

Marcelo Augusto Garcia Júnior

Desenvolvimento de novas moléculas bioativas baseadas em componentes salivares, bioinformática e inteligência artificial: Estratégias de diagnóstico salivar e atividade antiviral para Mpox, Norovirus e o SARS-CoV-2

Development of New Bioactive Molecules Based on Salivary Components, Bioinformatics, and Artificial Intelligence: Salivary Diagnostics and Antiviral Activity Strategies Against Mpox, Norovirus, and SARS-CoV-2

Tese apresentada ao Programa
de Pós-Graduação em
Odontologia Universidade
Federal de Uberlândia, para
Defesa de Doutorado em
Odontologia na Área de Clínica
Odontológica Integrada.

Uberlândia, 2024

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Uberlândia, 2024



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Iniciando os trabalhos o(a) presidente da mesa, Dr(a). Robinson Sabino da Silva, apresentou a Comissão Examinadora e o candidato(a), agradeceu a presença do público, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação do Discente e o tempo de arguição e resposta foram conforme as normas do Programa.

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DEDICATÓRIA

Dedico a minha tese à todas as
pessoas que tiveram seus sonhos
interrompidos pela pandemia de
COVID-19.

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Se você pode sonhar, pode você fazer

- Tom Fitzgerald

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RESUMO

Esta tese destaca avanços significativos para detecção salivar dos vírus Mpox, Norovirus e o SARS-CoV-2 e o potencial desenvolvimento de inovadoras moléculas bioativas antivirais baseadas em dockings *in silico* de peptídeos salivares e inteligência artificial. Os objetivos específicos incluem analisar a replicação do Mpox, sua potencial transmissão oral e as perspectivas da aplicação de tecnologias ômicas no diagnóstico salivar de Mpox; desenvolver um dispositivo eletroquímico portátil, modificado com peptídeos salivares naturais e acoplado a algoritmos de inteligência artificial, para detecção sensível e específica de norovírus; criar e testar um dispositivo eletroquímico portátil com algoritmos de aprendizado de máquina e peptídeos gerados por inteligência artificial para a detecção de SARS-CoV-2; e explorar peptídeos salivares naturais e artificiais como agentes antivirais, avaliando sua capacidade de inibir a entrada e replicação do vírus nas células hospedeiras. Inicialmente, discutimos o potencial de usar saliva como ferramenta diagnóstica para a mpox, enfatizando sua presença em lesões da mucosa oral e fluidos corporais, e sugerindo a aplicação de tecnologias Ômicas para detecção não invasiva. Em um estudo focado no norovírus, utilizamos bioinformática e IA para selecionar peptídeos salivares (NVp1, NVp2, NVp3) que melhoraram a detecção eletroquímica do vírus com alta acurácia, sensibilidade e especificidade. Outro estudo direcionado ao SARS-CoV-2, onde peptídeos modelados por IA foram usados em um biossensor eletroquímico, alcançando 100% de sensibilidade, 80% de especificidade e 90% de acurácia para detecção salivar de COVID-19, validado por algoritmos de aprendizado de máquina. Além disso, exploramos peptídeos salivares para bloquear a interação da proteína Spike do SARS-CoV-2 com receptores ACE2, demonstrando significativa atividade antiviral e redução da replicação viral em variantes. Por fim, peptídeos gerados por IA, BIAI1, BIAI2 e BIAI3, mostraram alta eficácia antiviral contra variantes do SARS-CoV-2, reduzindo significativamente os títulos virais e a morte celular, indicando seu potencial para aplicações profiláticas, diagnósticas e terapêuticas. Coletivamente, esses estudos destacam a promessa de integrar bioinformática, IA e diagnósticos salivares para detecção viral rápida, precisa e não invasiva, bem como estratégias de intervenção.

Palavras chave: Diagnóstico salivar; Inteligência artificial; SARS-CoV-2; Norovírus; Mpox.

ABSTRACT

This thesis highlights significant advancements in salivary detection of Mpox, Norovirus, and SARS-CoV-2, as well as the potential development of innovative bioactive antiviral molecules based on in silico docking of salivary peptides and artificial intelligence. The specific objectives include analyzing Mpox replication, its potential oral transmission, and the prospects of applying omics technologies in the salivary diagnosis of Mpox; developing a portable electrochemical device, modified with natural salivary peptides and coupled with artificial intelligence algorithms, for sensitive and specific detection of norovirus; creating and testing a portable electrochemical device with machine learning algorithms and artificial intelligence-generated peptides for the detection of SARS-CoV-2; and exploring natural and artificial salivary peptides as antiviral agents, evaluating their ability to inhibit viral entry and replication in host cells. Initially, we discuss the potential of using saliva as a diagnostic tool for mpox, emphasizing its presence in oral mucosal lesions and body fluids, and suggesting the application of omics technologies for non-invasive detection. In a study focused on norovirus, we used bioinformatics and AI to select salivary peptides (NVp1, NVp2, NVp3) that improved the electrochemical detection of the virus with high precision, sensitivity, and specificity. Another study targeted SARS-CoV-2, where AI-modeled peptides were used in an electrochemical biosensor, achieving 100% sensitivity, 80% specificity, and 90% accuracy for salivary detection of COVID-19, validated by machine learning algorithms. Additionally, we explored salivary peptides to block the interaction of the SARS-CoV-2 Spike protein with ACE2 receptors, demonstrating significant antiviral activity and reduced viral replication in variants. Finally, AI-generated peptides, BIAI1, BIAI2, and BIAI3, showed high antiviral efficacy against SARS-CoV-2 variants, significantly reducing viral titers and cell death, indicating their potential for prophylactic, diagnostic, and therapeutic applications. Collectively, these studies highlight the promise of integrating bioinformatics, AI, and salivary diagnostics for rapid, accurate, and non-invasive viral detection, as well as intervention strategies.

Keywords: Salivary diagnosis; Artificial intelligence; SARS-CoV-2; Norovirus; Mpox.

INTRODUÇÃO, REFERENCIAL TEÓRICO E PROPOSIÇÃO

INTRODUÇÃO

A formatação desta tese foi realizada de acordo com o modelo alternativo proposto pelo Programa de Pós-Graduação em Odontologia da Universidade Federal de Uberlândia, recomendando que os resultados obtidos durante a realização da pós-graduação sejam apresentados na forma de artigos científicos, sendo cada artigo um capítulo diferente da tese.

A tese foi organizada nas seguintes seções:

- Introdução e Fundamentação Teórica, onde é apresentada uma revisão da literatura sobre os temas abordados ao longo dos capítulos da tese;
- Proposição, onde são expostos os objetivos dos trabalhos abordados na tese;
- Capítulos de 1 a 5, que contemplam os cinco manuscritos elaborados aceitos, enviados e a serem submetidos;
- Conclusão, que corresponda à uma síntese dos principais resultados dos estudos apresentados nos capítulos anteriores;
- Referências bibliográficas, onde estão incluídas as referências utilizadas na confecção do referencial teórico.

O primeiro capítulo, intitulado "*Oral Infection, Oral Pathology and Salivary Diagnostics of Mpox Disease: Relevance in Dentistry and OMICs Perspectives*", tem como objetivo destacar a relação entre a infecção pelo vírus mpox e o papel da saliva como ferramenta diagnóstica, considerando a abordagem molecular atual e a aplicação de tecnologias ômicas. Esse capítulo está publicado (doi: 10.3390/ijms241814362) na revista "International Journal of Molecular Sciences" (Fator de impacto: 4,9).

O segundo capítulo, intitulado "*Bioinformatics Driven Approach for Selection of Salivary Peptides to Detect Norovirus in Saliva Using Electrochemical Biosensors supported by Neural Network machine learning algorithm*" teve como objetivo selecionar peptídeos naturais na saliva usando bioinformática e aplicá-los em um dispositivo eletroquímico de ponto de

atendimento para diagnóstico salivar de infecções por norovírus. Essa pesquisa venceu o prêmio “Fórum Científico” na 41ª Reunião Anual da Sociedade Brasileira de Pesquisa Odontológica (SBPqO). O artigo será submetido à revista "Journal of Dental Research (JDR)" (fator de impacto: 5.7).

O terceiro capítulo, intitulado "*Artificial Intelligence Bioinspired Peptide for Salivary Detection of SARS-CoV-2 in Electrochemical Biosensor*" busca criar um peptídeo bio-inspirado por meio de uma plataforma de inteligência artificial para o diagnóstico salivar da COVID-19, utilizando um dispositivo eletroquímico portátil. Essa pesquisa foi apresentada na 40ª Reunião da Sociedade Brasileira de Pesquisa Odontológica (SBPqO) e concorreu ao prêmio “Fórum Científico”. O artigo será submetido à revista "Talanta" (fator de impacto: 5.6).

O quarto capítulo, intitulado "*Synthetic Mimotopes of Salivary Peptides Against SARS-CoV-2 in vitro*" visa selecionar peptídeos salivares de alta afinidade para avaliar seu potencial em bloquear a interação Spike-RBD/ACE2 do SARS-CoV-2 e avaliar seu desempenho antiviral. Essa pesquisa foi premiada na 39ª Reunião da Sociedade Brasileira de Pesquisa Odontológica (SBPqO) com o IADR Unilever Hatton Divisional Award e concorreu ao IADR Hatton Award na 2023 IADR/LAR General Session & Exhibition with WCPD. O artigo está submetido à revista "Journal of Dental Research (JDR)" (fatos de impacto: 5.7).

O quinto manuscrito, intitulado "*Artificial Intelligence Generated Peptides Against SARS-CoV-2 in vitro*" utiliza a plataforma de inteligência artificial SAGAPEP para gerar peptídeos de alta afinidade e examinar sua capacidade de interromper a interação viral Spike-RBD/ACE2, medindo sua eficácia antiviral contra variantes do SARS-CoV-2. Pretende-se submetê-lo à revista "Science Advances" (fator de impacto: 11.7).

A relevância dos estudos apresentados nesta tese para as ciências da saúde, especialmente na Odontologia, reside na aplicação inovadora de ferramentas diagnósticas e terapêuticas no campo das doenças infecciosas. O uso de peptídeos salivares para diagnóstico e inibição viral, abordado em diferentes capítulos, demonstra o potencial da saliva como uma matriz biológica versátil, não apenas para detecção precoce de patógenos, mas também para o desenvolvimento de intervenções terapêuticas, como no caso do SARS-CoV-2.

