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Tese de doutorado

Diagnóstico molecular alternativo, desenvolvimento e aplicação de biomaterial para remoção de SARS-CoV-2 em água

Victor Dos Santos Barboza

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A Mãe Natureza é um serial killer. Não há melhor que ela. Nem mais criativa. E como todos os outros; Não resiste à tentação de ser pega; Então ela deixa migalhas. Estudamos décadas para reconhecer essas migalhas como pistas. E, às vezes, o que achávamos ser o aspecto mais brutal do vírus, nada mais é que sua vulnerabilidade; Ela adora disfarçar a fraqueza em força.

(Filme- Guerra Mundial Z)

Resumo

Barboza, Victor Dos Santos. **Diagnóstico molecular alternativo, desenvolvimento e aplicação de biomaterial para remoção de SARS-CoV-2 em água.** 109f. Tese para o doutorado – Programa de Pós-Graduação em Parasitologia e Microbiologia, Instituto de Biologia, Universidade Federal de Pelotas, Pelotas, 2024.

Em dezembro de 2019, o Centro Chinês de Controle de Doenças (CDC da China) relatou um surto de pneumonia na cidade de Wuhan (província de Hubei, China) que assombrou o mundo, resultando em uma pandemia global. Esse surto foi causado por um betacoronavírus chamado coronavírus da síndrome respiratória aguda grave 2 (SARS-CoV-2). Vários desses casos foram observados em profissionais de saúde que trabalhavam em hospitais e prestavam atendimento na linha de frente da pandemia, com isso produzimos o 1° capitulo na qual investigamos novas técnicas para diagnóstico molecular rápido, preciso e de baixo custo para COVID-19 em profissionais da saúde, mediantes aos resultados obtidos para especificidade e sensibilidade do Ensaio de amplificação isotérmica mediada por loop de transcrição reversa (RT-LAMP) surgiu como uma ferramenta de diagnóstico molecular rápido para COVID-19 em profissionais de saúde. Contúdo, após termino da pandemia ocasionada pelo SARS-Cov-2, uma nova preocupação para o controle de surtos a partir de águas residuais, se fez necessário. Relatos da presença de RNA do SARS-CoV-2 resiliente em águas durante alguns dias sem completa degradação despertou o interesse por novas pesquisas. Diante disso, surgiu à ideia dos capítulos 2 e 3 nesse trabalho. No capítulo 2, visamos a remoção de SARS-CoV-2 de água contaminada "*in natura*" a alga *Hydrocotyle ranunculoides*. Tal estudo revelou uma consideravel taxa de remoção de SARS-CoV-2 de 88,28% na água. No capítulo 3, buscamos desenvolver um modelo experimental para mensurar a redução de SARS-CoV-2 em água, e para isso, foi utilizado um criogel (C) incorporado com óleo essencial de *Rosa centifolia* (RCEO). Constamos que criogel incorporado com o óleo (RCEO) exibiu uma remoção da quantidade de carga viral de SARS-CoV-2 presente na água após filtração, sendo estimada em redução de 32 vezes. Portanto, estes estudos abrangem as preocupações que acometeram o mundo devido a surgimento, transmissão e infecção ocasionado pelo SARS-CoV-2, tendo o capítulo 1, diagnóstico de COVID-19 e os capítulos 2 e 3 destacando de utilização de compostos naturais como uma excelente alternativa para redução e descontaminação do SARS-COV-2 em águas.

Palavras chaves: Biologia molecular, Diagnóstico molecular, Doenças infeciosas, Água residual, Química verde

Abstract

Barboza, Victor Dos Santos. **Alternative molecular diagnosis, development and application of biomaterial for removal of SARS-CoV-2 in water.** 109p. Doctoral thesis – Postgraduate Program in Parasitology and Microbiology, Institute of Biology, Federal University of Pelotas, Pelotas, 2024.

In December 2019, the Chinese Center for Disease Control (China CDC) reported an outbreak of pneumonia in the city of Wuhan (Hubei province, China) that haunted the world, resulting in a global pandemic. This outbreak was caused by a betacoronavirus called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Several of these cases were observed in health professionals who work in hospitals and provide care on the front line of the pandemic, so we produced the 1st chapter in which we investigated new techniques for rapid, accurate and low-cost molecular diagnosis of COVID-19 in healthcare professionals, based on the results obtained for specificity and sensitivity of the Reverse Transcription Loop-Mediated Isothermal Amplification Assay (RT-LAMP), emerged as a rapid molecular diagnostic tool for COVID-19 in healthcare professionals. However, after the end of the pandemic caused by SARS-Cov-2, a new concern for controlling outbreaks from wastewater became necessary. Reports of the presence of resilient SARS-CoV-2 RNA in waters for a few days without complete degradation sparked interest in further research. Given this, the idea for chapters 2 and 3 in this work came up. In chapter 2, we aimed to remove SARS-CoV-2 from contaminated water using “in natura” the algae *Hydrocotyle ranunculoides*. This study demonstrated a considerable SARS-CoV-2 removal rate of 88.28% in water. In chapter 3, we sought to develop an experimental model to measure the reduction of SARS-CoV-2 in water, and for this, a cryogel (C) incorporated with Rosa centifolia essential oil (RCEO) was used. We found that cryogel incorporated with oil (RCEO) exhibited a removal of the amount of SARS-CoV-2 viral load present in the water after filtration, estimated to be a 32-fold reduction. Therefore, these studies cover the concerns that have affected the world due to the emergence, transmission and infection caused by SARS-CoV-2, with chapters 1, diagnosis of COVID-19 and Chapters 2 and 3 highlight the use of natural compounds as an excellent alternative for reducing and decontaminating SARS-COV-2 in water.

Keywords: Molecular biology, Molecular diagnosis, Infectious diseases, Wastewater, Green chemistry

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1. Introdução

Um surto de pneumonia, relatado pelo Centro Chinês de Controle de Doenças na cidade de Wuhan, província de Hubei em Wuhan, China, em dezembro de 2019 assombrou o mundo (ZHU et al., 2020; JIN et al., 2020). Esse surto resultou em uma pandemia global causada por um Betacoronavírus (Chan et al., 2020; CHANG et al., 2006; SHEIKH et al., 2020), que o Comitê Internacional de Taxonomia de Vírus denominou como coronavírus da síndrome respiratória aguda grave 2 (SARS-CoV-2) devido à sua proximidade genética e parentesco com a síndrome respiratória aguda grave (SARS-CoV) (CARTER et al., 2020; KAUSHIK et al., 2020; WANG et al., 2020).

Desde o início da pandemia já foram registrados 772.116.517 milhões de casos confirmados de infecção pelo SARS-CoV-2, com 6.981.263 milhões de mortes em todo o mundo (OMS, 2024). No Brasil, o primeiro caso de infecção por SARS-CoV-2 foi confirmado em 25 de fevereiro de 2020 (JESUS et al., 2020) e desde então, o número de casos confirmados chega a 38.592.310 milhões e o número de mortes por COVID-19 somam mais 710.427 mil (MINISTÉRIO DA SAÚDE, 2024).

Parte desses casos foi observado em profissionais de saúde que atuam em hospitais e prestam atendimento na linha de frente da pandemia (DROR et al., 2020; PETERSEN et al., 2020; HEATH et al., 2020; KURSUMOVIC et al., 2020; GODDERIS et al., 2020; REMUZZI et al., 2020). Tais profissionais eram diagnosticados rotineiramente pela técnica de transcrição reversa da cadeia da polimerase em tempo real (RT-PCR) (BOUSTEAD et al., 2020; MONCUNILL et al., 2021).

Embora a transcrição reversa da cadeia da polimerase em tempo real quantitativa (RT-qPCR) venha sendo utilizada para a detecção do SARS-CoV-2 por ser sensível e específica, o método possui algumas limitações (CORMAN et al., 2020**)**. Devido ao aumento dos casos em todo mundo e necessidade de diagnóstico confirmatório, existiu uma escassez de reagentes disponíveis para a realização da RT-qPCR. Dessa forma ocorreu um aumento no custo para execução dos testes e a alta demanda pelos insumos fez com que o prazo para entrega dos kits comerciais se estendesse por pelo menos 60 a 90 dias.

Nesse contexto, o ensaio de amplificação isotérmica mediada por loop de transcrição reversa (RT-LAMP) pode ser uma alternativa interessante, tendo em vista que tem se mostrado um método sensível, específico, versátil e robusto. O ensaio RT-LAMP simplifica os procedimentos de execução da técnica, pois simplifica os

procedimentos de extração, reduz o tempo de detecção, utiliza equipamentos mais simples como bloco de incubação de amostras e é baseado em ensaio colorimétrico (SILVA et al., 2019; AHN et al., 2019; YAN et al., 2020).

De acordo com a Organização Mundial da Saúde (OMS) no que diz respeito à testagem dos profissionais da área saúde, por estarem na linha de frente ao enfrentamento da COVID-19 devem ser monitorados e testados periodicamente, tendo em vista o custo elevado das reações pelo método RT-qPCR, acarretaria numa limitação no método. Essa preocupação ocorre, devido à necessidade desses profissionais serem isolados e adequadamente tratados quando ocorre a confirmação da infecção por SARS-CoV-2. Para contornar essa problemática abordada, o monitoramento da presença do SARS-CoV-2 em amostras clínicas desses profissionais, pode ser realizado através dos métodos de amplificação poderiam ser utilizados alternativos para o diagnóstico da doença (ALPDAGTAS et al., 2020; FALZONE et al., 2021).

À medida que a pandemia passou, foi encontrado um novo desafio: o SARS-CoV-2 foi detectado em águas residuais em todo o mundo, atingindo até domínios públicos e esgotos domésticos (KUMAR et al. 2021a). Além disso, a consciência do risco de infecção através de águas residuais aumentou ainda mais quando começaram a ocorrer possíveis transmissões de COVID-19 através de esgotos (YUAN et al. 2020; GRAHAM et al. 2020).

Alguns estudos passaram a avaliar o risco a que estão expostos os trabalhadores de estações de tratamento de água e esgoto ao entrarem em contato com água contaminada, sugerindo a necessidade de adoção de protocolos de tratamento de águas residuais e esgoto e também de práticas para conter esse vírus potencialmente pandêmico (ZANETI et al. 2021; GHOLIPOUR et al. 2021; DHAMA et al, 2021).

Enquanto a discussão sobre a remoção do SARs-CoV-2 da água e do esgoto já era considerada, um novo cenário preocupante se apresentava: o risco toxicológico para ambientes aquáticos causado por fragmentos Spike do SARS-CoV-2 (CHARLIE-SILVA et al. 2021). Esses fragmentos causaram desequilíbrio REDOX e neurotoxicidade aguda em girinos (*Physalaemus cuvieri*) e demonstraram através de estudos *in silico* que ocorrem interações entre peptídeos e acetilcolinesterase e enzimas antioxidantes. Assim, a contaminação de ambientes aquáticos por partículas

de SARS-CoV-2 pode apresentar um risco ecotoxicológico e ambiental adicional (CHARLIE-SILVA et al. 2021).

Por fim, mediante a crise sanitária que acarretou em impactos duradouro na saúde da população, nas esferas econômicas e sociais, e visto as incertezas quanto ao comportamento e mutações do SARS-CoV-2 no ambiente (HU et al. 2021), se fez necessário desenvolver materiais alternativos com potencial para remoção/redução do vírus no ambiente, produzidos de forma sustentável, em grande escala e com baixo custo.

2 Objetivos

2.1 Objetivo Geral

- Avaliar novos métodos de diagnóstico molecular para COVID-19 em profissionais da saúde.
- Investigar a capacidade da *H. ranunculoides* na remoção de SARS-CoV-2 na água.
- Desenvolver um criogel incorporando o óleo essencial de *Rosa centifolia* para redução de SARS-CoV-2 na água.

2.2 Objetivos Específicos

- Determinar a especificidade e sensibilidade do ensaio de RT-LAMP desenvolvido durante a pandemia do SARS-CoV-2;
- Visualizar as morfologias dos poros da superfície da *H. ranunculoides;*
- Relatar a variação da perda de massa da *H. ranunculoides*, em função do decorrer do tempo e temperatura submetida;
- Verificar as propriedades térmicas da *H. ranunculoides*;
- Distinguir os grupos funcionais da *H. ranunculoides*;
- Caracterizar os compostos majoritários da *R. centifolia*;
- Observar a morfologia da superfície do criogel incpororado pelo óleo essencial *Rosa centifolia*;
- Analisar a variação da perda de massa do RCEO, em função do decorrer do tempo e temperatura submetida;
- Analisar a densidade, porosidade, solubilidade em água e propriedades mecânicas dos criogéis;
- Executar a técnica de RT-qPCR na água filtrada pelo C-RCEO;

3 Revisão da Literatura

3.1 Coronavírus

Apesar do esforço do poder de saúde pública e do progresso significativo em diferentes partes do mundo em conter o desenvolvimento das doenças infeciosas (SHEN et al., 2020), surtos repetidos nas últimas décadas, incluindo coronavírus (De WIT et al., 2016), gripe aviária (CHATZIPRODROMIDOU et al., 2018) e chikungunya (WEAVER et al., 2015), demonstraram que os agentes infecciosos estão em constantes mutações (YOUNES et al., 2020).

Recentemente, no final de 2019, uma nova doença respiratória de rápida disseminação foi detectada na cidade de Wuhan, província de Hubei, China, acometendo mais de 216 países no mundo (WHO, 2020; CARTER et al., 2020). Dados completos do genoma viral identificou que a doença é causada por um novo vírus de RNA relacionado à família Coronaviridae, nomeado de síndrome respiratória aguda grave coronavírus 2 (SARS-CoV-2), posteriormente intitulado como novo coronavírus (COVID-19) (WHO, 2020), classificado no gênero *Betacoronavirus*, na ordem dos nidovirales (Yang e Wang, 2020; ZHU et al., 2020). Trata-se de um vírus envelopado e seu material genético é do tipo RNA de sentido positivo, que funciona também como RNA mensageiro, sendo traduzido pelos ribossomos da célula hospedeira, envolto em fita simples (SAWICKI et al., 2007; KAUSHIK et al., 2020).

3.2 Genoma do SARS-CoV-2

O SARS-CoV-2 é um vírus com diâmetro de 60 nm a 140 nm genoma do SARS-CoV-2, constituído por dois terços do RNA genômico que codifica duas grandes poliproteínas sobrepostas, a estrutura de leitura aberta (ORF) 1a e ORF1b, que são processadas pela polimerase de RNA dependente de RNA (RdRp) e outras proteínas não estruturais (Nsps), na qual estão associadas tanto ao mecanismo de síntese de RNA quanto o da modulação da resposta do hospedeiro (WANG et al., 2020; ROMANO et al., 2020). O outro terço do genoma codifica quatro proteínas estruturais sendo a primeira a proteína “spike” (S), que é uma das proteínas imunodominantes vitais do CoVs capazes de induzir respostas imunes do hospedeiro (LI., 2016), através dos seus ectodomínios constituídos subunidades de S1 e S2, responsáveis por

realizar a ligação e fusão ao receptor do hospedeiro (BELOUZARD et al., 2012), a segunda proteína "envelope" (E), que se liga ao nucleocapsídeo e atua como um organizador central da montagem do coronavírus, tendo como função de moldar definitivamente o envelope viral de (NEUMAN et al., 2011; NA et al., 2005), por terceiro a "membrana" (M), está associada a virulência do vírus (DEDIEGO et al., 2007) e atua de forma multifuncional na montagem, patogênese e liberação do virus (NIETO et al., 2014), a quarta que se trata da proteína "nucleocapsídeo" (N), a qual desempenha função de formar complexos com o genoma viral, assim facilitando a interação da proteína M, aumentando a eficiência da transcrição do vírus e outras proteínas acessórias (CHANG et al., 2006; SHEIKH et al., 2020).

3.3 Transmissão do SARS-CoV-2

Existem diversas formas de transmissão do SARS-CoV-2, dentre elas é possível citar a transmissão de humano para humano que ocorre através da dissipação de gotículas respiratórias expelidas por um indivíduo, aumentando assim o risco de contrair a doença (CARLOS et al., 2020; CHAN et al., 2020). Além disso, uma outra forma de transmissão da COVID-19 se dá através da zoonose (XIAO et al., 2020). Todavia, embora vários relatórios tenham mencionado que um portador da doença COVID-19 deve estar presente para que seja transmitido o SARS-CoV-2 existem formas adicionais de transmissão viral que foram observadas ao longo dessa pandemia, como por exemplo a detecção de SARS-COV-2 em amostras fecais de pacientes hospitalres, indicando a capacidade do vírus de proliferar no trato digestivo, indicando assim um uma rota de transmissão fecal-oral (ZHANG et al., 2020; CHEN et al., 2020; CHEUNG et al., 2020).

