with an additional emphasis on sequencing-free variant discrimination. For this, the following specific objectives were designed:

1 Characterize SARS-CoV-2 presence in air in indoor and outdoor environments of hospital settings, public transport and public spaces, including restaurant terraces and crowded urban areas, in Portugal, Spain and Norway;

2 Test an alternative methodology to cell culture for assessing SARS-CoV-2 viral viability in air samples;

3 Develop and validate a novel detection tool for SARS-CoV-2.

3. Thesis structure

The first chapter is a comprehensive review of the current knowledge on SARS-CoV-2, particularly focusing on airborne transmission, environmental factors, and detection challenges.

The chapters two to six present the published research articles within the aims of the thesis. These articles detail studies on SARS-CoV-2 contamination in hospitals, public transport systems, and outdoor public spaces across Portugal, Spain, and Norway. A novel detection tool for viral RNA and variant profiling is also presented.

The final chapter synthesizes the findings from the published research, discusses the implications for public health and future pandemics, and offers conclusions and recommendations for ongoing research and surveillance efforts.

CHAPTER 2

Evidence of Air and Surface Contamination with SARS-CoV-2 in a Major Hospital in Portugal (Article 1)

Environmental Dissemination of SARS-CoV-2 in a University Hospital during the COVID-19 5th Wave Delta Variant Peak in Castile-León, Spain (Article 2)

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Article

Evidence of Air and Surface Contamination with SARS-CoV-2 in a Major Hospital in Portugal

Priscilla Gomes da Silva [1,2,3], José Gonçalves [4,5], Ariana Isabel Brito Lopes [6], Nury Alves Esteves [6], Gustavo Emanuel Enes Bamba [6], Maria São José Nascimento [7], Pedro T. B. S. Branco [3], Ruben R. G. Soares [8], Sofia I. V. Sousa [3] and João R. Mesquita [1,2,*]

1 ICBAS–School of Medicine and Biomedical Sciences, Porto University, 4050-313 Porto, Portugal; up202002072@edu.icbas.up.pt
2 Epidemiology Research Unit (EPIunit), Institute of Public Health, University of Porto, 4050-600 Porto, Portugal
3 LEPABE–Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, 4200-465 Porto, Portugal; up200606911@fe.up.pt (P.T.B.S.B.); sofia.sousa@fe.up.pt (S.I.V.S.)
4 Institute of Sustainable Processes, University of Valladolid, 47011 Valladolid, Spain; zemcg5@gmail.com
5 Department of Chemical Engineering, University of Valladolid, 47011 Valladolid, Spain
6 Unidade Local de Saúde do Alto Minho E.P.E., 4904-858 Viana do Castelo, Portugal; ariana.lopes@ulsam.min-saude.pt (A.I.B.L.); nury.esteves@ulsam.min-saude.pt (N.A.E.); gustavo.bamba@ulsam.min-saude.pt (G.E.E.B.)
7 Faculty of Pharmacy, University of Porto, 4050-313 Porto, Portugal; saojose@ff.up.pt
8 Department of Biochemistry and Biophysics, Science for Life Laboratory, Stockholm University, SE-106 91 Stockholm, Sweden; ruben.soares@scilifelab.se
* Correspondence: jrmesquita@icbas.up.pt

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Abstract: As the third wave of the COVID-19 pandemic hit Portugal, it forced the country to reintroduce lockdown measures due to hospitals reaching their full capacities. Under these circumstances, environmental contamination by SARS-CoV-2 in different areas of one of Portugal's major Hospitals was assessed between 21 January and 11 February 2021. Air samples (n = 44) were collected from eleven different areas of the Hospital (four COVID-19 and seven non-COVID-19 areas) using Coriolis® µ and Coriolis® Compact cyclone air sampling devices. Surface sampling was also performed (n = 17) on four areas (one COVID-19 and three non-COVID-19 areas). RNA extraction followed by a one-step RT-qPCR adapted for quantitative purposes were performed. Of the 44 air samples, two were positive for SARS-CoV-2 RNA (6575 copies/m^3 and 6662.5 copies/m^3, respectively). Of the 17 surface samples, three were positive for SARS-CoV-2 RNA (200.6 copies/cm^2, 179.2 copies/cm^2, and 201.7 copies/cm^2, respectively). SARS-CoV-2 environmental contamination was found both in air and on surfaces in both COVID-19 and non-COVID-19 areas. Moreover, our results suggest that longer collection sessions are needed to detect point contaminations. This reinforces the need to remain cautious at all times, not only when in close contact with infected individuals. Hand hygiene and other standard transmission-prevention guidelines should be continuously followed to avoid nosocomial COVID-19.

Keywords: SARS-CoV-2; environmental contamination; air samples; surface samples

1. Introduction

SARS-CoV-2 infection causes respiratory illness ranging from mild to severe disease and death, with some infected people being asymptomatic [1]. According to the World Health Organization (WHO), evidence shows that SARS-CoV-2 spreads mainly between people who are standing near one another. In these situations, aerosols or droplets produced by an infected person and which contain the virus are inhaled or come directly into contact with the nose or mouth of a susceptible person, particularly in poorly-ventilated and crowded indoor environments [2].

Respiratory droplets formed from respiratory secretions and saliva are emitted through talking, coughing, sneezing and even breathing, and have a diameter ranging from <1 µm to >100 µm [3]. Respiratory droplets are generally defined as particles that fall to the ground (or any surface) more quickly under the influence of gravity due to their larger size; typically, these are particles > 5–10 µm that fall within 2 m of the source. When these particles settle on surfaces, the contaminated surfaces are then called fomites. Once emitted by humans, these respiratory droplets tend to reduce in size due to evaporation, after which they are termed as droplet nuclei or aerosols, which can be defined as particles that remain suspended due to size and/or environmental conditions; typically, these are particles $\leq$ 5 µm that stay suspended in air for longer, eventually falling to the ground if the air is motionless for long enough (at least 30 min) [3]. Considering that aerosols are small enough to remain suspended in air, they can accumulate in poorly-ventilated spaces and in turn be inhaled at both short and long ranges by a susceptible person, indicating the importance of improving and ensuring good indoor ventilation in the context of COVID-19 [4]. Moreover, the produced aerosols contain much higher viral loads when compared to viral loads in droplets [5–8]. Hence, proper ventilation can reduce surface contamination by removing virus particles before they can land on surfaces [9].

Despite droplet and fomite transmissions being considered the probable main modes of transmission for SARS-CoV-2, these alone cannot account for superspreading events [10–12], or for differences in transmission between indoor and outdoor environments [8]. SARS-CoV-2 modes of transmission are now distinguished as inhalation of virus, deposition of virus on exposed mucous membranes, and touching mucous membranes with soiled hands contaminated with virus [13]. However, individuals who come into contact with potentially infectious surfaces or aerosols containing viral particles often have close contact with an infected person, making it difficult to distinguish the source of infection as being airborne or through fomites [14], particularly in healthcare institutions where infected individuals continuously excrete high viral loads into the environment, potentially adding to the occupational risk of healthcare professionals [15]. In fact, while the risk of transmission via environmental contamination of SARS-CoV-2 is considered to be generally low [16], a number of factors may increase this risk, particularly considering hospital environments where a high number of symptomatic patients with active infection and increased viral shedding might be present when compared to the situation in the community outside the hospital environment [17].

At the beginning of this study, 21 January 2021, there were 151,226 active cases of COVID-19 in Portugal and 702 people admitted in intensive care units (ICUs) all over the country [18]. To the best of the authors' knowledge, no study on environmental contamination with SARS-CoV-2 in hospital settings has been performed in Portugal; hence, this study aimed to assess air and surface contamination in different areas of a major Hospital in Portugal during the peak of the third wave of COVID-19 in the country (late December 2020 to mid-February 2021). The study also aimed at assessing the performance and suitability of two air samplers (Coriolis® µ and Coriolis® Compact) for SARS-CoV-2 air monitoring. The results may be relevant in establishing interventions to prevent healthcare workers' exposure to SARS-CoV-2 and to optimize and better understand the extent of environmental viral contamination of surfaces in healthcare settings.

2. Materials and Methods

Sampling sites

Environmental sampling took place in a Hospital in Portugal that serves around 2.2% of the Portuguese population, between 21 January and 11 February 2021. Air samples (n = 44) were collected from four COVID-19 and seven non-COVID-19 areas. COVID-19 areas included the COVID-19 ICU, intermediate COVID-19 ICU, COVID-19 nursing area and the COVID-19 testing room. Non-COVID-19 areas included the respiratory diseases observation room, respiratory diseases waiting room, clinical decision unit, non-respiratory

diseases patients' waiting room, urgent care (recovery area), the Hospital's outside entrance atrium and the Hospital's staff cafeteria.

Surface sampling (n = 17) was performed on four areas, of which three were non-COVID-19 areas (non-respiratory disease waiting room, staff cafeteria, and outside entrance atrium) and one COVID-19 area (the COVID-19 testing room). Further details about each sampling site are summarized in Tables 1 and 2.

Table 1. Air ventilation details about the sampling sites.

	Hospital Area	Type of Ventilation and Pressure	People with Access to This Area
COVID-19 areas	COVID-19 ICU	Mechanic ventilation, negative pressure	Patients and Hospital staff
	Intermediate COVID-19 ICU	Mechanic ventilation, negative pressure	Patients and Hospital staff
	COVID-19 nursing area	Mechanic ventilation, negative pressure	Patients and Hospital staff
	COVID-19 testing room	Mechanic ventilation, negative pressure	Patients and Hospital staff
Non-COVID-19 areas	Respiratory diseases observation room	Natural ventilation, neutral pressure	Patients and Hospital staff
	Respiratory diseases waiting room	Natural ventilation, neutral pressure	Patients, Hospital staff and patients' companions
	Non-respiratory diseases waiting room	Natural ventilation, neutral pressure	Patients, Hospital staff and patients' companions
	Clinical decision unit	Natural ventilation, neutral pressure	Patients and Hospital staff
	Urgency care (recovery area)	Natural ventilation, neutral pressure	Patients and Hospital staff
	Hospital's outside entrance atrium	Natural ventilation, neutral pressure	Open to the general public
	Hospital staff's cafeteria	Natural ventilation, neutral pressure	Hospital staff

Table 2. Details of the air samples' collections for SARS-CoV-2 RNA detection in the Hospital.

Device	Sample ID	Date of Collection	Hospital Area	Sampler Location	Sampling Parameters
Coriolis® Compact	C1	21 January 2021	ICU COVID-19	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	50 L/min, 60 min
	C2	21 January 2021	ICU intermediate COVID-19	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	50 L/min, 60 min

Table 2. *Cont.*

Device	Sample ID	Date of Collection	Hospital Area	Sampler Location	Sampling Parameters
	C3	21 January 2021	Nursing area COVID-19	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	50 L/min, 60 min
	C4	27 January 2021	Respiratory diseases observation room	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	50 L/min, 60 min
	C5	27 January 2021	Respiratory diseases waiting room	Air sampler placed approximately 1.3 m above the floor in the center of the room	50 L/min, 60 min
	C6	27 January 2021	Clinical decision unit	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	50 L/min, 60 min
	C7	2 February 2021	COVID-19 testing room	Air sampler placed approximately 1.3 m above the floor in the center of the room	50 L/min, 60 min
	C8	2 February 2021	Non-respiratory diseases waiting room	Air sampler placed approximately 1.3 m above the floor in the center of the room	50 L/min, 60 min
	C9	2 February 2021	Urgency Care (Recovery area)	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	50 L/min, 60 min
	C10	11 February 2021	Hospital's outside entrance atrium	Air sampler placed approximately 1.3 m above the floor in the center of the room	50 L/min, 60 min
	C11	11 February 2021	Hospital employe's cafeteria	Air sampler placed approximately 1.3 m above the floor in the center of the room	50 L/min, 60 min
Coriolis® µ	M1	21 January 2021	ICU COVID-19	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	100 L/min, 10 min
	M2	21 January 2021	ICU COVID-19	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	200 L/min, 10 min

Table 2. *Cont.*

Device	Sample ID	Date of Collection	Hospital Area	Sampler Location	Sampling Parameters
	M3	21 January 2021	ICU COVID-19	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	300 L/min, 10 min
	M4	21 January 2021	ICU intermediate COVID-19	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	100 L/min, 10 min
	M5	21 January 2021	ICU intermediate COVID-19	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	200 L/min, 10 min
	M6	21 January 2021	ICU intermediate COVID-19	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	300 L/min, 10 min
	M7	21 January 2021	Nursing area COVID-19	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	100 L/min, 10 min
	M8	21 January 2021	Nursing area COVID-19	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	200 L/min, 10 min
	M9	21 January 2021	Nursing area COVID-19	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	300 L/min, 10 min
	M10	27 January 2021	Respiratory diseases observation room	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	100 L/min, 10 min
	M11	27 January 2021	Respiratory diseases observation room	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	200 L/min, 10 min
	M12	27 January 2021	Respiratory diseases observation room	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	300 L/min, 10 min

Table 2. *Cont.*

Device	Sample ID	Date of Collection	Hospital Area	Sampler Location	Sampling Parameters
	M13	27 January 2021	Respiratory diseases waiting room	Air sampler placed approximately 1.3 m above the floor in the center of the room	100 L/min, 10 min
	M14	27 January 2021	Respiratory diseases waiting room	Air sampler placed approximately 1.3 m above the floor in the center of the room	200 L/min, 10 min
	M15	27 January 2021	Respiratory diseases waiting room	Air sampler placed approximately 1.3 m above the floor in the center of the room	300 L/min, 10 min
	M16	27 January 2021	Clinical decision unit	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	100 L/min, 10 min
	M17	27 January 2021	Clinical decision unit	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	200 L/min, 10 min
	M18	27 January 2021	Clinical decision unit	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	300 L/min, 10 min
	M19	2 February 2021	COVID-19 testing room	Air sampler placed approximately 1.3 m above the floor in the center of the room	100 L/min, 10 min
	M20	2 February 2021	COVID-19 testing room	Air sampler placed approximately 1.3 m above the floor in the center of the room	200 L/min, 10 min
	M21	2 February 2021	COVID-19 testing room	Air sampler placed approximately 1.3 m above the floor in the center of the room	300 L/min, 10 min
	M22	2 February 2021	Non-respiratory diseases waiting room	Air sampler placed approximately 1.3 m above the floor in the center of the room	100 L/min, 10 min
	M23	2 February 2021	Non-respiratory diseases waiting room	Air sampler placed approximately 1.3 m above the floor in the center of the room	200 L/min, 10 min

Table 2. *Cont.*

Device	Sample ID	Date of Collection	Hospital Area	Sampler Location	Sampling Parameters
	M24	2 February 2021	Non-respiratory diseases waiting room	Air sampler placed approximately 1.3 m above the floor in the center of the room	300 L/min, 10 min
	M25	2 February 2021	Urgency Care (Recovery area)	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	100 L/min, 10 min
	M26	2 February 2021	Urgency Care (Recovery area)	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	200 L/min, 10 min
	M27	2 February 2021	Urgency Care (Recovery area)	Air sampler placed approximately 1.3 m above the floor and 2 m from a patient bed, in the center of the room	300 L/min, 10 min
	M28	11 February 2021	Hospital's outside entrance atrium	Air sampler placed approximately 1.3 m above the floor in the center of the room	100 L/min, 10 min
	M29	11 February 2021	Hospital's outside entrance atrium	Air sampler placed approximately 1.3 m above the floor in the center of the room	200 L/min, 10 min
	M30	11 February 2021	Hospital's outside entrance atrium	Air sampler placed approximately 1.3 m above the floor in the center of the room	300 L/min, 10 min
	M31	11 February 2021	Hospital staff's cafeteria	Air sampler placed approximately 1.3 m above the floor in the center of the room	100 L/min, 10 min
	M32	11 February 2021	Hospital staff's cafeteria	Air sampler placed approximately 1.3 m above the floor in the center of the room	200 L/min, 10 min
	M33	11 February 2021	Hospital staff's cafeteria	Air sampler placed approximately 1.3 m above the floor in the center of the room	300 L/min, 10 min

Collection of air and surface samples

Air samples were collected using two cyclonic microbial air samplers, a Coriolis® µ and a Coriolis® Compact (Bertin Instruments, Montigny-le-Bretonneux, France). Using the Coriolis® µ, three consecutive air samplings were collected from each of the eleven areas of the Hospital for 10 min each with an airflow rate of 100 L/min (total of 1 m^3), 200 L/min (total of 2 m^3) and 300 L/min (total of 3 m^3), respectively. Air samples with the Coriolis® µ were collected on wet medium, with 4 mL of sterile phosphate buffered saline (PBS) added to the collection cones before sampling. With the Coriolis® Compact, one air sampling

was performed in the same eleven areas for 60 min, with an airflow rate of 50 L/min (total of 3 m^3). Air samples with Coriolis® Compact were collected on dry medium, with 4 mL of sterile PBS added to the collection cones after sampling. Both Coriolis® samplers were placed side by side at 1.3 m height using a portable table, and the three consecutive Coriolis® µ samplings were performed simultaneously within the 60-min sampling periods of the Coriolis® Compact.

Surface samples were collected on 10 cm × 10 cm surface areas (100 cm^2 area per sampling) using sterile flocked plastic swabs previously wetted on PBS and immediately placed in PBS (4 mL).

All samples were stored at 4 °C before being taken to the laboratory facilities, and were processed within 8 h. Details about the characteristics of the air and surface samples collected are summarized in Tables 2 and 3, respectively.

Table 3. Details of the surface samples' collections for SARS-CoV-2 RNA detection in the Hospital.

Sample ID	Hospital Area	Collection Date	Sample Location
S1	COVID-19 testing room	2 February 2021	Hand sanitizer dispenser
S2			Instruments' counter
S3			Glove box
S4			Wall of a COVID-19 testing booth
S5			Paper dispenser
S6	Non-respiratory disease waiting room	2 February 2021	Faucet handle
S7			Vending machine (buttons)
S8			Bathroom: flush button
S9			Bathroom: inside doorknob
S10			Bathroom: outside doorknob
S11	Hospital's outside outside entrance atrium	11 February 2021	Statue (approx. 3 m away from outside entrance)
S12			ATM (buttons)
S13			Fire extinguisher (approx. 3 m away from outside entrance)
S14	Hospital staff's cafeteria	11 February 2021	Soap dispenser
S15			Faucet handle
S16			Paper dispenser
S17			Table sign on a table *

* Table sign: a sign with precautions to avoid contamination was placed on the table.

RNA extraction and detection of SARS-CoV-2

RNA extraction was performed using the GRS Viral DNA/RNA Purification Kit (GRISP, Porto, Portugal) according to the manufacturer's instructions. RNA extraction was performed on 200 µL of sample suspensions as previously described [19]. A one-step RT-qPCR reaction aimed at two viral gene targets (N1 and N2) using viral target-specific primers and Taqman probe technology based on a previously described protocol [20] was

used (Xpert qDetect COVID-19, GRISP, Porto, Portugal). For the CFX Real-Time PCR (qPCR) Detection System (Bio-Rad, Hercules, CA, USA), the Bio-Rad CFX Maestro 1.0 Software version 4.0.2325.0418 was used to control the runs and remotely analyze the data. Each RT-qPCR run included ssDNA targets for both N1 and N2 regions (positive controls) and a no-template control. Reactions were set up and run with initial conditions of 15 min at 45 °C and 2 min at 95 °C, then 45 cycles of 95 °C for 15 sec and 55 °C for 30 sec. A standard curve was constructed using the ssDNA targets for both N1 and N2 regions in a 10-fold serial dilution mixture starting at 200,000 copies/µL, in order to quantify the number of viral gene copies present in each sample from the measured Ct values; the limit of detection (LOD) was 1.3 copies/µL for N1 and 3.2 copies/µL for N2. Air sample results are expressed in copies/m^3, and surface sample results in copies/cm2.

3. Results

Of the 44 air samples collected in eleven different areas of the Hospital, only two (C1 and M1) were positive for SARS-CoV-2 RNA (Table 4). They were both from the same place, the COVID-19 ICU, and were collected at the same time; C1 (viral loads of 6000 and 6575 copies/m^3 for N1 and N2 genes, respectively) was collected during 60 min sampling with the Coriolis® Compact at an airflow rate of 50 L/min (total of 3 m^3), while M1 (viral loads of 6362.5 and 6662.5 copies/m^3 for N1 and N2 genes, respectively) was collected with the Coriolis® µ during the first 10 min of the Coriolis® Compact collection period, at an airflow rate of 100 L/min (total of 1 m^3). The two other Coriolis® µ consecutive samples (M2 and M3) collected within the 60-min time frame of the Coriolis® Compact (air flow rates of 200 L/min and 300 L/min, respectively) were both negative for SARS-CoV-2 RNA.

Table 4. Details of Hospital area, sampling location and viral genome copy numbers of the positive air and surface samples.

	Sample ID	Hospital Area	Sample Location	Copy Number (N1 Gene)	Copy Number (N2 Gene)
Air samples	C1	COVID-19 ICU	Air sampler placed approximately 1.3 m above the floor and 2 m from intubated patients beds, in the center of the room	6000 copies/m^3	6575 copies/m^3
	M1			6362.5 copies/m^3	6662.5 copies/m^3
Surface samples	S4	COVID-19 testing room	Wall of a COVID-19 testing booth	200.6 copies/cm^2	No amplification detected
	S6	Non-respiratory disease patients' waiting room	Faucet handle	179.2 copies/cm^2	No amplification detected
	S8		Bathroom: flush button	No amplification detected	201.7 copies/cm^2

Of the 17 surface samples collected in four different areas of the Hospital, three were positive for SARS-CoV-2 RNA, with viral loads of 200.6 copies/cm^2 (COVID-19 testing room, wall of a testing booth), 179.2 copies/cm^2 (non-respiratory disease waiting room, faucet handle), and 201.7 copies/cm^2 (non-respiratory disease waiting room, bathroom's flush button). The three samples amplified only one of the two target genes (N1, N1 and N2 respectively). Details on the Hospital area, sampling location and viral genome copy numbers of the positive air and surface samples are summarized in Table 4.

4. Discussion

The present study aimed to evaluate SARS-CoV-2 environmental contamination of air and surfaces in a major Hospital in Portugal during the third wave of the COVID-19 pandemic. Eleven different areas of the Hospital were selected to be assessed for air contamination, including four COVID-19 and seven non-COVID-19 areas. SARS-CoV-2 RNA was only detected in the air of the COVID-19 ICU. The viral load of these air samples collected with the Coriolis® Compact and Coriolis® µ ranged from 6000 to 6662.5 copies/m^3. Interestingly, only the first sample of Coriolis® µ, collected during the first 10 min of the 60-min time frame of the Coriolis® Compact, was SARS-CoV-2 RNA positive. The two other consecutive samplings of Coriolis® µ performed within the 60-min time frame of the Coriolis® Compact were negative for SARS-CoV-2 RNA in spite of a higher airflow rate (air flow rates of 200 L/min and 300 L/min, respectively), suggesting a point contamination which could be explained by the fact that, when the sampling period started, a patient had just been intubated. This intubation can explain the presence of aerosols containing virus during the first 10 min of Coriolis® µ, sampling and the negative results in the second and third samples, considering that aerosols containing SARS-CoV-2 may have been removed by the rooms' ventilation system by the time the other two samplings took place.

In this study, we aimed to assess the performance and suitability of both air samplers for SARS-CoV-2 air monitoring. The results of this study suggest that Coriolis® µ and Coriolis® Compact samplers seem robust for SARS-CoV-2 air sampling, as both were able to detect SARS-CoV-2 RNA in the air of the COVID-19 ICU. However, longer collection times are more likely to cover point contaminations, as was seen with the 60-min collection with Coriolis® Compact. During this collection, three consecutive samplings of Coriolis® µ were performed, with only the first providing a positive air sample.