Além disso, a utilização de tecnologias emergentes, como inteligência artificial e biossensores eletroquímicos, reforça a interseção entre inovação tecnológica e prática clínica, evidenciando a aplicabilidade odontológica ao permitir que dentistas atuem de maneira mais integrada no diagnóstico de doenças sistêmicas, através de abordagens não invasivas, contribuindo para a saúde geral dos pacientes.

REFERENCIAL TEÓRICO

A saliva é um fluido transparente que apresenta uma composição muito diluída, sendo formado por mais de 99,5% de água, e o restante de sua composição formado por componentes orgânicos e inorgânicos (Humphrey and Williamson, 2001). A saliva é composta de mais de 12.000 peptídeos, 3.000 proteínas, 780 lipídeos, 3.000 mRNA, ~50 microRNAs, 198 RNAs extracelulares, centenas de lipídeos, metabólitos, DNA, ureia, eletrólitos, glicose, e mais de 700 espécies de microrganismos, como vírus, fungos e bactérias (Caixeta et al., 2021; Dawes and Wong, 2019; Lee et al., 2022).

Nesse contexto, a potencial utilização da saliva no diagnóstico de infecções virais ganhou atenção devido à simplicidade de sua auto-coleta de forma não-invasiva e indolor e ao risco reduzido de infecção para os profissionais de saúde (Caixeta et al., 2023). Além disso, componentes salivares específicos também desempenham papel antiviral crucial (Farshidfar and Hamedani, 2021; Mohtasham et al., 2023).

Fisiologia da secreção salivar

A saliva é uma secreção exócrino muco-serosa, hipotônica em relação ao plasma, proveniente de glândulas salivares parótidas, submandibulares e sublinguais e também por glândulas salivares menores distribuídas na mucosa oral. As glândulas salivares maiores são formadas por células acinosas rodeadas por uma camada de células mioepiteliais conectadas a uma estrutura ductal formada por células epiteliais de ductos intercalados, estriados e excretores. Este sistema ductal pode modificar a saliva primária e realizar sua condução até a secreção na cavidade oral (Atyeo et al., 2024).

Dentro da cavidade oral, a saliva se mistura com o fluido gengival crevicular, componentes da película adquirida na estrutura dental, microrganismos orais, debris celulares, e restos de alimentos constituindo a saliva total (Humphrey and Williamson, 2001). Sabe-se que a saliva tem papel fundamental para manutenção da saúde oral e que suas múltiplas funções são desempenhadas pela sua característica fluida e pelos componentes salivares

orgânicos e inorgânicos específicos. A saliva atua de forma extensiva no paladar, mastigação, formação do bolo alimentar, digestão enzimática, deglutição e fala (Pedersen et al., 2018). Além disto, as funções da saliva envolvem a higienização da cavidade oral com a remoção de alimentos, atuação na solubilização de substâncias presentes na cavidade oral, na lubrificação da mucosa oral, na proteção dos dentes pela neutralização dos ácidos, na manutenção da saturação de fosfato de cálcio relacionada à hidroxiapatita e a participação na formação da película adquirida. Além disto, destaca-se o papel da saliva na ação antimicrobiana em bactérias, fungos e vírus (Humphrey and Williamson, 2001).

A secreção salivar é majoritariamente controlada pelo sistema nervoso autônomo que inerva as glândulas salivares. O mecanismo fisiológico de secreção salivar é um arco reflexo formado por uma via aferente e outra eferente. A via aferente é formada por receptores e seus respectivos nervos que conduzem os impulsos elétricos gerados por estímulos mecânicos da mastigação e químicos da gustação e olfação, até o centro da salivação na região da ponte e bulbo. A via eferente é formada pelo sistema nervoso autônomo, simpático e parassimpático, que inerva as glândulas salivares separadamente. O estímulo parassimpático desencadeado pela acetilcolina promove principalmente a formação de água de forma mais abundantemente, enquanto o estímulo simpático promove a secreção da saliva com maior viscosidade e maior concentração de proteínas, no entanto em menor volume. Este arco reflexo também recebe importante influência do sistema nervoso central (Humphrey and Williamson, 2001; Pedersen et al., 2018).

O fluxo salivar é influenciado por um grande número de fatores, incluindo o grau de hidratação, exposição à luz, estimulação prévia a estímulos em glândulas salivares, ao ritmo circadiano, tamanho das glândulas salivares, ao uso de drogas lícitas e ilícitas, e a presença de infecções, incluindo infecções virais. O fluxo salivar não estimulado é variável, com média de 0,3 a 0,4 mL/min. O efeito dos estímulos na secreção salivar é muito amplo com uma série destes produzindo fluxos de saliva inferiores a 2 mL/min e diferentes tipos de estímulos mastigatórios produzindo fluxos de saliva entre 3 e 5 mL/min. Destaca-se o efeito

da infusão de ácido cítrico à 5% diretamente na cavidade oral que pode provocar um fluxo salivar superior a 7 mL/min . Embora exista uma variação ampla na população, estima-se que o volume secretado de saliva diariamente é de cerca de 600 mL. (Dawes, 2008; Watanabe and Dawes, 1988). Isto representa uma enorme capacidade secretora, dado o tamanho relativamente pequeno da glândula salivar em comparação com outras glândulas secretoras (Di Pasquale et al., 2020).

Saliva como fluido diagnóstico e plataformas de diagnóstico

A saliva é um fluido de diagnóstico promissor, oferecendo inúmeras vantagens em relação às análises de sangue tradicionais (Dawes and Wong, 2019). Além disso, a saliva contém numerosas biomoléculas que podem refletir a saúde oral e sistêmica (Dawes and Wong, 2019). Dentre essas vantagens, destaca-se a possibilidade de auto-coleta pelo próprio paciente de forma conveniente, indolor, não invasiva e com redução significativa do risco de infecções nosocomiais comparando com outros biofluidos tradicionais como o sangue e o swab de nasofaringe. Desta forma, a saliva tem sido considerada um biofluido profícuo para aplicações em larga escala e triagem de doenças orais e sistêmicas (Caixeta et al., 2022, 2021). Outra vantagem da utilização da saliva é a presença de uma grande quantidade de biomarcadores que podem indicar várias doenças orais e sistêmicas, incluindo diferentes tipos de câncer, distúrbios autoimunes e doenças infecciosas (Ferreira et al., 2020; Garcia-Junior et al., 2023; Zhang et al., 2016). Nesse contexto, os recentes avanços nas tecnologias de detecção melhoraram significativamente a sensibilidade dos diagnósticos salivares, permitindo a detecção precoce de doenças e a monitorização dos resultados do tratamento (Dawes and Wong, 2019). Assim, à medida que tecnologias avançam, o diagnóstico salivar se mostra preparado para revolucionar a prática clínica, oferecendo um meio não invasivo e eficiente de detecção e monitorização de doenças (Nonaka and Wong, 2023).

Os principais desenvolvimentos recentes no diagnóstico salivar incluem a publicação do proteoma salivar humano e a integração de nanotecnologias para dispositivos portáteis para detecção no ponto de atendimento (*point of*

care) (Miller et al., 2010). O desenvolvimento de métodos econômicos de estabilização de analitos e de técnicas de pré-amplificação de ácidos nucleicos também colaborou para o aumento na acurácia dos diagnósticos salivares (Bonne and Wong, 2012). Além disso, a saliva contém numerosas biomoléculas que podem refletir a saúde oral e sistêmica (Dawes and Wong, 2019). Avanços tecnológicos recentes, como a sequenciação de RNA e a biópsia líquida, expandiram as aplicações potenciais do diagnóstico salivar, abrindo novos horizontes para a medicina personalizada e a detecção precoce de doenças (Zhang et al., 2016).

A integração das genômicas, transcriptômicas, proteômicas, metabolômicas e do microbioma aumentou significativamente a sensibilidade e a especificidade na detecção de biomarcadores salivares (Cuevas-Córdoba and Santiago-García, 2014). Essas técnicas permitem a identificação de numerosos componentes salivares, incluindo DNA, RNA, proteínas, metabólitos e microrganismos, que podem servir como potenciais biomarcadores para várias doenças orais e sistêmicas (Spielmann and Wong, 2011). Especialmente no diagnóstico de câncer oral e em infecções virais, as abordagens ômicas da saliva têm se mostrado promissoras na detecção precoce e no prognóstico do desfecho clínico dos pacientes (Aro et al., 2019; Garcia-Junior et al., 2023). Com o avanço da descoberta de moléculas derivados de DNA, peptídeos e proteínas com expressão diferenciada na saliva de determinadas doenças, novos biomarcadores salivares apresentam potencial capacidade de detecção em sensores eletroquímicos, o que pode favorecer sua utilização para diagnóstico não-invasivo de doenças orais e sistêmicas, incluindo vírus emergentes.

Sensores eletroquímicos

Os sensores eletroquímicos têm demonstrado um potencial significativo na evolução do diagnóstico salivar de doenças sistêmicas através de métodos não invasivos, rápidos e econômicos (Li et al., 2024; Mani et al., 2021). Eles consistem em dispositivos analíticos que convertem reações eletroquímicas em sinais quantificáveis, utilizando tipicamente três eletrodos: um eletrodo de referência, um contra-eletrodo e um eletrodo de trabalho (Pereira et al., 2024).

Essa quantificação ocorre através da conversão de alterações biológicas em sinais eletrônicos, caracterizando-se como uma poderosa ferramenta para aplicações clínicas devido ao seu baixo custo, sensibilidade e versatilidade (Baranwal et al., 2022; Kimmel et al., 2012; Ronkainen et al., 2010). O desenvolvimento destes sensores envolve uma organização encadeada dos materiais eletrônicos e das configurações dos dispositivos para otimizar a geração de sinais (Cui, 2017).

Recentes avanços nos materiais também melhoraram a seletividade, a estabilidade e a sensibilidade dos sensores eletroquímicos (Chen and Chatterjee, 2013; Dong et al., 2023). Nesse contexto, com a aplicação técnicas eletroquímicas, é possível detectar diversos biomarcadores de doenças como diabetes mellitus, diferentes tipos de câncer e Doença de Alzheimer, além de infecções virais como SARS-CoV-2 (Liu et al., 2023; Mani et al., 2021). Além disto, a integração com plataformas portáteis e smartphones também pode facilitar a democratização dos testes nos pontos de atendimento de difícil acesso e viabilizando avanços das aplicações em telemedicina (Khan et al., 2017; Liu et al., 2023). Dessa forma, os sensores eletroquímicos oferecem uma detecção ultrasensível de marcadores químicos e biomarcadores proteicos, permitindo o diagnóstico precoce de doenças e cuidados de saúde personalizados (Barry et al., 2009; Chen and Chatterjee, 2013; Kim et al., 2021).