3.4 Patogenia do SARS-COV-2

O SARS-CoV-2 tem como células alvo as células epiteliais nasais e brônquicas e pneumócitos, onde se liga através da proteína S ao receptor da enzima conversora de angiotensina 2 (ECA2) presente nessas células (TIAN et al., 2020; BARTON et al., 2020; LETKO et al., 2020; HOFFMANN et al., 2020). A serina protease transmembrana tipo 2 (TMPRSS2), presente na célula hospedeira, promove a

absorção viral por clivagem da ECA2 e ativação da proteína S do SARS-CoV-2, que conduz a entrada do coronavírus nas células hospedeiras (ZHOU et al., 2020; ZHU et al., 2020 HOFFMANN et al., 2020). Devido ao ECA2 ser um homólogo da enzima conversora de angiotensina (ECA), desempenha um papel fundamental no sistema renina-angiotensina aldosterona (SRAA), envolvendo a regulação da pressão arterial e homeostase eletrolítica (LAN et al., 2020; TIAN et al., 2020). Os maiores efeitos oriundos da ECA2 incluem vasoconstrição, reabsorção renal de sódio e excreção de potássio, síntese de aldosterona, elevação da pressão arterial e indução de vias pró-inflamatórias e pró-fibróticas (DONG et al., 2020; BOURGONJE et al., 2020; PACES et al., 2020). Devido ao SARS-CoV-2 competir com a angio II pela ECA2 para entrar nas células, acaba desregulando assim o SRAA, aumentando a biodisponibilidade de angio II e consequentemente favorecendo eventos pro-inflamatórios e pro-fibróticos (TIAN et al., 2020; LETKO et al., 2020; LU e SUN, 2020; RIZZO et al., 2020). Em estágios posteriores da infecção, quando a replicação viral acelera, a integridade da barreira epitelial é comprometida (KINARET et al., 2020). Além das células epiteliais, o SARS-CoV-2 infecta as células endoteliais de capilares pulmonares, acentuando a resposta inflamatória e desencadeando um influxo de monócitos e neutrófilos (BARTON et al., 2020; XU et al, 2020; WIERSINGA et al., 2020). Em pacientes graves com COVID-19, esta resposta imune é excessiva e, portanto, descrita como uma tempestade de citocinas, que precipita o início da síndrome de resposta inflamatória sistêmica (YAO et al., 2020; JASON et al., 2020; MCGONABLE et al., 2020). Vale ressaltar, que um dos fatores que podem contribuir para respostas sistêmicas à infecção por SARS-CoV-2 é a resposta imunológica (PADOAN et al., 2020; PACES et al., 2020; JARJOU et al., 2021), além disso a presença da ECA2 na mucosa oral e nasal, alvéolos pulmonares, células epiteliais do intestino delgado, células endoteliais vasculares, células musculares lisas, miócitos cardíacos, túbulos renais e epiderme (OKBA et al., 2020; RIZZO et al., 2020; BOURGONJE et al., 2020).

Estudos relataram que um período médio de incubação do SARS-CoV-2 no hospedeiro é de aproximadamente 4 a 5 dias (LI et al., 2020; ZHU et al., 2019; PUNG et al., 2020) com intervalos que podem chegar até 12-14 dias, período em que os primeiros sintomas levam para aparecer desde a infecção (LAUER et al., 2020; FLORINDO et al., 2020).

Indivíduos infectados com SARS-CoV-2 podem demonstrar sintomas mais comuns como febre, tosse seca, fadiga e perda do paladar ou olfato, existem também os sintomas menos comuns que são dores e desconforto no corpo e os sintomas mais graves, que estão associados aos sintomas respiratórios, acarretando em alguns casos no desenvolvimento da síndrome do desconforto respiratório agudo (CHEN, N et al., 2020), a qual resulta em falência de órgãos e, eventualmente, leva à óbito (HUANG et al ., 2020; BHATRAJU et al., 2020; TAY et al., 2020; BLANCO et al., 2020; HOLSHUE et al., 2020; ZHOU et al., 2020; TAJBAKHSH et al., 2021; HIGGINS et al., 2021). No entanto, alguns pacientes podem permanecer completamente assintomáticos, referindo a não demonstrarem sintomas a infecção, mas que tem potencial de transmitir o vírus, o que contribuiu para o aumento do número de casos de COVID-19 (BAI et al., 2020; ROTHE et al., 2020; ZHENG., 2020).

3.5 Epidemiologia da COVID-19

Desde o final da pandemia até presente momento foram registrados 551.226.298 milhões casos confirmados de infecção pelo SARS-CoV-2, com 6.345.595 milhões mortes em todo o mundo (WHO, 2024). No Brasil, o primeiro caso de infecção por SARS-CoV-2 foi confirmado em 25 de fevereiro de 2020 (JESUS et al., 2020) e desde então, o número de casos confirmados chega 38.592.310 milhões e o número de mortes por COVID-19 somam 710.427 mil (MINISTÉRIO DA SAÚDE, 2021) conforme observado no quadro 1

Quadro 1 -Número de casos e óbitos por COVID-19 em diferentes regiões do Brasil*

Região do Brasil	Casos	Mortes	Incidência/100mil hab	Mortalidade/100mil hab
Centro-oeste	4.502.576	66.915	27628,1	410,6
Sul	8.181.899	112.693	27294,8	375,9
Norte	2.968.945	51.964	16108,4	281,9
Nordeste	7.543.389	136.250	13217,4	239,6
Sudeste	15.395.501	342.114	17421,4	387,1

*Os dados de casos e óbitos de COVID-19 nas regiões do Brasil referente à Semana Epidemiológica. Ministério da Saúde e Secretaria de Vigilância Sanitária (SVS) do Brasil. 11/03/2024 -12:14

3.6 Variantes do SARS-CoV-2

Com o avanço da COVID-19, estudos têm relatados novas variantes do SARS-CoV-2, que causam severos problemas nos setores da saúde e socioeconômico (TANG et al., 2020; HARVEY et al., 2021; MEO et al., 2021). Neste âmbito, desde o início da pandemia de SARS-COV-2, a partir do ano 2019 foram identificados 5 VOCs (variantes de preocupação) como Alpha (B.1.1.7) (RAMBAUT et al., 2020), Beta (B.1.351) (TEGALLY et al., 2020), Gama (P.1) (FUJINO et al., 2021), Delta B.1.617.2 (CHERIAN et al., 2021), Omicron (D614G)(MEO et al., 2021) oriundas do Reino Unido, África do Sul, Brasil e Índia. Além destes, as VOIs atualmente designados (variantes de interesse) e as VOMs (variantes sob monitoramento) surgem como um alerta quanto à patogenicidade (WHO., 2021; MARIANA et al., 2022), uma vez que, estes COVs têm sido responsáveis por um número expressivo de casos e aumento da transmissibilidade entre a população (PLANTE et al., 2021; ZHOU et al., 2021).

No Brasil, foram notificados mais de 30 milhões de casos confirmados de COVID-19, ultrapassando 600 mil óbitos, sendo mais de 2 milhões de casos e 39 mil óbitos no Estado do Rio Grande do Sul, segundo as autoridades. A resposta de anticorpos, virulência, potencial de reinfecção e eficácia da vacina contra as variantes de emergência, ainda não são totalmente conhecidos, representando um risco para futuros surtos e eficácia em programas de vacinação (POLACK et al., 2020; ANDERSON et al., 2020; HARVEY et al., 202).

O recente surgimento de novas variantes vem demonstrando motivos de preocupação devido as mutações na estrutura, mutações em genes e proteínas do SARS-CoV-2 (DAVIES et al., 2021; LEUNG et al., 2021), dentre essas alterações, de acordo com a literatura, embora cada variante tenhas suas respectivas mutações, como por exemplo, deleção do H69/V70 e P681H/R na cepa da B.1.1.7 (LIU et al., 2021; STARR et al., 2021), enquanto que na cepa da B.1.617 apresenta múltiplas mutações na proteína S incluindo o D111D, G142D, L452R (HOFFMANN et al., 2020; PEACOCK et al., 2021). Importante destacar, que o surgimento de novas mutações impacta diretamente no índice de transmissibilidade, taxa de infecção e gravidade da doença (VOLZ et al., 2021; SABINO et al., 2021; FARIA et al., 2021; NAVECA et al., 2021; ZHANG et al., 2021; MAHASE et al., 2021; WALL et al., 2021; MCCALLUM et al., 2021), HARVEY et al., 2021; PLANTE et al., 2021; ZHOU et al., 2021; STARR et al., 2021; PEACOCK et al., 2021).

3.7 Desenvolvimento de vacinas frente a covid-19

Atualmente, diversas vacinas consideradas eficazes e seguras contra o COVID-19 foram licenciadas para o uso em nível nacional e internacional, sendo uma das alternativas preventivas mais significativas para conter o avanço da doença e também limitar as graves consequências da pandemia para a saúde global (PORMOHAMMAD et al., 2021; DONG et al., 2020; VAN RIEL et al., 2020; WHO., 2021) As principais vacinas estão apresentadas no quadro 2.

Quadro 2. Principais vacinas licenciadas para imunização da covid-19 no Brasil.

Vacina	**Fabricante**	**Eficácia (IC50 95%)**
BNT16b2	Pfizer/BioNtech	95% (90,3-97,6)
mRNA-1273	Moderna	94,1% (89,3-96,8)
Vacina AZD1222 ChAdOx1 nCoV-19	AstraZeneca/Universidade de Oxford	70,4% (54,8–80,6)
Ad26.COV2.S	Johnson & Johnson	66,1% (55–74,8)
CoronaVac	Sinovac Biotech	51% (50,7%-62,3%)
Covaxina	Bharat Biotech	77,8% (65,2–86,4%)

Adaptado: FIOLET et al., 2022; PORMOHAMMAD et al., 2021; POLACK et al., 2020; KNOLL E WONODI., 2021; SADOFF et al., 2021; WHO., 2021)

Como mostrado no quadro 2, é possível observar a eficácia dos diferentes fabricantes de vacinas, o que também pode influenciar indiretamente a capacidade de diminuir a transmissão do vírus. Neste sentido, as vacinas de vetores virais como Oxford-ChAdOx1-nCoV-19, Johnson e Johnson, Sputnik-V, Ad-5 Vector da CanSino são vacinas recombinantes que usam um vírus modificado, contendo material genético extra que codifica de forma mais intensa a Proteína de pico viral do SARS CoV2 (BALL., 2021; LI et al., 2020), enquanto que as vacinas de RNA mensageiro (mRNA) como Pfizer-BioNTech, Moderna-mRNA-1273 permitem a expressão do antígeno de pico SARS-CoV-2, que ativa a resposta imune (YAN et al., 2021; LIU et al., 2020; SHIH et al., 2020), sendo que nas nossas células ocorre o processo de síntese da proteína a qual atua como antígeno estimulando a resposta imune humoral, todavia vacina à base de proteína NVX-CoV2373 Covovax da Novavax e Corbevax utilizam fragmentos de proteína do vírus SARS-CoV-2, para desencadear a resposta imune protetora contra a infecção (KHALAJ., 2020; PROMPETCHARA et

al., 2020). Além disso, posteriormente foi licenciada a vacina inativada Covaxin que utiliza o virus inativado para induzir resposta imune protetora (MULLARD., 2021; GOLDBERG et al., 2021; DOLGIN et al., 2021) semelhante a infecção natural do SARS-CoV-2.

3.8 Diagnóstico da COVID-19

A análise do sequenciamento do genoma completo do novo coronavírus, permitiu a seleção de genes específicos do vírus, permitindo a detecção rápida e precisa do patógeno causador é essencial no controle do surto entre portadores assintomáticos e indivíduos que apresentam sinais da doença (HARAHWA al., 2020; CORMAN et al .,2020), pois a identificação dos portadores pode interromper a infecção e transmissão da comunidade alvo e permitir o rastreamento de contatos e o tratamento oportuno (AHN et al., 2019; CHAN et al., 2020).

A partir do sequenciamento genético do SARS-CoV-2 (ZHOU al., 2020), foram propostas diferentes formas de detecções visando principalmente ácidos nucleicos virais específicos (teste molecular), proteínas (teste de antígeno) ou anticorpos anti-SARS-CoV-2 (teste sorológico) (ALPDAGTAS et al., 2020; FALZONE et al., 2021), baseados na RT-PCR (YAN et al ., 2020), amplificação isotérmica de ácido nucleico (transcriptase reversa- amplificação isotérmica mediada por loop (RT-LAMP) (NAGURA al., 2020) e repetições palindrômicas curtas regularmente Inter espaçadas agrupadas (CRISPR) (HUANG et al., 2020; AGUIAR et al 2020). Quando comparados com outros testes disponíveis, esses métodos são muito mais sensíveis, e pode detectar vírus muito mais cedo em amostras clínicas (HARAHWA al., 2020).

Dentre os testes moleculares utilizados para o diagnóstico de COVID-19, temos ensaio o RT-LAMP, um teste de diagnóstico comumente utilizado nos pontos de atendimento (SILVA et al., 2019; AHN et al., 2019). Vale ressaltar que a otimização dos primers facilita a análise do resultado para interpretação de amplificação do gene alvos, neste caso o gene ORF1ab COVID-19 (AHN et al., 2019).

Outra abordagem foi relatada por Yan et al (2020), que avaliaram o ensaio RT-LAMP para detecção dos genes ORF1ab e S de SARS-CoV-2 (detecção fluorescente) em comparação com RT-qPCR de 130 *swabs* e amostras de lavado bronco alveolar

obtidas de indivíduos com pneumonia e suspeita de COVID-19. Os resultados mostraram que a sensibilidade e especificidade de ambos os métodos foram de 100%.

O teste baseado em ácido nucleico viral, que utiliza RT-qPCR, é o método de triagem de primeira linha de escolha para detecção de SARS-CoV-2 (WANG et al., 2020), sendo considerado o teste “padrão ouro” devido a sua sensibilidade e detecção rápida (HENTZIEN et al., 2020; LI et al., 2020). Além disso, a técnica de RT-qPCR é o método mais adequado, pois permite a detecção e quantificação viral (CORMAN et al., 2020; SIMOSKA et al., 2020). Neste contexto, os testes de RT-qPCR são usados para a identificação e diferenciação do SARS-CoV-2 nas amostras/espécimes coletados de pacientes sintomáticos e assintomáticos, detectando sequências de RNA específicas do SARS-CoV-2 (SINGHAL, 2020). Em geral, esses testes envolvem três etapas essenciais: extração do RNA viral dos espécimes coletados, posteriormente é realizada transcrição reversa de RNA viral para DNA de fita simples (cDNA) utilizando a enzima transcriptase reversa e, por fim, a amplificação do cDNA acoplado à detecção fluorescente (KUBINA et al., 2020).

Há ferramentas complementares no diagnóstico de SARS-CoV-2 em hospitais, como a imagem radiológica usando tomografia computadorizada (TC) de tórax, na qual fornecem 98% de sensibilidade na triagem de infecções por SARS-CoV-2 (CHAN et al., 2020; LEI et al., 2020). No estudo de Fang et al. (2020), a taxa de sensibilidade de 51 TCs de tórax de pacientes com COVID-19, foram comparadas com os resultados de RT-PCR, e verificou-se que as TCs de tórax foram capazes de detectar evidências de anormalidades, indicando pneumonia viral em pacientes mesmo quando seus resultados de RT-PCR que eram incialmente negativos. Embora o RT-PCR seja um método altamente sensível para detecção de SARS-CoV-2, certas deficiências podem levar a resultados negativos, como coleta inadequada (CHUNG et al., 2020, XIE et al., 2020). Isso ressalvas a importância do uso de diversos métodos de detecção para garantir um diagnóstico preciso do paciente (LI et al., 2020).

O Antígeno SARS-CoV-2 e detecção baseada em imunoensaio da infecção por SARS-CoV-2, baseados em antígeno, como ensaios imunofluorescentes, ensaios imunocromatográficos, quimioluminescentes e ensaios imunossorventes enzimáticos (ELISA) também são métodos confiáveis para a detecção de infecções por SARS-CoV-2 (DOMENICO et al., 2021). Uma vez que, esses kits disponíveis comercialmente são geralmente compatíveis com uma variedade de amostras

clínicas, como *swabs* nasofaríngeos, *swabs* nasais e saliva, detectando principalmente a presença de dois principais antígenos SARS-CoV-2 (ALPDAGTAS et al., 2020).