To assess contamination of surfaces, four different areas of the Hospital were selected, one COVID-19 area and three non-COVID-19 areas. SARS-CoV-2 RNA was detected on the wall of one of the testing booths (where patients wait for nasopharyngeal sample collection), which was somewhat expected due to the fact that during sampling there were possibly-infected individuals constantly coming in to collect nasopharyngeal samples for testing, all of them reporting as symptomatic (presenting respiratory symptoms such as coughing or sneezing). Moreover, this is the only room where patients remove their masks, likely increasing the viral load in indoor air. Contamination in this room might have happened through respiratory aerosols or droplets from the infected patients that settled on the surfaces sampled, considering that neither the patients or healthcare staff touch the walls in this area, ruling out the possibility of direct touch contamination in this case. SARS-CoV-2 viral RNA was detected in the non-respiratory disease waiting room as well. This was one of the non-COVID-19 areas sampled, and therefore the presence of viral RNA was not expected. This was a positive pressure room, which ideally prevents unfiltered air from outside the room from coming inside [21]. Nevertheless, viral RNA was detected in two surface samples in this area, namely on a faucet handle located in the middle of the waiting room and on the flush button of a bathroom in this area. As these are frequently-touched surfaces, the most likely explanation for viral RNA presence is direct touch contamination. Nonetheless, SARS-CoV-2 excretion in stools has been described, and the flushing could generate contaminated aerosols that could ultimately deposit on these surfaces [22,23]. These results highlight the importance of hand and general hygiene in public toilets as well as the need for enhanced disinfection protocols in all areas of the Hospital, not only those dedicated to COVID-19 patients.

Airborne transmission of SARS-CoV-2 is now widely accepted as a mode of transmission of SARS-CoV-2 [2,4,8,12]. This route dominates under certain environmental conditions, particularly indoor environments without proper ventilation [24–29], with a recent study demonstrating that some fraction of the RNA-containing aerosols emitted from infected people contain intact, replication-competent virions [30]. On the contrary, current evidence suggests that transmission through contaminated surfaces is rare; however, when it comes to healthcare settings where COVID-19 patients are being treated, espe-

cially in ICUs, aerosol-generating medical procedures take place which could potentially exacerbate the contamination of air and surfaces in the surrounding area [31]. To avoid nosocomial infection of healthcare workers, non-COVID-19 patients, and visitors by these virus-laden aerosols as well as to avoid contamination of medical equipment and surfaces, it is imperative that hospital ventilation works properly [4].

COVID-19 patients that need to undergo intubation and extubation are usually placed in negative-pressure isolation rooms, which is considered to be safer [32]. Negative-pressure rooms have a ventilation system in which air flows from the exterior to the interior [21]. This keeps aerosolized viruses from spreading through the heating, ventilation, and air conditioning system. Under these conditions, if an opening exists, air will flow from the surrounding areas into the negatively pressurized space [4]. In the Hospital assessed in this study, the COVID-19 ICU, which was the only negative room sampled, had twelve air changes per hour (ACH), in compliance with the safety rules for these types of rooms [31].

Since the beginning of the pandemic, many articles have been published on detection of SARS-CoV-2 in air samples [24,25,33–35]; however, some inherent problems with air sampling for the detection of viruses have come up, such as the limited diversity of monitored spaces (most studies are done in indoor healthcare facilities), limited number of samples, diversity of methodologies (there is no gold-standard protocol for air sampling of SARS-CoV-2 and other airborne viruses), not all studies performing both surface and air sampling simultaneously, and lastly, the fact that most of these studies were performed during the first wave when little was known about the virus, which could have led to errors in methods and molecular analysis.

This study faced some limitations that are worth highlighting. This was an observational study at a single hospital, which means that the results may not be generalizable to other healthcare facilities. Additionally, no assessment of virus viability was performed, as no BSL3 facility was available to perform such experiments. As a result, the findings in this study, although reflecting the real extent of environmental contamination with SARS-CoV-2 in the Hospital, do not necessarily amount to an infection risk assessment for air and surfaces. Moreover, no surface sampling was performed in the COVID-19 ICU, and we do not have individual data on patients, particularly those who occupied the COVID-19 ICU at the time of sampling. Patients with severe infection influence the viral load in droplets and exhaled aerosols; therefore, it is important that in future studies these individual patient data are acquired in order to allow better interpretation of results. Nevertheless, this study is an important addition to the growing literature on the detection of SARS-CoV-2 RNA in air and on surfaces.

5. Conclusions

The present study showed SARS-CoV-2 hospital environmental contamination both in air and on surfaces in locations where both COVID-19 and non-COVID-19 patients were present. This reinforces the need to remain cautious at all times, not only when in close contact with infected individuals. Hand hygiene and other standard transmission-prevention guidelines should be continuously followed in order to avoid nosocomial COVID-19. Further studies combining air and surface sampling with virus viability assays are still needed to fully elucidate the real risk of air and environmental transmission in healthcare facilities.

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Environmental Dissemination of SARS-CoV-2 in a University Hospital during the COVID-19 5th Wave Delta Variant Peak in Castile-León, Spain

Priscilla Gomes da Silva [1,2,3,4,5,*], José Gonçalves [6,7], Andrés Torres Franco [6,7], Elisa Rodriguez [6,7], Israel Diaz [6,7], Antonio Orduña Domingo [8], Sonsoles Garcinuño Pérez [9], Gabriel Alberto March Roselló [9], Carlos Jesús Dueñas Gutiérrez [10], Maria São José Nascimento [11], Sofia I.V. Sousa [4,5], Pedro Garcia Encina [6,7] and João R. Mesquita [1,2,3,*]

1 ICBAS—School of Medicine and Biomedical Sciences, Porto University, 4050-313 Porto, Portugal
2 Epidemiology Research Unit (EPIunit), Institute of Public Health, University of Porto, 1800-412 Porto, Portugal
3 Laboratório para a Investigação Integrativa e Translacional em Saúde Populacional (ITR), 1800-412 Porto, Portugal
4 LEPABE—Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, 1800-412 Porto, Portugal
5 ALiCE—Associate Laboratory in Chemical Engineering, Faculty of Engineering, University of Porto, 1800-412 Porto, Portugal
6 Institute of Sustainable Processes, Valladolid University, Dr. Mergelina S/N., 47011 Valladolid, Spain
7 Department of Chemical Engineering and Environmental Technology, University of Valladolid, Dr. Mergelina s/n., 47011 Valladolid, Spain
8 Microbiology Service, Valladolid University Clinical Hospital (HCUV), Faculty of Medicine, University of Valladolid, 47011 Valladolid, Spain
9 Microbiology Service, Valladolid University Clinical Hospital (HCUV), 47011 Valladolid, Spain
10 Internal Medicine, Infectious Diseases Section, Valladolid University Clinical Hospital (HCUV), 47011 Valladolid, Spain
11 Faculty of Pharmacy, University of Porto, 1800-412 Porto, Portugal
* Correspondence: up202002072@edu.icbas.up.pt (P.G.d.S.); jrmesquita@icbas.up.pt (J.R.M.)

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Abstract: The dominant SARS-CoV-2 Delta variant (B.1.617.2) became the main circulating variant among countries by mid 2021. Attention was raised to the increased risk of airborne transmission, leading to nosocomial outbreaks even among vaccinated individuals. Considering the increased number of COVID-19 hospital admissions fueled by the spread of the variant, with Spain showing the highest COVID-19 rates in mainland Europe by July 2021, the aim of this study was to assess SARS-CoV-2 environmental contamination in different areas of a University Hospital in the region of Castile-León, Spain, during the peak of the 5th wave of COVID-19 in the country (July 2021). Air samples were collected from sixteen different areas of the Hospital using a Coriolis® μ air sampler. Surface samples were collected in these same areas using sterile flocked plastic swabs. RNA extraction followed by a one-step RT-qPCR were performed for detection of SARS-CoV-2 RNA. Of the 21 air samples, only one was positive for SARS-CoV-2 RNA, from the emergency waiting room. Of the 40 surface samples, 2 were positive for SARS-CoV-2 RNA, both from the microbiology laboratory. These results may be relevant for risk assessment of nosocomial infection within healthcare facilities, thus helping prevent and minimize healthcare staff's exposure to SARS-CoV-2, reinforcing the importance of always wearing appropriate and well-fit masks at all times and proper PPE when in contact with infected patients.

Keywords: SARS-CoV-2; airborne transmission; nosocomial transmission; COVID-19; air sampling; airborne SARS-CoV-2

1. Introduction

In the beginning of the COVID-19 pandemic, it was thought that SARS-CoV-2 transmission occurred through direct, indirect, or close contact with infected people, mostly by droplets and fomites [1] and regular and thorough hand hygiene, wearing masks, and social distancing proving to be effective ways for preventing SARS-CoV-2 infection [2–5].

As the COVID-19 pandemic progressed and more data about SARS-CoV-2 became available, alarms were raised on the importance of airborne transmission [6], especially considering that SARS-CoV-2 replication occurs primarily in the respiratory tract [7,8] and its high degree of genetic similarities with SARS-CoV [9], which had been reported as being airborne in several studies during the SARS outbreak of 2002/2003 [10–14]. Currently, airborne transmission of SARS-CoV-2 has been accepted as a mode of transmission [15,16], being frequently associated with poorly ventilated and/or crowded indoor environments, where people tend to stay for longer periods of time [16].

In addition to that, the emergence of SARS-CoV-2 variants of concern that caused more infections and spread faster led to infection waves worldwide. Among these variants, the Delta variant (B.1.617.2), which was first reported in India in October 2020 and classified as a "variant of concern (VOC)" by the WHO on 11 May 2021 [17], rapidly spread around the globe, becoming the dominant variant circulating among countries by mid 2021 [18], and being responsible for the record-high cases reported in most European countries during 2021 [19]. This was attributed mainly to the fact that the Delta variant is more transmissible and is excreted in higher viral loads in those infected than the previously identified VOCs [20–22]. Moreover, some studies have reported that Delta have higher aerosol and surface stability, meaning that it might be more easily transmitted via long-range aerosols [23,24]. In fact, attention was raised to the increased risk of airborne transmission, as well as nosocomial infections with Delta [25], which has inclusively led to vast nosocomial outbreaks even among vaccinated individuals [26–28].

Considering the increased number of COVID-19 hospital admissions fueled by the spread of the Delta variant, with Spain showing the highest COVID-19 rates in mainland Europe by July 2021 [29], the aim of this study was to assess SARS-CoV-2 environmental contamination (both air and surfaces) in different areas of a University Hospital in the region of Castile-León, Spain, during the peak of the 5th wave of COVID-19 in the country (July 2021), caused by the Delta variant.

2. Materials and Methods

2.1. Sampling Sites

Environmental sampling took place in a Hospital in Castile-Leon, northern Spain, between 27 and 29 July 2021. Air (n = 21) and surface (n = 40) samples were collected from the following areas of the hospital: microbiology laboratory, emergency department, COVID-19 patients' triage, COVID-19 patients' triage waiting room, COVID-19 observation wards, non-COVID-19 intensive care unit (ICU), COVID-19 ICU, COVID-19 ICU's pharmacy, internal medicine nurses' room (non-COVID area), corridor of internal medicine department (non-COVID area), internal medicine's doctor's room (COVID-19 area), internal medicine department's corridor (COVID-19 area), internal medicine's nurses' room (COVID-19 area), internal medicine's waiting room, internal medicine oncology department's corridor, internal medicine's oncology department's waiting room. Further details about each sampling site are summarized in Tables 1 and 2.

Table 1. Details from air samples locations.

Air Sampler	Sample Location	Function of the Area	Hospital Area
Coriolis μ	Entrance	Room where the Hospital main entrance is located	Microbiology diagnostics
	Sample handling room	Room where clinical samples from patients are handled for molecular testing	Microbiology diagnostics
	Extraction room	Room where DNA/RNA extraction of clinical samples to be tested through PCR is performed	Microbiology diagnostics
	Waiting room (clean area)	Waiting room for patients that have tested negative for COVID-19	Emergency Department
	Reception	Reception of the emergency department where all patients arriving at the hospital stay in the beginning prior to triage	Emergency Department
	Triage	Room where patients are brought to be categorized based on their symptoms	Emergency Department
	COVID-19 waiting room	Waiting room only for patients diagnosed with COVID-19	Emergency Department
	COVID-19 area	Room where only COVID-19 patients are being treated	Emergency Department
	Corridor between triage and COVID-19 area	Corridor located continuously after the triage room and before the COVID-19 area	Emergency Department
	Non-COVID-19 area	Room where only patients who have tested negative for COVID-19 are being treated	ICU
	Pharmacy	Room where medications are stored	ICU
	COVID-19 area	Room where only COVID-19 patients are being treated	ICU
	Non-COVID-19 area: Nurses' room	Designated room for nurses where they can go to during their work breaks	Internal Medicine
	Corridor	Main corridor within the Internal Medicine	Internal Medicine
	Non-COVID-19 practitioners' room	Designated room for doctors who are treating patients who are not infected with COVID-19	Internal Medicine
	COVID-19 practitioners room	Designated room for doctors who are treating patients who are infected with COVID-19	Internal Medicine
	10th floor corridor	Main corridor of the 10th floor (clean area)	Internal Medicine
	10th floor nurses room	Designated room for nurses where they can go to during their work breaks	Internal Medicine
	8th floor waiting room	Waiting room for Internal Medicine patients	Internal Medicine
	6th floor oncology room	Waiting room for oncology patients	Internal Medicine
	Corridor of oncology waiting room	Corridor outsider the oncology waiting room	Internal Medicine

Table 2. Details from surface samples collection.

Sample Location	Hospital Area
Sample reception room: table	Microbiology diagnostics
Reception room: door handle	Microbiology diagnostics
Entrance door: inside handle	Microbiology diagnostics
Sample handling room: keyboard	Microbiology diagnostics
Sample handling room: buttons from laminar flow cabinet	Microbiology diagnostics
Table where samples are placed before opening	Microbiology diagnostics
Touchscreen of MagNA machine (nucleic acid extraction machine) in extraction room	Microbiology diagnostics
Buttons of KingFisher machine (nucleic acid extraction machine) in extraction room	Microbiology diagnostics
COVID-19 samples' freezer: handle	Microbiology diagnostics
Vending machine: button	Emergency Department
Coffee machine: button	Emergency Department
Door handle from door leading to non-COVID area	Emergency Department
Door handle from toilet's door in the non-COVID area	Emergency Department
Wall where patients stay in the non-COVID area	Emergency Department
Table where medication is prepared	Emergency Department
Triage: Buttons Spot Vital Signs	Emergency Department
Phone	Emergency Department
Door handle to door leading to common room	Emergency Department
Chair in COVID-19 waiting room	Emergency Department
Door handle from the toilet's door in the COVID-19 area	Emergency Department
Touch screen from vital signs device in the COVID-19 area	Emergency Department
Toilet's flush button in the COVID-19 area	Emergency Department
Gloves box in the COVID-19 area	Emergency Department
Door handle of staff's toilet	Emergency Department
Toilet's flush button in staff's toilet	Emergency Department
Staff's table in the non-COVID-19 area	ICU
Computer's keyboard in the non-COVID-19 area	ICU
Phone in the non-COVID area	ICU
Pharmacy's table	ICU
Staff's computer's keyboard in the COVID-19 area	ICU
Phone in the COVID-19 area	ICU
Table in the non-medical staff's room in the COVID-19 area	ICU
Microwave's handle in the non-medical staff room of COVID-19 area	ICU
11th floor, nurses room: microwave handle	Internal Medicine
11th floor: computer's keyboard in corridor	Internal Medicine
11th floor, medical doctors room: phone	Internal Medicine
10th floor, medical doctors room: phone	Internal Medicine
10th floor: computer's keyboard corridor	Internal Medicine
Microwave handle nurses room	Internal Medicine
8th floor: small table in waiting room	Internal Medicine
Toilet's flush button	Internal Medicine
Entrance door's handle	Internal Medicine
6th floor: toilet faucet	Internal Medicine
6th floor: toilet flush button	Internal Medicine
Waiting room: small table	Internal Medicine
5th floor traumatology and orthopedics' toilet: sink faucet	Internal Medicine
5th floor traumatology and orthopedics' toilet: flush button	Internal Medicine
5th floor traumatology and orthopedics': exit door handle	Internal Medicine

2.2. Collection of Air and Surface Samples

Air samples were collected using one Coriolis® μ (Bertin Instruments, Montigny-le-Bretonneux, France) cyclonic microbial air sampler. One air sampling was collected from each of the above-mentioned areas of the Hospital for 10 min each with an airflow rate of 300 L/min (total of 3 m^3). In every sampling location, the sampler was placed in the middle of the room or as close to the middle of the room as possible, with their air inlets at approximately 1.3 m height, which is approximately the distance from the ground to the nose of a seated person. The samples were collected on wet medium, with 6 mL of sterile

phosphate buffered saline (PBS) added to the collection cones before sampling. After every sample, the cleaning and decontamination of the sampler were performed according to the manufacturer's instructions. Briefly, a wipe dampened with a surfactant–water solution was used to clean the external parts of the air sampler. After that, the sampler was wiped down with a soft cloth to remove any excess product.

Surface samples were also collected from each of the above-mentioned areas of the hospital on 10 cm × 10 cm surface (100 cm^2) using sterile flocked plastic swabs previously wetted on PBS and immediately placed in vials containing 4 mL of PBS. The surfaces sampled in every room were the ones pointed by the healthcare workers present in every sampled room as frequently touched surfaces.

The researchers responsible for performing the sampling were wearing KN95 masks and gloves at all times, with masks changed every 4 h, gloves changed after handling each sample, and hand washing between handling each sample.

All samples were stored at 4 °C until transportation to the laboratory facilities and were processed within 24 h.

2.3. RNA Extraction and Detection of SARS-CoV-2

RNA extraction was performed using the NucleoSpin® mini kit for viral RNA/DNA purification (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. To determine the extraction efficiency of the samples, The Mengovirus Extraction Control kit KMG (BioMérieux, Marcyl'Etoile, France) was used according to the manufacturer's instructions. The nucleic acids extracted from the air and swab samples were tested for SARS-CoV-2 using the TaqPath™ 1-Step RT-qPCR Master Mix (ThermoFisher catalog #A15300). The reaction aimed at two viral gene targets (N1, N2) using viral target-specific primers and Taqman probe technology (IDT Technologies, San Diego, CA, USA) according to a previously described protocol [30,31]. For the QuantStudio™ 1 Real-Time PCR System (ThermoFisher catalog #A40427), the QuantStudio™ Design & Analysis Software v1.5.1 was used to control the runs and remotely analyze the data. Each RT-qPCR run included triplicates for each sample, including negative and positive controls, and a negative control of RNA isolation.

Reactions were set up and run with initial conditions of 15 min at 50 °C and 2 min at 95 °C, then 45 cycles of 95 °C for 3 s and 55 °C for 30 s [30,31]. A standard curve was constructed using the ssDNA targets for both N1 and N2 regions in a ten-fold serial dilution mixture starting at 2 × 105 copies/µL to quantify the number of viral gene copies present in each sample from the measured CT values. The limit of detection (LOD) was 10 copies/µL for N1 and 10 copies/µL for N2. Air sample results are expressed in copies/m^3, and surface sample results in copies/cm^2.

3. Results

The negative controls of isolation and non-template controls that were added to monitor each sampling run were negative, discarding the possibility of contamination during RNA extraction and during the preparation of the plate for RT-qPCR. The Ct values of the mengovirus RNA varied only slightly between samples, confirming that the RNA isolation was almost optimal and that there was little or no inhibition in the RT-qPCR. The positive controls added to the RT-qPCR reactions for each gene behaved as expected with very low inter-reaction variation.

Of the 21 air samples collected in 16 different areas of the hospital, only 1 was positive for SARS-CoV-2 RNA, namely a sample from the emergency's waiting room, with an average copy number of 8.30 gene copies/µL.

Of the 40 surface samples collected, 2 were positive for SARS-CoV-2 RNA, both from the microbiology laboratory, with an average copy number of 3.65 and 3.57 gene copies/µL, respectively. One of the samples was from the buttons of the laminar flow cabinet (sample handling room) and the other from the handle of a freezer containing COVID-19 samples (See Table 3 below).

Table 3. Values of gene copies/L and standard deviation for the positive air and surface samples tested in triplicate for the N1 region.

	N1				
Sample	**Result 1 (Copies/µL)**	**Result 2 (Copies/µL)**	**Result 3 (Copies/µL)**	**Average Copies/µL**	**Standard Deviation**
Emergency's waiting room	-	4.38	3.92	8.30	0.645
Buttons from the laminar flow cabinet (sample handling room)	1.96	1.55	1.82	3.65	0.468
Handle of freezer for COVID-19 samples	2.20	1.37	-	3.57	1.167

4. Discussion

In Spain, the fifth wave of COVID-19 caused by the Delta variant reached its peak by the end of July 2021 [32]. Because the Delta variant is more contagious and its reproductive number higher than the previous VOCs [33], attention was brought to an increased risk in airborne transmission, as well as nosocomial infections [25].

Indeed, a nosocomial cluster of COVID-19 caused by the Delta variant was reported in a major acute care hospital in Singapore [28]. This cluster comprised 47 cases and happened despite enhancement of infection control measures for COVID-19 undertaken by this hospital. Turbulent air flow and swabs from air exhaust filters showed to be positive for SARS-CoV-2. Another nosocomial outbreak caused by the Delta variant in a highly vaccinated population was described in Israel in 2021 [26]. This cluster had 42 infected patients, staff, and family members, of which 39 were fully vaccinated. Moreover, all cases were linked and traced to one patient, with several transmissions having occurred between people who were wearing face masks. Similarly, a study in Finland has reported an outbreak caused by the SARS-CoV-2 Delta variant in a secondary care hospital, which resulted in 58 infections, including 18 deaths in patients and 45 infections among healthcare staff [34]. This outbreak occurred despite the use of personal protective equipment (PPE), a high vaccination coverage, and universal masking by the healthcare staff.

These studies reflect the higher transmissibility of the Delta variant, which in turn can lead to an increase in the risk for airborne transmission and nosocomial infections even when appropriate PPE are being used, and among fully vaccinated individuals as well. With that in mind, it was our goal to investigate the presence of SARS-CoV-2 in indoor air and surfaces of a major hospital in the region of Castile-León in Spain, in order to assess if hospital measures to reduce nosocomial infection in place were efficient at preventing infection with the high transmissible Delta variant. During the collection period, the cumulative incidence rate (IR) and the cumulative mortality rate (MR) at 14 days at the national level was ~800 cases per 100,000 habitants and ~150 deaths per 100,000 habitants, respectively [35].

We found in our study three samples positive for SARS-CoV-2 RNA, namely one air sample from the emergency's waiting room and two surface samples from the hospital's microbiology laboratory, where COVID-19 nasopharyngeal swab samples from COVID-19 suspected patients were processed and analyzed. The low number of positive samples found are not surprising, though. Unlike the pre-vaccination period of the COVID-19 pandemic, the number of hospitalized people and people admitted to the ICU greatly decreased due to the high COVID-19 vaccination rate in Spain by 29 July 2021, with 56% of the population with at least one dose of the vaccine at the time this experiment ended [36]. The benefits of a high vaccination coverage can be seen in the evolution of cumulative hospitalization rates and cumulative ICU admissions at 7 days per 100,000 habitants in

Spain during this period (end of July 2021), figured at ~10 cases per 100,000 habitants, in contrast to January and February 2021, when the number of vaccinated people was still low (2.7% of population with at least one dose of the vaccine), and it figured at ~40 cases per 100,000 habitants [35]. Taking that into consideration, a great degree of air and environmental contamination with SARS-CoV-2 was not expected.

Of note, data on SARS-CoV-2 testing of suspected COVID-19 patients, as well as sequencing of positive samples, were provided by the Hospital. During the week this experiment took place, 4734 people suspected of being infected with SARS-CoV-2 have been tested for COVID-19 in this hospital, of which 725 tested positive (15.3%). Of the 725 positive patients, 639 were from primary care, and 86 came from the clinical hospital (9 admitted, 17 from the polyclinics and 60 from emergencies). Of these 725 positive cases 547 cases (75.4%) were from the delta variant (absence of 69–70 deletion, and presence of N501Y mutation), 160 cases (22.1%) were from the alpha variant (presence of deletion 69–70, and presence of N501Y mutation), and 18 cases (2.5%) were from other strains of the virus which are neither delta nor alpha. Out of these 725 positive samples, a random sample constituting of 55 positive patients, was further analyzed through whole genome sequencing (NextSeq Illumina). Table 4 shows the information about the variants identified through sequencing of samples from SARS-CoV-2 positive patients at the hospital during the week the experiment took place.

Table 4. Variants identified through sequencing of samples from SARS-CoV-2 positive patients at the hospital during the week the experiment took place.