Entretanto, os sensores eletroquímicos para diagnóstico salivar apresentam alguns desafios técnicos, uma vez que a preparação da amostra e dos elementos de biorreconhecimento são cruciais para melhorar o desempenho do sensor (Li et al., 2024). Apesar destes desafios, os sensores eletroquímicos oferecem potencial para diagnósticos não invasivos, rápidos e econômicos (Mani et al., 2021). Ademais, a integração com microfluídica e o desenvolvimento dispositivos *lab-on-a-chip* são ferramentas promissoras para melhorar a eficiência dos sensores e permitir a monitorização contínua (Li et al., 2024; Swetha et al., 2022).

Inteligência artificial dirigida para diagnóstico de doenças

A inteligência artificial (IA) constantemente mais integrada no avanço

tecnológico possibilitando a realização de procedimentos computacionais coletar e interpretar grandes conjuntos de metadados com intuito de obter conhecimentos que provavelmente não fossem possíveis utilizando apenas métodos científicos tradicionais (Deng, 2018; Kallem, 2012; Wang et al., 2023). Os algoritmos de IA funcionam por meio da identificação de correlações estatísticas em grandes conjuntos de dados, permitindo-lhes aprender e resolver problemas de forma autônoma (Deng, 2018). A IA é cada vez mais aplicada em várias áreas, onde se apresenta de forma muito promissora para melhorar os cuidados com a saúde (van Assen et al., 2020; Sher et al., 2020).

Nesse contexto, as redes neurais (*Neural Networks*), as máquinas de vetores de suporte (*Support Vector Machines - SVM*) e o AdaBoost são alguns dos algoritmos de aprendizagem automática proeminentes, apresentando abordagens distintas. As redes neurais, inspiradas no cérebro humano, processam a informação através de nós interligados (Sawakar et al., 2006). As máquinas de vetores de suporte têm por objetivo encontrar um hiperplano que separe ao máximo os pontos de dados no espaço de características, utilizando funções de kernel para casos não lineares (Gou et al., 2023). O AdaBoost treina iterativamente classificadores fracos, ajustando os pesos das amostras para se concentrar em instâncias mal classificadas (Gou et al., 2023). Estudos comparativos demonstraram que as SVM têm frequentemente um desempenho superior ao das redes neurais (Tsiligaridis, 2018). No entanto, pesquisadores desenvolveram métodos para combinar essas abordagens, como a maximização da margem em redes neurais inspiradas em SVMs e AdaBoost (Romero et al., 2004). Além disso, algoritmos de árvores de decisão têm sido usados para melhorar a classificação de SVMs (Tsiligaridis, 2018). As técnicas de *boosting* também têm sido aplicadas a redes neurais, demonstrando um melhor desempenho em várias tarefas (Schwenk and Bengio, 2000).

Associados ao diagnóstico salivar, a IA e a aprendizagem automática (AM) emergem como ferramentas poderosas, melhorando a descoberta, validação e análise de biomarcadores, e aumentando a sensibilidade, especificidade e acurácia dos testes de diagnóstico (Adeoye and Su, 2024; Patil et al., 2022). Classificadores de aprendizagem automática que utilizam dados

metatranscriptômicos salivares também têm demonstrado uma elevada acurácia no diagnóstico do câncer oral, com potencial para sua detecção precoce (Banavar et al., 2021). Além disso, abordagens baseadas em IA também têm se mostrado promissoras no combate infecções virais (Rosa et al., 2021). Assim, nosso grupo de pesquisa tem indicado que a integração da IA no diagnóstico salivar pode conduzir a ferramentas de rastreamento mais precisas e não invasivas para várias doenças sistêmicas, incluindo diabetes mellitus, Chikungunya vírus, Zika vírus e diferentes tipos de câncer (Caixeta et al., 2023a, 2020; Guevara-Vega et al., 2024; Oliveira et al., 2023; Vitorino et al., 2023).

Patogênese viral em glândulas salivares

A cavidade oral é uma via de transmissão viral epidemiologicamente relevante, uma vez que diversos vírus são transmitidos na saliva (Atyeo et al., 2024; Garcia-Junior et al., 2023; Ghosh et al., 2022). Alguns vírus têm tropismos bem estabelecidos para as glândulas salivares, sendo que estudos recentes destacam o papel das glândulas salivares como possíveis reservatórios de vírus com capacidade de replicação neste território (Ghosh et al., 2022). A compreensão da variedade de vírus que estão relacionados com as glândulas salivares, bem como das respostas às estas infecções, pode fornecer informações importantes sobre a relevância da interação vírus-saliva (Atyeo et al., 2024). Assim, apesar da sólida presença de vírus na saliva já ter sido relatada na literatura (Atyeo et al., 2024; Bellocchio et al., 2023; Garcia-Junior et al., 2023; Ghosh et al., 2022), mais estudos são necessários para uma compreensão abrangente das vias moleculares pelas quais os vírus entram e replicam nas glândulas salivares.

COVID-19

Considerando a que infecções virais apresentam importância global, sabe-se que o SARS-CoV-2, vírus responsável pela COVID-19, já causou mais de 37,5 milhões de casos no Brasil e mais de 775 milhões no mundo, resultando em quase 7 milhões de mortes (World Health Organization, 2023a). A doença varia de síndromes respiratórias assintomáticas e graves e pode levar a

consequências a longo prazo, como fadiga, dificuldades de sono, distúrbios psicológicos, comprometimento das capacidades pulmonares e morte (Andrade et al., 2021). O SARS-CoV-2 faz parte da família *Coronaviridae*, e possui proteínas estruturais como a proteína Spike (S), a proteína matriz (M) e a proteína do envelope (E) (Zawilska et al., 2021). O vírus entra principalmente nas células hospedeiras através do domínio de ligação ao receptor da proteína Spike que interage com o receptor ACE2, que é altamente expresso na mucosa oral, tornando-a um local favorável para a infecção (Grosche et al., 2023; Surboyo et al., 2021).

Estudos têm demonstrado uma elevada concordância (>90%) entre a o diagnóstico salivar de COVID-19 e as amostras da nasofaringe na detecção do RNA do SARS-CoV-2 (Caixeta et al., 2023). A saliva possui potencial para identificar portadores assintomáticos e pacientes acometidos pela doença em diferentes estágios (Kurzanov et al., 2020; Sapkota et al., 2021). A presença de receptores ACE2 nas glândulas salivares torna-as um reservatório potencial para o SARS-CoV-2 (Shaukari et al., 2021). Apesar das suas vantagens, o diagnóstico salivar ainda enfrenta desafios na padronização e validação (Caixeta et al., 2021). A identificação de novos biomarcadores salivares especialmente baseado em metabolômica podem ser aplicados para o desenvolvimento de testes salivares rápidos para uma melhor identificação e prognóstico da COVID-19 (Sapkota et al., 2021; Sri Santosh et al., 2020).

Infecção por mpox

O mpox, é outro vírus de reconhecido impacto global nos serviços de saúde. O mpox foi originalmente designado como “monkeypox”, foi descoberto em macacos cynomolgus em 1958 e mais tarde renomeada pela OMS em 2022 (Chauhan et al., 2023). O primeiro caso humano surgiu na República Democrática do Congo em 1970, com surtos inicialmente confinados no continente africano (Ladnyj et al., 1972). Entre 1970 e 2000, foram confirmados menos de 1.000 casos, principalmente em regiões endêmicas, mas o número aumentou para mais de 18.000 entre 2000 e 2019, com alguns casos detectados fora da África (Chauhan et al., 2023). Nos anos de 2022 e 2023, o

mpox propagou-se em escala mundial, com mais de 88.000 casos e 149 mortes em mais de 110 países, o que levou a OMS a declarar uma Emergência de Saúde Pública de Preocupação Internacional em julho de 2022 (Garcia-Junior et al., 2023). Os surtos recentes são em grande parte atribuídos à transmissão entre humanos, com casos significativos notificados nos EUA, no Brasil e em Espanha (World Health Organization, 2023b). A estratégia da OMS para o controle do mpox inclui a vigilância e o envolvimento da comunidade, embora a erradicação continue a ser um desafio global (World Health Organization, 2023b). Atualmente, dois clados virais são responsáveis pelas infecções, sendo que propagação do mpox está potencialmente ligada à diminuição da vacinação contra a varíola e à atividade enzimática do hospedeiro que afeta o genoma viral (Isidro et al., 2022).

Os testes de diagnóstico atuais para o mpox baseiam-se principalmente em métodos moleculares, sendo a PCR em tempo real a técnica mais utilizada para detectar o vírus em amostras humanas (Chauhan et al., 2023). Embora o swab de lesões cutâneas continue sendo a amostra recomendada para o diagnóstico, estudos recentes exploraram métodos de amostragem alternativos. Os testes PCR em tempo real baseados na saliva têm mostrado resultados promissores, com elevada sensibilidade e concordância com os testes de esfregaço de lesões (Allan-Blitz et al., 2023; Coppens et al., 2023). As amostras de saliva demonstraram cargas virais mais elevadas em comparação aos swabs de orofaringe e ao plasma sanguíneo (Coppens et al., 2023). Outras abordagens de diagnóstico incluem ensaios serológicos, isolamento de vírus e microscopia eletrônica (Chauhan et al., 2023). A aplicação de diagnósticos salivares na detecção do mpox oferece potenciais vantagens, como a amostragem não invasiva e a possibilidade de detecção precoce antes do início da erupção cutânea (Garcia-Junior et al., 2023).

Os surtos simultâneos de mpox em vários países em 2022/2023 despertaram preocupações quanto às possíveis rotas de infecção, especialmente entre os profissionais de saúde da linha de frente que estão em contato direto com os pacientes. Estes profissionais, incluindo cirurgiões-dentistas, podem contribuir para reduzir a disseminação do mpox ao

compreenderem novos mecanismos de transmissão por meio da saliva, gotículas respiratórias, procedimentos que produzem aerossóis e contato face a face indicados no recente surto de mpox de 2022/2023. Embora estudos adicionais sejam necessários para entender a entrada do mpox nas células hospedeiras e a replicação viral na cavidade oral e glândulas salivares, sabe-se que a expressão de laminina, condroitina, sulfatos de heparina, e glicosaminoglicanos podem indicar a interação com as proteínas de superfície do mpox, permitindo a adesão viral e posterior replicação em células orais que expressam essas proteínas (Garcia-Junior et al., 2023).