3.9 Macrófita *Hydrocotyle ranunculoides*

A espécie *H. ranunculoides*, conhecida como pennywort flutuante, é uma macrófita aquática flutuante da família Araliaceae e ocorre em uma ampla diversidade de ambientes, sendo nativa das Américas e invasora na Europa (WALSH e MAESTRO, 2017). É uma planta perene com características rizomatosas e rasteiras e, assim como outras plantas daninhas aquáticas, *H. ranunculoides* tolera grandes quantidades de nutrientes, sendo facilmente encontrada em cursos d'água eutróficos (SIMBANEGAVI et al. 2018). Já foi relatada por vários estudos a capacidade desta planta aquática em remover nutrientes e outros contaminantes, incluindo metais pesados, através de vários mecanismos como rizofiltração e fitoextração (DEMARCO et al. 2018; CUSTODIO et al. 2020). Biofiltros recentes de baixo custo utilizando macrófitas aquáticas foram propostos utilizando adsorventes produzidos com espécies de *Hymenachne grumos* "in natura" e nas formas de carvão ativado. Os adsorventes produzidos alcançaram potencial de remoção superior a 98% para SARS-CoV-2 e a proposta visava a aplicação de dispositivos flutuantes utilizando os adsorventes produzidos e plantas vivas para descontaminação de cursos d'água e tratamento de águas residuais (DEMARCO et al. 2022).

3.10 *Rosa centifolia*

A espécie da *R. centifolia* trata-se de uma planta do reino Platae, filo Tracheophyta, classe Magnolipsida, ordem Rosales família Rosaceae e gênero Rosa L. sendo proveniente do Marrocos e cultivada principalmente na cidade Grasse na França. É considerada um híbrido complexo entre *Rosa gallica*, *Rosa mochata* e *Rosa canina* (IWATA et al., 2000). Apresenta aspecto arbustivo com uma variação de crescimento de 1,5 a 2 metros de altura, espinhentos, folhas peninérveas, apresenta de 5 a 7 folículos com aspectos verdes acinzentados. Devido a sobreposição de suas

pétalas finas formam flores rosas redondas e globulares (PHILLIPS e RIX, 2004; JINTEDRA et al., 2012).

O gênero *Rosa* é amplamente cultivada como rosa de jardim, representando uma das mais importantes plantas ornamentais, promovendo benefícios importantes para saúde como um alimento funcional, apresentando funções afrodisíaca, anti-inflamatória, antioxidante e antimicrobiana. Além disto ameniza gastrites e dispepsias (CAI et al., 2005; ORHAN et al., 2007; WENZING et al., 2008; KUMAR et al., 2010). Essa planta contém terpeno, mirceno, vitamina C, taninos e proantocianidinas (CANALES, 2005; e JINTEDRA et al., 2012). Os compostos fenólicos, nesse caso as antocianinas presentes nas pétalas da rosa, são consumidos através de chás e bolos (VINOKUR et al., 2006; KUMAR et al., 2010).

Kondo et al. (2011) demonstraram que o extrato de pétalas de *R. centifolia* possui ação inibitória de diacetilglicerol acetiltransferase, sendo benéfico na manutenção e controle do metabolismo lipídico.

Azmi et al. (2012), constatou que o extrato de *R. centofilia* pode ser utilizado como um biossorvente no tratamento de águas subterrâneas. Ela foi capaz de remover ferro e manganês, resultando na obtenção de água com níveis aceitáveis de qualidade conforme estabelecido pela OMS.

3.11 Presença de SARS-CoV-2 em água residual

Durante a pandemia, a principal tarefa dos profissionais de saúde é realizar testes de diagnóstico laboratoriais nos indivíduos; no entanto, é relativamente demorado e trabalhoso. Portanto, a monitorização de águas residuais é frequentemente recomendada como um sistema de alerta precoce para monitorizar o surgimento e o ressurgimento de surtos, enquanto os funcionários governamentais podem utilizá-lo para identificar populações-alvo para testar e desenvolver medidas para conter e mitigar surtos (AGUIAR-OLIVEIRA et al. 2020). Além disso, a monitorização de águas residuais tem a vantage de se obter uma amostragem fácil e com capacidade de estimar o estado geral da área de captação (SHAH et al. 2022).

A presença de material genético viral nas fezes de indivíduos com a doeça ou que tiveram resultado positivo pode refletir a abundância de RNA do SARS-CoV-2 contido em águas residuais não tratadas (KUMAR et al., 2021; GRAHAM et al., 2020;

YUAN et al., 2021) e águas superficiais nas quais as águas residuais são descarregadas diretamente (DHAMA et al., 2021). O monitoramento de enterovírus e patógenos bacterianos na água é uma prática epidemiológica reconhecida dentro da estratégia de vigilância em saúde pública (ASGHAR et al., 2014; MOAZENI et al., 2017). Nesta base, estudos realizados na Dinamarca (RASMUSSEN et al., 2023), Itália (LA ROSA et al., 2023) e Inglaterra (TOLEDO et al., 2022) mostraram que a vigilância do SARS-CoV-2 baseada na água poderia ser usada como um método complementar ao diagnóstico clínico e ao sistema de alerta precoce para a reemergência local da COVID-19, contudo a presença do SARS-CoV-2 nas águas residuais torna-se uma preocupação crítica e um risco toxicológico para ambientes aquáticos, conforme constatado no Charlie et al. (2021), além de ter constado a presença de fragmentos do SARS-CoV-2 na água residual acarretando num desequilíbrio redox e neurotoxicidade aguda em girinos (*Physalaemus cuvieri*) e demonstrou através de estudos *in silico* uma interações entre peptídeos e acetilcolinesterase e enzimas antioxidantes. Assim, a contaminação de ambientes aquáticos por partículas de SARS-CoV-2 pode apresentar um risco ecotoxicológico e ambiental (NAVARRO et al., 2021; HAAS et al., 2021; MATHERI et al., 2023).

Assim, monitoramento de águas residuais pode complementar os testes clínicos, fornecendo monitoramento em larga escala por meio de métodos não invasivos, eficientes e econômicos (SHAH et al. 2022). Portanto, o monitoramento ambiental e os testes de água estão se tornando cada vez mais importantes para a detecção do SARS-CoV-2 em situações alarmantes de saúde pública. A epidemiologia baseada em águas residuais (WBE) também está sendo desenvolvida como uma nova ferramenta para a análise de biomarcadores em tubulações de águas residuais.

4. CAPÍTULO 1

4.1 Manuscrito 1

O artigo intitulado “Reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay as a rapid molecular diagnostic tool for COVID-19 in healthcare workers” é apresentado conforme publicado na Revista Journal of Clinical Virology Plus, ISSN: 2667-0380, com fator de impacto 1.7, tendo sido aceito em 6 de janeiro de 2023

Reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay as a rapid molecular diagnostic tool for COVID-19 in healthcare workers

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Abstract

In December 2019, the Chinese Center for Disease Control (CDC of China) reported an outbreak of pneumonia in the city of Wuhan (Hubei province, China) that haunted the world, resulting in a global pandemic. This outbreak was caused by a betacoronavirus named severe acute respiratory syndrome coronavirus 2 (SARS-CoV2). Several of these cases have been observed in healthcare professionals working in hospitals and providing care on the pandemic's frontline. In the present study, nasopharyngeal swab samples of healthcare workers were used to assess the performance of the reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay and subsequently compared with the real-time reverse-transcription quantitative PCR (RT-qPCR) method. Thus, in this study, we validated a method for detecting SARS-CoV-2 based on RT-LAMP that can be used to diagnose these workers. The methodology used was based on analyzing the sensitivity, specificity, evaluation of the detection limit, and cross-reaction with other respiratory viruses. The agreement was estimated using a dispersion diagram designed using the Bland-Altman method. A total of 100 clinical specimens of nasopharyngeal swabs were collected from symptomatic and asymptomatic healthcare workers in Pelotas, Brazil, during the SARSCoV-2 outbreak. RT-LAMP assay, it was possible to detect SARS-CoV-2 in 96.7% of the healthcare professionals tested using the E gene and N gene primers approximately and 100% for the gene of human β-actin. The observed agreement was considered excellent for the primer set of the E and N genes (k = 0.957 and k = 0.896), respectively. The sensitivity of the RT-LAMP assay was positive for the primer set of the E gene, detected to approximately 2 copies per reaction. For the primer set of the N gene, the assay was possible to verify an LoD of approximately 253 copies per reaction. After executing the RT-LAMP assay, no positive reactions were observed for any of the virus respiratory tested. Therefore, we conclude that RT-LAMP is effective for rapid molecular diagnosis during the COVID-19 outbreak period in healthcare professionals.

Keywords: COVID-19; RT-LAMP; SARS-CoV-2; healthcare workers

1. Introduction

An outbreak of pneumonia, reported by the Chinese Center for Disease Control [1] in the city of Wuhan, Hubei province in Wuhan, China, in December 2019 haunted the world [2,3]. This outbreak resulted in a global pandemic caused by a betacoronavirus, which the International Committee for Virus Taxonomy named as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) due to its genetic proximity and kinship with SARS-CoV [4].

SARS-CoV-2 is the causal agent for COVID-19, and the World Health Organization classifies this virus as an airborne pathogen transmitted by asymptomatic, pre-symptomatic, and symptomatic individuals through close contact via exposure to infected droplets and aerosols and resulted in a global pandemic [1–4].

It was noticed 624.599,147 million more cases have been confirmed worldwide, with approximately 6.567,281 million confirmed deaths. In Brazil, infected patients have already exceeded 34.746,762 million, with more than 687,144 thousand death. More than 2.739,695 million cases have already been reported in the state of Rio Grande do Sul, with more than 41.153 thousand deaths caused by SARS-CoV-2 [5].

A portion of these cases has been observed in healthcare professionals working in hospitals and providing care on the pandemic's frontline [6–10]. Such professionals are routinely diagnosed by the technique of reverse transcription of the polymerase chain in real time (rRT-PCR) [11–16]. The most explored SARS-CoV-2 genomic regions in molecular tests using rRT-PCR comprise the genes: RdRp, E, N, and S are reported in different studies [17–21].

Diagnostic tests for SARS-CoV-2 are essential to identify infected patients, monitor the beds of the intensive care units (ICU), for epidemiological studies, in public health interventions, in the accurate tracking of the population, in the social isolation of patients, thus contributing to controlling SARS-CoV-2 transmission in the population [22–24] Such strategies have become paramount for controlling and managing the pandemic since only recently have specific vaccines emerged to immunize the population [25–27].

However, because of the increasing number of new cases worldwide due to the emergence of SARS-CoV-2 variants like P1.0 and P2.0, molecular diagnostic tests have become increasingly scarce [28–30]. It is also notable that there is a lack of options for monitoring SARS-CoV-2 infections among healthcare professionals in hospitals. In this context, the reverse transcription loop-mediated isothermal amplification assay (RTLAMP) could be an interesting alternative, bearing in mind that it has proven to be a highly sensitive,

specific, versatile, and robust method. The RT-LAMP assay simplifies the procedures for performing the technique, such as simplifying the extraction procedures, reducing the detection time, using simpler equipment as a sample incubation block, and developing the colorimetric assay [31–36].

Considering that such assay has not yet been described for monitoring SARS-CoV-2 infections in healthcare workers, we hereinafter validated a method for detecting SARS-CoV-2 based on isothermal amplification mediated by a reverse transcription loop (RT-LAMP). In addition, we determined the LoD of the method for the set of primers used to develop the method and estimated the number of viral copies found per reaction.

2. Material and methods

2.1 Samples

A total of 100 clinical specimens (nasopharyngeal swabs collected in 2 mL of molecular grade phosphate-buffered saline) were collected from symptomatic and asymptomatic healthcare workers in Pelotas, Brazil, during the SARS-CoV-2 outbreak (November 2020 to January 2021). All healthcare workers included in the study performed their activities in different sectors of the School Hospital (HE) of the Federal University of Pelotas (UFPel). The study protocol (4.124.248) was approved by the Ethics Committee on Research with Human Beings at the Federal University of Pelotas (FAMED/UFPel), and all procedures followed the ethical guidelines for human experimentation.

2.2 Virus isolates

The positive controls used were SARS-CoV-2, kindly provided by Prof. Dr. Edison Durigon of the University of São Paulo (USP/SP). Human adenovirus (HAdV), human rhinovirus (HRV), human influenza A virus (FLUAV), and rotavirus (RV), were isolated from a clinical sample collected from the environment and identified at the Microbiology Laboratory (FEEVALE/RS). Hepatitis C virus (HCV), human immunodeficiency virus (HIV), hepatitis B virus (HBV), and human cytomegalovirus (CMV) were clinical samples kindly provided by Dr. Marne Dias Real from the Clinical Analysis Laboratory of General Hospital of Santa Maria (HGe/RS). Canine coronavirus (CCoV) was kindly provided by Dra. Silvia de Oliveira Hübner, Virology and Immunology Laboratory of the Federal University of Pelotas (UFPel/RS).

Respiratory syncytial virus (RSV), herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), human parainfluenza virus (HPIV), and human papillomavirus (HPV) are clinical samples identified in the Laboratory of Biochemistry and Molecular Biology of Microorganisms of the Federal University of Pelotas (UFPel/RS). Bovine herpesvirus 5 (BoHV-5) was kindly provided by Dr. Eduardo Flores, Virology Laboratory of the Federal University of Santa Maria (UFSM/RS).

2.2 DNA and RNA extraction

Nucleic acids were extracted from viral isolates using the BioGene kit (Quibasa-Bioclin, Belo Horizonte, MG, Brazil) for DNA/RNA viral extraction, as recommended by the manufacturer. Total RNA was isolated from 200 μL of the sample using the IndiMag® Pathogen kit (Indical Bioscience, Germany), according to the manufacturer's instructions. Purified RNA was eluted in 100 μL AVE buffer and divided into two parts for further analysis through the RT-LAMP assay and RT-qPCR reaction. RNA samples were quantified, and their purity (260/280 and 260/230 ratios) was examined using a UV spectrophotometer NanoDrop® Lite (Thermo Fisher Scientific, Waltham, USA). Samples were stored at -80º C until further use.

2.3 Real-time reverse-transcription quantitative PCR (RT-qPCR)

For comparative RT-qPCR analysis, the samples were analyzed using the nCoV19 CDC kit, which includes two targets for detecting SARS-CoV-2 (N1 and N2) and an RNaseP (RP) target for RNA extraction quality control. As per the manufacturer's instructions, a volume of 5 μL of RNA was mixed with 15 μL of the master mix. The thermal cycler conditions were reverse transcribed for 10 min at 45 °C and initial denaturation for 10 min at 95 °C, followed by 40 cycles of 95 °C for 3 s and 55 °C for 30 s. RT-qPCR was run on an Agilent AriaMx Real-Time PCR System (Agilent Technologies). The cycle threshold (C_t) for each target gene (N1, N2, and RP) in all samples from healthcare workers was recorded (Supplementary Information). The primers and probes used for real-time PCR are listed in **Table 2**. The selection of positive samples among the 100 health workers evaluated by RT-qPCR was used to estimate viral copies within the average of each C_t range. The C_t values were separated in the following intervals: 17–18, 19–20, 21–22, 23–24, 25–26, 27–28, 29–30, 31–32, 35–36, and 37–38.

2.4 RT-LAMP

As shown in **Table 1**, two sets of LAMP primers were used for the E and N (Nsp3_1-61) target genes of the SARS-CoV-2 genome available at NCBI (GenBank accession number NC_045512.2), and one set of LAMP primers targeting the human β-actin gene was included as an internal control for the RT-LAMP assay in this study [92]with modification. Six primers, including the forward primer (F3), backward primer (B3), forward inner primer (FIP), backward inner primer (BIP), forward loop primer (LF), and backward loop primer (LB) for each target gene, were used in the RT-LAMP assay. The preparation of primer mix used for each of the target genes was made before the reactions as follows: 10× E primer mix (F3, 0.1 μM; B3, 0.1 μM; FIP, 0.8 μM; BIP, 0.8 μM; LF, 0.2 μM; LB, 0.2 μM), 10× Nsp3_1-61 primer mix (F3, 0.4 μM; B3, 0.4 μM; FIP, 0.8 μM; BIP, 0.8 μM; LF, 0.2 μM; LB, 0.2 μM), and a 10× β-actin primer mix (F3, 0.2 μM; B3, 0.2 μM; FIP, 0.4 μM; BIP, 0.4 μM; LF, 0.2 μM; LB, 0.2 μM), respectively. The RT-LAMP assay was carried out using WarmStart™ Colorimetric LAMP 2× Master Mix kit (DNA & RNA) (New England Biolabs, Inc) following the manufacturer's recommended protocol, with a reduced reaction final volume. A 6.25 μL reaction mixture (2× MasterMix, 6.25 μL; 10× primer mix, 2.65 μL; RNA target, adding RNase-free molecular grade water to obtain a 12.5 μL final volume per reaction) was mixed homogeneously and centrifuged for 5s. RT-LAMP assays were performed in a heating block set at 65 °C for 30 minutes. The color change (from orange to yellow after successful amplification) reaction was evaluated by the naked eye for each tested sample.