Variant	Delta (70.9%)			Alpha (21.8%)	Beta (3.6%)	Lambda (3.6%)
Lineage	B.1.617.2	AY12	AY4	(B.1.1.7)	(B.1.351)	(C.37.1)
Number of cases	31	4	4	12	2	2
Total number of cases	55					

When taking a closer look at these results, it is important to highlight the fact that, in the microbiology laboratory of the hospital, there were two surface samples positive for SARS-CoV-2 RNA, with no positive air sample in this environment. As such, the positive surface samples are likely the result of deposition of airborne particles or even contamination of surfaces by clinical samples since it is in that area of the hospital that SARS-CoV-2 molecular diagnosis is performed using the same combinations of primers/probes as we used in this study.

When it comes to the positive air sample from the emergency's waiting room, it should be highlighted that during the whole duration of sampling this room was empty, suggesting that the positive air sample is likely the result of suspended SARS-CoV-2 particles in the air emitted by patients who have been in the room previously to our air sampling.

It is worth to mention that the same air sampling methodology has been previously used in both healthcare and non-healthcare facilities and successfully detected the presence of SARS-CoV-2 RNA in indoor and outdoor air samples [37,38], including negative pressure rooms [37], although with a low rate of positivity just as in this study. Nonetheless, the absence of detectable SARS-CoV-2 in air samples might be due to many factors, among them (1) infected patients were not present in the sampled environments long enough to shed a measurable amount of virus; (2) patients in this hospital were all complying with preventive guidelines and were wearing high quality masks such as KN95 and PFF2 masks; (3) the specific transmission prevention rules in place at this hospital such as a "clean area" where patients who have tested negative for COVID-19 through RT-PCR were taken to, which was completely isolated from the "contaminated area" where patients with RT-PCR confirmed COVID-19 were taken to, control measures over capacity and attendance per day in order to reduce the circulation of people in the hospital, as well the use of properly pressurized rooms (positive or negative) according to the type of patients present in each area/room of the hospital.

Negative pressure rooms incorporate a ventilation system designed so that air flows from the corridor into the negative pressure room, with COVID-19 patients usually kept in isolation wards with this type of pressurization in order to prevent the contaminated air to escape from these areas to other parts of the hospital [39]. Therefore, detection of SARS-CoV-2 in the air of negative pressure rooms with COVID-19 patients is to be expected.

Other alternative approaches to predict the dispersion of SARS-CoV-2 in the air in different environments could be computational fluid dynamics stimulation models [40,41], risk assessment analysis [42–44], and modelling of aerosol transport and virus exposure with numerical simulations [45]. More conclusions could be drawn if other similar studies are performed during the circulation of other variants of concern, so a comparison between patterns of SARS-CoV-2 distribution in these environments could be compared in different waves caused by different variants.

Nonetheless, it has been demonstrated that expiratory aerosol particles can escape from surgical masks due to imperfect sealing [46,47], making surgical masks less efficient when it comes to preventing transmission of more transmissible variants such as the Delta variant, and, more recently, the Omicron (B.1.1.529) and all its sub-lineages [48–51]. That coupled to the fact that current health policies in many countries are still dealing with the consequences of taking too long to acknowledge airborne transmission of SARS-CoV-2, make the surge of these new more transmissible VOCs lead to much higher quantum production rates, suggesting that the pandemic is now driven mainly by the airborne route of transmission [52]. Taking all that into consideration, and considering that SARS-CoV-2 has now become part of our lives and from now on we will continue to co-exist with it [53], public health policies should pay close attention to current infection prevention guidelines and determine the necessary updates in said guidelines in order to minimize airborne transmission of new VOCs [53], especially when it comes to wearing masks in closed spaces and healthcare settings. In this regard, the recommendation for use of more efficient masks (e.g., KN95 and FFP2 masks) should be maintained, as this is one of the most efficient ways of preventing widespread transmission of SARS-CoV-2 through the airborne route in all environments.

5. Conclusions

Considering that COVID-19 is an airborne disease, an in light of the surge of new variants highly transmissible and less susceptible to vaccines, as well as the current lift of restrictions worldwide, it is essential that the prevention guidelines for COVID-19 maintain the recommendation for the use of more efficient masks such as KN95 or FFP2 models in healthcare settings, as these have been proven to be more effective than surgical and cloth masks when it comes to these more transmissible variants. The results may be relevant for risk assessment of nosocomial infection within healthcare facilities, thus helping prevent and minimize healthcare staff's exposure to SARS-CoV-2, reinforcing the importance of always wearing appropriate and well-fit masks at all times and proper PPE when in contact with infected patients.

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CHAPTER 3

Detection of SARS-CoV-2 in the Indoor and Outdoor Areas of Urban Public Transport Systems of Three Major Cities of Portugal in 2021 (Article 3)

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Article

Detection of SARS-CoV-2 in the Indoor and Outdoor Areas of Urban Public Transport Systems of Three Major Cities of Portugal in 2021

Priscilla Gomes da Silva [1,2,3,4,*], José Gonçalves [5], Maria São José Nascimento [6], Sofia I. V. Sousa [4,7] and João R. Mesquita [1,2,3,*]

1 ICBAS—Instituto de Ciências Biomédicas Abel Salazar, School of Medicine and Biomedical Sciences, University of Porto, 4050-313 Porto, Portugal
2 Epidemiology Research Unit (EPIUnit), Instituto de Saúde Pública, University of Porto, 4050-600 Porto, Portugal
3 Laboratório para a Investigação Integrativa e Translacional em Saúde Populacional (ITR), 4050-313 Porto, Portugal
4 LEPABE—Laboratório de Engenharia de Processos, Ambiente, Biotecnologia e Energia, Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, 4200-465 Porto, Portugal; sisousa@fe.up.pt
5 Department of Chemical Engineering and Environmental Technology, Institute of Sustainable Processes, University of Valladolid, 47011 Valladolid, Spain; zemcg5@gmail.com
6 Faculty of Pharmacy, University of Porto, 4050-313 Porto, Portugal; saojose@ff.up.pt
7 ALiCE—Associate Laboratory in Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal
* Correspondence: up202002072@edu.icbas.up.pt (P.G.d.S.); jrmesquita@icbas.up.pt (J.R.M.)

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Abstract: Airborne transmission is mainly associated with poorly ventilated and crowded indoor environments where people stay for long periods of time. As such, public transport is often perceived as having a high risk for the transmission of SARS-CoV-2. Considering that data on the detection of SARS-CoV-2 in public transport systems are scarce, we performed air sampling for SARS-CoV-2 in indoor and outdoor spaces of public transport systems in Portugal. Air (n = 31) and surface (n = 70) samples were collected using a Coriolis® Compact microbial air sampler and sterile flocked plastic swabs, respectively. Samples were extracted and analyzed through RT-qPCR. Only two air samples from an outdoor and a partially open space were found to be positive for SARS-CoV-2 RNA. No positive surface samples were detected. These results indicate that the viral concentration in ambient air in public transport systems is linked to the number of people present in that environment and whether they are wearing properly fitting masks. Considering the current lifting of COVID-19 restrictions around the world, it is essential that people continue to wear masks in both indoor and outdoor environments, especially in crowded spaces. More studies on this topic are needed to fully elucidate the real risk of infection in outdoor spaces.

Keywords: SARS-CoV-2; air sampling; airborne transmission; environmental contamination; indoor air; outdoor air

1. Introduction

The main modes of transmission for respiratory viruses are reported in the literature as direct, via physical contact between an infected individual and a susceptible individual; indirect, via contact with contaminated surfaces or objects (fomites); or through the air via contaminated aerosols that might be suspended and therefore inhaled by susceptible people [1]. Regarding SARS-CoV-2, much attention was given to the fomite transmission route and the cleaning of surfaces in the first year of the pandemic. However, it is widely accepted that COVID-19 rarely spreads through surfaces [2], with the focus turning to the airborne transmission route as the key way SARS-CoV-2 has spread worldwide [3].

Now, two years since the pandemic started, airborne transmission is widely recognized as a major mode of transmission for SARS-CoV-2 [4,5], and is mainly associated with poorly ventilated indoor environments [6], especially crowded indoor environments where people might spend long periods of time [7]. This is particularly relevant in public transport, with people who need to use these systems to commute to work showing concern about their safety during the pandemic [8]. It is noteworthy that it has been reported that the chances of those using public transport acquiring an acute respiratory infection increased up to six times during influenza outbreaks [9]. As such, public transport is often perceived as posing a higher risk for the transmission of respiratory viruses. These environments are usually crowded and it is more difficult to maintain social distancing in them [1,10]. Consequently, a significant change in people's mobility patterns and usage of public transport systems was seen during the pandemic, as people started to avoid using public transport systems for commuting to reduce their risk of infection with COVID-19 [11–13].

Despite this, only a few studies have been published on SARS-CoV-2 transmission in public transport systems. An study conducted by Luo et al., 2020 [14], reported a SARS-CoV-2 outbreak event linked to bus journeys, where 234 individuals were epidemiologically linked to the bus journeys of a person infected with COVID-19, of which 12 tested positive for COVID-19. Another study carried out by Harris et al, 2020 [15], reported that the New York City's subway system played a significant role in SARS-CoV-2 transmission during the first wave of the disease in March 2020.

Moreover, regarding the environmental contamination and presence in the air of SARS-CoV-2 in public transport systems, only three studies where viral RNA presence was detected have been published so far, to the best of our knowledge. The first one, by Di Carlo et al., 2020 [16], assessed the presence of SARS-CoV-2 RNA in the air and on surfaces frequently touched by passengers inside a city bus during its normal operation. However, after two weeks of measurements, the virus was not detected in any sample. In the study by Troko et al., 2021 [8], the presence of SARS-CoV-2 RNA in the air and on surfaces on public buses and subway trains was assessed, with viral presence being detected in both surface and air samples. The study conducted by Comunian et al., 2021 [17], collected aerosol samples from both indoor (hospital facilities) and outdoor public spaces, including a bustling bus station. Although SARS-CoV-2 RNA was detected in the indoor areas studied, no viral presence was found in the bus station. Nevertheless, due to experimental limitations, only the presence of viral RNA was assessed in all these studies and viral viability could not be studied; thus, no assumptions could be made regarding whether the virus was infectious or not [18].

Considering that data on the detection of SARS-CoV-2 in public transport systems are still scarce, this study aimed to perform air sampling and tests for SARS-CoV-2 in the indoor and outdoor spaces of public transport systems in three major cities of Portugal during the period following the 3rd wave of lockdown release. During this time, the effective reproduction number R(t) for SARS-CoV-2 was 1.03 in the northern region and 1.00 in the central region of Portugal, respectively [19]. Moreover, considering that virus-laden aerosols that might have been suspended in the air could have already settled onto surfaces prior to our sampling, we have also performed surface sampling inside public transport vehicles.

2. Materials and Methods

2.1. Sampling Sites

Air and surface sampling was performed between 5 April 2021 and 30 April 2021 in the indoor and outdoor areas of the urban public transport systems of three major cities of Portugal, one from the northern region (City A) and two from the central region (City B and C). Air samples (n = 31) were collected in 4 different train stations and 15 bus stations during the rush hour in the morning, between 7 and 9 am. Surface samples (n = 70) were collected inside 14 different trains during their journeys to and from the three cities between 6 and 12 am. Details of the sampling sites can be found in Table 1.

Table 1. Details of air and surface sampling sites in the public transport systems of three major cities in Portugal, 2021.

	Cities (Region)	Public Transport Systems (Total Number)	Sample Locations (Number of Samples)	Sampling Location Type	Total Number of Samples
Air sampling	City A (North)	Train station (n = 2)	Entrance hall (n = 1) Waiting room (n = 1) Middle platform (n = 1) Front of the station (n = 1)	Indoor Indoor Outdoor Outdoor	13
		Bus station [a] (n = 5)	Bus stop (n = 1)	Outdoor	
	City B (Center)	Train station (n = 1)	Entrance hall (n = 1) Waiting room (n = 1) Middle platform (n = 1) Front of the station (n = 1)	Indoor Indoor Outdoor Outdoor	9
		Bus station [a] (n = 5)	One sample in each for a total of 5 samples	Outdoor	
	City C (Center)	Train station (n = 1)	Entrance hall (n = 1) Waiting room (n = 1) Middle platform (n = 1) Front of the station (n = 1)	Indoor Indoor Outdoor Outdoor	9
		Bus station [a] (n = 5)	One sample in each station (n = 1)	Outdoor	
Surface sampling	City A to City B City B to City A City B to City C City C to City B	Trains (n = 14)	Window (n = 1) Arm rest (n = 1) Grab handles [b] (n = 2) Door button (n = 1)	Indoor	70

[a] Bus stations were single-bus stations in central areas of the city. [b] Handles are located on the top of seats so that people travelling standing up can use them to hold on safely while on the train.

2.2. Collection of Air and Surface Samples

Air samples were collected using the Coriolis® Compact (Bertin Instruments, Montigny-le-Bretonneux, France) cyclonic microbial air sampler with an airflow rate of 50 L/min (total of 1.5 m^3) for 30 min. The sampler was placed at an approximately 1 m height in every sampling site. Air samples were collected on a dry medium, with 4 mL of sterile phosphate-buffered saline (PBS) added to the sterile collection cones after each sampling. After every sampling, the cleaning and decontamination of the sampler were performed according to the manufacturer's instructions. Briefly, a wipe dampened with a surfactant–water solution was used to clear the external parts of the Coriolis compact. After that, the sampler was wiped down with a soft cloth to remove any excess.

Surface samples were collected on 10 cm × 10 cm surface areas (100 cm^2) using sterile flocked plastic swabs previously wetted on PBS and immediately placed in vials containing 4 mL of PBS. All samples were stored at 4 °C in sterile 1.5 mL RNAse-free Eppendorfs until transportation to the laboratory facilities and processed within 24 h in order to ensure the minimum loss of viral RNA.

It should be noted that the person performing the sampling was wearing KN95 masks and gloves at all times, with their mask being changed every 4 h, their gloves being changed after handling each sample, and their hands being washed between handling each sample.

2.3. RNA Extraction and RT-qPCR

RNA extractions were performed using the GRS Viral DNA/RNA Purification Kit (GRISP, Porto, Portugal) according to the manufacturer's instructions. RNA extraction was performed using 200 µL of sample suspension, as previously described [20]. A one-step RT-qPCR reaction targeting two viral gene targets (N1 and N2) was used (Xpert qDetect COVID-19, GRISP, Porto, Portugal). Selected targets, corresponding primer sequences, and probes were chosen based on a previously described protocol [21]. The CFX Real-Time PCR (qPCR) Detection System (Bio-Rad, Hercules, CA, USA) with the CFX Maestro 1.0 Software version 4.0.2325.0418 (Bio-Rad, Hercules, CA, USA) was used to control the runs

and remotely analyze the data. Each RT-qPCR run included ssDNA targets for both the N1 and N2 regions (positive controls) and a no-template control. The PBS used for the collections was also tested through RT-qPCR in order to show that no virus was present in it and therefore exclude the possibility of false positives through the contamination of the suspension media. Reactions were set up and run with the initial conditions of 15 min at 45 °C and 2 min at 95 °C, then 45 cycles of 95 °C for 15 s and 55 °C for 30 s. A standard curve was generated using ssDNA targets for both the N1 and N2 regions in a 10-fold serial dilution mixture starting at 200,000 copies/µL in order to quantify the number of viral gene copies present in each sample from the measured Ct values, with limit of detections (LODs) of 1.3 copies/µL for N1 and 3.2 copies/µL for N2. Air sample results are expressed in copies/m^3 and surface sample results in copies/cm^2.

3. Results

During the sampling period of this study, Portugal was between two waves of COVID-19 outbreaks and the rate of transmission of SARS-CoV-2 was considered low, with a national incidence of 64.3 cases of COVID-19 per 100,000 inhabitants [22]. The effective reproduction number R(t) for SARS-CoV-2 was 1.03 and 1.00 for Portugal's northern and central regions, respectively [19].

Of the 31 air samples collected in the public transport systems, only two (both from the train station of City A, one collected in front of the station (open outdoor area) and the other in the entrance hall (partially open)) were positive for SARS-CoV-2 RNA, which corresponds to a positivity of 9.5% (2 out of 21) of the outdoor air samples, compared to 0% (0 out of 10) of the indoor air samples. Both positive air samples were taken on the same day, with an interval of ~10 min between each other. The sample from the front of the train station was amplified for target genes N1 (8500 gene copies/m^3) and N2 (6025 gene copies/m^3), while the sample from the entrance hall of the train station was amplified only for N2 (6312 gene copies/m^3). Positivity for only one region has often been reported in other studies, which is likely due to primer mismatching [23].

Of the 70 surface samples collected in different trains going to and coming from the three cities, none were positive for SARS-CoV-2 RNA.

4. Discussion

Studies on SARS-CoV-2 in outdoor public locations are scarce and the results are contradictory, with some reporting high rates of SARS-CoV-2 RNA positivity in these environments [8] and others reporting no viral presence at all [16,17,24,25]. In the present study, out of 31 air samples collected in public transport systems, only 2 were found to be positive for SARS-CoV-2 RNA, with all surface samples being negative for the presence of viral RNA. Regarding surface samples, this result is not unexpected considering the infection prevention measures enforced in Portugal at the time, in which train surfaces were disinfected before passengers were allowed to go in, added to the fact that all passengers were wearing surgical or KN95 masks at all times when inside the trains, therefore decreasing the probability of viral shedding to the environment inside trains. The two positive samples were taken from City A at the same train station, with the first sample being taken in an outdoor open space. This train station is located at the center of the City A and is composed of an entrance hall connected to the train platforms in the back, which are in the open air. Moreover, the main entrance doors remain open, therefore allowing for constant natural ventilation within the station. These two samples were taken in the morning in the rush hour—i.e., between 7 and 9 am—when the train station is crowded and is more full of people due to work commutes. In this case, the large number of people passing by during the sampling period can explain the detection of SARS-CoV-2 in the air. The presence of SARS-CoV-2 in public spaces is reported to be directly related to the number of infected people in that environment—i.e., the more people are present, the more likely it is that high viral concentrations of the virus might be present [26,27]. It is of note

that the 14-day cumulative incidence rate per 100,000 inhabitants in the northern region of the country, where City A is located, was 64.7 when the positive sample was collected.

The first issue to be considered when interpreting the discrepant results of our study and previous studies on this subject is the lack of a standard methodology for sampling air when it comes to SARS-CoV-2. Additionally, different research groups use different parameters in their sampling, such as airflow rate, sampling duration, sample storage, and air sampler type, which should be taken into consideration. The air sampler itself is a very important factor, as air samplers with different collection methodologies (cyclone, impinger, impactor, filter, water condensation) might have different efficiency recovery rates for sampling SARS-CoV-2. Second, it should be considered that the sampling protocols used in these studies are implemented in different countries and/or cities and at different months throughout 2020 and 2021, which means that the meteorological conditions and epidemiological situation in each studied location will vary greatly. Furthermore, no control samples were used during our experiment. as the air sampling took place in indoor and outdoor public spaces where people were present at all times; therefore, it was not possible to perform air sampling in a controlled environment with clean air.

The global positivity in this study was low. Notwithstanding, the spaces where viral RNA was detected in air were either partially open (entrance hall of the train station) or completely open to the outdoor air (front of the train station), where more people not wearing masks were present, in contrast to the air of closed spaces where everyone was wearing a mask at all times and no positive surface sample could be detected. The presence of viral RNA in outdoor air, where ventilation is optimal, suggests that the presence of SARS-CoV-2 in the air is not linked to proper ventilation but rather to the number of people present in that environment and if they are wearing properly fitting masks or not, especially considering that no viral RNA could be detected in the public indoor spaces of the urban public transport systems, where, according to Decree-Law No. 78-A/2021 in force in Portugal at the time, the use of proper masks (of surgical grade or higher) was mandatory, with penalties that varied between EUR 100 and 500 for those who failed to follow the law, reinforcing the notion that the proper use of masks greatly decreases the amount of virus emitted to the environment.

It is important to highlight the value of maintaining preventive measures such as the regular disinfection of public transport system areas, proper ventilation, good hand hygiene, and wearing well-fitting masks at all times. However, even though the risk of infection in these environments is potentially low, the results presented here show that the risk of airborne transmission of COVID-19 in public transports exists in certain circumstances—namely, when infection prevention measures such as proper ventilation, wearing appropriate masks, and keeping at least 1 m of space between people are not strictly followed. Additionally, caution is needed when interpreting the results of this study because positive samples do not necessarily correlate to infectious SARS-CoV-2 particles. Moreover, even if viable particles were present, the viral loads found in the air could have been below the minimum infective dose of SARS-CoV-2 needed to cause COVID-19.

5. Conclusions

In this study, we report the detection of SARS-CoV-2 RNA in the outdoor environments of the urban public transport systems of Portugal. These outdoor environments were crowded and the majority of people were not wearing protective masks. Considering the current lifting of COVID-19 restrictions all around the world, it is essential that people continue to wear masks in both indoor and outdoor environments, especially in crowded spaces, as wearing a mask decreases the risk of transmission through air. More studies including data about viral viability, duration of exposure, frequency of exposure, number of people wearing masks, and distribution of virus-laden particles of different sizes are needed to fully elucidate the real risk of infection in outdoor spaces.

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CHAPTER 4

SARS-CoV-2 in outdoor air following the third wave lockdown release, Portugal, 2021 (Article 4)

SARS-CoV-2 presence in outdoor air of public spaces in Valladolid during winter, 2021 (Article 5)

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SARS-CoV-2 in outdoor air following the third wave lockdown release, Portugal, 2021

Priscilla Gomes da Silva[1,2,3,4,5], Maria São José Nascimento[6], Sofia I. V. Sousa[6,5] and João R. Mesquita[1,2,3,*]

Abstract

Aiming to contribute with more data on the presence of SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) in outdoor environments, we performed air sampling in outdoor terraces from restaurants in three major cities of Portugal in April 2021, following the third wave lockdown release in the country. Air samples (n=19) were collected in 19 restaurant terraces during lunch time. Each air sample was collected using a Coriolis Compact air sampler, followed by RNA extraction and real-time quantitative PCR for the detection of viral RNA. Viral viability was also assessed through RNAse pre-treatment of samples. Only one of the 19 air samples was positive for SARS-CoV-2 RNA, with 7337 gene copies m^{-3} for the genomic region N2, with no viable virus in this sample. The low number of positive samples found in this study is not surprising, as sampling took place in outdoor settings where air circulation is optimal, and aerosols are rapidly dispersed by the air currents. These results are consistent with previous reports stating that transmission of SARS-CoV-2 in outdoor spaces is low, although current evidence shows an association of exposures in settings where drinking and eating is possible on-site with an increased risk in acquiring SARS-CoV-2 infection. Moreover, the minimal infectious dose for SARS-CoV-2 still needs to be determined so that the real risk of infection in different environments can be accurately established.

DATA STATEMENT

All the data used in this study are available upon request.

HIGHLIGHTS

- The overall rate of sample positivity in outdoor air was low
- No viable virus was detected in the positive sample
- RNAse pretreatment of samples can be an alternative to culture for assessing viral viability
- SARS-CoV-2 was present only in a crowded outdoor space

INTRODUCTION

The World Health Organisation (WHO) has outlined three transmission modes as responsible for SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) infection, namely respiratory droplets, aerosols and fomites [1]. The relative impact of each of these transmission modes is critical to disease control and mitigation strategies [2]. Now, more than 2 years after the pandemic started, and with the accumulation of new data about the virus and dynamics of transmission, the fomite route has been ruled out

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Author affiliations: [1]ICBAS – School of Medicine and Biomedical Sciences, Porto University, Porto, Portugal; [2]Epidemiology Research Unit (EPIUnit), Instituto de Saúde Pública da Universidade do Porto, Porto, Portugal; [3]Laboratório para a Investigação Integrativa e Translacional em Saúde Populacional (ITR), Porto, Portugal; [4]LEPABE – Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Porto, Portugal; [5]ALiCE – Associate Laboratory in Chemical Engineering, Faculty of Engineering, University of Porto, Porto, Portugal; [6]Faculty of Pharmacy, University of Porto, Porto, Portugal.
***Correspondence:** João R. Mesquita, jrmesquita@icbas.up.pt
Keywords: SARS-CoV-2; airborne transmission; outdoor air; COVID-19; air sampling.
Abbreviations: NTC, no template control; PoC, point of care; RP, RNAse P; R(t), effective reproduction number; RT-qPCR, real-time quantitative PCR; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; WHO, World Health Organisation.
Four supplementary tables are available with the online version of this article.
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as a major contributor to the spread of the virus worldwide [3], and the airborne route has been established as the predominant mode driving the pandemic [4].