Infecção por norovírus

Além do SARS-CoV-2 e do mpox, o norovirus (NoV) também tem ganho notoriedade ao redor do mundo, uma vez que ele é uma das principais causas de gastroenterite aguda (GA) em escala mundial, afetando todas as faixas etárias, mas particularmente bebês, idosos e indivíduos imunocomprometidos (Zhang et al., 2024). Além disso, ele é responsável por custos significativos para a saúde e a sociedade, incluindo 4,2 mil milhões de dólares em custos diretos de saúde e 60,3 milhões de dólares em custos sociais anualmente (Bartsch et al., 2016; Zhang et al., 2022). Em 2019, o NoV causou mais de 130 000 mortes, principalmente entre crianças com menos de cinco anos e adultos com mais de 70 anos (Zhang et al., 2022). O vírus propaga-se rapidamente em ambientes fechados, com maior incidência nas estações mais frias e através de alimentos e água contaminados (Yu et al., 2022). Os principais surtos, especialmente em creches e escolas, devem-se principalmente à transmissão de pessoa para pessoa, tendo recentemente sido descoberta uma nova rota de infecção oral-oral (Ghosh et al., 2022; Yu et al., 2022). O NoV é um vírus RNA não envelopado, altamente contagioso, da família *Caliciviridae*, classificado em dez genogrupos com base na proteína do capsídeo VP1 e nas regiões da RNA polimerase dependente de RNA, sendo os genogrupos GI, GII, GIV, GVIII e GIX os que afetam os seres humanos (Chhabra et al., 2019).

As abordagens de diagnóstico atuais para norovirus incluem microscopia eletrônica, RT-PCR, RT-PCR em tempo real, ensaios enzimáticos e tecnologias

moleculares (Pang and Lee, 2015; Rabenau et al., 2003). Embora as amostras de fezes continuem sendo o principal material de diagnóstico, os ensaios de anticorpos salivares representam uma alternativa não invasiva promissora (Moe et al., 2004; Pisanic et al., 2019). Estes testes salivares demonstram uma elevada sensibilidade e especificidade na detecção de infecções recentes por norovírus e podem identificar corretamente os genótipos infectantes (Griffin et al., 2015). Apesar dos recentes avanços no diagnóstico salivar de infecções por norovírus, métodos portáteis, de baixo custo e baixa complexidade ainda são escassos e representam um campo a ser explorado (Zaczek-Moczydlowska et al., 2021).

Atividade antiviral da saliva

No cenário das infecções virais, é importante ressaltar que a saliva possui vários peptídeos antimicrobianos (PAM) de ocorrência natural que desempenham funções importantes na defesa cavidade oral, como histatinas, defensinas, catelicidinas e aglutinina salivar (Mohtasham et al., 2023; Mori et al., 2006). Os PAMs da saliva têm atividades antibacterianas, antivirais e antifúngicas e servem como linha inicial de defesa contra infecções microbianas (Gallo et al., 2002; Niu et al., 2021). Alguns PAMs salivares, como as histatinas, podem também ligar metais, o que aumenta a sua ação antibacteriana (Melino et al., 2014). Além disso, peptídeos salivares também têm se mostrado promissores na sua ação antiviral contra vírus como o SARS-CoV-2 (Mohtasham et al., 2023). Assim, a utilização do peptidoma salivar apresenta uma rica alternativa para seleção de moléculas com potencial para atendimentos de clínica odontológica personalizada e para medicina de precisão (Vitorino et al., 2014).

De modo a potencializar a utilização de peptídeos salivares, o design de peptídeos com maior energia de interação molecular tem o desafio de avaliar uma quantidade gigantesca de possibilidades, neste sentido a utilização de frameworks de algoritmo genético assistido por Surrogate tem se mostrado eficaz para selecionar e descobrir moléculas com potencial terapêutico e para utilização em diagnóstico (Basith et al., 2020). Esses algoritmos podem atuar

classificando os peptídeos com base na sua capacidade de ligação com moléculas alvo (Basith et al., 2020). Com a utilização dessa técnica, peptídeos têm sido desenvolvidos de forma a mimetizar a interação de proteínas receptoras com proteínas virais do SARS-CoV-2 (Dhingra et al., 2023). Ademais, aprendizado de máquinas também tem sido utilizado para prever novas sequências de peptídeos que tem maior interação e que podem atuar na infecciosidade viral. (Kaygisiz et al., 2023). Diversas técnicas computacionais foram criadas para identificar e otimizar possíveis interações de peptídeos com proteínas do envelope viral, com especial ênfase na prevenção do processo de fusão (Xu et al., 2012).

Neste contexto, esta tese de doutorado tem o objetivo de analisar a replicação do Mpox, sua potencial transmissão oral e a aplicação de tecnologias ômicas no diagnóstico salivar de Mpox, bem com, o objetivo de investigar a aplicação de peptídeos salivares de ocorrência natural e peptídeos gerados por bioinformática e inteligência artificial como uma ferramenta alternativa para detecção eletroquímica de norovírus e COVID-19, bem como a potencial ação antiviral desses peptídeos salivares.

PROPOSIÇÃO

Esta tese visa explorar e desenvolver métodos avançados de diagnóstico e terapias baseados em saliva para infecções virais de importância global, como SARS-CoV-2, mpox e norovírus, utilizando técnicas ômicas, dispositivos eletroquímicos portáteis, e inteligência artificial. Especificamente, a tese abordará os seguintes objetivos:

- Investigar a replicação do Mpox, sua potencial transmissão oral e as perspectivas da aplicação de tecnologias ômicas no diagnóstico salivar de Mpox.
- Desenvolver e avaliar um dispositivo eletroquímico portátil, modificado com peptídeos salivares naturais, acoplado a algoritmos de inteligência artificial, para a detecção sensível e específica de norovírus.
- Criar e testar um dispositivo eletroquímico portátil vinculado a algoritmos de aprendizado de máquina utilizando peptídeos gerados por inteligência artificial para a detecção de SARS-CoV-2.
- Explorar o potencial de peptídeos salivares naturais como agentes antivirais contra o SARS-CoV-2, avaliando sua capacidade de inibir a entrada e replicação viral nas células hospedeiras.
- Desenvolver peptídeos antivirais gerados por inteligência artificial e testar sua eficácia contra o SARS-CoV-2, analisando sua habilidade de bloquear a entrada e replicação viral nas células hospedeiras.

CAPÍTULO 1

*Oral Infection, Oral Pathology and Salivary
Diagnostics of Mpox Disease: Relevance in
Dentistry and OMICs Perspective*



Review

Oral Infection, Oral Pathology and Salivary Diagnostics of Mpox Disease: Relevance in Dentistry and OMICs Perspectives

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Abstract: In this narrative review, we aim to point out the close relationship between mpox virus (MPXV) infection and the role of saliva as a diagnostic tool for mpox, considering the current molecular approach and in the perspective of OMICs application. The MPXV uses the host cell's rough endoplasmic reticulum, ribosomes, and cytoplasmic proteins to replicate its genome and synthesize virions for cellular exit. The presence of oral mucosa lesions associated with mpox infection is one of the first signs of infection; however, current diagnostic tools find it difficult to detect the virus before the rashes begin. MPXV transmission occurs through direct contact with an infected lesion and infected body fluids, including saliva, presenting a potential use of this fluid for diagnostic purposes. Currently available diagnostic tests for MPXV detection are performed either by real-time quantitative PCR (RT-qPCR) or ELISA, which presents several limitations since they are invasive tests. Despite current clinical trials with restricted sample size, MPXV DNA was detected in saliva with a sensitivity of 85%–100%. In this context, the application of transcriptomics, metabolomics, lipidomics, or proteomics analyses coupled with saliva can identify novel disease biomarkers. Thus, it is important to note that the identification and quantification of salivary DNA, RNA, lipid, protein, and metabolite can provide novel non-invasive biomarkers through the use of OMICs platforms aiding in the early detection and diagnosis of MPXV infection. Untargeted mass spectrometry (MS)-based proteomics reveals that some proteins also expressed in saliva were detected with greater expression differences in blood plasma when comparing mpox patients and healthy subjects, suggesting a promising alternative to be applied in screening or diagnostic platforms for mpox salivary diagnostics coupled to OMICs.

Keywords: monkeypox; saliva; oral pathology; multiomics; proteomics; dentistry; virology



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1. Introduction

The mpox disease was first detected in cynomolgus monkeys in 1958 and exhibited skin lesions as a typical clinical feature. Due to the pioneering discovery in monkeys, the term “monkeypox virus” was coined in 1958, and it was later renamed as “mpox” by the World Health Organization (WHO) in November 2022 and included in the ICD-10 [1,2]. The first reported mpox disease case consisted of an infant from the Democratic Republic of the Congo in 1970, and since then, there have been several other mpox virus (MPXV) outbreaks, frequently limited to Africa, but only recently became a global threat [3,4].

From a historical perspective, from 1970 to 2000, less than 1000 cases were confirmed, mostly in endemic regions caused by mpox viral clade I, without detection in other continents. Subsequently, the number of confirmed mpox cases increased to over 18,000 between 2000 and 2019 with predominant detection in Africa and confirmed cases in North America, Europe, and Asia [2,5,6]. The first human detection of mpox outside the African endemic regions was described in the United States in 2003 [7]. In this mpox outbreak, there was an outbreak recording 47 cases in the United States that were traced back to prairie dogs kept in contact with exotic rodents imported from Ghana [6]. Until November 2021, reports of human-to-human transmission of mpox were still incipient, except for a cluster of cases in the United Kingdom, when a family member was infected during a trip to Nigeria and transmitted the virus to other two relatives [8].