2.5 Sensitivity and number of viral copies

The sensitivity of the RT-LAMP assay was evaluated using E and N primer sets. The total RNA of the SARS-CoV-2 control was used to prepare nine serial 10-fold dilutions with an isolated sample of SARS-CoV-2. After performing the RT-LAMP assay, the tubes were developed as described above, and the number of viral copies was estimated. Subsequently, the detection limit of the copies of the target RNA per reaction was determined. All nine diluted samples were tested by gold standard rRT-PCR, and the resulting cycle threshold (Ct) values were compared to determine the number of viral copies.

2.6 Specificity

The specificity of the RT-LAMP assay was evaluated after the extraction of genetic material specific to each virus. Fifteen different viral isolates were used: HAdV, HRV, FLUAV, HCV, HIV, HBV, RV, CMV, RSV, BoHV-5, CCoV, HSV-1, HSV-2, HPIV, and HPV, as described above. SARS-CoV-2 viral RNA was used as the positive control. Viruses HAdV, HRV, FLUAV, RSV, and RV have been in house assay, according to Menezes [93], Demoliner [94] and Bortagaray [95]. HPIV was determined by RT-qPCR, as described by Vaucher [96]. HSV-1 and HSV-2 were determined by PCR as described by Sartori [97]. BoHV-5 was determined according to the method described by Spilki [98]. CCoV was determined by RT-PCR in house method according to HCV and HIV were evaluated in-house by RT-qPCR for the termination of viral load, and HBV and CMV were evaluated in-house by PCR. SARS-CoV-2 according to the nCoV19 CDC [99] kit by real-time RT-qPCR using the TaqMan protocol.

2.7 Statistical analyses

Statistical analyses were performed using GraphPad Prism version 4.0. All tests were two-sided, with *p*-values of <0.05, denoting statistical significance. The comparison between the range of C_t of the RT-PCR tests of the samples and the number of viral copies was made with a paired-samples Student's t-test. The reliability assessment between the rRT-PCR and RT-LAMP techniques was performed considering the dichotomous variables using the *Kappa* index (*k*). Bland-Altman analysis was used to represent the degree of agreement between the rRT-PCR and the RT-LAMP SARS-CoV-2 based on the mean difference and standard deviation (SD) of the dilutions of the positive control of the SARS-CoV-2 employed in the sensitivity test.

3 Results

3.1 RT-LAMP, RT-qPCR, and overall agreement rate (OAR)

In the present study, samples of nasopharyngeal swab healthcare workers were used to assess the performance of the RT-LAMP assay and subsequently compared with the RT-qPCR method. The results of the RT-LAMP tests with the three sets of tested primers were satisfactory, as shown in **Figure 1**.

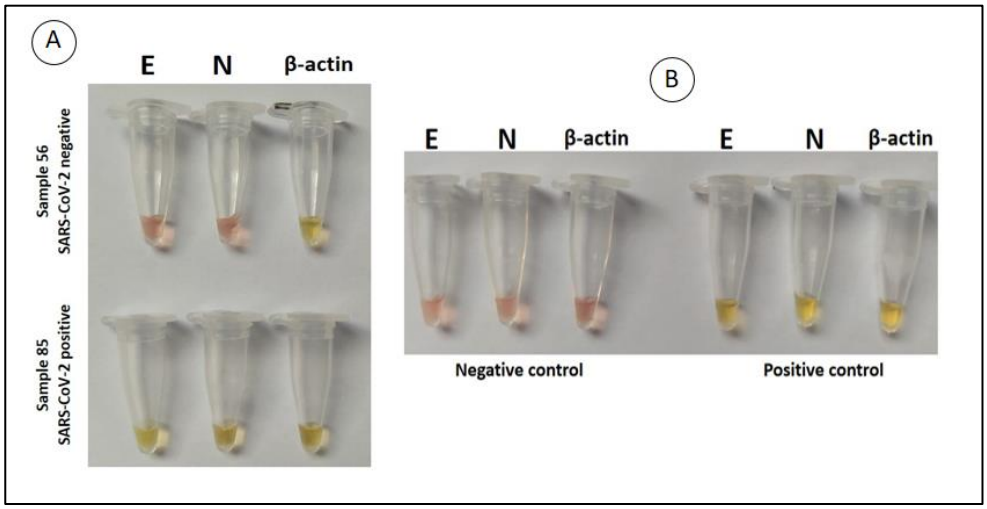

Figure 1.Colorimetric RT-LAMP assay using E, N and human β-actin primer set. A) Representative samples obtained from healthcare workers used in present study. B) Negative control lacking SARS-CoV-2 viral RNA, and a Positive control containing SARS-CoV-2 viral RNA.

With the RT-LAMP test, it was possible to detect SARS-CoV-2 in 60% (60/100) of the healthcare professionals tested using the E gene and N gene primers approximately and 100% (100/100) for the gene of human β-actin. In the RT-qPCR, positive results were observed in 62% (62/100) of health workers and 38% (38/100) of negative results when using the primer sets of the N1 and N2 genes to detect SARS-CoV-2. Few results from the analyzed samples were inconsistent between the two trials. In the RT-LAMP assay, for primer sets for the E and N genes, 3.33% (2/60) and 7.01% (4/57) of the negative results were observed among healthcare workers. These same results were positive on RT-qPCR for both N1 and N2 genes (**Supplementary data**).

Comparing the dichotomous variables of the RT-LAMP and RT-qPCR assays, the overall agreement rate between the two methods was determined by calculating the kappa index (*k*). For the primer set of the E and N genes, the observed agreement was considered excellent (k = 0.957 and k = 0.896), respectively.

3.2 Sensitivity of the RT-LAMP assay

Standard curves were generated to quantify the sensitivity of RT-qPCR. Nine-fold serial dilutions of standard controls from 10^{0} to 10^{-8}) were prepared, starting at $3.12x10^{6}$ viral copy equivalents per reaction (SARS-CoV-2) (**Figure 2**). The standard control was run in duplicate, with an efficiency of 111.07 % (R2 = 0.982, slope = − 3,082) for N1 and 158.27 % (R2 = 0.966, slope = − 2,427) for gene N2. No template control (NTC) was used in each run to ensure the

absence of contamination in the assay. From the standard curve of each gene, viral quantification of SARS-Cov-2 was performed, taking into account the C_t value of each dilution (**data not shown**).

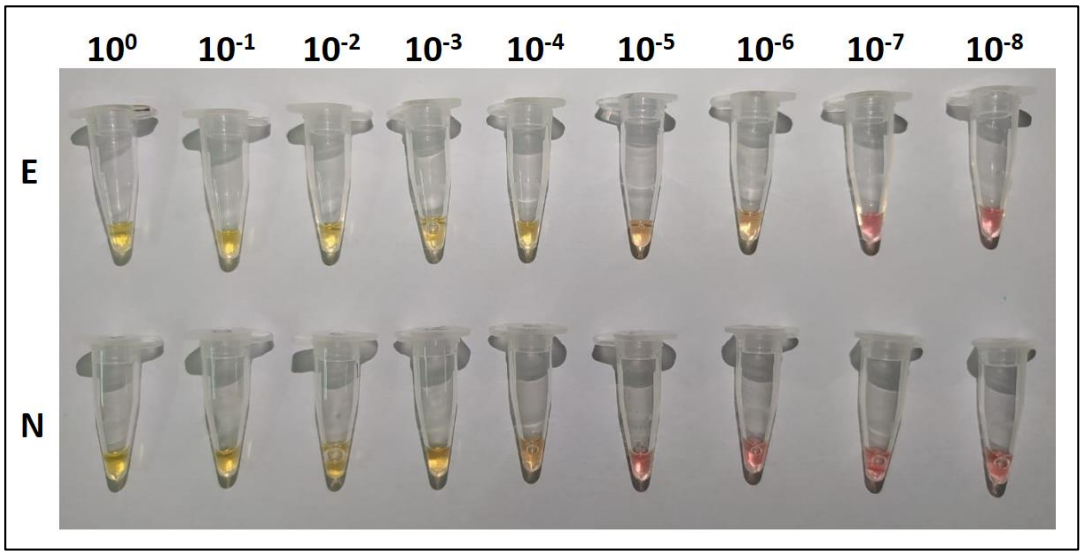

Figure 2.Sensibility detection of RT-LAMP assay using E and N primer set. Serial dilution assay using 50 ng of SARS-CoV-2 viral RNA as initial template.

3.3 Specificity of the RT-LAMP assay

The specificity of the RT-LAMP assay was determined using 15 different viral isolates. After the execution of the RT-LAMP assay, no positive reactions were observed for any of the viruses tested, and only a positive reaction occurred for SARS-CoV-2, included as a positive control in the test (**Figure 3**).

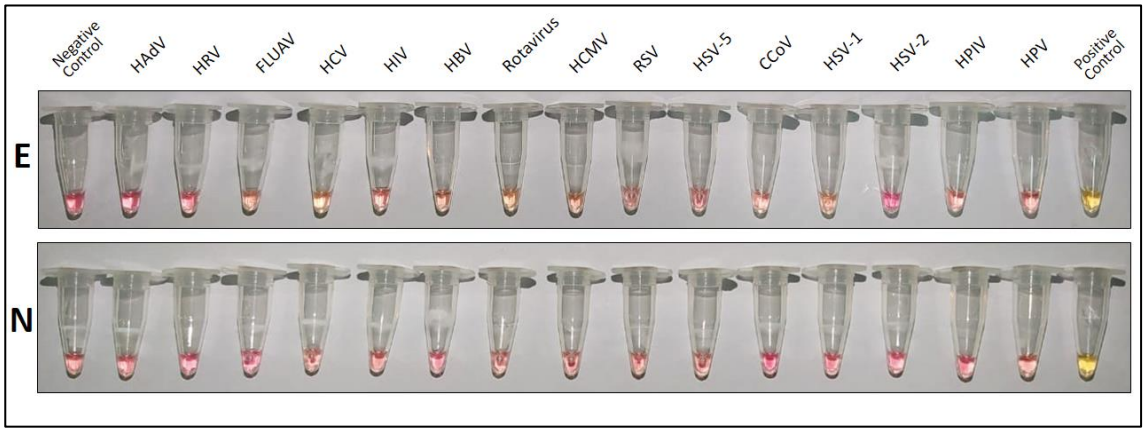

Figure 3. Specificity detection of RT-LAMP assay using E and N primer set. Negative control lacking SARS-CoV-2 viral RNA, Human Adenovirus (HAdV), Human Rhinovirus (HRV), Human Influenza A Virus (FLUAV), Hepatitis C virus (HCV), Human Immunodeficiency Virus (HIV), Hepatitis B virus (HBV), Rotavirus, Human Cytomegalovirus (HCV), Respiratory Syncytial Virus (RSV), Bovine herpesvirus 5 (BHV-5), Canine Coronavirus (CCoV), Herpes simplex virus type 1 (HSV-1), Herpes simplex virus type 2 (HSV-2), Human Parainfluenza virus (HPIV), Human Papillomavirus (HPV), and Positive control containing SARS-CoV-2 viral RNA.

3.4 Agreement between RT-LAMP and RT-qPCR assays assessed by the Bland-Altman method

The C_t values of the dilutions of the SARS-CoV-2 RNAs determined by RT-qPCR were used to calculate the number of viral copies in the RT-LAMP assay. From these results, the correlation between the two assays was assessed. The agreement was estimated using a dispersion diagram designed using the Bland-Altman method.

Figure 4 shows the viral copy number (log_{10}) detected. It can be seen in the Bland-Altmann diagram that the viral copy number in the RT-LAMP for the primer set of the E gene showed a strong correlation with the values observed in the RT-qPCR. When compared to RT-qPCR, it is evident that RT-LAMP using primers set for the N gene demonstred a lower viral copy number for SARS-CoV-2. However, the RT-LAMP assay for the primer set of the E gene showed a significantly higher agreement with the RT-qPCR for detecting SARS-CoV-2. Overall, our results demonstrate that RT-qPCR has a higher sensitivity when compared to viral copy number of SARS-CoV-2 RNA in relation to the two RT-LAMP assays.All data were analyzed within a 95% confidence interval.

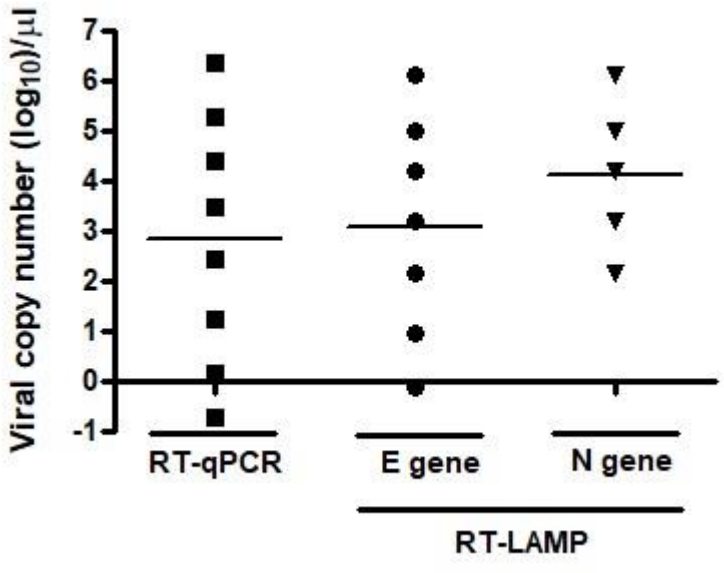

Figure 4: Agreement between the RT-qPCR and RT-LAMP assay for the primer set of the E and N gene by assessing the viral copy number (log_{10}), using the Bland-Altmann method. The difference in the log number of viral copies determined in the assays are located on the vertical axis, and the mean value is on the horizontal axis.

3.5 Determination of viral RNA copies limits for RT-LAMP

From the RT-qPCR tests of health professionals and the determination of the number of viral RNA copies/µl, it was possible to estimate the limit of detection (LoD) per reaction of the RT-LAMP assay (Table 3). The highest viral RNA copies number observed in RT-LAMP compared to RT-qPCR was detected in sample number 100, with a Ct range of 17–18. This value corresponded to approximately 2.328.000 copies per reaction. On the other hand, the smallest number can be detected in sample 2, in the range of 37–38 Ct, corresponding to approximately 2 copies per reaction. This previously estimated LoD value can be attributed to the reaction to the E gene in the RT-LAMP assay. As for the N gene in the RT-LAMP assay, it was possible to verify an LoD of approximately 253 copies per reaction.

Table 3: Estimated limits of detection (LoD) for RT-LAMP. Mean and standard deviation (SD) of Cts for the N1 and N2 genes of all health professionals who tested positive for SARS-CoV-2 and determination of agreement with RT-LAMP.

	RT-qPCR				RT-LAMP	
Cts	**Média Cts N1**	**Viral copy number/µl**	**Média Cts N2**	**Viral copy number/µl**	**E**	**N**
17-18	**ND**	**ND**	17.87 ± 0.62	**5.82×10^5**	**+**	**+**
19-20	19.68 ± 0.43	1.48×10^5	19.42 ± 0.31	1.49×10^5	+	+
21-22	21.64 ± 0.50	3.69×10^4	22.07 ± 0.59	2.47×10^4	+	+
23-24	23.52 ± 0.63	9.27×10^3	24.11 ± 0.63	6.11×10^3	+	+
25-26	25.84 ± 0.75	1.55×10^3	26.06 ± 0.75	1.54×10^3	+	+
27-28	28.29 ± 0.45	2.91×10^2	28.08 ± 0.78	3.86×10^2	+	+
29-30	30 ± 0.58	9.6×10^1	30.15 ± 0.58	**9.50×10^1**	+	+
31-32	32.27 ± 0.49	1.81×10^1	31.63 ± 0.57	4.70×10^1	+	-
35-36	36.39 ± 0.51	0.112×10^1	35.57 ± 0	0.22×10^1	+	-
37 – 38	37.09 ± 0	**7.5×10^{-1}**	ND	**ND**	+	-

ND - not detected;

4. Discussion

The present study describes a quick and simple method to diagnose SARS-CoV-2 in nasopharyngeal swab samples from healthcare professionals. RT-LAMP was developed to rapidly detect SARS-CoV-2, using different genes, human β-actin, E, and N genes.

The sensitivity found in our study was 156 viral copies/µL, which is in agreement with most RT-LAMP assays having a range of 100–200 copies per reaction. Nagura [85] used various molecular techniques, including RT-qPCR and RT-LAMP, to diagnose COVID-19 in 103 saliva samples from symptomatic and asymptomatic patients, and found that RT-LAMP had a sensitivity of 70.9%, corroborating the various reports that demonstrate a high sensitivity

of RT-LAMP. For instance, Huang [78] reported a sensitivity of 80 copies of viral RNA per mL. Park [92] used the Nsp3 gene to detect SARS-CoV-2, which had a detection limit of 100 copies per reaction. In turn, Yan [100], using ORF1ab and the S gene, obtained 20–200 viral copies per reaction in clinical patient samples. A sensitivity of up to 10 copies/reaction has also been observed in other studies [101.102].