Consistent with an airborne pathogen, it has been demonstrated that transmission of SARS-CoV-2 is higher indoors than outdoors [5] and can be substantially reduced by indoor ventilation [6]. Outdoor spaces have some advantages over indoor ones when considering the transmission risk of SARS-CoV-2, as they allow for physical distancing, more airflow, better ventilation and the absence of recycled air – all these features, in theory, will help to minimize airborne transmission [7]. This is reflected in prevention guidelines and mitigation strategies, which recommend keeping social gatherings in outdoor environments whenever possible to decrease the risk of infection [8, 9]. Nonetheless, the risk of infection in outdoor spaces should not be underestimated when considering other factors that might affect transmission in these environments, notably the number of people present, wind speed, temperature and relative humidity of the air [10, 11].

Some studies have been published reporting SARS-CoV-2 presence in outdoor environments [12–18], but little is known about the dynamics of transmission outdoors and which factors might reduce safety in these settings [8]. Although SARS-CoV-2 presence in indoor and outdoor air has been extensively studied, little is known about the viability of the virus in the air, mainly because it requires a BSL-3 laboratory facility where cell culture viability assays need to be carried out [19]. Considering alternative methods such as nuclease pretreatments can be used to assess viral viability [20].

Aiming to contribute with more data on the presence and viability of SARS-CoV-2 in outdoor environments, this study reports the results of an air sampling campaign performed in crowded outdoor terraces of restaurants in three major cities of Portugal (one from the North and two from the Centre) in April 2021 following the third wave lockdown release.

METHODS

Air samples (n=19) were collected in 19 restaurant terraces, with nine samples from the city in the North region and ten from two cities in the Centre region (five samples in each city). During the sampling period, the effective reproduction number $R(t)$ for SARS-CoV-2 was 0.99 and 1.04 in the North and the Centre region of Portugal, respectively [21].

All samples were collected during lunch when the maximum capacity of users was present. The area and density of people on each terrace are summarized in Table 1. Each air sample was collected using one (n=1) Coriolis Compact (Bertin Instruments) microbial air sampler placed over the restaurant table for 30 min with an airflow rate of 50 l min^{-1} (total of 1.5 m^3). The sampler was placed a distance of 1.5 m from every restaurant's terrace and at a height of approximately 1.3 m at all sampled locations so as not to bother people during their meal, as the sampler makes a loud noise during air sampling. Air samples were collected on a dry medium, with 4 ml of sterile PBS added to the collection cones after each sampling. After every sampling, the sampler was cleaned and decontaminated according to the manufacturer's instructions. Briefly, a wipe dampened with a surfactant–water solution was used to clear the external parts of the Coriolis Compact. After that, the sampler was wiped down with a soft cloth to remove any excess.

RNA extractions were performed using the GRS Viral DNA/RNA Purification Kit (GRISP) by using 200 µl of sample suspension, as previously described [22, 23] and according to the manufacturer's instructions. Briefly, lysis of viral particles was done using a lysis buffer, with the buffer system being optimized to allow selective binding of nucleic acids to the glass fibre matrix of the spin column [24]. Contaminants were removed using two washing buffers followed by centrifugation steps. The purified RNA was subsequently eluted in 50 µl of RNase-free water.

To detect SARS-CoV-2 RNA, a commercial one-step real-time quantitative PCR (RT-qPCR) targeting two viral genes (N1 and N2) using viral target-specific primers and Taqman probe technology was used (Xpert qDetect COVID-19; GRISP). The human RNase P gene (RP) was not included in the PCR runs as this study consists of environmental samples, therefore excluding the need of a control for human cells. Selected targets, corresponding primer sequences and probes were according to the protocol published by the US CDC [25]. Sequencing details for the N1 and N2 genes can be found in Table S1 (available with the online version of this article). Each run included a SARS-CoV-2-positive control consisting of a plasmid harbouring the complete nucleocapsid gene (N) from SARS-CoV-2 (binding sites for primers and probes can be found in the online supplementary materials), and a no-template control (NTC) consisting of nuclease-free water. The PBS used for the air sampling was also tested through RT-qPCR to verify that no virus was present in it and therefore exclude the possibility of false positives through contamination of the suspension media. A standard curve was generated using ssDNA targets for both the N1 and N2 regions in a 10-fold serial dilution mixture starting at 200 000 copies μl^{-1} for quantification of the number of viral gene copies present in each sample from the measured Ct values, with limit of detections (LODs) of 1.3 copies μl^{-1} for N1 and 3.2 copies μl^{-1} for N2.

The viral viability of the positive air samples was evaluated by RT-qPCR preceded by a nuclease pretreatment using a previously described method with some minor modifications [26]. Briefly, prior to RNA extraction, three aliquots of the positive sample were pretreated with 8 µl of RNAse A (GRISP) in the following concentrations, 1, 10 and 50 µg μl^{-1}, and incubated at 37 °C for

Table 1. Area, number of people present and density of people for every sampled terrace

	Terrace	Area (m^2)	No. of people present	Density of people (m^{-2})
City A – North Region	1	328.4	110	0.33
	2	372.5	50	0.13
	3	82.5	25	0.30
	4	83.9	35	0.41
	5	176.5	45	0.25
	6	316.4	40	0.12
	7	44.6	20	0.44
	8	26.4	15	0.56
	9	31.4	25	0.79
City B – Centre Region	1	106.3	40	0.37
	2	44.6	30	0.67
	3	9.9	12	1.21
	4	8.5	8	0,94
	5	82.7	40	0.48
City C – Centre Region	1	169.1	48	0.28
	2	7.02	12	1.70
	3	177.8	120	0.67
	4	35.4	24	0.67
	5	95.8	40	0.41

30 min. An aliquot of the untreated sample was also tested in each reaction to assess viral load differences and hence quantify the amount of viable virus present in the sample.

RESULTS AND DISCUSSION

Of the 19 air samples, only one was positive for SARS-CoV-2 RNA, with 7337 gene copies m^{-3} for the genomic region N2. PCR results for the standard curve and positive control, as well as for the tested samples can be found in Tables S2 and S4. Positivity for only one region has often been reported in other studies, probably due to primer mismatching [27]. Genetic variations in the viral genome at primer/probe binding regions resulting from mutations can result in these potential mismatches and false-negative results [28]. This sample was collected on 5 April 2021, on terrace number 1 of City B in the Centre region, on a sunny day with an air temperature of 15.2 °C during the lunch hour, and density of people of 0.37 m^{-2}. At the moment of air sampling, all tables in the restaurant were at full capacity, with most people at the tables not wearing masks because they were eating and drinking. Notwithstanding this, the Centre region of Portugal, where the only positive sample was collected, presented a higher $R(t)$ than the North region (1.08 and 0.98, respectively) at the time the air sampling campaign took place.

The low number of positive samples in our study was expected and can be explained by optimal air circulation in outdoor restaurant terraces and the fact that the virus is highly diluted in the air, as well as the fact that aerosols are rapidly dispersed by air currents [29]. Environmental factors such as UV radiation, temperature and humidity also contribute to the low risk of transmission in outdoor environments, as they can help inactivate viable viruses in the air [30, 31]. We did not retrieve data regarding the vaccination status of individuals sitting at the terrace tables that could ultimately aid in interpreting the environmental findings of this study. Of note, only 6% of the country's population had a complete vaccination status, that is two doses of the vaccine [32]. Comparative information regarding the vaccination status in Portugal until 11 April 2021 can be found in Table S2.

The RNAse pretreatment method for assessing viral viability assumes that viable viruses with intact capsids and envelopes could not have their genetic material degraded by RNAse [33]. Therefore, after pretreatment, amplification during RT-qPCR would come only from viable viruses, excluding the need for cell culture in BSL-3 facilities [33]. In this study, no amplification was observed in any of the samples after pretreatment with 1, 10 and 50 μg ml^{-1} RNAse, indicating that the viruses present in

this sample probably did not have an intact capsid, allowing the RNAse to degrade their genetic materials, and resulting in no amplification during RT-qPCR. Nonetheless, the non-treated samples still showed amplification when tested at the same time as the RNAse-pretreated samples.

The concentrations of RNAse used in this study are based in a study by Marti *et al.*, in which DNase and RNase pretreatments were used to detect intact particles. In the present study, three concentrations of RNase A that specifically degrades ssRNA were tested (1, 10 and 50 µg ml^{-1}). Of these, 10 µg ml^{-1} was considered more appropriate to treat viral-eluted samples from fresh produce. This was due to the fact that although 50 µg ml^{-1} RNAse degraded 7 log_{10} of free RNA from the genomes of inactivated viruses, it was not possible to inactivate the enzyme at this concentration before extracting genomes from intact viruses. Moreover, the results in this study confirmed that RNase at 10 µg ml^{-1} was able to degrade more than 5 log_{10} of inactivated virus without affecting the RNA from undamaged virus [26].

More recently, it has been shown that the efficiency of removal of non-viable viruses by nuclease pretreatments was generally <30% as predicted by cell culture [34], with previous studies also reaching similar conclusions [35, 36]. In one study, it was determined that viruses which had been inactivated with UV, chlorine and 72 °C yielded a true-negative RT-PCR after undergoing composite enzymatic digestion with both proteinase K at 20 U and RNase at 100 ng followed by incubation at 37 °C for 30 min prior to RNA extraction [37]. Additionaly, in a different study by the same author, it was shown that the capsid from viruses which had undergone heat inactivation at 37 °C were still capable of protecting their viral genome from RNase [38].

When interpreting the results of our study, although they indicate that non-infectious viruses were present in the SARS-CoV-2 RNA-positive air sample, one cannot exclude the possibility that the collection process could have contributed to the inactivation of SARS-CoV-2, as previously suggested [39], or that any thermal inactivation might have occurred during the enzymatic pretreatment as a result of the long exposure at 37 °C [33]. Nonetheless, previous reports on viable SARS-CoV-2 recovered from air samples are in accordance with the fact that no viable virus was present in the positive sample in this study, as it had only 7337 gene copies m^{-3} and much higher values are reported for viable virus recovered from air samples in the literature. In a study by Lednicky *et al*., culturable virus could be recovered from four samples with 16 000, 30 000, 44 000 and 94 000 gene copies m^{-3} of air, respectively [40]. Another study by the same author reported that culturable virus could be recovered from an air sample with 31 400 gene copies m^{-3} of air [41]. In a different study, it was reported that culturable virus could be recovered from an air sample with 1010 000 gene copies m^{-3} of air [42].

It is important to highlight that the effectiveness of a pretreatment method can only be determined for culturable viruses, and, ideally, viral culture should be performed together with nuclease pretreatments for a more precise interpretation of the results [34].

During the sampling period of this study, April 2021, the North and Centre regions had a 14 day cumulative incidence rate below 60 cases per 100000 inhabitants [43] and the reproduction number $R(t)$ was 0.99 and 1.04 in the North and the Centre region of Portugal, respectively [21]. Officially registered epidemiological data on COVID-19 showed a national increasing tendency in $R(t)$ between 11 February and 8 April 2021 [44], a period that included the national lockdown release following the third wave of COVID-19 in Portugal, when only restaurants, pastry shops and cafes with open terraces were allowed to reopen, only outdoors, and with groups limited to a maximum of four people.

Evidence has been found supporting the low risk of holding mass-gathering events in outdoor settings and an increase in COVID-19 cases associated with spreading events where no measures had been implemented [45]. In an international prospective cohort study, individual-level factors associated with an increased risk for COVID-19 were an increase in the number of non-household contacts, attending events of at least 10 people and restaurant visits [46]. In another study, COVID-19 infection was associated with dining at a restaurant (any area designated by the restaurant, including indoor, patio and outdoor terraces) in the 2 weeks preceding illness onset [47]. The role of different settings and activities in acquiring SARS-CoV-2 infection was also assessed in a nationwide online case-control study in France, showing an increased risk of infection associated with having frequented bars and restaurants [48]. The effect of two mass-gathering outdoor events on the incidence of SARS-CoV-2 infection was also analysed in northeast Spain, showing that those attending outdoor music festivals had a two-fold higher incidence of COVID-19 than that of a population-based control group [45].

It is important to emphasize that the minimal infectious dose of SARS-CoV-2 still has not been determined, with most of what is known on this topic coming from studies with influenza viruses [49–51]. Answering this question is still essential for performing an accurate risk assessment of the real risk of infection with SARS-CoV-2 in both outdoor and indoor settings. Without this crucial piece of information, most of what has been published so far on the airborne transmission of the virus comes from correlation and observation-based studies and not quantitative models of the physical processes of transmission, which would be more appropriate regarding public policies intended to prevent transmission in the community [30].

However, although our results are consistent with previous reports on the lower incidence of SARS-CoV-2 in outdoor air, an association of exposures in bars, restaurants and coffee shops with an increased risk in acquiring SARS-CoV-2 infection has been pointed out in previous studies, showing that exposures and activities where wearing masks and social distancing are difficult to maintain might be important risk factors for acquiring COVID-19 [45–48].

It is important to emphasize the limitations of our study, namely the low number of sampled terraces and the lack of a BSL-3 laboratory facility to confirm the results of viral viability with cell culture assays, as per regulations by the WHO. However, although SARS-CoV-2 infectiousness of the positive air sample could not be confirmed by this gold standard method, we tentatively used the pretreatment with RNase as an alternative method, which could be considered as an option over cell culture in situations where rapid detection of viable virus is needed, such as point-of-care (PoC) diagnosis and outbreak investigations [34]. More community exposure studies in these outdoor settings that include viral viability assessment are still needed, and determination of the minimum infectious dose of SARS-CoV-2 is urgently required to improve risk assessment and help to give better prevention strategies for SARS-CoV-2 airborne transmission.

Conclusion

The results of our study show that SARS-CoV-2 circulates in outdoor air and can be detected in outdoor environments, especially during exposures and activities where wearing masks and social distancing are difficult to maintain, such as when eating or drinking. Although wearing of masks is no longer mandatory in many countries, it might still be the most efficient way to avoid SARS-CoV-2 infection in situations such as those mentioned above. Moreover, nuclease pretreatments of samples prior to RNA extraction could be considered as an alternative over cell culture in situations where rapid detection of viable virus is needed, such as PoC diagnosis and outbreak investigations.

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Author contributions
P.G.S: Conceptualization, Methodology, Investigation and Writing – Original Draft. J.R.M: Conceptualization, Methodology, Writing – Review & Editing, Supervision. S.I. V. S: Methodology, Writing – Review & Editing, Supervision. M.S.J.N: Writing - Review & Editing, Formal Analysis.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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RESEARCH

SARS-CoV-2 RNA Presence in Outdoor Air of Public Spaces in Valladolid During Winter, 2021

Priscilla Gomes da Silva[1,2,3,4,5] · José Gonçalves[6] · Elisa Rodriguéz[7,8] · Pedro A. García-Encina[7,8] · Maria São José Nascimento[9] · Sofia I. V. Sousa[4,5] · João R. Mesquita[1,2,3]

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Abstract
As SARS-CoV-2 continues to evolve and herd immunity establishes, an increasing number of asymptomatic infections have been reported, increasing the risk of airborne spread of the virus. Most of the studies regarding SARS-CoV-2 RNA presence in air refer to indoor environments, with few studies having reported SARS-CoV-2 RNA in outdoor air. The aim of this study was to assess the presence of SARS-CoV-2 RNA at two different settings, crowded outdoor versus empty outdoor environments in Valladolid, Spain, during winter 2021. Using a Coriolis® air sampler, samples were taken from nine different locations within the city center. RNA extraction and a one-step RT-qPCR were carried out. Six out of the 20 air samples were found to be positive, and they were all obtained from crowded outdoor environments. These results highlight that although in less quantity, SARS-CoV-2 RNA is still present in outdoor air, especially at moments of relaxed mitigation efforts and depending on the number of people present.

Keywords SARS-CoV-2 · Airborne SARS-CoV-2 · Airborne transmission · COVID-19 · Public health

Introduction

Three years have passed since the World Health Organization (WHO) declared the COVID-19 pandemic (WHO, 2023a), and even though there are now highly effective vaccines and high vaccination coverage in most parts of the world (WHO, 2024), new waves of COVID-19 are still emerging worldwide, mainly due to breakthrough infections in vaccinated individuals and reinfections (Doke et al., 2023). These waves are driven mainly by the rise of new variants of concern such as Omicron (B.1.1.529), which are more transmissible and can evade our immune system more easily (Tan et al., 2023). As of May 2023, the XBB.1 descendant lineages (XBB.1.5, XBB.1.16, and XBB.1.9) predominate globally (WHO, 2023b).

The WHO reported three modes of transmission for SARS-CoV-2 infection: through respiratory droplets, aerosols and fomites (WHO, 2021). SARS-CoV-2 is transmitted from person to person either by direct (person-to-person contact and droplet spread) or by indirect contact with surfaces (fomites) where virus-containing droplets expelled by an infected person have been deposited (Morawska & Cao, 2020), whereas airborne transmission can happen via droplets or aerosols generated during coughing, sneezing,

✉ João R. Mesquita
jrmesquita@icbas.up.pt

1 ICBAS – School of Medicine and Biomedical Sciences, Porto University, Porto, Portugal

2 Epidemiology Research Unit (EPIunit), Institute of Public Health, University of Porto, Porto, Portugal

3 Laboratório Para a Investigação Integrativa E Translacional Em Saúde Populacional (ITR), Porto, Portugal

4 LEPABE – Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Porto, Portugal

5 ALiCE – Associate Laboratory in Chemical Engineering, Faculty of Engineering, University of Porto, Porto, Portugal

6 MARE - Marine and Environmental Sciences Centre, ARNET - Aquatic Research Network Associate Laboratory, NOVA School of Science and Technology, NOVA University Lisbon, Caparica, Portugal

7 Institute of Sustainable Processes, Valladolid University, Valladolid, Spain

8 Department of Chemical Engineering and Environmental Technology, University of Valladolid, Valladolid, Spain

9 Faculty of Pharmacy, University of Porto, Porto, Portugal

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talking, singing or breathing (Jones & Brosseau, 2015). This airborne spread of an infectious pathogen is caused by the dissemination of aerosols that remain infectious when suspended in air over long distances and periods of time (WHO, 2020b).

The probability of airborne transmission is contingent on several still-uncertain parameters, including virus-laden aerosol concentrations, viability and longevity, and the minimum infectious dose required for infection (Buonanno et al., 2020; Contini & Costabile, 2020). Three factors significantly affect the viral load of aerosols discharged from a SARS-CoV-2 infected person: when disease has started; the severity of the illness; and physiological characteristics that control the rate at which aerosols and therefore viral particles are emitted (Cox et al., 2023). Nonetheless, viral dispersion strongly influences SARS-CoV-2 transmission (Poydenot et al., 2022), thus, experimental studies of aerosol dispersion would help avoid and control future public health emergencies (Fen et al., 2023).

Still, as SARS-CoV-2 keeps changing and herd immunity builds, more and more cases of asymptomatic infections are reported, making airborne transmission more likely (Lee et al., 2020). It is also important to consider the role that asymptomatic cases play in the spread of COVID-19 in the community (Gao et al., 2021), as it is particularly important in crowded places, where the chance of transmission significantly increases (Zhang et al., 2023).

In one study, about half of patients who were diagnosed with COVID-19 claimed to be unsure about whether they had contact with an infected person or not (Tenforde et al., 2020), raising the possibility that these patients' lack of information about previous COVID-19 exposures was due to the fact that the exposure happened in environments generally accepted as safe, such as outdoor environments (Clouston et al., 2021). Outdoor social events, such as backyard barbecues, viewing outside events, standing in line, or socializing outdoors, may be sensitive to factors that may affect their protective properties, such as air turnover rate and number of people present (Clouston et al., 2021).

However, most of the studies regarding SARS-CoV-2 RNA presence in air refer to indoor environments and healthcare-related facilities, with only a few studies until this date having reported SARS-CoV-2 RNA presence in outdoor environments (Chirizzi et al., 2021; Dunker et al., 2021; Passos et al., 2021; Pivato et al., 2021; Silva et al., 2023; Tao et al., 2022). Notably, outdoor airborne transmission has been pointed as playing a significant role in the COVID-19 outbreak that occurred in northern Italy in the winter of 2020 (Conticini et al., 2020; Setti et al., 2020a; Setti et al., 2020b).

With that in mind, the aim of this study was to assess the presence of SARS-CoV-2 RNA in outdoor air environments in the city of Valladolid, Castille y León, Spain, during winter 2021, at a time when COVID-19 reported cases were starting to increase in the country (The New York Times, 2023). We also aimed to compare SARS-CoV-2 RNA presence in air at two different settings, crowded outdoor environments versus empty outdoor environments, as this data might be relevant for prevention strategies and public health.

Materials and Methods

Sampling Locations

Air sampling was performed in December 2021, in outdoor areas of the city of Valladolid, Castille y León, Spain. Air samples ($n = 20$) were collected from nine different locations, with each location being sampled twice, namely in the afternoon, between 13:00 and 17:00, when most of the stores were closed and streets were mostly empty, with no people or only one person at most passing by during all the sampling periods—designated from now on as "empty-street scenario" –, and again after this period, between 18:00 and 23:00, when stores were open again and streets had a big influx of people passing by in every direction, with at least 30 people passing by the sampler every minute with less than 1 m distance between them – "crowded-street scenario". Sampling locations are represented in Fig. 1. At one of the locations (number 9), two samples were collected in each period of the day because it was the place with more density of people. Of note, there were at least 50 people close to the sampler during the whole sampling time on location 9.

Air Sampling

Air samples were collected using the Coriolis® Compact (Bertin Instruments, Montigny-le-Bretonneux, France) dry cyclonic air sampler with an airflow rate of 50 L/min for 20 min (total of 1 m^3). The sampler was placed at approximately 1.5 m height in every sampling site. Air samples were collected at first in an empty sterile collection cone. After each sampling, 4 mL of sterile phosphate buffered saline (PBS) (GRISP, Porto, Portugal) was added to the collection cone. All samples were stored at 4°C until transportation to the laboratory facilities and were processed within 24 h.

Following manufacturer directions, the sampler was cleaned and decontaminated after each sampling. Surfactant–water wipes cleaned the Coriolis Compact's exterior. After that, the sampler was wiped clean.

During air sampling KN95 masks and gloves were used by the operator, with masks replaced after each sampled area and gloves after each sample. Hands were properly washed and disinfected between handling of each sample.

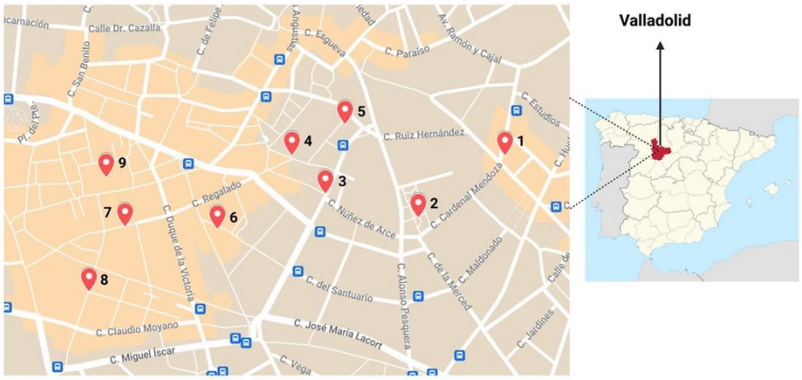

Fig. 1 Sampling locations: in the city center of Valladolid: 1—street; 2—public square; 3—street; 4—stairs of a church; 5—university' square; 6—front of the post-graduate students' dormitory; 7—commerce street; 8—commerce street; 9—Christmas market at the city's main square. Figure created with BioRender.com

RNA Extraction and RT-qPCR

RNA extractions were performed using the GRS Viral DNA/RNA Purification Kit (GRISP, Porto, Portugal) according to the manufacturer's instructions. RNA extraction was performed using 200 µL of samples suspensions as previously described (Santarpia et al., 2020). A one-step RT-qPCR targeting the two viral genes N1 and N2 of SARS-CoV-2 was used (Xpert qDetect COVID-19, GRISP, Porto, Portugal). The selected targets, corresponding primer sequences and probes are based on a protocol previously described (CDC, 2020). The CFX Real-Time PCR (qPCR) Detection System (Bio-Rad, USA) with the Bio-Rad CFX Maestro 1.0 Software version 4.0.2325.0418 was used to control the runs and remotely analyze the data. Each RT-qPCR run included ssDNA targets for both N1 and N2 regions (positive controls) and a no-template control. Reactions were set up and run with initial conditions of 15 min at 45 °C and 2 min at 95 °C, then 45 cycles of 95 °C for 15 s and 55 °C for 30 s. A standard curve was generated using ssDNA targets for both N1 and N2 regions, in a tenfold serial dilution mixture starting at 200,000 copies/µL for quantification of the number of viral gene copies present in each samples from the measured Ct values, with a limit of detection (LOD) of 1.3 copies/µL for N1 and 3.2 copies/µL for N2. All samples were tested in duplicates, and air sample results were expressed in copies/uL.