In the 2022/2023 simultaneous multi-country outbreaks of mpox over 88,000 cases and 149 deaths have been reported in more than 110 countries worldwide until July 2023 [9–11]. In this context, the United States has been the country with the highest number of cases reporting more than 30,000 cases, followed by Brazil and Spain with about 26,000 and 7000 cases, respectively [11]. On 23 July 2022, the worldwide mpox epidemic was designated a Public Health Emergency of International Concern (PHEIC) by the WHO. However, on 11 May 2023, the Emergency Committee on mpox reported that the outbreak is no longer a PHEIC because of a consistent drop in cases. A strategy plan and technical guide materials for mpox were prepared by the WHO, pointing to surveillance, diagnostics, risk communication, and community involvement as crucial for preventing recurrence. Although longer-term control and eradication are difficult, continued efforts lower the likelihood of a worldwide mpox comeback [11]. According to WHO, there are two different circulating clades of the virus causing current infections: clade I, associated with lower transmission; and clade II, related to higher spreading and most of the worldwide spread of the virus [9,10,12]. Altogether with the decrease in smallpox vaccination, the current MPXV outbreak is potentially related to the action of host apolipoprotein B mRNA-editing catalytic polypeptide-like 3 (APOBEC3) enzymes in the viral genome [13]. The unprecedented 2022/2023 simultaneous multi-country outbreaks of mpox registered a high level of human-to-human transmission, which was related to a high index of case fatality rates and organ damage issues [2]. Now, due to the 2022/2023 mpox outbreak, most of the cases do not present a relation to traveling to endemic regions, and human-to-human transmission is mostly present in lesions on the tongue, oral, and perioral regions, despite sexual transmission and HIV co-infection risk factors are still in need of elucidation [14,15].

Within this context, this narrative review aims to approach MPXV structure and replication alongside insights into oral transmission and symptoms, placing saliva at the core of diagnostic perspectives. Additionally, we describe the current methods of mpox diagnostic using molecular tools and present the perspective of Omics application in mpox salivary diagnostics and screening.

2. Study Design and Search Strategy

We conducted a narrative review of the literature following the PRISMA (Preferred Reporting Items for Systematic Review and Meta-analyses) guidelines when applicable. The aim of our review was to assess research papers—including various types of studies—that would enlighten the relationship between MPXV and saliva in order to better understand the potential use of saliva coupled with OMICs as a means of mpox diagnosis. We also included articles related to MPXV structure and replication, oral transmission, and systemic and oral symptoms.

From October 2022 to August 2023, the search for review was performed with the following keywords: “monkeypox” and/or “mpox”, “saliva”, “multiomics”, “proteomics”, “diagnostics” and “dentistry” into PubMed, MEDLINE, and Google Scholar databases. Further to original research articles of different types of studies, review articles, and national (UK and US) or international (WHO) guidelines were consulted, as well as gray

literature. MeSH terms were chosen as the general controlled vocabulary and keywords, while language, year, and publication type restrictions were not applied to the searches.

3. Viral Structure, MPXV Entry into Host Cells, and MPXV Replicative Cycle

MPXV belongs to the Orthopoxvirus genus and Poxviridae family, characterized by having an oval-shaped structure and double-stranded DNA with 200 genes packed within 200,000 base pairs [6]. Specifically, this large genome is composed of a linear double-stranded DNA coupled with a hairpin on both 5' and 3' ends by a covalent interaction with inverted tandem repeats (ITS). It was suggested that MPXV is the largest virus capable of infecting human cells with a significant tissue tropism. As expected, several mammalian cell lines permit a viral entry [16]. Considering the two existent clades of MPXV, although infection and mortality rates are different, their genomic sequence is only 0.5% different in regions that have important virulent genes [17–19]. The WHO indicates that the global 2022/2023 mpox outbreak was caused by MPXV clade IIb. MPXV also contains two distinct viral particles: the mature virion (MV) and the enveloped virion (EV), and either of them uses different mechanisms to enter the host cells [20]. While the MV contains more than 20 viral proteins on the surface of its single lipid membrane, EV contains the MV structures plus a secondary outer covering membrane presenting 6 viral proteins. The intracellular mechanisms responsible for producing EV and MV present distinct viral protein compositions. In this context, although the majority of the MPXV virions are MVs, an effective immune defense response against mpox needs specific antibodies targeting both MV and EV [21,22].

About 50% of the genes in the genome of MPXV genome are responsible for its replication, and the remaining genes are related to interactions between the virus and the host cell [6,23]. The interaction between the surface components in the viral structure and the host cell, allowing the viral membrane to fuse with plasma or endosomal membrane in host cells, is required for viral replication. The surface proteins A26, A27, D8, and H3 can interact with glycosaminoglycans in host cells promoting cellular attachment [24,25]. Therefore, these surface proteins have already been considered targets for effective orthopoxvirus vaccines, including against MPXV [23].

In order to understand the viral entry mechanisms for MPXV, it is essential to understand the interactions between glycosaminoglycans and viral surface proteins. In this context, A26 interacts with laminin on its alpha 1 and gamma 2 subunits—expressed in humans by *LAMA1* and *LAMC2* genes; A27 and H3 proteins interact with heparan sulfate, specifically on sulfatases 1 and 2—coded by *SULF1* and *SULF2* genes; D8 interacts with chondroitin sulfate on its proteoglycan 4 receptor—coded by *CSPG4* gene [23,25–27]. Considering MPXV viral replication mechanisms, Figure 1 illustrates MPXV structure with surface proteins A26, A27, D8, and H3 and its respective interaction with genes/proteins of glycosaminoglycans that were described in oral mucosa, salivary gland, and tongue. Since the MPXV mechanism of entry into cells is not completely elucidated, it is difficult to measure the importance of these glycosaminoglycans on specific regions of the human body, however, these genes and/or receptors are located in the oral mucosa, salivary glands, and tongue, which suggests that the oral cavity and related tissues could play an important role on MPXV infection (Tables 1 and 2) [28,29].

After MPXV entry into the host cell, the viral particle can use the rough endoplasmic reticulum to adjust cytoplasmatic machinery, where its replication takes place. Nevertheless, MPXV still needs host cell ribosomes to perform mRNA translation and virion synthesis, allowing genome encapsulation for a posterior cellular exit. It also needs cytoplasmatic proteins of host cells to take apart their endoplasmic reticulum membrane to create their replication system [30].

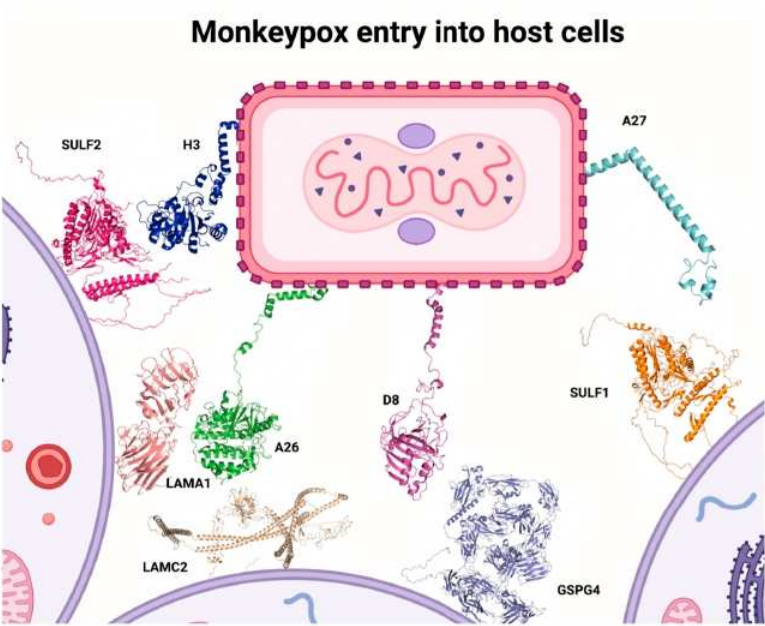


Figure 1. MPXV structure with surface proteins A26, A27, D8, and H3 and its respective interaction with glycosaminoglycans (created by the authors).

Table 1. Expression of genes for proteins receptors in human anatomical entities for Poxvirus based on Bgee platform.

Receptor	Gene Name	Uberon	Anatomical Entity	Expression Score
Laminin (Laminin subunit alpha 1)	LAMA1	UBERON:0001830	Minor salivary gland	68.59
Laminin (Laminin subunit gamma 2)	LAMC2	UBERON:0001830 UBERON:0007371	Minor salivary gland Superior surface of tongue	79.86 59.56
Chondroitin Sulfate (Chondroitin sulfate proteoglycan 4)	CSPG4	UBERON:0001830 UBERON:0007371	Minor salivary gland Superior surface of tongue	70.60 70.23
Heparan sulfate (Sulfatase 1)	SULF1	UBERON:0001830 UBERON:0007371 UBERON:0011876	Minor salivary gland Superior surface of tongue Body of tongue	77.09 67.66 66.51
Heparan sulfate (Sulfatase 2)	SULF2	UBERON:0001830 UBERON:0001723 UBERON:0011876 UBERON:0007371	Minor salivary gland Tongue Body of tongue Superior surface of tongue	93.40 88.59 88.55 87.51

Table 2. Expression of proteins receptors for Poxvirus based on the Human Protein Atlas.

Receptor	Gene Name	Anatomical Entity	RNA Expression				Protein
			Consensus nTPM	HPA pTPM	GTEX pTPM	FANTOM5 Scaled Tags Per Million	
Laminin (Laminin subunit alpha 1)	LAMA1	Oral mucosa Salivary gland Tongue	No data 1.3 0.0	No data 1.3 0.0	No data 1.0 No data	No data 4.1 0.6	Not detected Low No data
Laminin (Laminin subunit gamma 2)	LAMC2	Oral mucosa Salivary gland Tongue	No data 12.9 2.3	No data 3.5 2.3	No data 12.9 No data	No data 1.3 6.3	Not detected Low No data
Chondroitin Sulfate (Chondroitin sulfate proteoglycan 4)	CSPG4	Oral mucosa Salivary gland Tongue	No data 4.7 10.0	No data 1.9 10.0	No data 4.7 No data	No data 13.0 34.8	Low Medium No data

Table 2. Cont.

Receptor	Gene Name	Anatomical Entity	RNA Expression				Protein
			Consensus nTPM	HPA pTPM	GTEEx pTPM	FANTOM5 Scaled Tags Per Million	
Heparan sulfate (Sulfatase 1)	SULF1	Oral mucosa	No data	No data	No data	No data	Not detected
		Salivary gland	4.3	2.4	4.3	8.8	Not detected
		Tongue	11.2	11.2	No data	34.1	No data
Heparan sulfate (Sulfatase 2)	SULF2	Oral mucosa	No data	No data	No data	No data	Not detected
		Salivary gland	34.0	9.9	34.0	18.3	High
		Tongue	22.5	22.5	No data	22.4	No detected

4. Transmission

Poxviruses present two different types of hosts in their cycles: reservoir hosts and zoonotic hosts—the first ones carry the virus and can infect others, whereas the last ones develop the disease. When it comes to MPXV, its reservoir hosts are rodents and squirrels, most specifically species found in the African continent; on the other hand, the zoonotic hosts are monkeys and humans, where the virus can replicate in most mammalian cultured cells [31]. MPXV can also be spread by human-to-human infection. According to the Centers for Disease Control and Prevention (CDC), the human-to-human transmission of MPXV occurs through direct contact with infected skin lesions or prolonged contact of large respiratory droplets with the mouth or nose, although a potential sexual transmission route in the recent 2022/2023 MPXV outbreak indicates amplification of its transmission pattern. Intimate contact is also capable of transmitting MPXV, since sexual relations, hugging, and prolonged face-to-face interaction may promote viral infection as well [14,32].