The specificity of the RT-LAMP assay was assessed using 15 different human respiratory virus isolates. No positive reaction was observed for any of the test viruses. Only SARS-CoV-2, included as a positive control in the test, exhibited a positive reaction. Recently published studies have demonstrated promising results for RT-LAMP specificity, which can reach a high specificity value. For instance, in the study by Chow [83], 100% specificity was observed for the 143 samples tested. No false positives were observed, even for the samples with other coronaviruses and human respiratory viruses. These results are reinforced by Mautner [103], who evaluated 21 human respiratory pathogens and found no nonspecific signals for the ORF8 target genes and N in the SARS-CoV-2 genome. A study by Coelho [104] found that out of 466 clinical samples tested, RT-LAMP demonstrated a specificity of 90.4%, while the study by Österdahl [105] showed 73.0% specificity in nasal and pharyngeal smears.

Until the present moment of writing this article, no available statistical tool enables a concurrent analysis for the RT-qPCR technique with RT-LAMP. As we can see in the studies by Coelho [104], Fowler [106], and Thi [107], who chose to stratify the samples into two groups to assess the sensitivity and specificity rates of RT-LAMP. These studies considered a C_t value lower than 30 as a result of high value and a C_t value higher than 30 as a result of low value to have a better statistical understanding concerning assay performance, method properties, and test limitations. Thus, to our knowledge, this is the first work to demonstrate the agreement between these molecular tests using a scatter diagram drawn by the Bland-Altman method.

5. Conclusions

Taken together, our data unequivocally demonstrated that RT-LAMP assay can be used as an alternative molecular biology technique for diagnosing COVID-19 in healthcare professionals. The herein validated RT-LAMP assay is a simple, rapid and safe method and showed a high sensitivity and specificity when compared to a widely used qRT-PCR.

Competing interests

The authors declare no competing financial interests.

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Attachment

Table 1. Primers for the detection of SARS-CoV-2 nos ensaios de RT-LAMP

E	**Sequence 5'-3'**
F3	TGAGTACGAACTTATGTACTCAT
B3	TTCAGATTTTTAACACGAGAGT
FIP	ACCACGAAAGCAAGAAAAAGAAGTTCGTTTCGGAAGAGACAG
BIP	TTGCTAGTTACACTAGCCATCCTTAGGTTTTACAAGACTCACGT
LF	CGCTATTAACTATTAACG
LB	GCGCTTCGATTGTGTGCGT
N(Nsp3_1-61)	**Sequence 5'-3'**
F3	ACCAGGAACTAATCAGACAAG
B3	GACTTGATCTTTGAAATTTGGATCT
FIP	TTCCGAAGAACGCTGAAGCGGAACTGATTACAAACATTGGCC
BIP	CGCATTGGCATGGAAGTCACAATTTGATGGCACCTGTGTA
LF	GGGGGCAAATTGTGCAATTTG
LB	CTTCGGGAACGTGGTTGACC
β-actin	**Sequence 5'-3'**
F3	AGTACCCCATCGAGCACG
B3	AGCCTGGATAGCAACGTACA
FIP	GAGCCACACGCAGCTCATTGTATCACCAACTGGGACGACA
BIP	CTGAACCCCAAGGCCAACCGGCTGGGGTGTTGAAGGTC
LF	TGTGGTGCCAGATTTTCTCCA
LB	CGAGAAGATGACCCAGATCATGT

Table 2. Comparison of the results obtained in the RT-LAMP assay against those obtained in the RT-qPCR technique.

Sample number	**RT-qPCR**			**RT-LAMP**		
	N1	**N2**	**RP**	**E**	**N**	**β-actin**
1	31.48	27.31	29.34	+	+	+
2	37.09	28.53	30.61	+	-	+
3	29.75	24.46	30.58	+	+	+
4	21.90	23.75	23.88	+	+	+
5	25.11	19.34	29.39	+	+	+
6	-	-	28.01	-	-	+
7	30.54	25.84	26.52	+	-	+
8	28.24	22.73	23.76	+	+	+
9	-	-	26.16	-	-	+
10	24.28	19.78	26.18	+	+	+
11	30.89	25.81	27.39	+	-	+
12	25.73	18.26	27.64	+	+	+
13	28.39	23.68	24.88	+	+	+
14	27.70	23.66	28.12	+	+	+
15	25.23	21.23	28.18	+	+	+

16	32.37	27.24	27.55	+	+	+
17	26.74	22.17	26.86	+	+	+
18	-	-	29.69	-	-	+
19	30.47	24.97	32.35	+	+	+
20	23.88	23.96	31.34	+	+	+
21	28.29	24.22	23.91	+	+	+
22	-	-	24.15	-	-	+
23	23.34	22.68	24.99	+	+	+
24	24.92	21.45	25.16	+	+	+
25	-	-	28.75	-	-	+
26	36.95	28.62	27.77	+	+	+
27	-	-	28.54	-	-	+
28	36.29	28.92	26.72	+	+	+
29	-	-	23.49	-	-	+
30	29.37	30.92	20.89	+	+	+
31	-	-	24.67	-	-	+
32	32.35	31.46	23.85	+	+	+
33	27.75	27.07	24.61	+	+	+
34	-	-	22.78	-	-	+
35	30.86	30.46	24.37	+	+	+
36	32.23	32.27	21.61	+	+	+
37	-	-	25.91	-	-	+
38	35.74	35.57	26.18	-	-	+
39	31.85	29.51	26.22	+	+	+
40	19.99	18.22	25.33	+	+	+
41	-	-	30.07	-	-	+
42	29.20	29.29	30.28	+	+	+
43	29.88	26.64	31.46	+	+	+
44	30.26	26.98	32.77	+	+	+
45	-	-	30.52	-	-	+
46	-	-	26.65	-	-	+
47	30.28	22.23	29.98	+	+	+
48	-	-	21.94	-	-	+
49	-	-	27.47	-	-	+
50	25.37	25.91	27.17	+	+	+
51	-	-	27.14	-	-	+
52	-	-	26.91	-	-	+
53	24.55	22.12	29.11	+	+	+
54	24.87	23.93	26.13	+	+	+
55	27.75	24.42	27.96	+	+	+
56	-	-	29,39	-	-	+
57	24.71	23.16	30,31	+	+	+
58	26.86	25.47	30,05	+	+	+
59	-	-	27,42	-	-	+

60	25.67	24.60	25,86	+	+	+
61	-	-	26,38	-	-	+
62	25.08	21.88	25,65	-	-	+
63	-	-	27,81	-	-	+
64	-	-	25,49	-	-	+
65	-	-	20,73	-	-	+
66	-	-	25,27	-	-	+
67	24.10	21.02	22,76	+	+	+
68	26.84	22.82	22,86	+	+	+
69	28.35	26.06	23,65	+	+	+
70	-	-	23,52	-	-	+
71	-	-	26,79	-	-	+
72	-	-	24,82	-	-	+
73	-	-	25,59	-	-	+
74	-	-	26,61	-	-	+
75	-	-	24,58	-	-	+
76	-	-	26,24	-	-	+
77	-	-	26,62	-	-	+
78	21.70	24.57	24,51	+	+	+
79	21.03	22.10	25,62	+	+	+
80	28.97	28.46	27,75	+	+	+
81	36.58	31.16	28,38	+	+	+
82	-	-	23,34	-	-	+
83	24.81	26.68	25,34	+	+	+
84	-	-	26,21	-	-	+
85	28.69	29.30	24,96	+	+	+
86	-	-	26,46	-	-	+
87	-	-	27,02	-	-	+
88	-	-	25,08	-	-	+
89	22.07	25.52	23,88	+	+	+
90	23.03	23.61	29,84	+	+	+
91	21.02	19.16	23,89	+	+	+
92	32.73	30.39	27,09	+	+	+
93	23.60	25.69	29,2	+	+	+
94	32.92	29.88	29,72	+	+	+
95	23.85	24.17	27,1	+	+	+
96	28.84	29.78	26,51	+	+	+
97	-	-	27,82	-	-	+
98	23.47	22.51	25,65	+	+	+
99	22.17	24.50	29,19	+	+	+
100	19.38	17.15	26,08	+	+	+

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5. CAPÍTULO 2

5.1 Manuscrito 2

O presente manuscrito “Removal of SARS-CoV-2 from contaminated water using *Hydrocotyle ranunculoides*” está formatado de acordo com as normas da revista a qual foi submetido: Results in Chemistry, ISSN 2211-7156 (Online), com fator de impacto 2.3.

Removal of SARS-CoV-2 from contaminated water using *Hydrocotyle ranunculoides*

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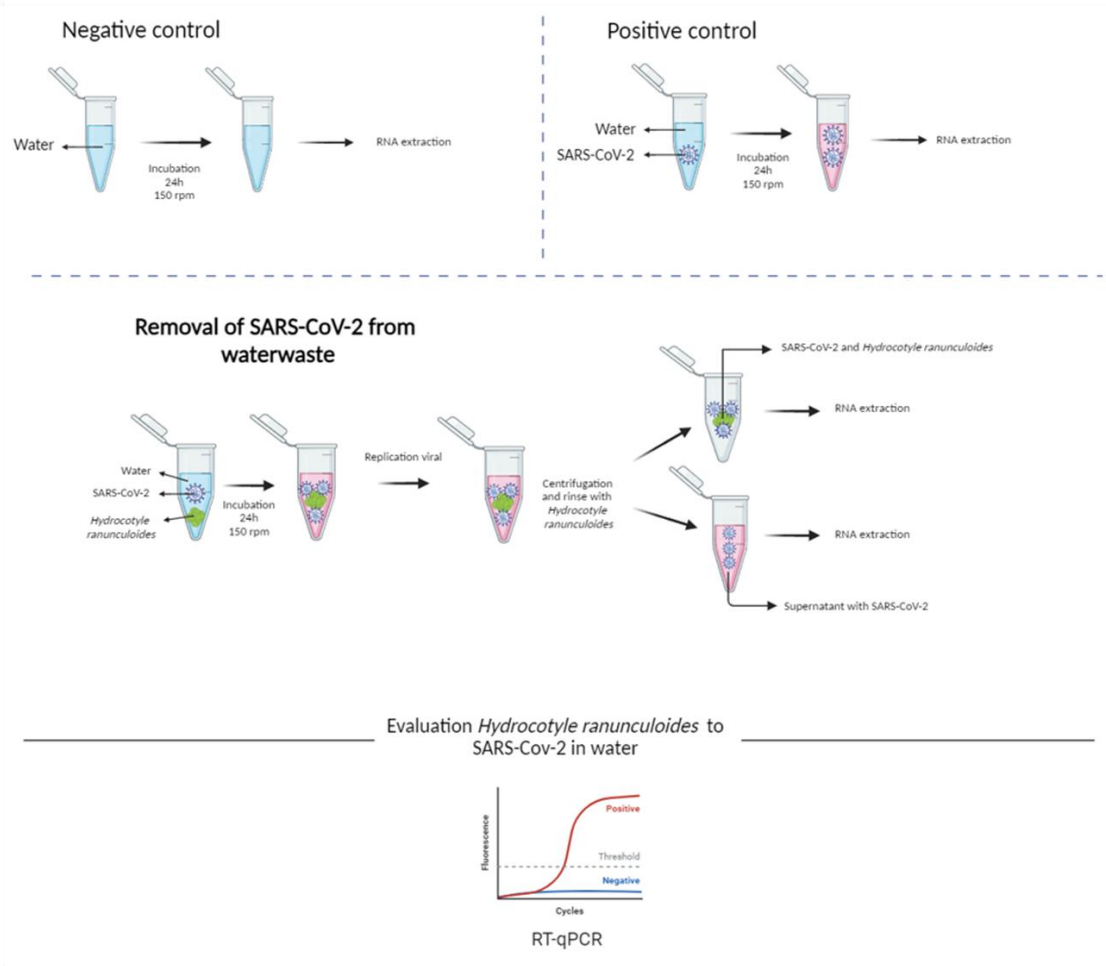
Graphical abstract



Highlights

- A native floating aquatic macrophyte was employed as a biomaterial in wastewater treatment.
- The presence of SARS-CoV-2 has been detected in wastewater, rendering it a potential source of contamination.
- The macrophyte *H. ranunculoides* performs a phytoremediation function in contaminated water.
- Development of a biomaterial based on *H. ranunculoides* has great potential for pathogen removal systems in wastewater

Abstract:

This short communication proposes the employment of molecular approaches to assess the aquatic floating macrophyte *Hydrocotyle ranunculoides* (*H. ranunculoides*) as a potential eliminator of SARS-CoV-2 from contaminated water. The functional group of *H. ranunculoides* sample was characterized through techniques including scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FT-IR) and Thermo Gravimetric Analysis (TGA). After a 24-hour incubation with an inactivated viral suspension, an assay was conducted to evaluate the potential of SARS-CoV-2 removal. The capacity for removing viral RNA was quantified using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) method. The results showed significant adsorption potential with a specific surface area of 8.24 m^2g^1 and a pore diameter of 4.36 nm. The FT-IR spectrum confirmed the presence of hydroxyl functional groups in *H. ranunculoides*, which potentially contribute to virus adsorption. Notably, the aquatic macrophyte exhibited an impressive SARS-CoV-2 removal rate of 88.28%. The RT-qPCR test additionally detected viral RNA presence. For the first time, this study highlights the removal potential of *H. ranunculoides* in the context of SARS-CoV-2 elimination.

Keywords: COVID-19; Macrophyte; Infectious diseases; Green chemistry; Green material; Water-waste.

1. Introduction

A particular concern in controlling a new outbreak is the likelihood of SARS-CoV-2 transmission through wastewater, as the RNA of the SARS-CoV-2 was discovered to be resilient in wastewater for days without substantial degradation [1]. Based on currently available data, there is a need for a better understanding of the wastewater significance as a potential source of infectious agents and as a risk factor for public health [2].

The advancement of environmental pollution the concept of green material, which has as its basic principles the practical use of raw materials, as well as avoiding the use of toxic and dangerous reagents and solvents, increasingly leads to the synthesis of more environmentally friendly chemical products, providing greater health security [3]

Considering that algae provide the pharmaceutical industry with a diversity of organic compounds that have different biological activities [4] added to the biodiversity of organic molecules using the precision of green chemistry [5], they emerge as a strategy as an alternative to conventional therapies for the prevention and treatment of diseases, as well as processes for removing matter and heavy metals from wastewater with phytoremediation and phytoadsorption processes [6,7,8]. For the first time, this study revealed that *H. ranunculoides* has the potential to remove SARS-CoV-2 from contaminated water.

2. Materials and methods

Inactivated (SARSCoV-2/SP02/human2020/Br, GenBank accession number MT126808.1) virus was kindly provided by Prof. Dr. Edison Luiz Durigon from the Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo (USP), Brazil. The macrophyte *H. ranunculoides* was collected as described by Demarco et al. (2018), at Pelotas municipality RS, Brazil. The biomass was rinsed with tap water followed by distilled water to eliminate any attached sediments. It was then dried at 65 °C, ground and passed through an 18-mesh sieve. The Thermogravimetric Analysis (TGA) analyses were performed using a Q5000 analyzer (TA Intruments Inc, USA) however differential scanning calorimetry (DSC) was subjected to three cycles of heating and cooling, and the analysis range was from -80 °C to the initial decomposition temperature of each sample. The heating rate was 10 °C/min and N_2 was used as the inert gas with a flow of 50 mL/min and both tests were analyzed using TA Universal Analysis 2000 software, version 4.5 (TA Intruments Inc, USA). Fourier-transform infrared spectroscopy (FT-IR) Each sample was scanned at 400–4000 cm^{-1}, 100 scans with a resolution of 1 cm^{-1} using the IR model Spirit (Shimadzu, Japan). The specific surface area of *H. ranunculoides* was obtained from the Brunauer, Emmett and Teller (BET) method, and the pore size distribution was obtained from Barrett-Joyner-Halenda (BJH) method (GEMINI 2390). The surface morphologies were evaluated using Scanning Electron Microscopy (SEM) (JEOL, JSM 6610 L V, Japan), using 15 kV and magnification of 50x at 1500x. *H. ranunculoides* in natura (10 mg) were properly dried at 37 °C for 2 h. The primer and probe used in PCR reactions were designed following the Centers for Disease Control and Prevention sequencing (2020). The reaction occurred with the following cycle 55 °C for 10 min for reverse transcription, followed by 95 °C for 3 min and 40 cycles of 95 °C for 15s, 58 °C for 30s (7500 Real-Time PCR System, Thermo Fisher Scientific, Waltham, USA). Data were expressed as mean ± SD for duplicates for each experimental point. Analysis of variance (ANOVA) was used to assess the data, followed by Tukey's test using GraphPad Prism 9.0.