Results

From the total of the 20 air samples tested, six were positive for SARS-CoV-2 RNA. In the first PCR run, all 14 negative samples did not amplify at all. In the second PCR run for the same samples, 11 did not amplify at all, two amplified with $Ct > 38$ for one of the targets (which is the cut-off value for the protocol we used), and one amplified only for target N2 with a $Ct > 38$. Considering the manufacturer's instructions about the cut-off value, and the fact that they amplified in only one of the PCR runs, we did not consider these three samples as positives.

None of the samples collected during the "empty-street scenario" (period of the day between 13:00 and 17:00) were positive for SARS-CoV-2 RNA, whereas all the positive samples were collected during the "crowded-street scenario" (period of the day between 18:00 and 23:00). Details about the air sampling (date, period of the day, location) and number of SARS-CoV-2 RNA copies of the positive samples are summarized in Table 1.

Discussion

At the time of this investigation, in early December 2021, there was a rapid increase in COVID-19 cases reports as a result of the rapid spread of the Omicron variant (B.1.1.529) worldwide (Khemiri et al., 2022). This variant

Table 1 Details about location and RT-qPCR results for the positive samples

Sample location number	Date of air sampling	Period of the day	Sampled location GPS coordinates	Ct (N1)	Gene copies/µL (N1)	Gene copies/m^3 (N1)	Ct (N2)	Gene copies/µL (N2)	Gene copies/m^3 (N2)
1	03/12/21	13:00–17:00	41.6523930, − 4.7181090	–	–	–	–	–	–
2	03/12/21	13:00–17:00	41.6511800, − 4.7203800	–	–	–	–	–	–
3	03/12/21	13:00–17:00	41.6516460, − 4.7228040	–	–	–	–	–	–
4	03/12/21	13:00–17:00	41.6523970, − 4.7236870	–	–	–	–	–	–
5	03/12/21	13:00–17:00	41.6530230, − 4.7236120	–	–	–	–	–	–
6	03/12/21	13:00–17:00	41.65094, − 4.72564	–	–	–	–	–	–
7	03/12/21	13:00–17:00	41.6509970, − 4.7280660	–	–	–	–	–	–
8	03/12/21	13:00–17:00	41.64971, − 4.729	–	–	–	–	–	–
9	03/12/21	13:00–17:00	41.65195, − 4.72856	–	–	–	–	–	–
10	03/12/21	13:00–17:00	41.65195, − 4.72856	–	–	–	–	–	–
1	03/12/21	18:00–23:00	41.6523930, − 4.7181090	36.65	3.61	45,125	36.75	3.31	41,375
2	03/12/21	18:00–23:00	41.6511800, − 4.7203800	35.77	7.65	95,625	36.77	3.25	40,625
3*	03/12/21	18:00–23:00	41.6516460, − 4.7228040	38.11	–	–	36.82	–	–
4	03/12/21	18:00–23:00	41.6523970, − 4.7236870	37	2.68	33,500	35.93	6.72	84,000
5*	03/12/21	18:00–23:00	41.6530230, − 4.7236120	36.28	–	–	38.90	–	–
6*	03/12/21	18:00–23:00	41.65094, − 4.72564	–	–	–	38.50	–	–
7	03/12/21	18:00–23:00	41.6509970, − 4.7280660	–	–	–	–	–	–
8	03/12/21	18:00–23:00	41.64971, − 4.729	32.38	141.25	1,765,625	32.14	173.34	2,166,750
9	05/12/21	18:00–23:00	41.65195, − 4.72856	35.03	14.52	181,500	36.39	4.51	56,375
10	05/12/21	18:00–23:00	41.65195, − 4.72856	32.53	123.42	1,542,750	32.47	130.41	1,630,125

– negative samples that did not amplify

*excluded samples due to Ct > 38 and/or amplification for only one of the target genes

had the capacity to infect six times as many people as Delta (B.1.617.2) over the same time and with an Rt of 1.4– to 3.1-fold higher than Delta (Callaway & Ledford, 2021; CDC, 2023; Tian et al., 2022). In addition, the B.1.1.529 Omicron variant showed to be able to propagate more easily among children than the previous variants (Chun et al., 2022).

Studying RNA detection of SARS-CoV-2 in different environments and matrices is crucial from a public health perspective for several reasons. Environmental monitoring, such as detecting SARS-CoV-2 RNA in wastewater and bivalve mollusks, provides an early warning of infection outbreaks in a community before clinical cases are identified, allowing for timely public health interventions to prevent the spread of the virus (Medema et al., 2020). Bivalve mollusks, which can accumulate viruses due to their ability to filter large amounts of water, can serve as indicators of virus

presence in aquatic environments (Lombardi et al., 2023). Similarly, understanding the presence of the virus on surfaces and in the air in various environments, like hospitals, public transportation, and schools, helps assess the risk of fomite and airborne transmission, leading to improved infection control measures (Morawska & Cao, 2020; Riddell et al., 2020).

In the current study, the presence of SARS-CoV-2 RNA was assessed in two different epidemiological contexts, "empty-street scenario" *versus* "crowded-street scenario", namely deserted streets versus busy streets. None of the air samples collected during the "empty-street scenario" were positive for SARS-CoV-2 RNA. However, the findings changed with samples collected during the "crowded-street scenario", as six samples from this scenario were found positive for SARS-CoV-2 RNA, representing 30% of all the air samples and 60% of samples collected on the "crowded-street scenario" timeframe.

In Spain, it is usual for stores to close between 14:00 pm and 17:00 pm, especially during the summer (Spain's official tourism website, 2023), with the majority of the streets ending up deserted during these hours. The air sampling campaign happened in December, when Christmas Markets were open and a great number of families with children were present outdoors in these places, contributing to an increased number of people on the streets at night, the timeframe where all the positive samples were collected. Events that attract a large number of people like Christmas markets, typically take place in confined spaces, either indoors or outdoors, for an extended period of time, during which attendees eat and drink in close quarters with one another. These conditions could increase the likelihood of SARS-CoV-2 spread and could destabilize the health system's ability to respond if a large number of individuals become infected (WHO, 2020a).

Environmental variables such as ultraviolet light (UV) exposure, temperature, relative humidity, wind currents, and ventilation systems can also influence air sampling results (Asif et al., 2022; Kumar et al., 2021), with SARS-CoV-2 showing to be considerably more resistant to environmental degradation than other enveloped viruses (Senatore et al., 2021). The environmental conditions in Valladolid during December are characterized by low temperatures ranging from 1.2 °C to 8.5 °C and high humidity levels averaging around 82% (Climate Weather, 2024; Weather Atlas, 2024). During both sampling days, THE average temperature was 9.7 °C and 10.7 °C, respectively, with average relative humidity of 84% and 73%, respectively (TuTiempo.net, 2021). Cold temperatures and high humidity are known to enhance the stability of the virus in aerosols, potentially increasing its persistence and transmissibility in the air (Audi et al., 2020a, 2020b). Moreover, the relatively low UV index (around 2) indicates minimal natural disinfection from UV radiation, which could further contribute to the prolonged viability of the virus on surfaces and in the air (Ratnesar-Shumate et al., 2020).

Moderate wind speeds in Valladolid during December, averaging 10 km/h, might also influence the dispersion of viral particles (Meteoblue, 2024). While higher wind speeds can disperse aerosols over larger areas, potentially reducing concentration, moderate winds may not significantly dilute viral loads in crowded outdoor settings (Dabisch et al., 2021). During the two sampling days, average wind speed was 21.4 and 20.8 km/h, respectively (TuTiempo.net, 2021). The combined effect of these factors suggests that winter conditions in Valladolid might facilitate airborne transmission of SARS-CoV-2, emphasizing the importance of indoor ventilation and adherence to public health measures during this season.

In a systematic review about the transmission of SARS-CoV-2 and other respiratory viruses in outdoor environments, authors concluded that the existing data support that outdoor environments likely pose a much smaller risk of SARS-CoV-2 transmission than indoor environments, mainly because outdoor environments allow for more physical distancing and better airflow and ventilation and less recycled air, all of which help to minimize airborne transmission (Bulfone et al., 2021b). In another study in which a simplified analytical model to compare the relative level of exposure occurring between comparable outdoor and indoor settings was developed the authors concluded that the risk of transmission outdoors is considerably lower than indoors (Rowe et al., 2021). However, authors adverted to situations of temperature inversion (when cool air is trapped at the ground beneath a layer of warm air) and low wind speeds, as it could result in levels of outdoor transmission close to those indoors, especially in crowded spaces (Rowe et al., 2021). In a study carried out in the municipality of Borriana, Spain, that attempted to calculate the odds of an outbreak of COVID-19 that occurred there and its correlation with large public gatherings, it was found that exposure during events where food and dancing took place played a major factor in the spread of COVID-19 (Domènech-Montoliu et al., 2021).

When looking at the findings of the present study, they are consistent with reports stating that risk of infection is lower outdoors, with SARS-CoV-2 RNA presence in air being directly related to the number of people present. In fact, when sampling took place with zero or only one person around, no SARS-CoV-2 RNA was detected, and when sampling took place in heavily crowded streets, where physical distancing of at least 1 m or more was not possible, more than half of the samples were positive for SARS-CoV-2 RNA.

Although the minimum infectious dose for SARS-CoV-2 in humans still hasn't been determined, it is generally assumed to be low due to the high transmissibility of

the virus (Karimzadeh et al., 2021). Research in hamsters suggests that low viral loads such as 10^3 or 10^5 50% tissue culture infective doses ($TCID_{50}$) could be sufficient to initiate infection with SARS-CoV-2 (Imai et al., 2020). Considering that the number of RNA copies/m^3 found in this study ranged from 2680 to 173,590, and presuming that the virus was still viable, would indicate a non-negligible risk of transmission, particularly in settings with high human density and limited ventilation. This is especially relevant considering that SARS-CoV-2 can remain viable and infectious in aerosols for extended periods under certain environmental conditions, as well as the fact that environments with lower temperatures and high humidity might be conducive to virus stability and transmission (Karimzadeh et al., 2021).

In addition to that, it is also important to highlight that in all locations where SARS-CoV-2 RNA was detected in air, there was a great number of children present, which constitutes a vulnerable group that requires attention, especially considering the emergence of more transmissible variants such as Omicron (Khemiri et al., 2022). SARS-CoV-2 RNA detection in air samples from environments with more children present was not unexpected, as increased vaccination rates in the general population usually results in an increase in infection rates among the unvaccinated parcel of the population such as children (Rotevatn et al., 2023).

The risk of acquiring SARS-CoV-2 infections in outdoor environments should not be overlooked if we consider that there is a lack of knowledge regarding the importance of outdoor environments as potential pathways for SARS-CoV-2 infection (Bulfone et al., 2021a). Elucidating more details of the dynamics of SARS-CoV-2 transmission outdoors is of particular interest in high-density cities, where, according to a first-principles model, the transmission of SARS-CoV-2 outdoors is suspected to have played a role in superspreading events (Huang et al., 2021)—whether these environments are crowded and if social distancing cannot be maintained, as well as the increased probability of being in contact with contaminated surfaces or items (fomites) and the number of asymptomatic people present (Senatore et al., 2021) are some examples of important details in order to elucidate the real dynamics of the virus in outdoor air.

Collectively, these results emphasize the importance of preventive measures such as wearing masks in order to avoid SARS-CoV-2 infection, even in outdoor settings. In addition to that, wearing masks can be an important tool to prevent the spread of other respiratory viruses such as respiratory syncytial virus (RSV) and influenza virus (flu), especially during winter when these viruses tend to circulate at the same time (Audi et al., 2020c; Furlow, 2023; Plantinga et al., 2023), resulting in a significant increase in respiratory disease burden worldwide in both high- and low-income countries (Ndumwa et al., 2022; Robinson, 2008; Salvi et al., 2018).

This study has some limitations that should be considered. Firstly, the small sample size may limit the generalizability of the findings, as only a limited number of samples were taken from two specific outdoor settings. Additionally, the study was conducted during the winter season in Valladolid, Spain, when environmental conditions like temperature, humidity, and UV exposure could affect viral persistence and detection differently compared to other seasons. These factors make it challenging to extend the results to other times of the year or geographical locations with varying climatic conditions. Further research incorporating a broader range of environmental conditions and viral viability assessment would enhance our understanding of SARS-CoV-2 behavior in outdoor air.

Conclusion

While outdoor environments generally pose a lower risk for SARS-CoV-2 transmission than indoor settings, this study detected SARS-CoV-2 RNA in air samples collected from crowded outdoor areas, suggesting that virus presence in the air is influenced by the number of people present. However, the study has limitations, including the small sample size and the fact that it was carried out in winter conditions in Europe, which cannot be generalized to other seasons or locations. Future studies about SARS-CoV-2 in outdoor are that include assessment of viral viability as well as environmental conditions such as humidity, temperature, UV radiation and wind speed are still needed, as these data would help us understand how different environmental factors at play can affect infectivity over time in different types of outdoor environments. In light of the COVID-19 pandemic, and in view of future worldwide respiratory viral pandemics that might surge, it is important for policy-makers and government authorities to promote education for the population about the importance of wearing good quality masks, as well as to provide a wider availability of these types of masks for the population in an accessible manner, as this measure alone could greatly reduce risk of acquiring respiratory infections not only in indoors but also in outdoors environments.

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Data Availability The data presented in this study are available upon request to the corresponding author.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics Approval Not applicable.

Consent to Participate Not applicable.

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CHAPTER 5

Airborne SARS-CoV-2 is more frequently detected in environments related to children and elderly but likely non-infectious, Norway, 2022 (Article 6)

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Airborne SARS-CoV-2 is more frequently detected in environments related to children and elderly but likely non-infectious, Norway, 2022

Priscilla Gomes da Silva[1,2,3,4,5], Mahima Hemnani[1,2,3], José Gonçalves[6], Elisa Rodriguéz[6], Pedro A. García-Encina[6], Maria São José Nascimento[7], Sofia I. V. Sousa[4,5], Mette Myrmel[7] and João R. Mesquita[1,2,3,8*]

Abstract

This study investigates the presence of SARS-CoV-2 in indoor and outdoor environments in two cities in Norway between April and May 2022. With the lifting of COVID-19 restrictions in the country and a focus on vaccination, this research aims to shed light on the potential for virus transmission in various settings. Air sampling was conducted in healthcare and non-healthcare facilities, covering locations frequented by individuals across different age groups. The study found that out of 31 air samples, only four showed the presence of SARS-CoV-2 RNA by RT-qPCR, with no viable virus detected after RNAse pre-treatment. These positive samples were primarily associated with environments involving children and the elderly. Notably, sequencing revealed mutations associated with increased infectivity in one of the samples. The results highlight the importance of considering children as potential sources of virus transmission, especially in settings with prolonged indoor exposure. As vaccination coverage increases globally, and with children still representing a substantial unvaccinated population, the study emphasizes the need to re-implement mask-wearing mandates indoors and in public transport to reduce virus transmission. The findings have implications for public health strategies to control COVID-19, particularly in the face of new variants and the potential for increased transmission during the autumn and winter seasons.

Keywords SARS-CoV-2, Airborne transmission, COVID-19, Indoor environments, Outdoor environments, Mitigation strategies, Vaccination, Mask mandates

*Correspondence:
João R. Mesquita
jrmesquita@icbas.up.pt
[1] ICBAS-School of Medicine and Biomedical Sciences, Porto University, Porto, Portugal
[2] Epidemiology Research Unit (EPIunit), Institute of Public Health, University of Porto, Porto, Portugal
[3] Laboratório para a Investigação Integrativa e Translacional em Saúde Populacional (ITR), Porto, Portugal
[4] LEPABE-Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, Porto, Portugal
[5] ALiCE-Associate Laboratory in Chemical Engineering, Faculty of Engineering, University of Porto, Porto, Portugal
[6] Institute of Sustainable Processes, Valladolid University, Dr. Mergelina s/n, Valladolid 47011, Spain
[7] Faculty of Pharmacy, University of Porto, Porto, Portugal
[8] Virology Unit, Norwegian University of Life Sciences, Ås, Norway

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Introduction

Almost three years have passed since the COVID-19 pandemic started in December 2019 [1]. During this time, much has been debated about the transmission of SARS-CoV-2, and how each transmission mode contributed to the worldwide spread of the virus, as well as all the factors involved in different transmission settings [2–4]. Now, it is well established that transmission through surfaces did not account for a great proportion of the infections, and that transmission occurs mainly through close contact, respiratory droplets and aerosols [5, 6], with indoor airborne transmission likely being the main driver of the pandemic [7–10].

Strict vaccination, surveillance and control policies helped to control viral spread, however, there is still a major gap in knowledge when it comes to airborne transmission, namely: how different environmental and meteorological parameters affect virus infectivity in the environment; how long the virus survives in the air; and more importantly, what is the minimum infectious dose of the virus. This information is essential for understanding airborne transmission dynamics and how we can prevent infection more efficiently [10–12]. There are several inherent difficulties in studying airborne SARS-CoV-2, e.g. the low virus concentration in air compared to clinical samples from the nasopharynx [13]; the variation of air-sampling techniques used, making generalization and interpretation of results difficult [14]; the lack of BSL-3 facilities for assessment of viral viability in air in most of the studies [15, 16], among others.

Although a high vaccination coverage was reached in most of Europe and America by the first trimester of 2022 [17], still new waves with record-high number of cases have been reported in that year, with the surge of a more transmissible variant of concern named Omicron (B.1.1.529) and its sub-lineages BA.4 and BA.5 31 [18]. Since distinct vaccine booster strategies are in place across the world that cover different age groups and individuals with different risk profiles, information regarding viral presence in air can provide a more in-depth characterization of the risk for COVID-19 infection.

In Norway, in early 2022, 86% of individuals above 16 years of age were vaccinated with two doses, and 86% of people ≥ 65 years old had received a booster dose [19]. No COVID-19 restrictions were in place other than the use of mask in healthcare facilities. As of 9th January 2022 in Norway, 72% of the entire population have been fully vaccinated. 86% of the people in the age group 16 years-old or older, and 88% of the people in the age group 18 years-old or older were fully vaccinated. Moreover, 82% of the people in the 16–17 years-old age group and 52% of people in the 12–15 years-old age group had been vaccinated with one dose, and 35% of people in the 16–17 years-old age group had been fully vaccinated [19].

With that in mind, we have performed air sampling in two cities in Norway in April and May 2022 in order to assess SARS-CoV-2 presence in different indoor and outdoor environments. Healthcare and non-healthcare related facilities were included, covering locations frequented by people of all age groups. Considering the complete lift of all restrictions in the country during that period, and the fact that the country had a low number of new daily infections, these results can be used as indicator of potential virus spread rates and the level of transmission risk in the country during that period.

Materials and methods

Air sampling

Air sampling (n = 31) was performed in the period of two weeks, between April and May 2022, in indoor and outdoor areas of two cities of Norway, Ås and Oslo, in healthcare and non-healthcare facilities, at periods of the day with increased movement of people.

Air samples were collected using the Coriolis® Compact (Bertin Instruments, Montigny-le-Bretonneux, France) air sampler with an airflow rate of 50 L/min. The duration of sampling was set according to the type of environment and permissions obtained for sampling, with slightly shorter sampling duration indoors where a high number of people was present. The minimum distance between the air sampling device and potential emission sources was 0.5 m in all sampling locations. The maximum distance varied depending on the size of the location and whether it was an open space or not, with distances of over 3 m between the air sampler and potential emission sources in open spaces such as the public parks and squares. Details about air sampling locations and settings are summarized in Table 1.

The sampler was placed at a height of approximately 1 m at all sampling sites. Air samples were collected on a dry medium, with 1.5 mL of sterile phosphate buffered saline (PBS) added to the collection cones after sampling. All samples were stored at 4 °C and transported to the laboratory within 4 h, where they were stored at − 80 °C until further processing.

RNA extraction and RT-qPCR

RNA extraction was performed using the GRS Viral DNA/RNA Purification Kit (GRISP, Porto, Portugal) according to the manufacturer's instructions using 200 µL of sample suspension, resulting in 50 µL of RNA eluate after extraction as previously described [20]. Two RT-qPCR reactions targeting N1 and N2 were used (Xpert qDetect COVID-19, GRISP, Porto, Portugal). The CFX Real-Time PCR (qPCR) Detection System

Table 1 Details about the air sampling settings and sampling sites

	Date	Sample number	Environment	Sampling site	Duration of sampling (min)	Period of the day
Non-healthcare facilities	24 April	1	Outdoor	Oslo Central Station	30	7:00–8:30am
		2	Outdoor	Statue in front of Oslo Central Station	30	8:30–9:00am
		3	Outdoor	Public Park	30	14:00–14:30pm
		4	Outdoor	Public Park	30	15:30–16:00pm
		5	Outdoor	Raadhus square	30	17:00–17:30pm
	25 April	6	Outdoor	Oslo Central Station	30	7:00–7:30am
		7	Outdoor	Statue in front of Oslo Central Station	30	8:30–9:00am
		8	Outdoor	Tram station	30	9:30–10:00am
		9	Outdoor	Public Park	30	10:30–11:00am
		10	Outdoor	Parliament square	30	13:00–13:30pm
	26 April	11	Outdoor	Oslo Central Station	30	7:00–7:30am
		12	Indoor	University Restaurant	50	11:50–12:40am
		13	Indoor	University Cafeteria	50	13:00–13:50pm
	27 April	14	Outdoor	Oslo Central Station	30	7:00–7:30am
		15	Indoor	University Restaurant	50	11:50–12:40am
		16	Indoor	University Cafeteria	50	13:00–13:50pm
	28 April	17	Outdoor	Oslo Central Station	30	7:00–7:30am
		18	Indoor	University Restaurant	50	11:50–12:40am
		19	Indoor	University Cafeteria	50	13:00–13:50pm
	29 April	20	Outdoor	Oslo Central Station	30	7:00–7:30am
		21	Indoor	Kindergarten	20	10:00–10:20am
		22	Outdoor	Kindergarten	20	10:40am-11:00am
		23	Indoor	Kindergarten	20	11:20am-11:40am
Healthcare facilities	02 May	24	Indoor	Nursing home	20	9:00–9:20am
		25	Indoor	Nursing home	20	9:40–10:00am
		26	Indoor	Nursing home	20	10:20–10:40am
		27	Indoor	Nursing home	20	11:00 –11:20am
		28	Indoor	Health Clinic	20	16:00–16:20pm
		29	Indoor	Health Clinic	20	16:40–17:00pm
		30	Indoor	Health Clinic	20	17:20–17:40pm
		31	Indoor	Health Clinic	20	18:00–18:20pm

(Bio-Rad, USA) with the Bio-Rad CFX Maestro 1.0 Software version 4.0.2325.0418 was used for data analysis. For each reaction, 4 µL of RNA was used, with every run including ssDNA N1 and N2 targets (positive controls) and a no-template control. Reactions were run for 15 min at 45 °C and 2 min at 95 °C, then 45 cycles of 95 °C for 15 s, and 55 °C for 30 s. All samples were run in duplicates.

A standard curve was generated using ssDNA targets for both N1 and N2 in a 10-fold serial dilution starting at 200,000 copies/µL, in order to quantify viral gene copies based on sample Ct values. The limit of detection (LOD) was 1.3 and 3.2 copies/µL for N1 and N2, respectively. Air sampling results are expressed in gene copies/m^3.

Viral viability

The viral viability was evaluated with a nuclease sample treatment prior to RNA extraction using a previously described method with minor modifications [21]. The RNAse pre-treatment assumes that viable viruses with intact capsids and envelopes will not have their genetic material degraded by RNAse and amplification during the RT-qPCR would indicate viable viruses [22]. Prior to RNA extraction, three aliquots of the air sample were pre-treated with 8 µL of RNAse A (GRISP, Porto, Portugal) with the following concentrations: 1 µg/µL, 10 µg/µL and 50 µg/µL, at 37 °C for 30 min. An untreated aliquot of each sample was also tested for comparison of RT-qPCR results between treated and non-treated samples.