Due to the recent COVID-19 pandemic, it is important to point out differences in transmissions of the diseases caused by SARS-CoV-2 and MPXV. Firstly, despite the 2022/2023 mpox outbreak, there is already valuable information available about MPXV due to previous research on other viruses from the same family. Secondly, its transmission does not happen in fast contact via respiratory aerosols—as it does with coronavirus: in MPXV it happens via larger droplets of body fluids, which are heavier and thus more difficult to spread through the air and by breathing. Thirdly, the odds of developing the asymptomatic disease are larger in COVID-19 than in mpox disease, which is justified due to mpox presenting visible signs of infection—even in mild infections cases—when compared to the SARS-CoV-2 infection, which does not exclude the possibility of presymptomatic transmission [19,33]. Finally, the presence of fewer strains than RNA viruses is expected, since MPXV is a double-stranded DNA virus [13,27]. However, there is still much that is yet to be discovered on MPXV, such as the possibility of asymptomatic transmission, the frequency of transmission via respiratory secretions, and if it can be transmitted via feces, urine, vaginal fluid, or semen [32]. It is also not clear what environmental circumstances are necessary for MPXV to remain infectious on the surface of objects since it is viable for periods up to 15 days, while other Orthopoxviruses presented longer periods—up to several weeks and months [19]. Although MPXV was detected in all saliva samples collected [34] and it presented higher viral load than oropharyngeal swabs [35], the precise role of MPXV-infected saliva in chains of transmission is yet to be determined, especially when droplets and aerosols are produced during dental clinical procedures [32].

5. Oral, Perioral, and Body Symptoms

Descriptive case series and cohort studies have shown that the presence of oral mucosa lesions associated with mpox infection is one of the first signs of infection. Moreover, oral and perioral lesions could appear in percentages that range from 5% to 70% of the cases [36–39]. In this context, the lymphadenopathy in the submandibular and cervical areas was described in the majority of the human mpox infections, when associated with inguinal swollen lymph nodes. Compared with smallpox diseases, lymphadenopathy was

reported as a characteristic symptom of mpox infection, and odynophagia secondary to mpox-associated ulceration may be present [40,41]. The heterogeneous presentations of the dermatologic, perioral, and oropharyngeal lesions related to MPXV can erroneously be confused with other sexually transmitted diseases [42], thus primary care healthcare workers need improved training in differential diagnosis to test adequately. Compared to other symptoms, oral and perioral lesions can be presented more often than arthralgia, back pain, and axillary lymphadenopathy [43]. Despite lesions caused by mpox infection usually being very heterogeneous, the oral ones seem to follow a well-circumscribed pattern with circular outliners [44]. Other systemic symptoms that can be present are headaches, sore throat, asthenia, myalgia, and proctalgia [33–35] (Figure 2).

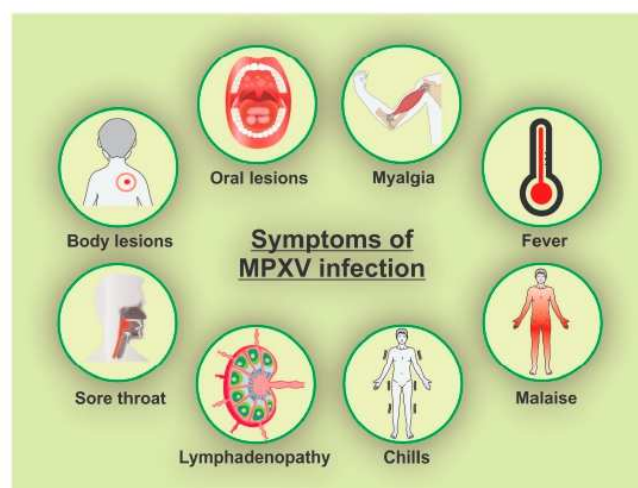


Figure 2. Main MPXV infection symptoms (created by the authors).

The identification of intra-oral mpox lesions is key to allowing healthcare workers to recognize the disease stage. During the enanthem stage—which consists of the appearance of a rash or lesions on the mucous membranes inside the body—the lesions first appear in the mouth or tongue, followed by the macular stage, when lesions present a macular appearance. After these first stages, which last one to two days, these lesions tend to progress from macular to raised papular lesions, and from papular to raised vesicular lesions filled with clear fluid—each of these stages also lasts one to two days. For the next five to seven days, the lesions progress into pustules and acquire deep seeded pustular appearance with opaque fluid, round shape, and firm-to-the-touch consistency; then, the lesions develop small depressions to their centers, maintaining this aspect until the end of the five to seven days. By the end of the pustular phase, the lesions begin to crust and to scab over. This process lasts 7–14 days until it is over, and the person is then no longer contagious [15,45].

6. Mpox Diagnosis Using Molecular Platforms

The molecular level comprehension of MPXV dynamics including the incubation stage, infectious period stage, and residual DNA shedding stage is pivotal to selecting the best-performing testing strategies for MPXV infection. The sample collection of suspected MPXV-infected subjects offers barriers due to a recent change in transmission mode (i.e., predominantly sexual transmission among MSM) [16]. Based on previous mpox outbreak data, the incubation period for MPXV was predicted to be around two weeks for droplet or noninvasive transmission routes [46]. A shorter incubation period of slightly more than one week was estimated for invasive exposures (mucous membrane) [47]. In the 2022/2023 simultaneous multi-country outbreaks of mpox the incubation stage was estimated to be around 8.5 days based on logarithmic analysis [16,48].

As aforementioned, other diseases with similar clinical characteristics reinforce the need for accurate diagnostic tests. Real-time polymerase chain reaction (RT-PCR) tests are a gold standard platform to perform an accurate and timely detection of MPXV, to support the breaking of chains of transmission. The endorsed specimen for molecular testing confirmation of MPXV is swabs of exudate from skin lesions. The WHO recommended an oropharyngeal swab as specimen type for mpox diagnosis in addition to skin exudate. Rectal swabs, genital swabs, urine, semen, and whole blood are specimens currently recognized for research; and serum and plasma for serology to contribute to diagnosis or research. Despite skin swabs being the standard samples used for MPXV testing, these lesions may be limited and located only in the anogenital area and asymptomatic infections can also occur in mpox patients [49,50]. Moreover, the use of oropharyngeal swabs associated with RT-PCR tests has already detected MPXV, which points out the importance of oral cavities in mpox diagnosis and viral load monitoring [2,51,52]. Until now, mpox diagnostics have been almost performed in symptomatic mpox patients with classical lesions. The RT-PCR testing in multiple samples indicates skin lesions with a sensitivity of 91–100%. It was assumed that the sensitivity of upper respiratory specimens including samples extracted from nasopharyngeal, rectal, and oral swabs, and seminal fluid and saliva reached between 69% and 100% in small-scale studies. RT-PCR analyses were also performed in whole blood, plasma, serum, and urine samples with highly restricted data available to estimate their accuracy properly. Altogether, it is expected that early detection of MPXV infectious, previous to the skin lesions, could be performed by body fluids with distinct sensitivity [16]. Besides, an extended shedding of more than three weeks in several biofluids collected from saliva, skin lesions, oropharyngeal swabs, nasopharyngeal swabs, and urine has been described, however, the clinical relevance and implications for mpox patient care remain unclear [16]. There is hope for the development of a multiplex real-time PCR test that can identify numerous poxviruses at the same time, including MPXV-specific detection [2].

The available diagnostic tests for MPXV detection are performed by real-time quantitative PCR (RT-qPCR). A brief overview of selected attributes of some scalable RT-qPCR assays commercially available to the global population is provided in Table 3 [2]. The target primers were engineered by conserved regions of the central coding and the ITS region. The *E9LNVAR* in the DNA polymerase gene, *B6R* in the envelope protein gene, and *F3L* in the ORF were selected in the central coding region; the *G2R* in a tumor necrosis gene was selected in the ITS region [53].

Table 3. Attributes of scalable qPCR assays commercially available for MPXV detection.

Brand Assay	Molecular Target	Processing Workload Capacity	Turnaround Strategy	Features/Analytical Performance
John Hopkins assay	MPXV (E9L), OPXV (B6R)	High-throughput	Variable by cycle threshold	LOD (95%) 100 copies/mL, analytical sensitivity, specificity is adequate and high reproducibility, tested in skin lesion swabs
US CDC assay	OPXV (E9L-NVAR), MPXV (B6R) (Multiplexed)	High-throughput	Variable by cycle threshold	LOD 16 copies/mL, assessed in Routine liquid transport media evaluated for Quantstudio 6
Novaplex MPX assay	MPXV	High-throughput	Variable by cycle threshold	100% sensitivity, evaluated for BioRad CFX 96
Bio-Speedy assay	MPXV (F3L)	High-throughput	Variable by cycle threshold	94% sensitivity, evaluated for BioRad CFX 96
ACON Biotech assay	MPXV (F3L)	High-throughput	Variable by cycle threshold	LOD 250 copies/mL, includes internal control, evaluated for BioRad CFX 96
Altona Diagnostics assay	OPXV	High-throughput	Variable by cycle threshold	Includes internal control, evaluated for BioRad CFX 96
Bioperfectus Technologies	MPXV (F3L)	High-throughput	Variable by cycle threshold	LOD 5 copies/reaction, includes internal control, evaluated for BioRad CFX 96

Table 3. Cont.