3. Results and discussion

The morphology of *H. ranunculoides* was confirmed by SEM allowing a specific view of the plant surface shown as shown in Fig 1. The specific surface area was 8.24 m^2g^{-1} with a pore diameter of 4.36 nm.

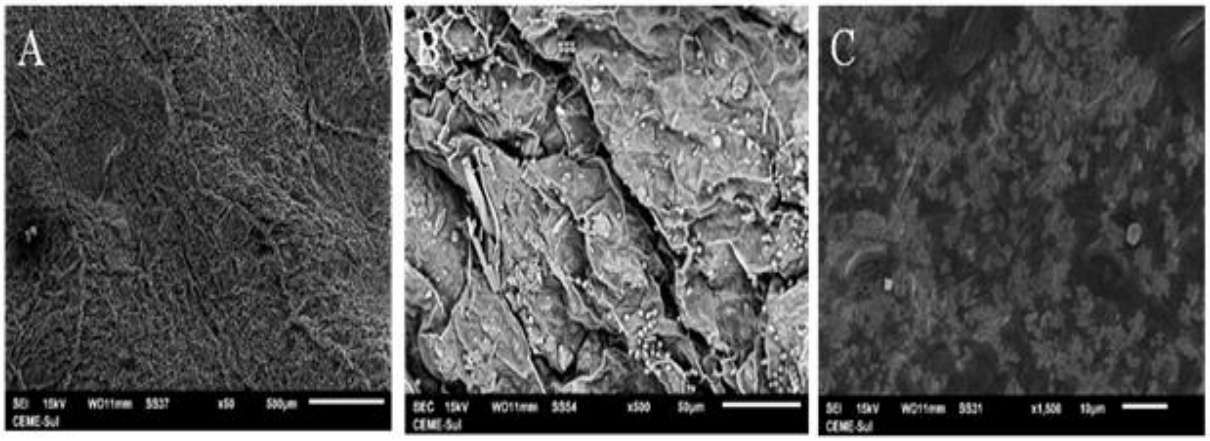


Fig. 1. A (50x), B(500x) and C(1.500x), Scanning electron microscopy images of *H. ranunculoides* dried biomass.

The FT-IR spectra of *H. ranunculoides* in Fig.2 suggest the presence of a large O-H band at 3294.11 cm^{-1} in both samples, two C-H stretching vibrations at 2908.37 cm^{-1} and 2852.07 cm^{-1}, with the latter peak being relatively small in amplitude. The C=O bond is identifiable at 1727.26 cm^{-1}, although at a small intensity, probably due to the presence of an acetyl [10],[11]. The peak at 1623.11 cm^{-1} may be a result of a double bond, representing either an interaction between C=C or a heterogeneous bond (C=O or C=N) [12]. The C-O bond can be verified at 1028-21 cm^{-1} and is likely derived from the cellulose matrix. The presence of these functional groups implies that adsorption is physical rather than chemical [13].

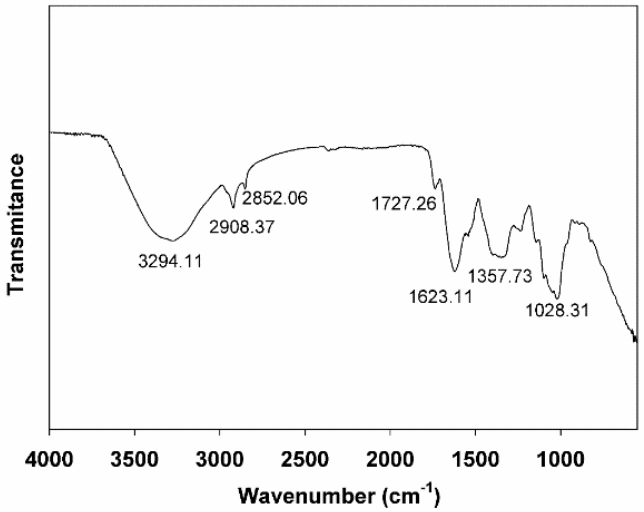


Fig. 2. Fourier-transform infrared spectroscopy of *Hydrocotyle ranunculoides* **.**

TGA study of *H. ranunculoides* Fig. 3. **(A)** revealed three significant weight decreases. Weight loss of 11% is observed at 42 °C. The most substantial sample decomposition occurred across two distinct phases: Td1 at 251 ºC and Td2 at 315 °C, resulting in a total weight loss of 52% within the analyzed temperature range. Demarco et al. (2023) performed TGA analyses of *H. ranunculoides* and the authors found similar results to those obtained in this study. The maximum decomposition temperature identified was 308 °C. Furthermore, it was reported that thermal degradation above 400 °C may be linked to carbon residue reactions for *H. ranunculoides* . In the DSC analysis in Fig. 2. **(B)**, no changes were observed in the molecular structure of the tested compound.

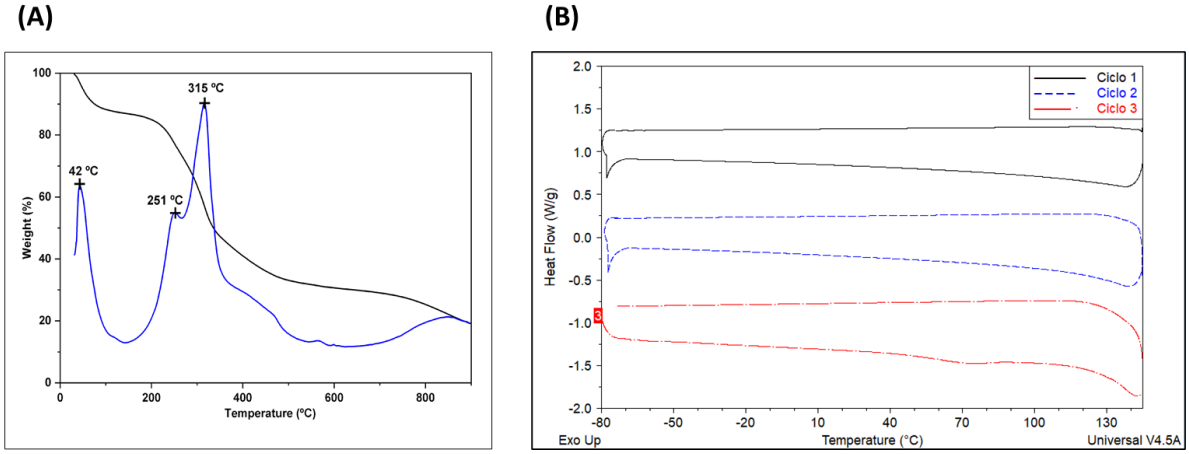


Fig.3. (A) Thermogravimetric Analysis and **(B)** differential scanning calorimetry of *H. ranunculoides* biomass

Figure 4 **(A)**. shows the cycle threshold (Ct) values detected in the supernatant for *Hydrocotyle. ranunculoides* was 20.24 ± 0.42, while the material had a value of 23.44 ± 0.73. Positive control has a Ct value of 18.85 ± 1.14 and negative control has no gene amplification and Figure 4 **(B)** showed results that the green material produced from *H. ranunculoides* promote high removal characteristics once after after 24 hours of incubation removed 88.28 ± 2.68%.

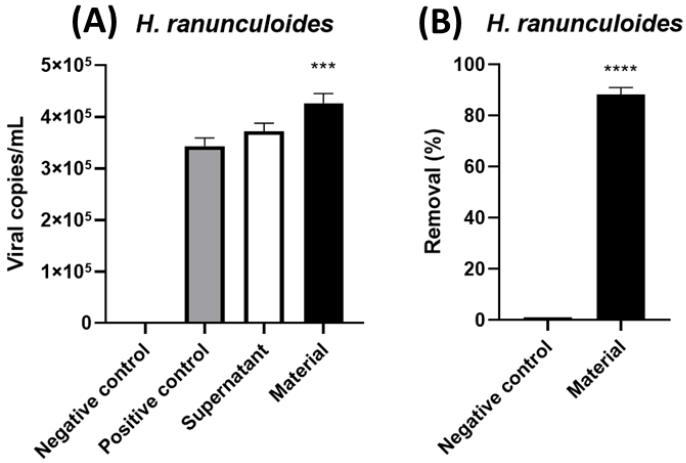


Fig. 4. (A) Ct values detected in *H. ranunculoides* values compared to negative control. **(B)** Removal properties of *H. ranunculoides* after 24 hours.

Several studies have been investigating this issue, aligning with our current work aiming to develop new biomaterials endowed with low cost and high efficiency for SARS-CoV-2 removal, as an alternative to conventional adsorbents. As observed in the study by Schoeler et al. (2023), using a polyurethane peach stone achieved removal 4,76 x 10^4 copies mL^{-1}, while polyurethane peach stone activated carbon reached removal 0.26 x 10^4 copies mL^{-1}, and when polyurethane commercial activated carbon, it was removal 0,90 x 10^4 copies mL^{-1}, it is also possible to observe this concern in the contamination of wastewater in the study Demarco et al. (2022) employed low-cost biofilters based on *Hymenachne grumosa* for SARS-CoV-2 removal in wastewater. It revealed the significant adsorption potential of the biofilter, achieving a removal 4.54 x 10^2 copies mL^{-1} for "in natura" *H. grumosa*, though 4.52 x 10^2 copies mL^{-1} for *H. grumosa* incorporated into activated carbon.

4. Conclusion

The green material produced from *H. ranunculoides* can be a promising alternative for the treatment of wastewater and aquatic environments contaminated with SARS-CoV-2, and the potential for use has been demonstrated in the field of biotechnology and in the diagnosis of infectious diseases.

Declaration of Competing 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.

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6. CAPÍTULO 3

6.1. Manuscrito 3

O artigo intitulado “Green Cryogel Development: Utilizing *Rosa centifolia* Essential Oil for SARS-CoV-2 Removal in Wastewater” é apresentado conforme as normais da revista International Journal of Biological Macromolecules, ISSN:0141-8130, com fator de impacto 8.2.

Green cryogel development: Utilizing *Rosa centifolia* essential il for SARS-CoV-2 removal in wastewater

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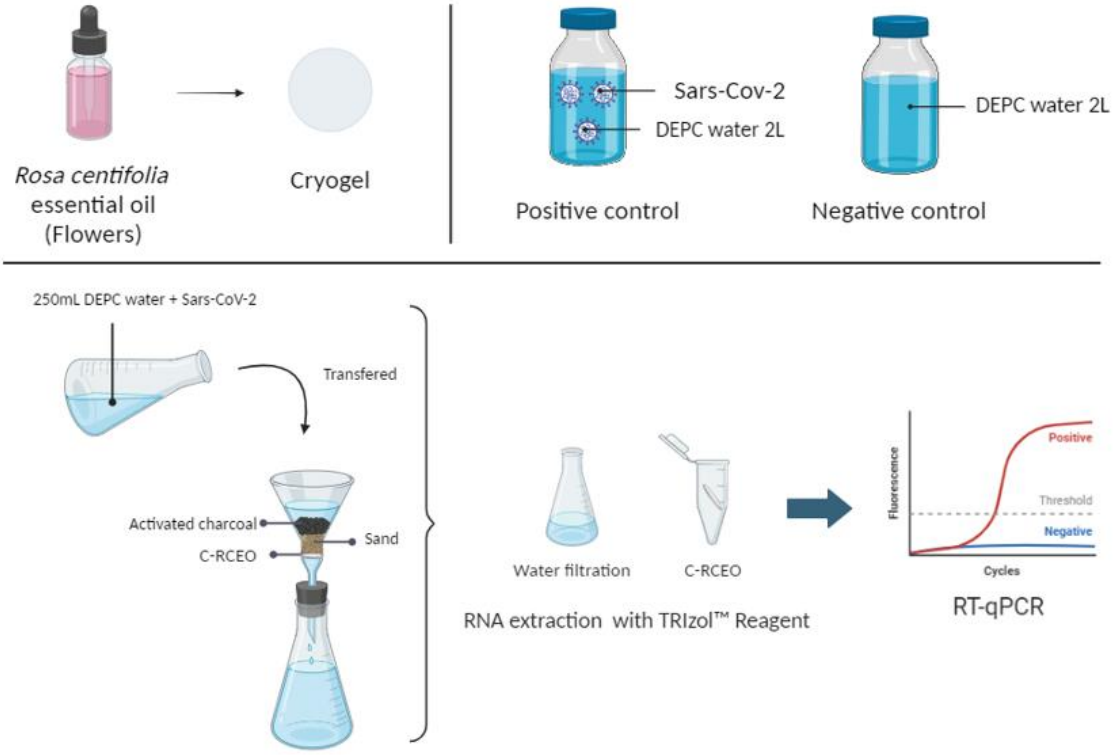
ABSTRACT

The COVID-19 pandemic was caused by SARS-CoV-2 with high serious health effects which led to widespread attempts to implement techniques for quantifying viral loads across various wastewater systems on a large scale. In this study, we propose the employment of molecular approaches to assess a new biomaterial, a cryogel containing *Rosa centifolia* essential oil (C-RCEO) as a potential removel of SARS-CoV-2 from contaminated water. The *Rosa centifolia* essential oil (RCEO) comes from a plant from the Rosaceae family, native from Morocco, widely cultivated as a garden rose, representing one of the most important ornamental plants. The characterization of RCEO was performed by gas chromatography with mass spectrometry (GC-MS), the thermal properties were evaluated by Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC). Cryogels with and without RCEO were characterized by density, porosity, water solubility, morphology and mechanical properties. After a 24-hour incubation with an inactivated viral suspension, an assay was performed to evaluate the C-RCEO potency for removing SARS-CoV-2 and subsequently the ability to remove viral RNA was then quantified using the occurrence in the polymerase chain. quantitative reverse transcription method (RT-qPCR). The majority composition of RCEO identified was citronellol (51.11%). Through the results obtained, we verified that in RCEO the maximum temperature before degradation occurs is 126 °C and no thermal events were observed in the analyzed temperature range from -80 °C to the initial decomposition temperature. Regarding cryogels, they showed characteristic behavior, with water absorption capacity of up to 893%, low density, high porosity and porous structure. When water contaminated with SARS-CoV-2 passed through the C-RCEO filter, the viral load of the virus was reduced by approximately 32-fold. Notably, RCEO exhibited an impressive amount of removal of SARS-CoV-2 present in water of approximately 32-fold. Therefore, this study highlights the possibility of using C-RCEO as an excellent alternative for reducing and decontaminating SARS-COV-2 in wastewater.

Keywords: COVID-19; Rose; Infectious diseases; Green chemistry; Cryogel; Waterwaste.

Graphical abstract



Highlights

- A plant native from Morocco was employed as a biomaterial in wastewater treatment.
- The presence of SARS-CoV-2 has been detected in wastewater, rendering it a potential source of contamination.
- A new applicability for a biomaterial composed of a cryogel incorporated with an essential oil in the reduction of Sars-Cov-2.
- The corn starch-based cryogel incorporated with RCEO has great potential for use in filtration devices in wastewater virus removal systems

1. Introduction

The presence of SARS-CoV-2 in wastewater, coupled with the poor quality of public drinking water supplies in developing countries, is concerning as it could become a new source capable of retaining and disseminating SARS-CoV in the environment [1,2,3]. RNA of SARS-CoV-2 is resilient in wastewater for days without substantial degradation, posing high ecotoxicological and environmental risks [4,5]. Furthermore, the wastewater generated represents a considerable portion of the water consumed, and its disposal may contain complex pharmaceuticals, antibiotics, narcotics, radioactive elements, hazardous compounds and pathogens [6,7,8]

Disinfection of wastewater contaminated by the virus may be an effective alternative in controlling the spread of SARS-CoV-2 [9], however, the specificities of the disinfection procedure or its effectiveness against the SARS-CoV-2 have not yet been elucidated [10]. In the study by Zhang et al. (2020) negative results for SARS-CoV-2 viral RNA in hospital septic tanks were reported using sodium hypochlorite at a concentration of 6,700 g/m^3. Nonetheless, the study reported that an overdose of sodium hypochlorite leads to a high level of disinfection by-product residues, generating environmental risks. Accordingly, a SARS-Cov-2 removal system in wastewater composed of different physical-chemical treatment steps can cause multiple barriers in the removal of the virus in rivers, lakes, groundwater, drinking water reservoirs, recreational water supplies services and waters [12,13,14]. An alternative to reducing or eliminating the use of toxic substances that lead to environmental contamination is the use of the principles of green chemistry, which replaces the use of solvents with natural compounds that may generate toxicity to a lesser extent [15,16].