Sequencing

Positive RT-qPCR samples were confirmed by heminested RT-PCR targeting a 398-bp fragment of the SARS-CoV-2 N gene [23]. Amplicons of expected sizes in the gel were purified with GRS PCR Purification Kit (Grisp, Porto, Portugal) and, bidirectional Sanger sequencing was performed with the specific primers of the target gene. Sequences were aligned with BioEdit Sequence Alignment Editor v7.1.9 software package, version 2.1 (Ibis Biosciences, Carlsbad, CA, USA) and compared with the sequences available in the NCBI (GenBank, Carlsbad, CA, USA) nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast, accessed on 9th November 2022).

Results

Out of 31 samples, four were positive for SARS-CoV-2 RNA by RT-qPCR when pre-treatment with RNAse was not performed. These samples were: sample 21 (kindergarten, indoor eating room) [6 gene copies/m^3], sample 22 (kindergarten, outdoor playground) [6 gene copies/m^3], sample 27 (nursing home, indoor cafeteria) [6 gene copies/m^3] and sample 28 (Health clinic, pediatrics waiting room) [5 gene copies/m^3]. Pre-treatment with the three RNAse concentrations followed by RT-qPCR showed that all the previously positive samples were negative.

Heminested RT-PCR followed by bidirectional sequencing allowed to retrieve one sequence. This sequence was obtained from sample 21 (GenBank accession number OQ296419). BLAST analysis showed 100% identity with a SARS-CoV-2 isolate from a human from Tokyo, Japan (OQ326844). Genomic sequence analysis identified two mutations in relation to the SARS-CoV-2 strain first detected in Wuhan in (NC_045512.2), both in the nucleocapsid (R203K and G204R) (Fig. 1).

Discussion

With all COVID-19 restrictions being lifted in Norway, and no obligation for mask use, not even in healthcare facilities, the present study aimed to assess the presence of SARS-CoV-2 in air samples from various indoor and outdoor spaces, including both healthcare and non-healthcare related facilities. We found that, out of 31 air samples, only four showed the presence of SARS-CoV-2 RNA by RT-qPCR, with no amplification after RNAse pre-treatment, strongly suggesting that no viable virus was present in these four samples.

The four positive samples came from a kindergarten (n = 2), a nursing home (n = 1) and from the pediatrics waiting room in a health clinic (n = 1). It should be noted that on the day of the sampling in the kindergarten, most of the children were showing respiratory disease symptoms but none was using facemasks. Moreover, at the nursing home one of the nurses' staff had tested positive for COVID-19 the previous day.

It is important to mention that the number of people present in each sampled location varied throughout sampling duration in every location, as sampled locations were either outdoor public spaces with a constant and heavy influx of people such as public parks or the train station, or indoor spaces with constant but not so heavy movement of people such as the health clinic, where patients would come and go constantly. Nonetheless, in the locations where SARS-CoV-2 RNA could be recovered from the air samples, the density of people present

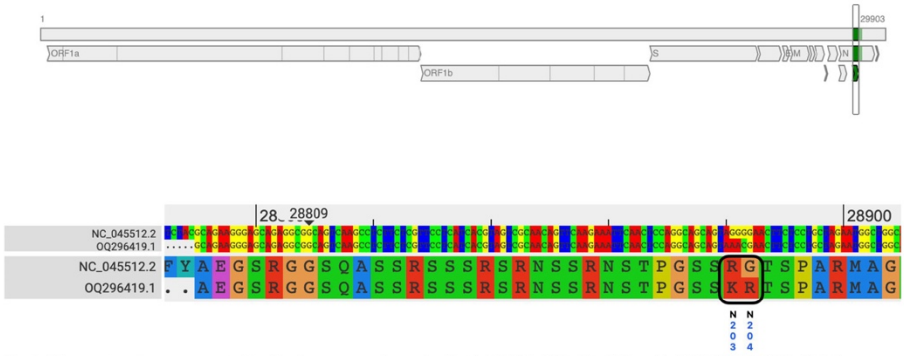

Fig. 1 Alignment and genome annotations for the sequenced sample with the SARS-CoV-2 isolate Wuhan-Hu-1 (NC_045512.2) highlighting the R203K and G204R mutations. Modified from Coronavirus Typing Tool, Version 1.25

was low (that is, less than 15 people present at the locations during sampling).

When looking at these results, although they indicate that non-infectious viruses were present in air samples, we cannot exclude the possibility that the collection process might have contributed to the inactivation of SARS-CoV-2 [24]. Multiple factors can potentially contribute to the decline in virus viability, including the collection process and prolonged airborne state (which increases the likelihood of surface damage due to impaction, exposure to harmful airborne contaminants, desiccation, or degradation) [25]. In the case of the Coriolis Compact, which is a cyclone sampler, a film of liquid is injected close to the cyclone's inlet to wet the cyclone walls, which are subsequently collected at the cyclone's base for analysis. This is thought to increase viability of collected viral or other microorganism particles that are being sampled, however shear forces may still decrease particles 'viability [26–28].

As the probability of detecting infectious SARS-CoV-2 is directly correlated with the amount of viral RNA detected by RT-qPCR, and infectious viruses are more likely to be detected when viral RNA is present in concentrations greater than 1×10^6 gene copies/mL [29], viable viruses were not expected to be present in our study based on the low numbers of gene copies/m^3 found. This is in keeping with previous studies on SARS-CoV-2 that identified viable virus in air samples [30–32], with 1000 gene copies/m^3 [31]. Of note, in another study it was estimated that an RNA concentration of 2.5×10^5 RNA copies/mL had less than a 5% success rate for isolating infectious virus [33]. Within this < 5% success rate of isolating infectious virus, another study reported a limit of 4.3×10^6 RNA copies/mL [34]. All these values are in accordance with other studies which have shown that samples with Ct values > 24 are unlikely to be virus positive after cultured [35]. However, it is important to emphasize that Ct measurements depend on the RT-qPCR assay and platform used [36].

Information about viability of SARS-CoV-2 viruses in air samples is very important when discussing risk assessment, as risk assessment studies are used by policymakers and health agencies to develop mitigation and preventative strategies to control viral transmission in the community, as well as its applications in occupational health [37]. In this context, the nuclease pre-treatment method offers an alternative to estimate the presence of infectious viruses in air samples when viral culture is not possible, allowing for a better interpretation of studies on SARS-CoV-2 presence in ar. Moreover, this method has the potential to enhance analyses aimed at supporting risk-based investigations, whether for preventing new outbreaks or managing recurrent ones, and can be applied across a wide range of scenarios [38].

SARS-CoV-2 typically causes mild illness and few deaths in children and adolescents when compared to adults [39]. However, these groups still remain susceptible to SARS-CoV-2 infection and may transmit the virus to other people, such as the elderly parcel of the population, which increases the burden of the disease on public health systems (World Health Organization (WHO) [40]). Interestingly, our results point to a link between SARS-CoV-2 presence in air in environments with more children under the age of three present, which, until this day, still makes up the age-group worldwide with more unvaccinated people against COVID-19 [41, 42].

Nonetheless, the sequenced sampled presented the nucleocapsid mutations R203K (28881G > A, 28882G>A) and G204R (28883G>C) that have been reported to increase the infectivity, fitness, and virulence of SARS-CoV-2 [43], being associated with increased infectivity of SARS-CoV-2 strains in the United States (USA), as well as being predominant in both the USA and Europe [44]. The mutation G204R is non-conservative, and the R203K mutation has been pointed to function as a non-conservative substitution due to the different size of the arginine (R) versus lysine (K) residues and the considerably different chemical features of the side-chain guanidinium group (arginine) versus the primary amine (lysine). It has been hypothesized that these mutations may influence disease severity by altering linker region flexibility and dynamics, which would in turn alter nucleocapsid function [45].

One of the significant unanswered questions about the COVID-19 epidemiology is related to the role of children in the transmission of SARS-CoV-2 [46], which is a group that comprises a significant share of the population in many countries (Charumilind et al. [47]). With milder symptoms, children are tested less often and cases may go unreported (World Health Organization (WHO) [40]), allowing the virus to reach more susceptible groups such as the elderly and immune-compromised. Recent reports have also suggested that the Omicron variant and its sub-lineages may lead to more frequent hospitalization in children, as children make up a larger part of patients hospitalized with COVID-19 than in previous infection waves caused by other variants of concern [41]. Moreover, when considering that SARS-CoV-2 RNA could be detected in a nursing home facility, it puts into question the prevention guidelines in place at the time, when it comes to the safety of the elderly population, as previous epidemiological data from Norway show that the majority of COVID-19 related deaths are among this risk group [48].

However, evidence to date still suggests that children are not among the main drivers of the pandemic [49]. That added to the fact that asymptomatic children have

significantly lower viral loads compared to children with symptomatic infections [50] have resulted in less effort trying to understand the role of children in the airborne transmission of SARS-CoV-2 [51].

In another study, it was highlighted that although estimates of children's susceptibility and infectivity are lower than those of adults within a household, it is important to remember that their role in the spread of SARS-CoV-2 is also affected by different contact patterns and hygiene habits outside the household (Dattner et al. [46]). More intense contact and mixing among children compared to adults in schools, e.g., could offset the effect of reduced susceptibility. In a review it is brought to attention the fact that roughly half of the United States population goes to school, works in a school, or is a first-degree contact of individuals that frequent these environments, suggesting that in-school transmission can impact the disease burden in surrounding communities [52]. This raises the alarm for the higher probability of virus spreading in school settings, as they have one of the main elements for a superspreading event, which is prolonged indoor exposure to other people. Whether these schools have appropriate ventilation or not is another factor that should be taken into consideration when evaluating exposure risk in school settings. Another important factor concerning children and increased risk of SARS-CoV-2 infection is that households with children in low income, urban communities have an extremely high household secondary attack rate, with children playing important roles as index cases [51].

This study sheds light on the presence of SARS-CoV-2 in indoor and outdoor environments, particularly focusing on healthcare and non-healthcare settings. With lifted COVID-19 restrictions and a significant percentage of the population vaccinated, our findings provide valuable insights into the dynamics of SARS-CoV-2 transmission at a time where all restrictions have been lifted and vaccination coverage is high.

Our air sampling in Norway took place in two cities, namely Oslo and Ås, in the end of April and beginning of May 2022. During this period, there had been a steady decline in the number of new patients admitted to Norwegian hospitals with COVID-19, with the number of new patients admitted per 100.000 people being highest in the age groups 75–84 and ≥ 85 years [19]. Of note, during this period, 58% of people who deceased due to COVID-19 complications died in a health institution other than a hospital, primarily in nursing homes. The Omicron variant BA.2 was the dominant sub-lineage in the country, accounting for 95–100% of all whole-genome sequenced samples during that period [19].

Considering that our sampling campaign took place at a moment of low-transmission in Norway, and that sampling covered various indoor and outdoor environments (public parks, public transport stations, university restaurants, a nursing home, a kindergarten, and a health clinic), the fact that SARS-CoV-2 RNA could be detected only in environments related to children and the elderly raises some issues when it comes to mitigation guidelines and prevention strategies.

First of all, as vaccination coverage is increasing around the world and we have started to normalize life towards the pre-COVID-19 time, COVID-19 cases are escalating and SARS-CoV-2 surveillance is reduced, painting out a coming challenging winter in Europe [53]. That being said, it is urgent that countries in the EU region relaunch mitigation efforts and are ready to respond to an increased burden on their health-care systems.

The application of what the WHO calls "Five Pandemic Stabilizers" (increased vaccination rates, a second booster dose to immunocompromised people and their close contacts, mask wearing indoors and in public transports, improving ventilation in crowded and public spaces, and applying rigorous therapeutic protocols for those at risk of severe disease) will be of the utmost importance in order to control virus transmission during autumn and winter [53].

When considering that children still represent the majority of the unvaccinated people not only in the EU region but in a global scale, and the fact that this group is often asymptomatic and less frequently tested, special attention should be given to the importance of re-implementing mask wearing indoors and in public transports, as this is still one of the most efficient interventions against SARS-CoV-2 airborne transmission [54, 55]. This would reduce transmission of the virus to children, which in turn would help prevent the virus spread from this group to at-risk groups such as the elderly and immunocompromised. Furthermore, considering the surge of Omicron and its sub-lineages that are more easily transmissible, reinstating mask mandates might be the best strategy to control community transmission of COVID-19 [55].

Conclusion

This study detected SARS-CoV-2 RNA in air samples from specific locations, such as a kindergarten, a nursing home, and a pediatrics waiting room in a health clinic. Importantly, the RNAse pre-treatment indicated that the virus in these samples was likely non-infectious, suggesting that the collection process may have contributed to the inactivation of the virus. Despite this, genetic sequencing revealed mutations associated with increased infectivity and virulence, raising concerns about the potential risks associated with infection. One notable aspect highlighted by this study is

the role of children in the transmission of SARS-CoV-2, particularly in settings with prolonged indoor exposure. Children, even if they experience milder symptoms, can still play a crucial role in spreading the virus to more vulnerable populations, such as the elderly. This emphasizes the importance of continued vigilance and public health measures, especially given the uncertainty surrounding new variants like Omicron and its sub-lineages. As we move forward, the study underscores the need for a balanced approach to COVID-19 prevention and control. Vaccination efforts are crucial, but it's essential to recognize that children, especially the younger ones, often remain unvaccinated and can still contribute to virus transmission. Therefore, reinstating mask mandates, improving ventilation in public spaces, and implementing rigorous therapeutic protocols are recommended as part of a comprehensive strategy to control the virus, particularly during the autumn and winter months. This research serves as a timely reminder that the fight against COVID-19 is ongoing, and we must adapt our strategies to evolving circumstances, including emerging variants and the role of different demographic groups in transmission. It underscores the importance of maintaining a multifaceted approach to minimize the risks associated with the virus and protect public health.

Author contributions
PGDS: Conceptualization, Methodology, Investigation and Writing - Original Draft. M.H: Investigation, Writing - Review & Editing. JG: Methodology, Writing - Review & Editing. ER: Writing - Review & Editing. PAGE: Writing - Review & Editing. MSJN: Writing - Review & Editing. SIVS: Methodology, Writing - Review & Editing, Supervision. MM: Methodology, Writing - Review & Editing. JRM: Conceptualization, Methodology, Writing - Review & Editing, Supervision.

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Availability of data and materials
The data presented in this study are available on request from the corresponding author.

Declarations

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare no competing interests.

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CHAPTER 6

Microfluidic toolbox using padlock probes and rolling circle amplification for direct detection and genotyping of viral RNA (Article 7)

Microfluidic toolbox using padlock probes and rolling circle amplification for direct detection and genotyping of viral RNA

João C. Varela[1, 2†*], Priscilla Gomes da Silva[3, 4, 5†], Hower Lee[6], João R. Mesquita[3, 4, 5], Aman Russom[1, 2], Ruben R. G. Soares[6], Mats Nilsson[6]

[1]Division of Nanobiotechnology, Department of Protein Science, Science for Life Laboratory, KTH Royal Institute of Technology, Solna, Sweden

[2]AIMES Center for the Advancement of Integrated Medical and Engineering Sciences at Karolinska Institutet and KTH Royal Institute of Technology, Stockholm, Sweden

[3]ICBAS-School of Medicine and Biomedical Sciences, University of Porto, Porto, Portugal

[4]Epidemiology Research Unit (EPIunit), Institute of Public Health, University of Porto, Porto, Portugal

[5]Laboratório para a Investigação Integrativa e Translacional em Saúde Populacional (ITR), Porto, Portugal

[6]Department of Biochemistry and Biophysics, Science for Life Laboratory Stockholm University, Solna, Sweden

[†]These authors contributed equally to this work;

[*]E-mail: joao.varela@scilifelab.se

ABSTRACT

Rolling circle amplification (RCA) combined with padlock probes presents a promising tool for direct detection and genotyping of viral RNA, offering advantages over conventional methods like RT-PCR. This isothermal process enables highly sensitive and specific amplification of nucleic acids without the need for thermal cycling, making it suitable for point-of-care applications. In this study, we demonstrate a microfluidic and RCA-based method for the direct detection of SARS-CoV-2 RNA and variant profiling, bypassing the reverse transcription step. Our approach allows for the identification of single nucleotide polymorphisms (SNPs) specific to viral variants, enhancing the detection sensitivity through the circle-to-circle amplification (C2CA) technique. This methodology shows potential as a robust, cost-effective platform for viral diagnostics, capable of being fully automated and integrated with miniaturized detection systems for efficient use in both resource-rich and resource-limited settings.

1. Introduction

In the past century alone, there have been more than six different influenza pandemics and epidemics [1], roughly 25 cases of animal-to-human transmission of Ebola viruses [2] and surge of at least seven coronaviruses that have caused disease and fatalities[3], with the recent COVID-19 pandemic serving as a reminder that rapidly emerging viral outbreaks are unavoidable[4].

The evolution and genetic diversity of viruses can significantly influence the dynamics of outbreaks and the effectiveness of control measures. This was clearly demonstrated during the COVID-19 pandemic, where the emergence of new variants highlighted these challenges to politicians, public health professionals, and the general public [5]. To accurately confirm infection with a specific variant, sequencing of the entire SARS-CoV-2 genome — or at least the whole or partial S-gene for current variants — is required. Genome sequencing remains the only reliable method for identifying and characterizing new variants and for unambiguously typing existing ones.

Although alternative methods such as diagnostic screening PCR-based assays have been developed for early detection of variants of concern, it is essential to perform confirmatory sequencing on at least a subset of viruses when using PCR-based assays, ensuring that these results can reliably indicate the circulation of variants of concern within the community [6].

Over the last decade, significant progress in the polymerase chain reaction (PCR) technology and other alternative isothermal methods for amplifying DNA and RNA targets have been made, with PCR remaining the gold-standard method[7,8]. In the particular case of viral diagnostics, the detection of point mutations holds significant diagnostic value[9], as these variations can be used to identify potentially concerning circulating variants [10]. While most mutations have minimal impact on the virus's characteristics, certain changes can influence factors like transmissibility, disease severity, or the efficacy of vaccines, treatments, diagnostic tools, and other public health interventions[11]. Therefore, identifying viral variants from a mixed population is essential for understanding viral evolution and genetic diversity, as well as assessing their health risk [10,12]. However, low viral loads in clinical samples can hinder the accuracy of variant identification in phylogenetic analyses and limit the detection of low-frequency variants, which are key for understanding the transmission chains of infections [13].

Despite the availability of various molecular diagnostic tests for viruses[14], these do not yet provide an adequate balance between high frequency screening and cost-

effectiveness and are not accessible for routine use in resource-limited settings, posing a challenge for the necessary coordinated global response to contain a pandemic or disease outbreak[15]. These technical problems led to the development of isothermal amplification techniques, such as Loop-Mediated Isothermal Amplification (LAMP), Rolling Circle Amplification (RCA) and Recombinase Polymerase Amplification (RPA) [16].

RCA is among the earliest and most widely used [17]. This method is based on the mechanism of rolling circle replication found in bacteria and viruses, yielding a long single-stranded concatemer DNA molecule with the circular DNA's complementary sequence repeated roughly 500–1,000 times, depending on the duration of amplification [18,19]. Padlock probes (PLPs) are linear oligonucleotides that undergo circularization through ligation in a strictly target-dependent fashion. When combined with RCA, it can offer highly multiplexed and specific bioanalytical assays for diagnostic applications [20].

The efficiency and fidelity of circle replication coupled with padlock probes (PLPs) has been used for viral diagnostics and subtyping, reaching sub-attomole sensitivity when combined with microfluidic detection devices [21,22]. This method allows the amplification of single target sequences into µm-sized ssDNA concatemers easily labelled and detected by standard fluorescence microscopy.

Besides high specificity, multiplexing stands as another advantage of PLP-based RCA, remarkably allowing the detection of hundreds of targets within a single sample without the need of Next Generation Sequencing (NGS)[23]. This can be achieved by integrating a barcode-like detection mechanism into the PLP backbone. Coupled with the single nucleotide specificity of the PLP hybridization and ligation, this setup enables the identification of SNPs, making it suitable for detecting different viral variants and potentially allowing the screening of multiple human respiratory viruses in a single assay, substantially cutting down the processing cost per sample. Moreover, by performing two consecutive rounds of RCA, known as circle-to-circle amplification (C2CA), the signal from a single RCP is amplified by a factor of 100 to 1000, effectively lowering the LoD to levels comparable to those of conventional PCR.

Considering the multiplexing capabilities and single nucleotide specificity, as well as the signal enhancement capability, here, we present a PLP-based RCA method for direct detection of SARS-CoV-2 RNA and variant profiling, without the need for a reverse transcription step. The described method represents a robust platform that can be fully automated and easily coupled with miniaturized fluorescent detectors, demonstrating

potential for viral diagnostics as a sensitive, simple and cost-effective point-of-care methodology.

2. Methods

2.1. Viral RNA Samples

Direct viral RNA detection on the microfluidic device with a single round of RCA was performed using a Synthetic Control 2 SARS-CoV-2 RNA (GISAID ID: MN908947.3, Twist BioScience). For the variant profiling experiments, trizol inactivated Vero E6 cell culture supernatants containing SARS-CoV-2 RNA (wuhan strain, B.1.1.7 and B.1.351 variants) were provided by the Swedish Health Agency (Folkhälsomyndigheten). RNA extraction from 500 µL of each supernatant was performed using a Zymo Direct-zol Miniprep Plus kit following the instructions provided by the manufacturer. The elution was performed in 50 µL of RNase/DNase-free water. The eluted RNA was quantified by a standard one-step RT-qPCR protocol for SARS-CoV-2 RNA detection optimized by FoHM using the TaqMan™ Fast Virus 1-Step Master Mix (Thermo Fisher Scientific) targeting the RdRp gene. For the CFX Real-Time PCR (qPCR) Detection System (Bio-Rad, Hercules, CA, USA), the Bio-Rad CFX Maestro 1.0 Software version 4.0.2325.0418 was used to control the runs and remotely analyze the data. In order to quantify the number of viral gene copies present in each sample from the measured Ct values, s standard curve was made from a Synthetic Control 2 SARS-CoV-2 viral stock of known concentration obtained from cell culture supernatant in a 10-fold serial dilution mixture starting at 106 copies/µL.

2.2. Probe Design

Different regions of the SARS-CoV-2 RNA genome were targeted for the two different used methodologies. For the hybridization-based RCA, probes were designed targeting a conserved Orf1ab genomic region encoding for RdRp: three ssDNA capture oligonucleotides containing a biotinylated poly-A tail at the 5'-end (biotinylated anchors) allow the capture of the target in streptavidin-coated agarose beads, while twelve L-shaped ssDNA oligonucleotides (L-probes) targeting this region, have an overhang sequence that enables PLP ligation.

Variant profiling was performed using a set of six PLPs (two targeting each variant) with a ribose on the 3'- end, targeting 30-bp long sequences in the S-region of the SARS-CoV-2 genome, containing single nucleotide polymorphisms specific for each of the selected variants. These probes were aligned so that the mutation to be detected would be complementary to the 3'- ribose of the PLP. While each variant-specific PLP contained a different detection sequence on the backbone, a primer-hybridization sequence and one restriction motif were added to all the probes, enabling the first and second round of RCA to be performed, respectively. A set of four biotinylated anchors targeting conserved sequences of the S-region were also used to capture the targets in the magnetic streptavidin-coated beads.

All probe sequences were blasted using BLASTn to confirm the specificity and checked for the formation of secondary structures with the mFOLD web tool. All used oligonucleotides were ordered from Integrated DNA Technologies (IA, USA), with their sequences listed in Table S1.

2.3. Microfluidic Device Fabrication and Bead Packing

Two different flow cell designs, previously described for diagnostic applications, were used in this work: a) two parallel 600 x 140 μm (width x height) cross-section columns, interconnected by channels with smaller 200 x 20 μm (width x height) cross-sections24, used for the on-chip, hybridization-based RCA; and b) straight flow cells with a 700 x 100 μm (width x height) cross-section and an interface with a 200 x 20 μm (width x height) cross-section21, used for detecting C2CA amplification products. In both designs, the height difference between the different cross-sections is intended to physically trap the agarose beads at the intersection. A schematic of both designs can be found in Figure S1.