Brand Assay	Molecular Target	Processing Workload Capacity	Turnaround Strategy	Features/Analytical Performance
DaAn Gene	MPXV (F3L)	High-throughput	Variable by cycle threshold	LOD 200 copies/mL, includes internal control, evaluated for BioRad CFX 96
Shanghai ZJ Bio-Tech Co assay	MPXV (F3L)	High-throughput	Variable by cycle threshold	LOD 5000 copies/mL, includes internal control, evaluated for BioRad CFX 96
Perkin Elmer Pkamp Monkeypox Virus RT-PCR RUO kit	MPXV (F3L)	High-throughput	Variable by cycle threshold	LOD 20 copies/reaction, includes internal control, evaluated for BioRad CFX 96
Sansure Biotech Monkeypox Virus Nucleic Acid Diagnostic Kit	MPXV (F3L)	High-throughput	Variable by cycle threshold	LOD 200 copies/mL, includes internal control, evaluated for BioRad CFX 96
Thermo-Fischer Taqman Monkeypox Virus Microbe Detection	MPXV (J1L)	High-throughput	Variable by cycle threshold	LOD < 10 copies/reaction, evaluated for BioRad CFX 96
TIB Mobiol LightMix Modular Orthopox Virus/Monkeypox Virus	OPXV (14kDa), MPXV (J2L/J2R)	High-throughput	Variable by cycle threshold	LOD < 10 copies/reaction, evaluated for BioRad CFX 96
FilmArray Sentinel Panel & FilmArray BioThreat Panel (BioFire)	OPXV	Low-throughput	60 min	No reagent preparation. One test each per assay.
BD Max	LDT	Low-throughput	180 min	Automated extraction/amplification. Permits to run 24 samples in 3 h.
Roche Omni Cobas 6800/8800 (Roche)	LDT, Cobas MPXV (Multiplexed)	Intermediate-throughput	210 min	Automated extraction/amplification. Permits to run 800 tests in 8 h
Alinity (Abbott Molecular)	LDT, Alinity m MPXV (Multiplexed)	Intermediate-throughput	<60 min	Automated extraction/amplification. Permits A random access, up to 300 tests in 8 h
Panther Fusion Open Access (Hologic)	LDT, can multiplex up to 5 targets	Intermediate-throughput	<140 min	Automated extraction/amplification. Permits random access, up to 800 tests in 8 h.

A multiplex real-time PCR assay for simultaneous detection and differentiation of mpox virus IIa, IIb, and I clades and the B.1 lineage was assembled to detect specific genomic regions (G2R and F3L) of MPXV with unique nucleotide differences between Clades I, IIa, and IIb. Given the rapid appearance of mutations in the 2022/2023 outbreak, permanent evaluation of primers should be performed when new mutations can reduce the performance of diagnostic tests [54]. In low- and middle-income countries with restricted economic resources or places with barriers to access RT-qPCR platforms including thermocycler with electricity supply and temperature control, loop-mediated isothermal amplification (LAMP) tests are an interesting alternative approach. Although it can offer some advantages, LAMP-based test assays still require a moderate laboratory structure. Novel tests based on recombinase polymerase amplification with clustered regularly interspaced short palindromic repeats (CRISPR) associated with lateral flow assays could significantly improve the access of MPXV detection in these areas [16].

Of being executed at BSL2 or preferably in non-controlled places can increase access to mpox tests. Some of these serological tests can inform about the assessment of population immunity, indicating both T cell and B cell immunity responses and also indicates the presence of neutralizing antibodies [55], however, to date, there is a strong limitation of MPXV antigens based on inactivated viral peptides. Another critical limitation is the potential presence of cross-reactivity with other Orthopoxviruses in these serological tests [16]. For IgM and IgG detection in human serum, most serologic techniques currently employ ELISA, where mpox-specific IgM antibodies can be found as early as five days after the beginning of rashes (Figure 3) [2]. In addition, next-generation sequencing technologies such as Ion Torrent PGM, Illumina, and Oxford Nanopore MinION are cutting-edge approaches for

mpox diagnosis. These methods enable whole-genome sequencing of MPXV in clinical samples, allowing researchers to get insight into the phylogenomics of circulating mpox strains [2]. Virus isolation and electron microscopy (EM) have also been used since they are critical in virus initial characterization. These procedures are additionally important for virological studies that report on the environmental persistence of the MPXV, as well as viral identification and characterization of poxviruses in experimental settings [51]. All MPXV specimens analyzed should be reported to national health authorities.

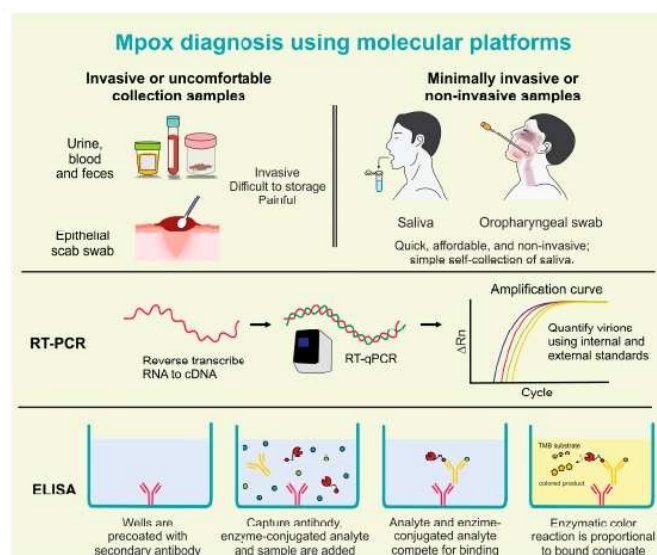


Figure 3. Mpox diagnosis using molecular platforms in invasive, minimally invasive, and non-invasive samples (created by the authors).

7. Mpox Salivary Diagnostics and Omics Perspectives on Mpox Research

Before indicating the characteristics of detection of MPXV in saliva, it is important to point out the limitations of the samples currently used for the diagnosis of mpox. In the context of mpox disease, the use of skin lesion exudate presents important barriers to its use in large-scale tests as a result of three main key factors: (i) these lesions can be established in intimate body regions such as the anogenital area, (ii) asymptomatic infections without skin lesions can also occur in mpox patients; and (iii) following the mpox lesions progression in macular, papular, vesicular to pustular stages, the sample collection is different, which may affect the sensitivity and accuracy of current diagnostic tests. In addition, an additional limitation of epithelial scab swab collection is the drastic reduction in lesions in more advanced stages of mpox diseases, which can affect the sensitivity of diagnostic tests. The collection of epithelial scabs using swabs also can present pain at the time of collection, which is another significant disadvantage. The diagnostic of several other infectious diseases occurs using blood samples; however, this collection is invasive, uncomfortable for suspected cases tested, difficult in storage, presents a risk of clotting or even pain at the time of collection, and this collection is not feasible in some healthcare centers [56]. These disadvantages drastically limit the large-scale tests that are essential for the control of infectious diseases.

Notwithstanding, saliva samples of infected patients have performed positively in detecting the virus, reinforcing the importance of oral cavity and saliva, which may be a screening or diagnostic specimen as well [35–37,45,57,58]. It can be easily self-collected and sample collection does not require expertise [59]. Saliva specimens can be collected in community and household areas where negative ventilation chambers are not available, minimizing the exposure of healthcare workers to the virus and the risk of respiratory cross-infection [60]. This may be relevant to increasing the safety of frontline healthcare workers while analyzing evidence for the contribution of the respiratory route to the transmission

of mpox is achieved. Although data analyses indicate little evidence, further large-scale clinical studies need to be performed to assess the potential impact of a respiratory route on mpox transmission [61]. The saliva collection is also simpler and more comfortable than the swab collection, which requires skilled staff with personal protective equipment [57,62]. Overall, saliva has several advantages as a diagnostic fluid due to its ease of collection, low cost, and non-invasive nature.

In a recent study during the 2022/2023 mpox outbreak, MPXV DNA was detected in saliva with 100% sensitivity, frequently with high viral loads compared to other samples [35]. Besides, a viral kinetics study showed that MPXV DNA was detected in saliva 76 days after symptom onset [63]. Another clinical study showed that 88.9% (16 of 18) of mpox-infected patients with skin lesion tests positive also presented the same result in a saliva-based analysis. As expected, in 100% of subjects with a negative test in skin lesions, the results with saliva tests for MPXV DNA detection were similar [64]. The correlation between skin and saliva tests for MPXV DNA detection was also higher in another study achieving 93.5% (29 of 31) of sensitivity to this mpox salivary diagnostics. Moreover, high salivary viral loads of MPXV DNA were detected using RT-qPCR in 85% (35 of 41) of these non-invasive samples. These data were accompanied by other relevant analyses revealing that infectious MPXV virions were rescued from 67% of saliva samples (22 of 33) of mpox patients [65].

There are several potential pathways for MPXV to be present in saliva, including infection of minor and major salivary glands, oral mucosal cells from macules and ulcers, lymph from maxillofacial and cervical lymphadenopathy, and gingival crevicular fluid containing serum-derived proteins [60]. Moreover, MPXV may not be positive in other bodily fluids and swab samples, portraying saliva as plausible and highly sensitive samples, especially when collected three or four days after the onset of symptoms [60]. Although recent advances have occurred in antibody tests for viral diseases, the previous cross-reactivity with antibodies induced by prior smallpox vaccination can disrupt the expected accuracy for MPXV antibody tests [35,40,66]. MPXV antigens and antibodies could be detected using a variety of techniques, including the complement fixation test, hemagglutination inhibition assay, enzyme-linked immunosorbent assay (ELISA), plaque reduction neutralization test, western blot, and electrochemiluminescence (ECL) assay [66]. Real-time PCR and viral isolation are not clearly correlated, especially because viral isolation has only been tested from skin lesion samples, lacking isolation in saliva samples [35]. Still, data on the utility of saliva for MPXV detection is limited, and there is a lack of standard operation procedures for collection methods.

The development of mpox detection using profitable OMICS platforms can be improved with the knowledge of basic molecular mechanisms explaining the pathophysiological routes to mpox-related biomarkers from peripheral tissues to saliva. Until now, there is a scientific gap about the salivary biomarkers of mpox, which limits translation outcomes for developing mpox detection platforms from peripheral tissues to the oral compartment [56].