Given this situation, the development of a sustainable green biomaterial can be achieved in the synthesis of cryogels based on cornstarch, which exhibit excellent physical and chemical properties, such as low density, high porosity and highwater absorption. Furthermore, these materials can protect and release bioactive compounds in a controlled manner in addition to being able to preserve and deliver them [17,18,19,20], as seen in the study by Dinu et al. (2021) that incorporated *Thymus vulgaris* essential oil into cryogels, thus adding antioxidant and antifungal activities in the material.

In our study, we used the essential oil of *Rosa centifolia* (RCEO), a plant from the Rosaceae family, native from Morocco, widely cultivated as a garden rose, representing one of the most important ornamental plants. Many studies report the presence of phytoconstituents such as terpenes, myrcenes, tannins, flavonoids, proanthocyanidins, anthocyanins [22,23,24,25] which are responsible for its anti-inflammatory [26] (, antioxidant [27]and antimicrobial activities [28]. According to Azmi et al. (2012), *Rosa centifolia* extract can be considered a promising biosorbent in the treatment of groundwater, removing iron and manganese, resulting in the obtaining of drinking water. Therefore, the present study aims to evaluate the synthesis of a starch based cryogel incorporating RCEO as its bioactive compound (C-RCEO) and its efficacy for removal of SARS-CoV-2 in wastewater.

2. Materials and methods

2.1. Sampling

2.1.1 Obtainment of SARS-CoV-2

For this experiment we used the inactivated SARS-CoV-2 virus (SARS.COV-2/SP02/human2020/Br, GenBank accession number MT126808.1) kindly provided by Prof. Edison Luiz Durigon from the Department of Microbiology at the Institute of Biomedical Sciences at the University of São Paulo (USP), Brazil, in which he composed the positive control (SARS-CoV-2 + Water).

2.1.2 Aquisition of essential oil of *Rosa centifolia*

The essential oil of *Rosa centifolia* was commercially acquired from the company Bioessencia (Jaú/SP – Brazil).

2.2 Characterization

2.2.1 Characterization of *Rosa centifolia* essential oil

The characterization of the *Rosa centifolia* essential oil was carried out at the Department of Chemistry, Heterocycle Chemistry Center (NUQUIMHE), Federal University of Santa Maria, using a gas chromatography (GC) system (Shimadzu QP2010 UltraPlus, Shimadzu, Japan) equipped with a mass detector spectrometer (Shimadzu Corporation, Japan) and Rxi-1MS capillary column (30 m × 0.32 mm × 0.25 µm, Restek). The ramp temperature was maintained at 40 ◦C for 5 min, increased to 50 ◦C at 3 ◦C/min, to 120 ◦C at 5 ◦C/min, to 175 ◦C at 7 ◦C/min, and finally at 230 ◦C at 10 ◦C/min, and then maintained under isothermal conditions for 8 min. Mass spectrometry (MS) was operated in full scan mode (m/z mass range 30–450). The GC-MS data were analyzed using LabSolution (GC-MS solution Version 4.11 SU2). The compounds were identified by comparing similarity indices and mass spectra with the National Institute of Standards and Technology (NIST 11) system database and retention index calculated from a homologous series of C_8–C_{40} hydro-carbons, according to Vandendool and Kratz (1963). Finally, the quantification was determined by internal standardization.

2.2.1 Thermogravimetric Analysis (TGA)

The TGA were performed using a TGA Q5000 analyzer (TA Intruments Inc., USA). The equipment was calibrated with $CaC_2O_4H_2O$ (99.9%) for accurate measurements. Thermal characterization assessments were conducted at a heating rate of 10 °C min^{-1} gradually ascending to 900 °C. Sample mass analyzed ranged from 3 to 6 mg. The inert atmosphere was composed of N_2 in a flow of 25 mL min^{-1}. Data were treated using the TA Universal Analysis 2000, version 4.5 (TA Instruments Inc., USA).

2.2.2 Differential Scanning Calorimetry (DSC)

The phase transitions were achieved by Modulated Temperature Differential Scanning Calorimetry (MTDSC) on a DSC Q2000 (TA Instruments, USA). The mass of LI used for each analysis was 1 - 5 mg and was weighed on a Sartorius balance (M500P) with an accuracy of ± 0.001 mg. Each sample was subjected to three heating and cooling cycles, the analysis range was -80 °C up to the initial decomposition temperature of each sample. The heating rate was 10 ºC/min and N_2 was used as the inert gas with a flow rate of 50 mL/min. The data was analyzed using TA Universal Analysis 2000 software, version 4.5 (TA Intruments Inc., USA).

2.3 Production of the cryogels

The cryogels were prepared with corn starch as a polymer matrix, as described by Cruz et al. (2023), with some modifications. The components were mixed in 50 mL of distilled water for 30 min at 90 ± 5 °C under stirring and heating. The proportion of the components used were 10% of starch, 0.25% of Tween 80 (U.S.P, batch 231595, Synth) and, 0.20% of glycerin (P.A., batch 246357, Synth). Shortly afterwards, the suspension was mixed in an Ultra-Turrax (IKA®, T18B, Werke, Germany) at 15,500 rpm for 2 min and then cooled to 60 ± 5 °C for the addition of essential oil in the concentration of 30% (v/w, EO volume to starch weight) to produce the RCEO-based cryogel (C-RCEO) (30%). A control was produced without EO, formed by corn starch-based control cryogel (CC).

The mixture (5 g) was added into lids (diameter 2.5 mm x height 0.5 mm) and placed in an ultra-freezer at -67 °C for 24 h. The physical crosslinking was achieved using five alternating cycles of freezing (Consul, CVU18GBBNA, Brazil) at -20 °C for 12 h and thawing at 25 ± 3 °C for 12 h. The cryogels were formed by lyophilization (Liotop K108, Brazil) with a condenser temperature of −100 °C and pressure of 100 µHg for 48h. Cryogels were stored in a hermetically sealed container within a desiccator until further analysis and application.

2.4 Composition of filter

The control filter was composed of corn starch-based cryogel (CC) (3.7g) and cryogel essential oil *rosa essential* (C-RCEO) (3.7g) followed by a layer of sand (103.80g) and then a final layer of activated carbon (48g).

2.4.1 Morphology of the cryogels

The morphology of the cryogels was analyzed by a scanning electron microscope (SEM, Jeol, JSM-6610LV, USA). The cryogels were prepared by cutting approximately 1-mm thick cross-sections from the center and then placed in a stainless-steel stub and sputter-coated with gold using a Sputtering (Denton Vacuum, Desk V, USA) [17]. The parameters used for the analysis were a voltage acceleration of 15 kV and a magnification of 50X and 100X.

2.4.2 Density and porosity of the cryogels

The cryogels were evaluated for density and porosity according to Oliveira et al (2020). The density was calculated from the size and weight of each cryogel (Equation 1), and the porosity was calculated according to Equation 2, where "*V*" is the volume (cm^3) of the cryogels, "*w*" is the weight (g) of the cryogels, and "*d*" is the density of the starch (corn starch = 0.5085 g/cm^3). To calculate the "V", the equation was used: $\pi.r^2.h$, where "*h*" is height.

$$Density = \frac{w}{V} \qquad \text{(Eq. 1)}$$

$$Porosity = \frac{V - (\frac{w}{d})}{V} x100 \qquad \text{(Eq. 2)}$$

2.4.3 Water absorption capacity of the cryogels

The water absorption capacity of the starch-based cryogels was determined by gravimetric analyses. The cryogels were weighed before and after immersion in water for 24 h, and the water absorption capacity was calculated according to Equation 3, where "*wet weight*" is the weight of the cryogel after submerging in water for 24 h and "*dry weight*" is the initial dry weight of the cryogel [33].

$$Water\ absorption\ capacity\ (\%) = \frac{Wet_{weigth} - Dry_{weight}}{Dry_{weight}} \times 100 \qquad \text{Eq. (3)}$$

2.4.4 Mechanical properties of the cryogels

The mechanical properties of the cryogels was analyzed using a texture analyzer (TA.XTplus, StableMicro Systems, UK), as described by Silva et al. (2020). Two compression cycles until 50% of the cryogels initial height at 5 mm/s were applied using a 36-mm diameter cylindrical probe; the parameters evaluated were hardness, springiness, and cohesiveness.

2.5. Virus removal experiment using qRT-PCR

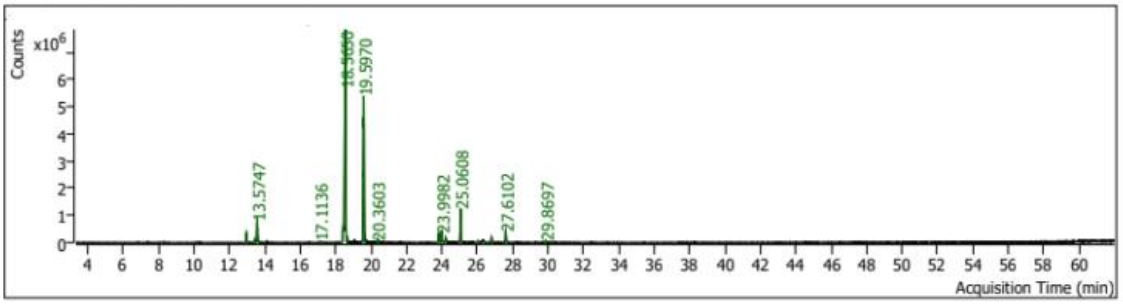
The primer and probe used in PCR reactions were designed in accordance with the Centers for Disease Control and Prevention sequencing (CDC 2020). Briefly, a reaction with a final volume of 25 µL was used, with the following volumes added to the concentrated master mix 1x: 5 µL of sample RNA, 12.5 µL of reaction buffer 2x, 1 µL of Superscript™ III One-Step with Platinum™ Taq DNA Polymerase (Invitrogen, Darmstadt, Germany), 0.4 mM of each dNTP, 0.4 µL of a 50 mM $MgSO_4$ solution (Invitrogen), 1 µg of non-acetylated bovine albumin (Roche), 10 µM of each primer 2019-nCoVN1-F2019-nCoV N1 (5'GACCCCAAAATCAGCGAAAT3'), 2019-nCoVN1-R2019-nCoV N1 (5'TCTGGTTACTGCCAGTTGAATCTG3'), 2019-nCoVN1-P2019-nCoV N1 probe (5'-FAM – ACCCCGCATTACGTTTGGTGGACC– BBQ 3'), and DEPC water. The reaction occurred with the following cycle: 55 °C for 10 min for reverse transcription, followed by 95 °C for 3 min and 40 cycles of 95 °C for 15s, 58 °C for 30s (7500 Real-Time PCR System, Thermo Fisher Scientific, Waltham, MA, USA).

3. Results and discussion

3.1. Characterization of RCEO

The results of the analyzes of RCEO chemical compounds are shown in Figure **1**, with the majority compounds being citronellol (51.11%) and geraniol (29.08%). The composition of RCEO corroborates previous research, which evaluated the constituents of rose essential oils, relating citronellol (35.20–52.54%) with a higher content of the main aromatic compounds, followed by geraniol (13.55–24.55%) [35,36,37,38]. Dobreva et al. (2021), who studied the chemical compostion of the *Rosa centifolia* extract, reported the presence of aliphatic hydrocarbons (40.98%) and monoterpenes (24.44%). These major compounds have a wide spectrum of pharmacological activities, including insecticidal [40], anti-inflammatory [41], antibacterial [42] , antifungal [43] and antiviral [44] actions. As the characterization chemical of RCEO is reported, its respective chemical compounds are presented in the study by Kumar et al. (2020), reporting the downregulate Angiotensin-Converting Enzyme 2, a SARS-CoV-2

spike receptor-binding domain, in epithelial cells, demonstrating that citronellol and its active derivatives are promising compounds in antiviral activity for SARS-COV-2.



Pk #	RT Min	Compound Name	CAS#	Peak Area	Component RI	Library RI	Match Factor	% of Total
1	12.9621	LINALOOL 11.4768	78-70-6	633635.3	1115	1101	98.21	0.89
2	13.4215	ROSE OXIDE<TRANS-> 12.7120	5258-11-7	271571.0	1126		97.06	0.38
3	13.5747	PHENYLETHYL ALCOHOL 13.0327	60-12-8	2475066.4	1129	1116	99.22	3.47
4	17.1136	TETRAHYDROGERANIOL 16.4610	1333-49-9	179425.5	1212	1197	92.07	0.25
5	18.4283	16.97 Nerol	106-25-2	1696855.0	1242		96.63	2.38
6	18.5650	CITRONELLOL 17.8369	106-22-9	36469708.9	1246		95.63	51.11
7	19.5970	17.95 Geraniol	106-24-1	20753172.2	1269		95.81	29.08
8	20.3603	CITRAL<E-> 18.7487 (GERANIAL)	141-27-5	160186.2	1287		92.13	0.22
9	23.8309	CITRONELLYL ACETATE 23.0722	150-84-5	898700.2	1368	1350	99.02	1.26
10	23.9982	EUGENOL 23.3291	97-53-0	1132250.1	1372	1356	99.35	1.59
11	24.2150	NERYL ACETATE 23.4587	141-12-8	400593.7	1377	1359	96.83	0.56
12	25.0608	GERANYL ACETATE 24.2999	105-87-3	3797999.1	1397	1379	99.40	5.32
13	26.0481	METHYL EUGENOL 25.3388 (4-ALLYL VERATROLE)	93-15-2	146119.5	1421	1404	95.52	0.20
15	26.8020	CARYOPHYLLENE<(E-)> 26.1165 (BETA-)	87-44-5	541429.6	1439		96.76	0.76
16	27.6102	CITRONELLYL PROPIONATE 26.8632	141-14-0	1241728.8	1459		97.37	1.74
17	29.8697	28.53 Zingiberene<alpha->	495-60-3	181805.0	1515		86.81	0.25

Fig. 1 Chemical composition of RCEO

3.2 TGA of RCEO

The thermal decomposition of the material occurs in a single step figure **2**, indicating its Td5% at 77 °C and ending its decomposition (Tf) at 236 °C, with 99% mass loss. The maximum decomposition temperature is 126 °C. The boiling points of the constituents of RCEO are lower than 230 ºC, which could explain the values obtained. Chiu et al. (2009) evaluated the thermal decomposition of lemon, rosemary, melaleuca, lavender and rose essential oils and found similar results to those obtained in this study.

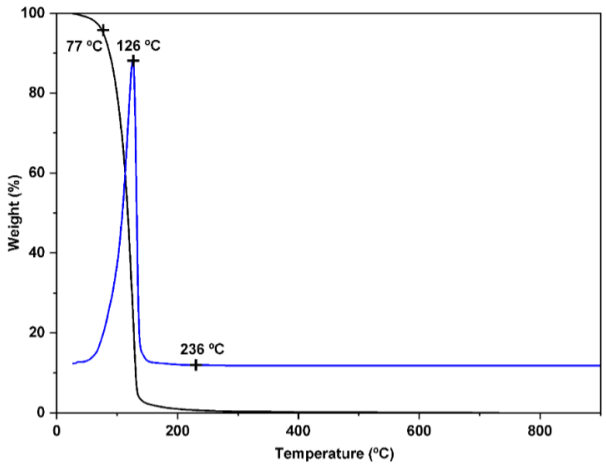


Fig. 2 Thermogravimetric analysis curve for RCEO

3.3 DSC of RCEO

According to the results obtained in Figure **3**, no thermal events were observed in the temperature range analyzed from -80 °C to the initial decomposition temperature of each sample.

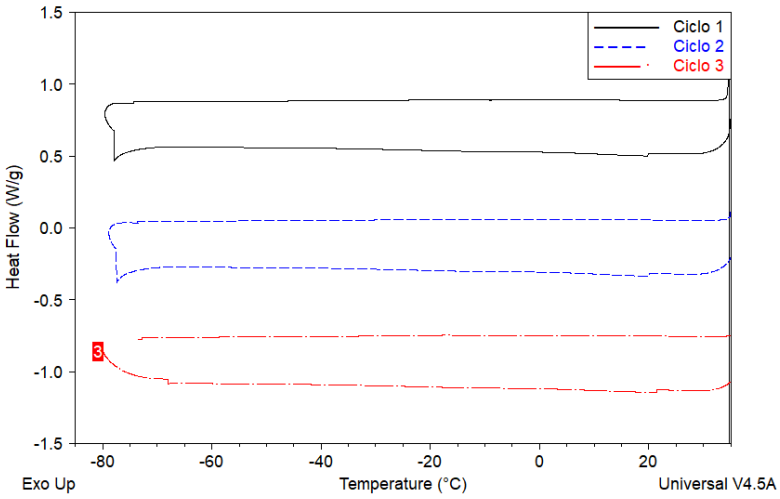


Fig 3. Differential scanning calorimetry curve for RCEO

3.4 Characterization of cryogels

The cryogels presented in Figure **4** a weight of 3.7 g and dimensions of 2.5 mm diameter x 0.5 mm height.