The microfluidic devices were fabricated in poly(dimethylsiloxane) (PDMS) using standard mold replication and soft lithography techniques in the same way as described in previous works [21,24]. The pre-polymer was first mixed with curing agent mixture in a ratio of 10:1 (w/w) (Sylgard 184, Down Corning), degassed for 30 minutes, poured into the two-level SU-8 mold for the design of choice, and cured at 65^{o}C for 90 minutes in a convection oven. Access holes were punched after peeling the PDMS from the mold, using a blunt 18-gauge syringe tip, and the microchannels were subsequently sealed against 700 μm thick glass slides (Corning microscope slides, plain) by exposing both surfaces to an oxygen plasma treatment for 30 s (Femto Science CUTE, 100 W, 80 Pa).

Bead packing was performed by flowing a solution of streptavidin-coated agarose beads with an average of 34 μm diameter (Streptavidin Sepharose High Performance, GE Healthcare) mixed in phosphate buffered saline (PBS) (137 mM NaCl, 10 mM phosphate, pH 7.4) at a ratio of 1:100 (V/V), at a flow rate of 10 μL/min, by applying negative pressure using a New Era Pump Systems NE-1200 12-channel syringe pump. This step was followed by a wash step with PBS at 10 μL/min for 2 min, with subsequent sample analysis following afterwards. For design a), the beads solution was flowed approximately for 2 min, while for design b) the channel was fully packed with beads. 1% BSA blocking solution in PBS (Thermo Fisher) was also flowed at 5 μL/min for 10 min right before starting the on-chip RCA experiments.

2.4. Hybridization-based Rolling Circle Amplification (HybRCA)

Hybridization of target SARS-CoV-2 RNA with the respective probes was first performed by mixing 1.5 μL of target with 3 nM of each different L-Probes and 10 nM of different Biotinylated Anchors, in 50 μL of capture solution mix, for a final concentration of 5x SSC, 30% formamide, 0.1 mg/mL salmon sperm DNA, 1 U/μL RiboProtect (Blirt, Gdansk), and nuclease-free water. This solution was first incubated at 65oC for 5 min outside of the chip, without RiboProtect, and subsequently flowed (upon addition of RiboProtect) at 2.5 μL/min for 15 min, at 45^{o}C. This was followed by an initial washing step where a washing solution (5x saline-sodium citrate (SSC; Invitrogen); 30% formamide; 0.5% Tween20) was flowed at 10 μL/min for 5 minutes, followed by a second washing step with PBS using the same flow conditions and time. PLP hybridization and ligation was performed by flowing the reactional mix containing 10 nM PLPs, 20% formamide, 200 μg/μL BSA, 1 U/ μL RiboProtect, 0.5 U/μL Tth DNA ligase (Blirt, Gdansk) in the respective ligation buffer (20 mM Tris-HCL pH 8.3, 25 mM KCl, 10 mM MgCl2, 0.5 mM NAD, and 0,01% Triton X-100), at a flow rate of 0,5 μL/min for 30 min, at 45^{o}C. Upon ligation, amplification was carried out by flowing a second reaction mix containing 5% glycerol, 250 μM dNTPs, 200 μg/μL BSA, 1 U/μL RiboProtect, 1 U/μL phi29 DNA polymerase (Blirt, Gdansk) in 1x polymerase buffer (50 mM Tris-HCL pH 8.3, 10 mM MgCl2, and 10 mM (NH4)2SO4), at a flow rate of 0.5 μL/min for 90 min, at 37^{o}C. The labeling of resulting RCPs was performed by flowing the final reaction mix consisting of 2x SSC, 20% formamide and 0.1 μM of detection oligonucleotides with a fluorophore (Cy5) at a flow rate of 5 μL/min for 10 min, at room temperature, followed by a washing step with a solution similar to the last reaction mix, without any detection oligonucleotides, at a flow rate of 10 μL/min for 5 minutes.

2.5. Circle-to-Circle Amplification (C2CA)

RNA samples were first hybridized with the respective biotinylated anchors by mixing 2 µL of the target with 100 nM of each biotinylated anchor in a solution comprised of 5x SSC, 0.5% Tween20, and 1 U/µL RiboProtect, for a total of 20 µL. This mixture was incubated for 5 min at 50oC, followed by a 20 min incubation time at room temperature, with 5 µL of streptavidin-conjugated magnetic beads (Dynabeads T1, Thermo Fisher) after these are washed three times with WTW buffer (10 mM Tris−HCl, pH 7.5, 5 mM EDTA, 0.1 % Tween 20, 100 mM NaCl). Following this step, the beads were washed once again with Wtw buffer, followed by a wash with PBS, and then incubated with a PLP-hybridization mix, consisting of a solution of 1 nM of each PLP, 2x SSC, 5% ethylene carbonate, 15 mM MgCl2, 0.1% (V/V) Tween20 and 1 U/µL RiboProtect for 30 min at 37ºC. Upon hybridization of the PLPs, the ligation is carried out by removing the supernatant and adding 20 µL of solution containing 5% PEG8000 (Sigma-Aldrich), 0.1 mM ATP, 500 mM Tris-HCl pH 7.5, 10 mM DTT, and 1 U/µL of homemade T4 RNA ligase 2 enzyme (Protein Science Facility, Karolinska Institutet), followed by incubation at 37ºC for 120 min. Before the first amplification round, 20 µL of solution containing 100 nM of the PLP primer, 2x SSC, 5% ethylene carbonate, 15 mM $MgCl_2$, 0,1% (V/V) Tween20 and 1.25% glycerol was added upon removing the previous supernatant and incubated for 30 min at 37ºC.

Amplification was then carried out by once again removing the supernatant and adding 15 µL of the first amplification mix, consisting of 50 mM Tris-HCL pH 8.3, 10 mM $MgCl_2$,10 mM $(NH_4)_2SO_4$, 5% glycerol, 0.25 mM dNTPs, 0.2 mg/mL BSA, 0.5 U/µL Phi29 DNA polymerase (Blirt, Gdansk), and 1 U/µL RiboProtect, followed by an incubation at 37ºC for 60 min, with a subsequent enzyme inactivation step at 60ºC for 10 min. After the first amplification, 5 µL of restriction solution is added to the solution to enable the second round of amplification. This solution consists of 120 nM of the restriction oligo, 50 mM Tris-HCl pH 8.3, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0,2 mg/mL BSA, 800 mU/µL AluI restriction enzyme (New England BioLabs), and is incubated at 37ºC for 10 min, followed by an enzyme inactivation step at 60ºC for 10 min. Upon digestion, the supernatant is collected and combined with the second amplification mix, for a final concentration of 50 mM Tris-HCL pH 8.3, 10 mM $MgCl_2$,10 mM $(NH_4)_2SO_4$, 0.68 mM ATP, 0.25 mM dNTPs, 0.2 mg/mL BSA, 0.05 U/µL T4 DNA ligase (Blirt, Gdansk), 0.5 U/µL Phi29 DNA polymerase, and incubated at 37ºC for 120 min, with a subsequent enzyme inactivation step at 60ºC for 10 min.

Obtained RCPs were then labelled with the respective labelling mix, for a final concentration of 5 nM of each detection oligonucleotide containing the respective fluorophore (Cy3, Cy5 or AF488), as well as 5 nM of a biotinylated oligonucleotide to allow the capture of the amplification product in the streptavidin-coated beads, 2x SSC, 20% formamide, and incubated at 37°C for 60 min.

2.6. Quantification of Rolling Circle Products (RCPs)

For the on-chip hybridization-based direct RNA detection, RCPs were quantified right after the final washing step by imaging the streptavidin-coated agarose beads with an inverted Nikon Ti-Eclipse fluorescence microscope (40× objective) equipped with a SOLA SE light engine and a TRITC, FITC and Cy5 filter cube. The acquired images were then analyzed using the ImageJ software (NIH, USA) by averaging the greyscale intensity in a designated area and comparing it to the fluorescent background on the channel.

RCP quantification for the variant detection experiments was performed as described elsewhere, using both Superfrost® Plus adhesion glass slides (VWR) and the microfluidic chromatography enrichment (μACE), coupled with fluorescence microscopy. Measurements using the glass slides were performed by placing 10 μL of the labelled RCP solution in the slide, covering it with a cover slip, and imaging it using a Zeiss Axio Imager Z2 epifluorescence microscope (20x objective) equipped with a Cy3, Cy5 and AF488 filter cube. Upon acquiring four independent 300 x 300 μm^2 images, RCPs were counted using the CellProfiler 4.2.5 software (Broad Insitute, USA), and the sum of the counts per glass slide was determined. For the microfluidic device, one single image of the streptavidin-coated beads was acquired using the aforementioned inverted Nikon Ti-Eclipse fluorescence microscope and its corresponding setting, and RCPs were quantified based on the average grey scale intensity of the bead-packed region, normalized for the fluorescence of the empty channel.

3. Results and Discussion

3.1. On-Chip detection of viral RNA using Hybridization-based Rolling Circle Amplification (HybRCA)

Expanding upon recent developments of RCA-based methods on-chip [25–30], we report herein a method for the PCR-free, direct detection of viral RNA on a microfluidic device. For this method, presented in Figure 1A, the target is (1) hybridized with three biotinylated "anchor" oligonucleotides – allowing the capture of the RNA in streptavidin-coated agarose beads inside the microchannels – and twelve L-shaped probes – containing one sequence targeting the RNA, and an overhang to allow the hybridization of a PLP – all targeting a conserved SARS-CoV-2 region encoding for RdRp. Upon (2) being captured in the respective beads inside the microfluidic device, (3) PLPs targeting the overhang of the L-probes are flowed through the microchannel and, if properly hybridized, circularized by the Tth DNA ligase. Upon specific ligation of the PLPs, (4) rolling circle amplification products (RCPs) are generated by the Phi29 DNA polymerase, resulting in one RCP per hybridized L-probe, for an expected total of twelve RCPs per target. Once the amplification is concluded (5) the amplification products are labelled with detection oligonucleotides containing a fluorophore, allowing their detection and quantification through fluorescent microscopy. Due to the multiple steps required to perform the on-chip amplification, it was necessary to confine the beads to prevent their movement during the assay, which could give rise to heterogeneous signals that could hinder the detection performance. To achieve this, the design from Ref. 38 was used and adapted for this diagnostic method, since this confines the beads in all directions, thus preventing movement during the assay, granting more homogeneous signals.

To test the performance of this method in the direct detection of viral RNA for diagnostic purposes and determine its limit of detection (LoD), a dilution series of synthetic SARS-CoV-2 RNA was detected using the aforementioned pool of probes. The obtained results, presented in Figure 1B, show that the on-chip HybRCA is capable of reliably detecting RNA concentrations down to 3000 copies/µL, with an estimated LoD of 1000 copies/µL after 90 min of a single round of isothermal amplification. Importantly, this method shows the potential to provide a qualitative "yes/no" answer for a patient with active infection while making use of a sub-cm-sized, robust microfluidic platform. Furthermore, the fast amplification combined with the simple and easily achievable isothermal requirements of the amplification assay, demonstrates the great potential of this assay in approaching diagnostics in both resource-rich and resource-limited settings. While the LoD of the method is not as low as the RT-qPCR gold standard for detecting viral RNA, it is comparable to that of the standard, one-round RCA methodologies[21,31]. However, since this methodology relies mostly on hybridization conditions of the probes, it does not yet allow for single nucleotide specific detection of RNA, since the ligation of the padlock probe is not done directly on the target. As such, this technology is more

directed for point-of-care viral diagnostics and not the profiling of pathogen variants characterized by their single nucleotide polymorphisms.

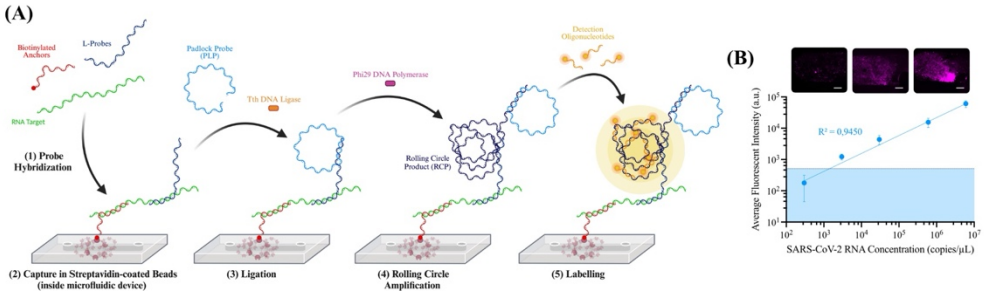

Figure 1: (A) Schematic of the hybridization-based rolling circle amplification (hybRCA), performed entirely inside a microfluidic device by capturing the RNA target in streptavidin-coated agarose beads using biotinylated "capture" oligonucleotides – the biotinylated anchors. **(B)** Dilution series of SARS-CoV-2 Control 2 RNA detected using HybRCA. The shaded region corresponds to the limit-of-detection of the method (average of the negative control plus three times the standard deviation; n = 3); representative images for 3 detected concentrations (300 copies/µL, 3000 copies/µL and 30000 copies/µL, respectively); scale bars correspond to 100 µm for each image.

3.2. Direct viral RNA genotyping resorting to Circle-to-Circle Amplification (C2CA)

RNA detection of pathogens typically requires a reverse transcription step that tends to increase the cost and complexity of the analysis. For PLP-RCA, this is especially problematic, since the lack of highly specific and efficient enzymes that can ligate the PLP in a DNA-RNA interface jeopardizes either the single nucleotide specificity inherently dependent on the proper ligation of the PLP, or the time necessary for the assay to take place, limiting its point-of-care applicability. Ligases typically used for the direct detection of RNA using RCA and PLPs include the Chlorella virus PBCV-1 DNA ligase (SplintR) and the T4 RNA ligase 2 (T4Rnl2). SplintR has been described as capable of ligating RNA-templated ssDNA with very high processivity but questionable specificity, typically requiring careful probe design or extra steps to ensure a high ligation accuracy [32,33]. On the other hand, T4Rnl2 has been shown to provide great ligation specificity when combined with the use of "chimeric" PLPs - a probe with a ribose at the 3'- end instead of a deoxyribose – especially when the probe targets the mutation at its 3´- end. However, the processivity of this enzyme is much lower compared to SplintR, resulting in increased assay times that may not result in enough amplification products

to ensure the detection of low target concentrations [33]. By performing two consecutive rounds of amplification, C2CA increases the sensitivity of the assay up to 1000 times per hour on the second round of amplification when compared to single-round RCA [19,21], helping to address the T4Rnl2 low processivity in the direct detection of RNA. Aiming to better understand the performance of C2CA when detecting RNA using T4Rnl2 in the first amplification round, dilution series of three SARS-CoV-2 variants (Wuhan strain – Wu; B.1.1.7 variant – Alfa; B.1.351 variant – Beta) were detected using a set of six "chimeric" PLPs, with two probes targeting each variant. A schematic of the used methodology is found in Figure 2A.

The results, summarized in Figure 2B for the analysis performed in the glass slides and Figure 2C when using μACE, shown a reliable detection of each individual variant down to a concentration of 100 copies/μL, with a LoD close to 10 copies/μL for all the variants. Furthermore, the obtained LoD matches previously determined values when using the same method for DNA detection, both when quantifying the amplification products through discrete counting using glass slides [21,34], and when determining the average fluorescent signal present in the streptavidin-coated beads inside the μACE[21], albeit with a noticeable decrease in the overall signal. It should be noted, however, that although no significant mismatches were observed in these experiments, T4Rnl2 has been described has being more specific and efficient when ligating certain nucleotides compared to others [33]. As such, this should be considered when designing future probes for targeting other mutations, since shifting the ligation site 1-2 bp away from the mutation could sometimes be enough to circumvent this limitation.

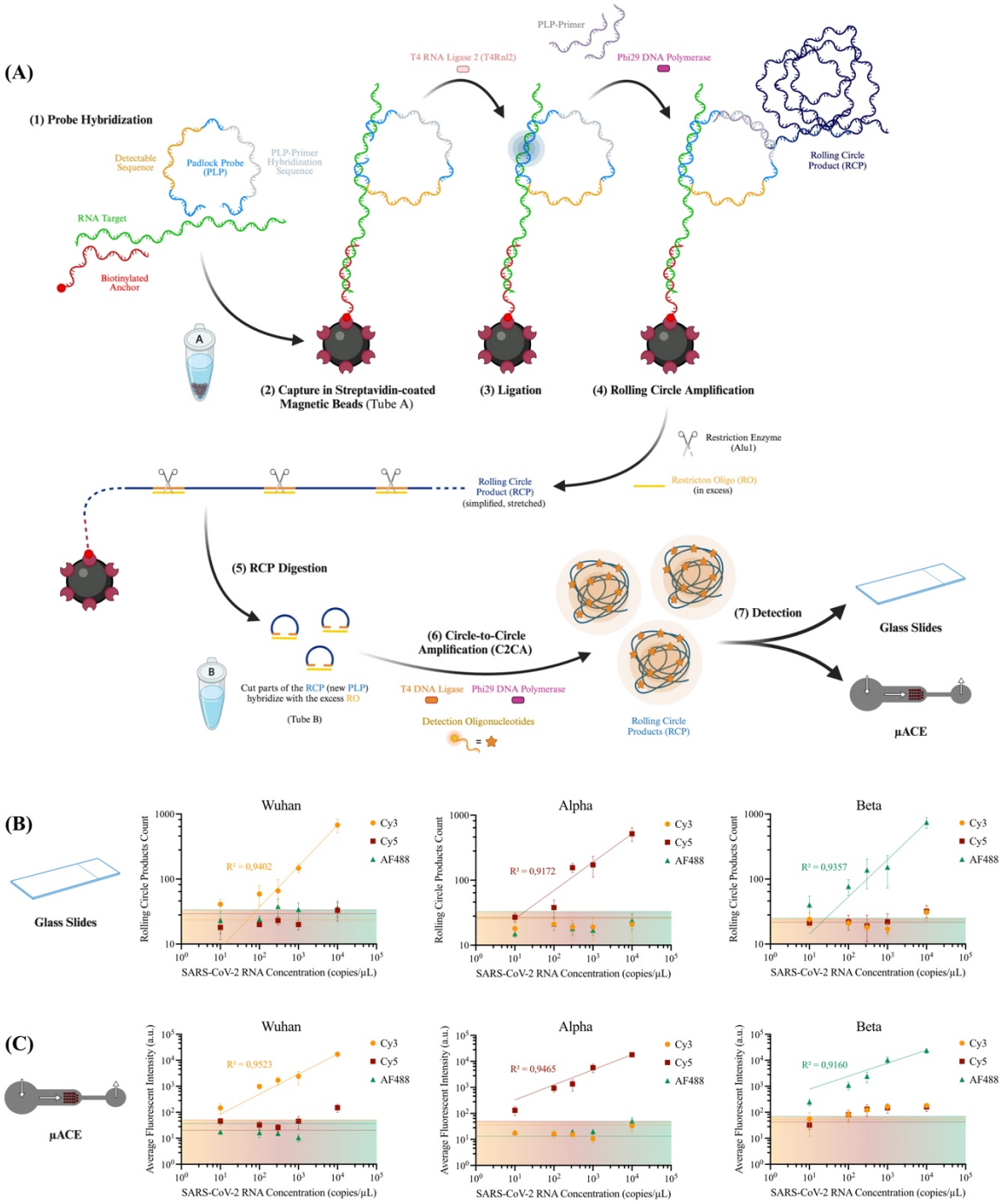

Figure 2: (A) Schematic of the Circle-to-Circle Amplification (C2CA) method used for RNA detection and variant profiling, using T4 RNA ligase 2 (T4Rnl2) as the RNA ligase of choice, combined with "chimeric" padlock probes (PLPs) in the first amplification round. Detection oligonucleotides (DO) with different fluorophores were used to detect the different SARS-CoV-2 variants: Cy3 for Wu (Wuhan strain); Cy5 for Alpha (variant B.1.1.7); and AF488 for Beta (variant B.1.351). **(B)** Counts of amplification products (RCPs) labelled with each of the different used DOs when detecting various concentrations of each SARS-CoV-2 variant. Each value corresponds to the sum of four

independent 300 x 300 μm^2 images of 10 µL of labelled solution in positively charged glass slides. (C) Average fluorescent intensity (a.u.) measured in the microchannels of the µACE for the different used DOs when detecting the various SARS-CoV-2 variants. Each value corresponds to the the average grey scale intensity of the bead-packed region, normalized for the fluorescence of the empty channel. The shaded-region corresponds to the LoD determined individually for each of the used DOs, (average of the negative control plus three times the standard deviation; n = 3).

3.3. Detection of Multiple Viral Strains – Variant Profiling using C2CA

To further test the capability of C2CA in detecting multiple single nucleotide polymorphisms specific to the three used viral variants, mixed solutions containing various combinations and concentrations of each variant were detected using this methodology. Quantification was done once again using both positively charged glass slides for discrete RCP counting, and µACE for determining the average fluorescent signal of the amplification products.

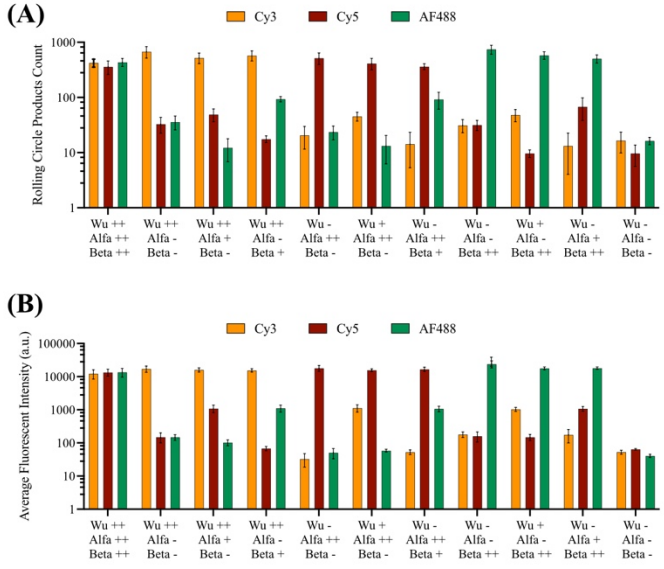

Figure 3: Registered signal obtained for each different DO when detecting various samples containing various concentrations and SARS-CoV-2 variants using (A) positively charged glass slides or (B) µACE as a detection method. The solutions contain either (-) none of the variant, (+) 10^2 copies/µL of the variant, or (++) 10^4 copies/µL of the variant (n = 3).

The results presented in Figure 3 highlight that T4Rnl2 enables the specific direct detection of each different RNA, while the quantified amplification products (counts in the glass slides, Figure 3A; average fluorescent signal, Figure 3B) allow the estimation of the amount of RNA present in each solution. Importantly, this demonstrates the potential of C2CA in not only detecting RNA directlty through the use of “chimeric” PLPs and T4Rnl2 in the first ligation step, but also in currently identifying different single nucleotide polymorphisms between different viral strains.

4. Conclusion

In this work, we aim to provide new molecular diagnostic tools combined with miniaturized devices to enable the detection and genotyping of viral pathogens through the direct detection of RNA. Here, we demonstrated that RCA, when combined with a microfluidic platform, allows the detection of RNA fragments down to a concentration of 10^2 copies/µL. The easily achievable isothermal requirements of this method (e.g: use of a thermal water bath), as well as its possible integration into a fully automated system in the future, shows the point-of-care potential of this technology, paving the way for a future diagnostic approach both in resource-rich and resource-limited setting alike. We also show the potential of C2CA as an alternative to next generation sequencing in identifying different viral variants, being able to detect single nucleotide polymorphisms when using T4Rnl2 and “chimeric” PLPs in the first round of amplification individually, down to a concentration of approximately 10 copies/µL, or in more complex solutions containing more than one variant. Furthermore, we emphasize the usefulness of the previously described µACE technology in not only facilitating the analysis and detection of amplification products, but also in bringing this lab-intensive method closer to point-of-care applications. We envisage that, upon these advances, a C2CA-compatible microfluidic device can be designed in the future as a point-of-care diagnostic tool for the direct detection and profiling of RNA from different pathogens.

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Supporting Information

ROLLING CIRCLE AMPLIFICATION AS A TOOL FOR DIRECT VIRAL RNA DETECTION AND VARIANT PROFILING

Table S1: List of biotinylated capture oligonucleotides (Biot-Anchor), padlock probes (PLP), L-shaped oligonucleotides (L-Probe), detection oligonucleotides (DO), PLP-primer and restriction oligonucleotide (RO) sequences used for each assay. Color codes highlight complementary regions, with green highlighting sequences complementary to the target, blue for complementary probes, and red for detection probes. Riboses are identified in the “chimeric” PLPs with a purple color.

Sequence ID (assay)	**Sequence (5' - 3')**
Biot-Anchor 1 (HybRCA)	**Biotin - AAAAAAAAAA TAGTGAACCGCCACACATGACCATTT CACTCAATACTTGAGCACACTCAT**
Biot-Anchor 2 (HybRCA)	**Biotin - AAAAAAAAAAAA TGTTTACGCAAATATGCGTAAAACT CATTCACAAAGTCTGTGTCAACA**
Biot-Anchor 3 (HybRCA)	**Biotin - AAAAAAAAAA GCTTCAGACATAAAAACATTGTTTTGAT AATAAAGAACTGACTTAAAGTT**
L-Probe 1 (HybRCA)	**ACATGTTGTGCCAACCACCATAGAATTTGCT TTTTAGTGT TACGATGTTGACCCCTGTA TTAGGGATGCGGGTAGTA**
L-Probe 2 (HybRCA)	**CATTTGAGTTATAGTAGGGATGACATTACGT TTTTAGTGT TACGATGTTGACCCCTGTA TTAGGGATGCGGGTAGTA**
L-Probe 3 (HybRCA)	**CAGATAGAGACACCAGCTACGGTGCGAGCTC TTTTAGTGT TACGATGTTGACCCCTGTA TTAGGGATGCGGGTAGTA**
L-Probe 4 (HybRCA)	**GCCATAATTCTAAGCATGTTAGGCATGGCTC TTTTAGTGT TACGATGTTGACCCCTGTA TTAGGGATGCGGGTAGTA**
L-Probe 5 (HybRCA)	**GCTACAACACGTTGTATGTTTGCGAGCAAGA TTTTAGTGT TACGATGTTGACCCCTGTA TTAGGGATGCGGGTAGTA**
L-Probe 6 (HybRCA)	**CAATTACTACAGTAGCTCCTCTAGTGGCGGC TTTTAGTGT TACGATGTTGACCCCTGTA TTAGGGATGCGGGTAGTA**
L-Probe 7 (HybRCA)	**CCCAACCCATAAGGTGAGGGTTTTCTACATC TTTTAGTGT TACGATGTTGACCCCTGTA TTAGGGATGCGGGTAGTA**
L-Probe 8 (HybRCA)	**GTGGCATCTCCTGATGAGGTTCCACCTGGTT TTTTAGTGT TACGATGTTGACCCCTGTA TTAGGGATGCGGGTAGTA**
L-Probe 9 (HybRCA)	**GACACTCATAAAGTCTGTGTTGTAAATTGCG TTTTAGTGT TACGATGTTGACCCCTGTA TTAGGGATGCGGGTAGTA**
L-Probe 10 (HybRCA)	**GAAACACACAACAGCATCGTCAGAGAGTATC TTTTAGTGT TACGATGTTGACCCCTGTA TTAGGGATGCGGGTAGTA**
L-Probe 11 (HybRCA)	**GAGGTCCTTTAGTAAGGTCAGTCTCAGTCCA TTTTAGTGT TACGATGTTGACCCCTGTA TTAGGGATGCGGGTAGTA**
L-Probe 12 (HybRCA)	**GCCGGCCCCTAGGATTCTTGATGGATCTGGGTTTTAGTGT TACGATGTTGACCCCTGTA TTAGGGATGCGGGTAGTA**
PLP (HybRCA)	**TACAGGGGTCAACATCGTA TGCGTCTATTTAGTGGAGCCTT AGTAGCCGTGACTATCGACT ACTACCCGCATCCCTAA**
DO (HybRCA)	**AGTAGCCGTGACTATCGACT - Cy5**

Sequence ID (assay)	**Sequence (5' - 3')**
Biot-Anchor 1 (C2CA)	**Biotin - TTTTT** AACATTAGTAGCGTTATTAACAA TAAGTAGGGACTGGGTC
Biot-Anchor 2 (C2CA)	**Biotin - TTTTT** CCAGTTTGCCCTGGAGCGATTTG TCTGACTTCATCACCTC
Biot-Anchor 3 (C2CA)	**Biotin - TTTTT** GAAACTTTTTGTTAGACTCAGTA AGAACACCTGTGCCTGT
Biot-Anchor 4 (C2CA)	**Biotin - TTTTT** AGCAGCAAGATTAGCAGAAGCTC TGATTTCTGCAGCTCTA
PLP 1-Wu (C2CA)	AGTGGGTTGGAAACC GTGTATGCAGCTCCTCAGTAATAGTGTCTTAC TGCGTCTATTTAGTGGAGCC GTAACCAACACCATrU
PLP 2-Wu (C2CA)	CCTGATAAAGAACAG GTGTATGCAGCTCCTCAGTAATAGTGTCTTAC TGCGTCTATTTAGTGGAGCC CTGTGCAGTTAACArU
PLP 1-Alfa (C2CA)	CAATGTCTCTGCCAA GTGTATGCAGCTCCTCAGTAATAGTGTCTTAC AGTAGCCGTGACTATCGAC CATCAGTAGTGTCArU
PLP 2-Alfa (C2CA)	AAGGATATCATTTAA GTGTATGCAGCTCCTCAGTAATAGTGTCTTAC AGTAGCCGTGACTATCGAC TTTGTCAAGACGTGrC
PLP 1-Beta (C2CA)	CACGCACTAAATTAA GTGTATGCAGCTCCTCAGTAATAGTGTCTTAC AGTCGATAGTCACGGCTACT AACCCTGAGGGAGArC
PLP 2-Beta (C2CA)	AACACCATTACAAGG GTGTATGCAGCTCCTCAGTAATAGTGTCTTAC AGTCGATAGTCACGGCTACT ACAATTAAAACCTTrU
PLP-Primer (C2CA)	TACTGAGGAGCTGCATACAC
RO (C2CA)	GTGTATGCAGCTCCTCAGTA
DO Biot-Common (C2CA)	GTAAGACACTATTACTGAGGATTTT - Biotin
DO Cy3-Wu (C2CA)	GGCTCCACTAAATAGACGCATTTT - Cy3
DO Cy5-Alfa (C2CA)	AGTCGATAGTCACGGCTACTTTT - Cy5
DO AF488-Beta (C2CA)	AGTAGCCGTGACTATCGACTTTT - AF488

CHAPTER 7

General discussion

Conclusion and perspectives for the future

1. General discussion

The study of airborne viral transmission, particularly with respect to SARS-CoV-2, has highlighted critical challenges in controlling infectious disease outbreaks. These challenges arise from the complexities of transmission mechanisms, the ambiguity in terminologies, and the limitations of current preventive strategies [1,2].

At the time this research PhD work started, early in the COVID-19 pandemic, there was strong and consistent evidence suggesting that SARS-CoV-2 was predominantly spread through the air [3,4]. Still, the WHO and other public health agencies around the world did not acknowledge the airborne transmission of SARS-CoV-2.

With that in mind, and in agreement with other specialists pointing to the probable airborne transmission of SARS-CoV-2, the first objective of this thesis was to investigate SARS-CoV-2 RNA presence in different environments, namely hospitals, public transport systems, and public spaces such as restaurant terraces and crowded urban areas in three countries: Portugal, Spain and Norway.

SARS-CoV-2 RNA detection in air samples in hospitals

Main conclusions from the studies:

- High Transmission Risk in Healthcare Settings: Detection of SARS-CoV-2 RNA in hospital air samples, particularly in COVID-19 ICUs, suggests a significant risk of nosocomial transmission.
- Impact of Variants: The results demonstrated that highly transmissible variants like Delta can lead to RNA detection even with high vaccination rates and stringent infection control measures.
- Surface Contamination: Though less common, the presence of viral RNA on surfaces in both COVID-19 and non-COVID-19 zones indicates potential for indirect transmission.
- Importance of Enhanced Preventive Strategies: The findings reinforce the need for effective masks, proper ventilation, and continuous infection control protocols in healthcare environments.

Considering that patients infected with SARS-CoV-2 would likely need to go to a hospital, the incidence of nosocomial infection with SARS-CoV-2 was expected to be high [5]. This type of transmission poses a risk of spreading the virus to the most vulnerable populations and can lead to broader consequences through interactions between hospitals and the community [6]. Moreover, nosocomial transmission was also known to have played a significant role in the prior outbreaks of MERS-CoV and SARS-CoV-1 [7,8]. In this context, we performed air sampling in two hospitals, namely one in Portugal [9] and one in Spain [10] during two different COVID-19 waves.

The first study assessed environmental contamination by SARS-CoV-2 in a major hospital in Portugal during the third wave of COVID-19, in January 2021, with the Alpha variant, also known as B.1.1.7, being the driver of this wave. It focused on both air and surface samples across eleven hospital areas, including COVID-19 and non-COVID-19 zones. Key findings revealed that SARS-CoV-2 RNA was detected exclusively in the air of the COVID-19 ICU, with viral loads ranging from 6000 to 6662.5 copies/m³. This detection was primarily noted during the first 10 minutes of air sampling with the Coriolis® μ, likely due to aerosol generation during a patient's intubation. On surfaces, SARS-CoV-2 RNA was found on a wall in a COVID-19 testing booth and in a non-COVID-19 waiting room, specifically on a faucet handle and a bathroom flush button, suggesting potential touch contamination or aerosol spread.

The second study was performed during the fifth wave of COVID-19 in Spain, in July 2021, which was marked by the highly transmissible Delta variant (B.1.617.2). In this study the presence of SARS-CoV-2 in indoor air and on surfaces in a major hospital in Castile-León was assessed. Despite rigorous infection control measures, the study identified three positive samples for SARS-CoV-2 RNA: one air sample from the emergency waiting room and two surface samples from the hospital's microbiology laboratory. The low number of positive samples might be linked with the high vaccination rates in Spain by late July 2021, which significantly reduced hospitalizations and ICU admissions compared to earlier waves. Also, even though immunization does not guarantee full protection against infection, it reduces the viral load, which may have contributed to the low number of positive samples.

The discussions and findings from both studies highlight the persistent risk of SARS-CoV-2 transmission, particularly in healthcare settings, even with stringent infection control measures in place. The detection of viral RNA in air and surface samples within hospitals, despite high vaccination coverage and the use of personal protective equipment, underscores the high transmissibility of variants like Delta and the potential for nosocomial infections. These findings emphasize the critical need for ongoing

vigilance and robust infection prevention protocols, including the use of highly efficient masks (e.g., KN95 and FFP2) and proper ventilation systems, especially in enclosed spaces and areas with high-risk patients.

Moreover, the studies illustrate that, while surface contamination may be less common, the potential for airborne transmission remains significant. This reinforces the importance of acknowledging and addressing the airborne route as a primary mode of SARS-CoV-2 spread, particularly considering new, more transmissible variants. The healthcare environments must continue to adapt, incorporating updated public health guidelines that prioritize the prevention of airborne transmission to protect both healthcare workers and patients. In conclusion, as SARS-CoV-2 continues to evolve, it is crucial to maintain and enhance preventive strategies to mitigate the spread of infection, ensuring safety in both healthcare settings and the wider community.

SARS-CoV-2 RNA detection in air samples in public transport settings

Main conclusions from the studies:

- Effectiveness of Preventive Measures: Low positivity rates in public transport air samples suggest that mask usage and ventilation significantly reduce airborne transmission risks.
- High-Risk Scenarios: The detection of viral RNA in busy train stations during rush hours highlights the increased risk in crowded and poorly ventilated settings.
- Residual Transmission Risk: Even with strict protocols, there remains a small risk of transmission in densely populated transport settings.
- Importance of Continued Adherence to Protocols: The study underscores the need for ongoing health and safety measures in public transportation to mitigate transmission risks.

SARS-CoV-2 is a respiratory virus, which makes it more rapidly and easily transmissible [11]. As public transportation is the only way of travel and commuting for a great portion of the population, it would be difficult to maintain the required distances in such confined spaces, either in a subway, a bus or a train [12]. Consequently, the extensive connectivity provided by public transport systems could enable the virus to spread rapidly, with the daily movement of people playing a role in local transmission [13]. The precise extent to which public transportation contributed to the spread of SARS-CoV-2, however, was not clear, with very few studies published on this topic [12–14] until summer 2021.

Aiming at better understanding the presence of SARS-CoV-2 in environments related to public transport, we performed air and surface sampling in public transport systems in Portugal during the fifth wave of the pandemic. In this study, both indoor and outdoor areas related to public transport were assessed. Out of 31 air samples collected, only two were positive, and no surface samples showed viral presence, underscoring the effectiveness of the infection prevention measures in place, such as thorough disinfection and mandatory mask-wearing on trains. Notably, the positive air samples were collected from a busy train station during morning rush hours, suggesting that high human movement might increase the likelihood of viral presence.

The low positivity rate in this study, particularly in outdoor and well-ventilated areas, confirms that proper ventilation combined with strict adherence to mask usage might significantly mitigate the risk of airborne transmission. However, the study also underscores that even in environments with robust preventive measures, there is a residual risk of transmission, especially in densely populated settings where mask usage and social distancing are not uniformly enforced. In conclusion, the results from this study highlighted the importance of maintaining stringent health and safety protocols in public transportation settings, including regular disinfection, proper mask usage, and maintaining physical distancing.

Air sampling in outdoor public spaces

Main conclusions from the studies:

- Lower Risk in Outdoor Settings: The results confirmed that outdoor transmission is generally less likely potentially due to natural ventilation, UV radiation, and environmental factors.

- Impact of Crowd Density: Crowded outdoor settings significantly increased the risk of airborne transmission, particularly when preventive measures like mask usage are not followed.

- RNA Viability: RNAse pretreatment results suggest that detected viral RNA in outdoor settings is often not viable, supporting the lower transmission risk outdoors.

- Adaptive Public Health Measures: The findings emphasize the importance of mask-wearing and social distancing in crowded outdoor environments, even after mask mandates are lifted.

Although the WHO did not acknowledge airborne transmission of SARS-CoV-2 as a mode of transmission until the end of 2021 [15], scientists and specialists generally agreed that COVID-19 was primarily spread through the air. Most infections occurred indoors, with outdoor transmission being less frequent [16]. This lower frequency of outdoor transmission is mainly due to improved airflow and natural ventilation that disperse viral particles. While several studies have been made on detection of SARS-CoV-2 in air samples from indoor environments [17–20], mainly in healthcare facilities, air monitoring of SARS-CoV-2 in outdoor non-healthcare environments were scarce [21–23]. With the goal of enhancing our understanding of SARS-CoV-2 dynamics in outdoor air, air sampling was conducted in public spaces across Portugal, Spain, and Norway during the spring and winter of 2021, and again in the spring of 2022. Additionally, given that the detection of RNA does not necessarily correlate to the viability of the virus, RNAse pretreatment of air samples was tested as an alternative to cell culture for assessing viral viability. This approach was implemented in the air samples collected during the studies in Portugal and Norway.

The first study was conducted in Portugal, in spring 2021, where out of 19 air samples collected from an outdoor restaurant terrace, only one was positive for SARS-CoV-2 RNA. This sample coincided with a high density of people in the vicinity of the air sampler, most of whom were not wearing masks due to eating and drinking. Despite the potential for higher virus transmission under these conditions, the general findings indicated a low risk of transmission in outdoor settings, credited to good air circulation and environmental factors like UV radiation and temperature that may have inactivated the virus. The study also explored the use of RNAse pretreatment to assess viral viability, which indicated that the detected virus was not viable, aligning with the notion that outdoor environments are generally lower-risk for COVID-19 transmission.

The second study, which took place in Spain, in winter 2021, was performed during the rapid spread of the Omicron variant (B.1.1.529) and assessed viral presence in two contrasting urban scenarios in Spain: empty and crowded streets. No virus was detected in air samples from empty streets, whereas a significant proportion of samples from crowded settings tested positive for SARS-CoV-2 RNA. This suggests that the risk of airborne transmission increases significantly with crowd density, which could potentially overcome the mitigative effects of being outdoors. The study highlighted the effectiveness of masks in reducing transmission, especially in conditions with inadequate air dispersion such as low wind days or temperature inversions that can keep viral particles concentrated near the ground.

The third study conducted in Norway in spring 2022, when restrictions had been

lifted, including the mandate for masks even in healthcare settings. The study focused on the detection of SARS-CoV-2 RNA in air samples collected from a mix of indoor and outdoor environments, including healthcare and non-healthcare facilities. Key findings from the study revealed that only four out of 31 air samples tested positive for SARS-CoV-2 RNA, none of which showed viral viability after RNAse pre-treatment. This suggests that while viral RNA was present, infectious viruses likely were not. The environments yielding positive results included places frequented by vulnerable populations: two samples from a kindergarten, one from a nursing home, and one from the pediatrics waiting room of a health clinic. Notably, the detection of RNA in these settings seems to be linked to higher densities of people, such as the full capacity observed in the kindergarten, which also reported symptomatic children. Despite low RNA counts suggesting an unlikely presence of infectious virus, the study points to the importance of continuous risk assessment and the development of mitigation strategies based on environmental viral presence data. The nuclease pre-treatment method provides a valuable tool in this context, offering an alternative to more complex viral culture methods for estimating the presence of infectious viruses.

Taken altogether, results across these three studies underscore the nuanced nature of SARS-CoV-2 transmission in various environments and under different epidemiological conditions. The detection of SARS-CoV-2 RNA in outdoor settings, such as restaurant terraces, is significantly influenced by factors like air circulation, UV radiation, and the density of people in the environment. Although RNA is detectable, it is likely not viable, as evidenced by RNAse pretreatment results. This supports the notion that outdoor transmission is less likely compared to transmission in indoor environments, thanks to natural ventilation and environmental factors that contribute to the rapid degradation of the virus.

The observed increase in RNA positivity during crowded events and in specific indoor settings amid the easing of restrictions underscores the crucial role of public health measures such as mask-wearing and social distancing. Notably, areas with dense movement of people and lax adherence to preventive measures exhibit higher RNA detection rates, emphasizing the necessity for ongoing public health vigilance even as restrictions are lifted.

In Norway, the absence of mask mandates in healthcare settings has correlated with the detection of RNA in environments frequented by the elderly and children, highlighting the potential risk of transmission among these high-risk groups. These findings indicate that particularly vulnerable settings involving unvaccinated or partially vaccinated populations require targeted strategies to effectively mitigate the spread of the virus.

The application of RNAse pretreatment serves as a practical method for assessing viral infectivity, especially in the absence of BSL-3 facilities. This approach provides a rapid assessment that can significantly inform and influence public health responses in real-time.

Moreover, the rapid proliferation of highly transmissible variants like Omicron necessitates adaptive and responsive public health strategies. The discussions illuminate the variable impact of such variants across different settings, prompting a crucial reassessment of existing prevention and control measures based on current and evolving epidemiological data. These insights highlight the ongoing need for strategic, data-driven approaches to managing public health in the face of an ever-changing viral landscape.

A new detection tool for viral RNA and variant profiling

Main conclusions from the studies:

- Challenges in Air Sampling: Low concentrations of viral RNA in air samples posed challenges for genome sequencing, crucial for understanding circulating variants.

- Innovative Detection Method: The development of the PLP-based RCA method allows for sensitive detection and variant profiling, addressing the limitations of traditional methods.

- Practical Applications: The microfluidic device offers a scalable and rapid diagnostic tool, ideal for Point-of-Care (PoC) settings and resource-limited environments.

- Future Surveillance: This advanced method positions itself as a crucial tool for future outbreak monitoring and response, particularly for identifying and managing emerging variants.

When it comes to air sampling for monitoring SARS-CoV-2 in indoor and outdoor environments, the low concentration of virus in air samples became challenging, as downstream molecular analysis usually requires high quantities of starting genetic material [24]. Therefore, genome sequencing of virus detected in air samples was not possible, making it difficult for a correct interpretation of results from air sampling studies

for SARS-CoV-2, as information about which variant was circulating during each experiment is extremely important from a public health and epidemiology standpoint. Considering these difficulties, we developed a PLP-based RCA method allowing both direct detection of SARS-CoV-2 RNA and variant profiling by detecting point mutations with high sensitivity.

In this study, we introduced an advanced RNA detection method utilizing a microfluidic device for signal read-out aiming at simplifying the complex process of RNA diagnostics. The method employs hybridization-based rolling circle amplification (HybRCA), capturing RNA on streptavidin-coated agarose beads and using biotinylated oligonucleotides and padlock probes (PLPs) to initiate the amplification. This technique proves capable of detecting as low as 1000 copies/μL of SARS-CoV-2 RNA, demonstrating a potential for rapid, qualitative diagnostic assessments directly on chip. The approach is particularly advantageous for PoC settings, offering a significant reduction in the complexity and requirements typical of standard diagnostic assays.

Further, the study explores C2CA to enhance sensitivity and specificity in RNA detection, overcoming limitations of single-round amplification by employing two consecutive amplification rounds. This method, utilizing T4 RNA ligase 2 (T4Rnl2) with chimeric PLPs, can detect less than 10 genome copies/μL and specifically identifies viral variants, showing potential for detailed variant profiling without extensive sequencing efforts.

In practical terms, the findings indicate that this platform can detect low concentrations of RNA and identify different SARS-CoV-2 variants using a robust, scalable approach suited for both high-resource labs and resource-limited settings. This positions the described methodologies as a promising tool for future outbreak surveillance and rapid response to emerging viral threats, emphasizing the importance of continuing development in diagnostic technology to enhance global pandemic preparedness.

2. Conclusions and future work

Studying the presence of SARS-CoV-2 in different environments during the COVID-19 pandemic has provided significant insights into its airborne transmission. This research has reinforced the understanding that SARS-CoV-2 can spread through the air, which ultimately contributed to the official recognition of airborne transmission by health authorities. This acknowledgment has been crucial, leading to the reformulation of mitigation strategies to better control the spread of the virus, such as the widespread use

of high-efficiency masks (e.g., N95), enhanced ventilation, and the implementation of social distancing measures. These measures are essential in controlling the virus within communities and should be considered fundamental in future respiratory disease outbreaks.

Moreover, the findings underscore the importance of incorporating air sampling programs into initial response plans for future outbreaks. Air sampling, as a non-invasive environmental monitoring method, could significantly aid in the early detection of airborne pathogens. These programs would not only provide real-time monitoring but also facilitate the swift implementation of public health interventions, thus mitigating the overall impact of any future respiratory disease outbreaks. For these studies to be effective and comparable, it is essential to standardize protocols. Future studies should always include detailed information regarding the type and brand of air samplers used, their location and position, the average flow rate and volume of air sampled, the date and time of sampling, and relevant environmental conditions such as wind direction, temperature and relative humidity. Additionally, the type of filter or collection medium used, and the storage and processing conditions of the samples should be meticulously documented.

In this context, longitudinal air sampling across diverse geographical locations becomes particularly important. This approach, involving continuous air sampling in various regions with differing climates, population densities, and cultural practices, is crucial for understanding how these factors influence airborne transmission. Such longitudinal studies would provide valuable data on the temporal and spatial dynamics of airborne pathogens, offering insights that can inform tailored public health interventions.

The research presented also highlights the need to assess the risks of airborne transmission for other respiratory pathogens. The methodologies developed in this study, including the novel RNA detection and viral profiling tool using PLPs, RCA and a microfluidic readout, could be adapted to monitor a broad spectrum of respiratory viruses. This adaptability is vital for establishing a proactive approach to emerging infectious diseases, ensuring that public health systems are better equipped to respond to future outbreaks.

Additionally, the development and expansion of collaborative global surveillance networks would be essential for real-time data sharing and coordinated response efforts. These networks would facilitate the global exchange of information on airborne pathogens, enabling quicker identification of emerging threats and more efficient deployment of resources to areas at high risk. By linking national and international health

agencies, these networks would enhance the collective capacity to monitor, detect, and respond to respiratory pathogens on a global scale.

Overall, this research underscores the ongoing need for robust monitoring of airborne viruses and the enhancement of infection control measures in both healthcare and public settings. The global recognition of the importance of airborne transmission, as highlighted by the COVID-19 pandemic, has already led to significant shifts in public health strategies. To further enhance our understanding and capabilities in preventing airborne transmission, future research should focus on expanding the geographical scope of air sampling studies, assessing the risks of other respiratory pathogens, and fostering collaboration through global surveillance networks.

Lastly, optimizing and refining point-of-care diagnostics, particularly those based on the PLP-based RCA method described in this study, could pave the way for more sensitive and specific tools, further enhancing our capacity to monitor and control airborne viruses effectively. The lessons learned from the COVID-19 pandemic should guide the development of better-prepared public health infrastructures and surveillance systems, ensuring a more robust response to future pandemics.

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