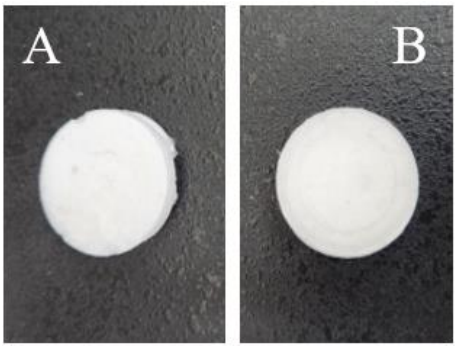


Fig. 4 Photograph of the control cryogel (A) and incorporated into the RCEO (B) produced from corn starch

The morphology of both cryogels (CC and C-RCEO) is shown in Figure **5**. A microporous structure is observed in both samples, this phenomenon being attributed to the formation and expansion of ice crystals during the freezing cycle phase, common in freeze-dried cryogels [17]. In general, control cryogel presented more well- homogeneous and uniform pores (Fig. **5**A), in contrast to the cryogel with RCEO, which presented slightly irregular and compact pores (Fig. **5**B). This behavior can be due to the amount of RCEO added, in which there may have been a destabilization in the three-dimensional network of the starch gel formed, or even an extrapolation in the capacity of the cryogel to trap RCEO, consequently resulting in pores poorly formed and agglomerated. In a comprehensive way, in comparison to other studies, variations in the microstructure of this type of material can be explained by several factors, such as the source of starch used, its concentration, the conditions of gelatinization and

retrogradation (crosslinking), in addition to considerations related to pressure, temperature and other relevant parameters [47].

Cryogels based on dextrin, chitosan and thyme essential oil studied by Dinu et al. (2021), revealed a layered lamella-like pore structure. these authors reported that pore sizes decreased with the addition of essential oils, indicating that this behavior can be due to the entrapment of the essential oil in the structural matrix of the cryogel.

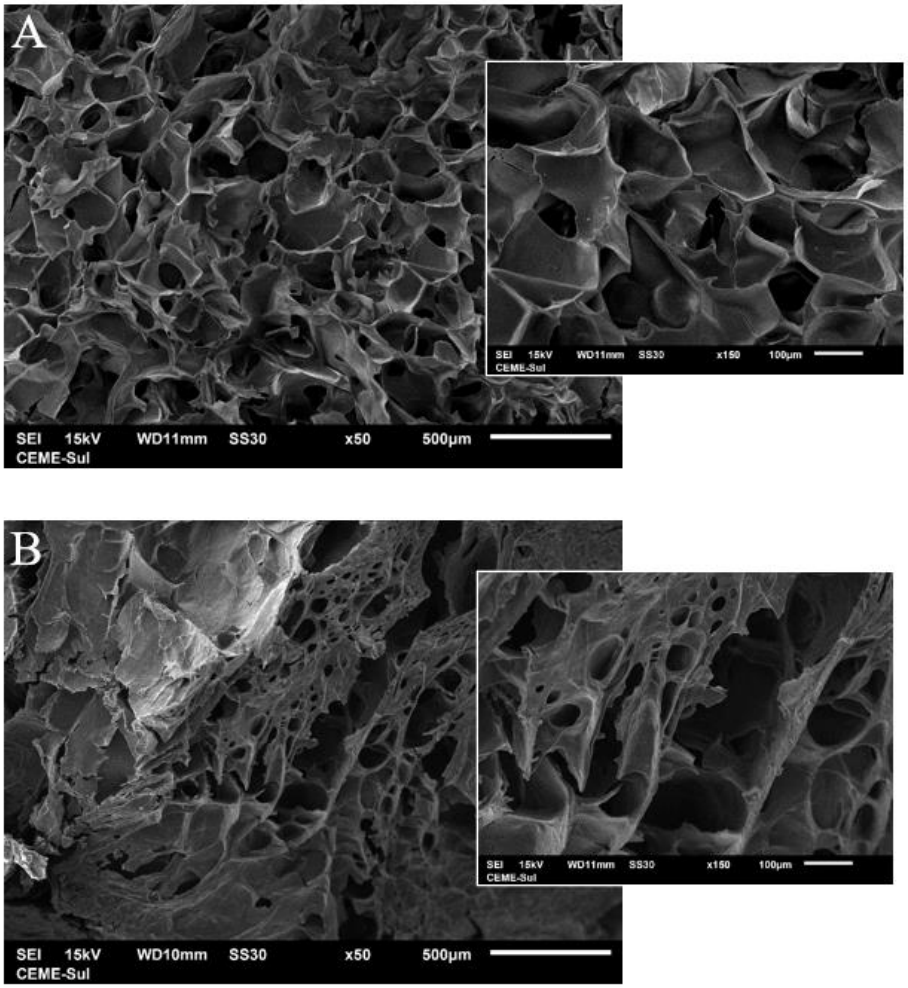


Fig. 5 Morphology of cryogels without RCEO (CC) (**A**) and with RCEO (**B**)

In Table **1**, data relating to the density, porosity and water absorption capacity of starch-based cryogels is presented. The density of control cryogels was significantly lower ($p < 0.05$), recording 0.096 ± 0.003 g/cm^3, compared to cryogels containing RCEO, which showed a density of 0.105 ± 0.001 g/cm^3. Regarding porosity, the control cryogel (CC) presented significantly ($p < 0.05$) greater porosity, when compared to cryogel with RCEO. This result of greater porosity corroborates with the morphology (Fig 3), since the pores of the control cryogels were more homogeneous and structured. In study of Cruz et al. (2023), corn starch cryogels incorporated with red onion peel extract demonstrated the same behavior as observed in a recent study. These authors reported that the incorporation of this extract resulted in an increase in density and a reduction in porosity. Furthermore, the corn starch cryogels produced in this study presented density and porosity similar to wheat starch aerogels, which exhibited a density ranging from 0.11 to 0.13 g/cm^3 and a porosity of 91.7% to 92.9% [48].

Regarding water absorption capacity, no significant difference was observed between cryogels ($p < 0.05$), ranging from 855 to 893%. This indicates that the addition of RCEO in the cryogel did not influence their ability to absorb water. Lower water absorption results were observed for corn starch cryogels incorporated with red onion extract [17], compared to the results of the present study. The water absorption capacity is an important parameter because it can influence the release behavior of the active ingredient, when added [49]. Starch cryogels tend to exhibit a highwater absorption capacity due to their predominantly amorphous structure and several hydroxyl groups [50].

Table. 1 Density, porosity, water absorption capacity, and mechanical properties of the CC and C-RCEO

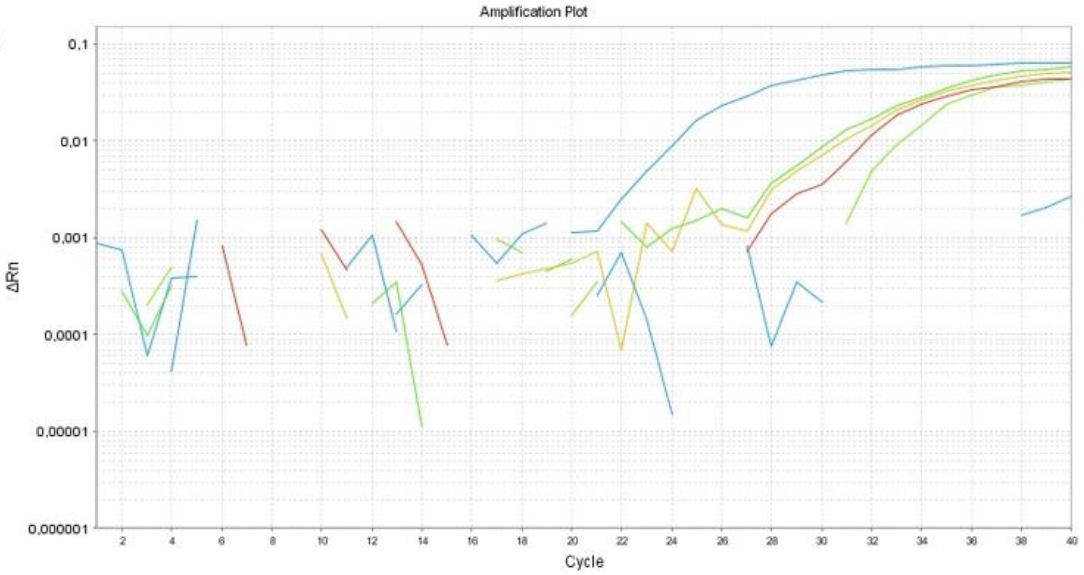
Cryogel	Density (g/ cm^3)	Porosity (%)	Water absorption capacity (%)	Mechanical properties		
				Hardness (g)	Springiness	Cohesiveness
CC	0.096 ± 0.003	81.07± 0.65	893.30 ± 34.0	141.7 ± 3.2	0.96 ± 0.01	0.80 ± 0.05**
C-RCEO	0.105 ± 0.001	79.45 ± 0.10*	854.5 ± 23.1*	179.3 ± 30.5*	0.95 ± 0.01	0.72 ± 0.01

Number of asterisks in the same column statistically varied from the means submitted by test t Student. * Values with an asterisk * ($p < 0.05$) and ** ($p < 0.005$).

3.7. SARS-CoV-2 removal

By analyzing the amplification plot Figure **6A**, it was possible to observe the presence of SARS- CoV-2 in the tested samples and in the negative control no amplification of the virus was observed. In the investigation of SARS-CoV-2 gene expression Figure **6B**, detected presenting cycle threshold (Ct) values for positive control Ct 27.76 ± 0.6 with a viral load log 5.4 x 10^4, negative control not detected amplification, for water passed through the CC-filter Ct 34.07 ± 0.24 with a viral load log 3.16 x 10^2, water passed through the RCEO -filter Ct 38.96± 0.79 with a viral load log 0.74 x 10^0, Biomaterial CC-filter Ct 33,51 ± 0,33 with a viral load log 3.46 x 10^2 and Biomaterial C-RCEO filter Ct 34.37 ± 0.62 with a viral load log 3.26 x 10^2. According to the results obtained, we found that passing water through the C-RCEO filter has the capacity to reduce the circulating amount of viruses in the analyzed wastewater by aproximity 32-fold.

A)



B)

Sample	Cts	Viral load/mL (10^n)
SARS-Cov-2 + Water (Positive control)	27.76 ± 0.6	log 5.4 x 10^4
DEPC (Negative control)	Not detected	-
Water passed through the CC-filter	34.07 ± 0.24	log 3.16 x 10^2
Water passed through the C-RCEO filter	38.96± 0.79	log 0.74 x 10^0
Biomaterial CC-filter	33,51 ± 0,33	log 3.46 x 10^2
Biomaterial C-RCEO filter	34.37 ± 0.62	log 3.26 x 10^2

Fig .6 A) Amplification plot of SARS-CoV-2 detection in wastewater. **B)** Determination viral load/mL of SARS-CoV-2 after wastewater filtration, biomaterials, positive control and negative control, using RT-qPCR. Viral load removed (copies mL^{-1}) = [viral load in supernatant / viral load in material].

Amidst of the COVID-19 pandemic, numerous cases have demonstrated the release of SARS-CoV-2 in human excreta, supporting the idea that wastewater can also contain viral RNA [51,52]. Recent studies have shown the detection of viral nucleotides in wastewater in countries with both high and low prevalence of COVID-19 cases. For instance, in Australia, with comparatively fewer reported cases, studies revealed the presence of SARS-CoV-2 in 22% of wastewater samples, with viral concentrations as low as 1.2×10^2 copies/L [53]. A similar study in the United Arab Emirates reported viral loads ranging from 7.5×10^2 to 3.4×10^4 copies/L in wastewater [54]. Similarly, Haramoto et al. (2020) showed that 20% of secondary treated effluent samples had a viral load of 2.4×10^3 copies/L.

The disinfection of SARS-CoV-2 in wastewater is a growing topic in the literature, which seeks to ensure the correct concentration of the active ingredient and the necessary contact time for an effective and safe effect in the disinfection process [56,57,58,59,60,61]. Wang et al. (2020) and Avila et al. (2020) address alternatives to mitigate the inactivation of SARS-CoV-2 in wastewater through the use of ultraviolet radiation and chlorine, however, precautions must be taken to avoid excessive exposure to ultraviolet radiation, as it causes damage

harmful to living organisms cells [64] (ZZZZ) and although the use of active chlorine is effective against SARS-CoV-2, an excessive dose of disinfectants generates potential biological risks [65,66].

4. Conclusion

Based on the results of the analysis of the genetic expression of SARS-CoV-2 in wastewater, we concluded that when the water is filtered through cryogel controlled, no detection of viral particles in the water, however when the C-RCEO is subjected to RT- qPCR, we verified the presence of viral particles, that is, C-RCEO is capable of retaining SAR-CoV-2 due to its chemical composition. This innovative approach appears as a new alternative in the applicability of cryogel associated with RCEO against SARS-Cov-2.

Author contribution

Victor Dos Santos Barboza[a]: Conceptualization, Methodology, Formal Analysis, Investigation, Data Curation, Validation; **Cleiton Jesus Andrade Pereira[a]:** Methodology, Writing - Original Draft, Writing - Review & Editing, Data Curation; **Vithor Parada Garcia[a]:** Visualization, Writing; **Mikaele Valério Tavares[a]:** Visualization, Writing; **Luane Garcia Pinheiro[a]:** Visualization, Writing; **Janice Luehring Giongo[a]:** Conceptualization, Supervision; **Nicolle Lima Bandeira[a]:** Formal Analysis, Investigation; **Valdir Dos Santos Barboza[a]:** Visualization, Formal Analysis; **Letícia Zibetti [b]:** Formal Analysis, Investigation; **Clarissa Piccinin Frizzo [b]:** Formal Analysis, Investigation; **Elder Pacheco da Cruz:** Visualization, Formal Analysis; **Elessandra da Rosa Zavareze[c]:** Conceptualization, Supervision; **Alvaro Renato Guerra Dias[c]:** Conceptualization, Supervision; **Rodrigo de Almeida Vaucher[a]:** Conceptualization, Methodology, Formal Analysis, Investigation, Project Administration.

Informed consent statement

All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript

Declaration of Competing 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.

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Attachment

Table. 2 CT values and correspondent viral copies mL

Cicle	Viral load/mL (10^n)	Viral load/mL
15	2.5×10^8	1250000000
16	1.25×10^8	625000000
17	6.25×10^8	3125000000
18	3.15×10^7	156250000
19	1.57×10^7	78645000
20	7.86×10^6	39300000
21	3.93×10^6	19650000
22	1.95×10^6	9825000
23	9.82×10^5	4914250
24	4.91×10^5	2456250
25	2.45×10^5	1228000
26	1.22×10^5	614000
27	6.14×10^4	307300
28	3.97×10^4	198500
29	1.53×10^4	76750
30	7.65×10^3	38250
31	3.83×10^3	19150
32	1.92×10^3	9600
33	4.7×10^2	2350
34	2.3×10^2	1150
35	1.15×10^2	575
36	6.2×10^1	310
37	3.1×10^1	155
38	1.5×10^0	7,5
39	0.7×10^{-1}	0,35
40	0.35×10^{-1}	0,175

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7. Considerações finais

A urgente necessidade em desenvolver técnicas alternativas para diagnóstico de SARS-CoV-2, bem como biomateriais capazes de minimizar a disseminação da COVID-19 em ambientes aquáticos contaminados por SARS-CoV-2, que representa uma grave ameaça à saúde da população foi o grande norteador do desenvolvimento dessa pesquisa. Os resultados dessa pesquisa comprovam o RT-LAMP pode ser uma alternativa para diagnóstico de COVID-19 em profissionais da saúde que atuam principalmente na de frente no combate de doenças infeciosas e visto a propagação do vírus na água, nossos resultados ressaltam utilização de compostos naturais atuando como agentes de descontaminação demonstrou ser uma perspectiva futura para a recuperação desses locais contaminados.

A pesquisa realizada abriu caminho para o desenvolvimento de novas tecnologias ambientais destinadas à reabilitação de ambientes aquáticos contaminados, empregando compostos naturais. Embora tenham sido identificados avanços significativos, existe uma vasta gama de materiais ainda a serem explorados. A integração desses compostos naturais pode otimizar a eficácia e a viabilidade das técnicas de biorremediação, promovendo simultaneamente a conservação dos ecossistemas e a salvaguarda da saúde pública. Assim, é crucial prosseguir com esses estudos para impulsionar a ciência e implementar soluções inovadoras e sustentáveis na remediação de ambientes aquáticos contaminados.

8. Referências

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