Salivaomics is a multidisciplinary integrative investigation of saliva components performed by omics platforms [56]. The concept of Salivaomics was first led by a research group at the University of California, Los Angeles (UCLA), which has performed pioneer analysis in the salivary genome, transcriptome, microRNA, proteome, metabolome, and microbiome in translational and clinical studies related to point-of-care salivary devices to detect oral and systemic diseases, including viral infections [67]. With the introduction of high-throughput omics technologies, several reports have been published to comprehensively catalog the salivary proteome in different diseases compared with healthy subjects. In addition, the fact that there is an overlap in protein content between saliva and plasma suggests that saliva could potentially serve as an alternative to blood tests for diagnostic purposes for viral infections [68]. This means that instead of having to draw blood, a simple saliva sample could be used to obtain important diagnostic insights. One explanation for the correlation between salivary and plasmatic proteins is that plasma may flow into saliva; another reason is that plasma and saliva may share important proteins that are

required for their physiological roles as bodily fluids [68]. Notwithstanding, viral infectious illnesses may induce changes in salivary protein expression, which can be detected on proteomics-based biomarker discovery [56,68,69]. Research on infectious diseases and chronic diseases based on large-scale multi-omics databases permits a significant improvement in the molecular and genetic features of diseases. It is expected that the integration of multi-omics analysis can promote significant improvement in the performance of diagnostic tests [70]. The integration of salivary omics datasets still presents challenges [67] that can be potentially resolved by artificial intelligence.

It is imperative to note that the identification and quantification of salivary DNA, RNA, protein, and metabolite can provide novel non-invasive biomarkers via omic platforms. The chewing and circadian physiological changes salivary composition, which can hinder the development of saliva-based diagnostic platforms [56]. To date, the framework with computational and informatics strategies does not present specific salivary biomarkers for mpox diseases. However, for transcriptomics, metabolomics, or proteomics analyses, several data of potential marker molecules will be described in these frameworks. A pioneering example is the Salivaomics Knowledge Base (SKB), which was validated at UCLA in an open biomedical initiative [67]. The SKB is a data repository to assist the analysis of miRNA, transcriptomics, metabolomics, proteomics, and also microbiome focused on saliva [67].

Using a mass spectrometry platform, the proteome of MPXV was capable of detecting 152 viral proteins, consistent with ~80% of proteome coverage. In summary, more than 1300 viral peptides were detected and 35 MPXV peptides from 13 viral proteins present higher intensity in mass spectrometry signature, suggesting potential biomarkers in this omics platform with fast and less laborious capacity to detect several viruses simultaneously [71]. A proteomic signature of mpox in blood plasma was obtained in tryptic-digested samples from mpox patients with mild severity of clinical symptoms including skin lesions and respective healthy subjects. It was shown that 56 proteins were differentially expressed in mpox patients compared to healthy subjects, 32 proteins presented higher expression in mpox patients, and the other 24 had lower expression [72]. We point out some proteins with the greatest expression differences comparing mpox patients and healthy subjects, including higher levels of some proteins detected also in saliva as C-reactive protein (CRP) [73], serum amyloid A1 (SAA1) [74], lipopolysaccharide-binding protein (LBP) [75], and leucine-rich alpha-2-glycoprotein 1 (LRG1) [76]. Considering that these proteins were highly expressed in the plasma of mpox patients using a proteomics-based biomarker discovery platform, it suggests that these proteins also detected in saliva are potential salivary biomarkers of mpox disease.

8. Measures to Dentists and Frontline Healthcare Workers

The 2022/2023 mpox outbreak is of interest to frontline healthcare workers, including dentists, since they are at risk of being infected, especially considering that the oral cavity is an area closely related to MPXV infection [50,58]. Studies have detected a high viral load of MPXV in saliva, nosocomial transmission of MPXV to healthcare workers has been presented, and it is known that human-to-human transmission can occur via direct contact with bodily fluids, therefore emphasizing the importance of dentists to be careful when assessing patients [39,58,59]. In this context, it is important to emphasize that wearing facemasks and protective goggles is mandatory for frontline healthcare workers, especially because there is the possibility of large saliva droplets coming in contact with the eyes, nose, or mouth of dentists, dental assistants, dental hygienists, and endoscopy suite staffing, which could present a significant risk for infection. In addition, when performing a procedure that generates aerosols in prolonged periods from oral secretions of MPXV-infected patients, the frontline healthcare worker is at risk of being infected by MPXV, reinforcing the aforementioned recommendation of facemask and protective goggles usage [28,40,41]. Nevertheless, all non-urgent dental appointments and endoscopic procedures should be canceled if the patient is infected by MPXV and, if the healthcare

professionals get exposed to the virus in the aforementioned scenarios, post-exposure prophylaxis should be performed [50,58]. These infection control measures should be performed without stigma with MSM [77].

Moreover, although human-to-human transmission is described, the mechanisms of how it occurs were not completely elucidated. It could occur via direct contact with contaminated surfaces and with mucocutaneous lesions, leading to viral entry and replication in oropharyngeal and oral mucosa [60]. In this context, A26, A27, D8, and H3 surface proteins of MPXV are critical for viral attachment to the host cell, since they interact with laminin, chondroitin sulfate, and heparan sulfate, which are glycosaminoglycans that are present on the tongue, salivary glands, and oral mucosa [23,25–27]. Thus, the oral cavity may play an important role in viral infection and replication, despite these mechanisms still being insipient. In addition, during the incubation stage, the virus circulates to lymph nodes after being absorbed by the mucosa, leading to primary viremia and viral replication on lymphoid organs and distant lymph nodes [57,60,78].

According to the CDC, the National Health Service (NHS), and other relevant dental associations, oral screening could play an important role in the early diagnosis of several infectious diseases, including mpox [2,42,43,45,79]. In addition, thorough patient assessment could lead to easier identification of risk factors, thus allowing the dentist to not only identify these lesions but also provide patient orientation, which could help slow down viral spread [36–39,41–43,80–82]. In this context, MPXV was usually detected in the saliva of mpox-infected patients with high viral load, suggesting a potential application in screening or diagnostic platforms for mpox salivary diagnostics.

9. Conclusions

The unprecedented 2022/2023 simultaneous multi-country outbreaks of mpox have raised several red flags regarding possible infection routes, especially by frontline healthcare workers who are in direct contact with patients. These professionals, including dentists, can contribute to decrease the spread of the MPXV by understanding novel transmission mechanisms through saliva, respiratory droplets, procedures that produce aerosols and face-to-face contact indicated in the recent 2022/2023 mpox outbreak. More studies are needed to understand viral entrance into host cells and viral replication in the oral cavity, especially approaching laminin, chondroitin, and heparan sulfates, and glycosaminoglycans that can interact with MPXV surface proteins allowing viral attachment and posterior replication in oral cells that express these proteins. The current methods of mpox diagnostics applying molecular methodologies present high accuracy, however, these tests are currently invasive, painful, and rely on the advanced phases of the disease. Notwithstanding, MPXV was detected in the saliva of mpox-infected patients with high viral load, indicating that novel salivary diagnostic platforms can be used in the early diagnosis of mpox. MPXV DNA was detected in saliva with a sensitivity of 85%–100%. In this context, OMICs technologies such as genomics, transcriptomics, and proteomics can be used in the perspective to study the MPXV and unique salivary components in mpox patients. Hence, OMICs technologies could play a central role to prevent future mpox outbreaks by providing detection of novel biomarkers of mpox diseases and aiding in the development of new diagnostic tools applied in saliva samples.

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CAPÍTULO 2

Bioinformatics Driven Approach for Selection of Salivary Peptides to Detect Norovirus in Saliva Using Electrochemical Biosensors supported by Neural Network machine learning algorithm

**Bioinformatics Driven Approach for Selection of Salivary Peptides to
Detect Norovirus in Saliva Using Electrochemical Biosensors supported
by Neural Network machine learning algorithm**

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ABSTRACT

Noroviruses infect around 700 million people globally every year, causing significant morbidity and mortality. Considering that noroviruses use the salivary glands as a reservoir with oral-oral transmission through saliva, our aim was to select naturally occurring peptides in saliva using the bioinformatics platform to implement in an electrochemical Point-of-Care device supported by machine learning algorithm for salivary diagnosis of norovirus infection. The molecular dockings by bioinformatics were used to select peptides based on interaction and molecular dynamics with the norovirus VP1 surface protein. The salivary peptides selected by artificial intelligence (NoroPep1, NoroPep2 and NoroPep3) were synthesized with thiol terminations and applied to printed electrodes covered with gold nanoparticles. Uninfected saliva or saliva infected with serial dilutions of norovirus were tested on the electrochemical platform using cyclic voltammetry (CV). Our platform was capable to discriminate serial dilutions of norovirus with sensitivity, specificity and accuracy of up to 90%. These results were validated with saliva from uninfected and infected mice with 85% sensitivity, 90% specificity and 87.5% accuracy. The Neural Network machine learning algorithm on the reduction curve of the voltammogram generated by saliva from uninfected and infected mice was able to achieve 100% sensitivity, 90.9% specificity and 94.1% accuracy. Our results suggest that the use of high-affinity bioinformatic-selected salivary peptides could be a promising platform for the development of portable electrochemical devices for the salivary diagnosis of norovirus.

Keywords: Norovirus; Artificial Intelligence; Salivary Diagnostics; Salivary Peptides

1. INTRODUCTION

Norovirus is the primary cause of acute gastroenteritis worldwide, accounting for almost 700 million cases of acute gastroenteritis and more than 200,000 fatalities each year in poor countries [1,2]. The virus affects individuals of all ages, with children under five years old bearing a disproportionately high burden, particularly in Africa [3]. Norovirus infections have significant economic implications, estimated at \$4.2 billion in direct health-care expenditures and \$60.3 billion in societal costs each year globally [4]. Norovirus is responsible for an estimated 900 deaths, 110,000 hospitalizations, and 2.3 million ambulatory clinic visits in the United States each year, with healthcare costs ranging from \$430 to \$740 million [5]. Viral genetic variety, fast evolution, and lack of a reliable cell culture technique make vaccine development difficult [6]. However, various vaccine candidates are being developed, which hold promise for future preventative methods [7].

Noroviruses are extremely infectious enteric infections that are assumed to be transmitted by fecal-oral transmission [3]. Viral particles are spread by a variety of channels, including person-to-person, food, water, and the environment [2,8]. However, a recent study has shown a new oral-oral transmission pathway via saliva [9,10]. Noroviruses can replicate in salivary glands at titers equivalent to levels found in the intestines [10]. This discovery associated with norovirus salivary secretion has implications for diagnostics, treatments, and sanitation strategies [10].

Norovirus diagnosis has progressed from electron microscopy to increasingly sensitive molecular techniques, with RT-PCR emerging as the gold standard [11]. This approach has better sensitivity than ELISA, which is excellent for large-scale testing but may require PCR confirmation for negative results [12]. Automated sample preparation paired with real-time PCR has increased efficiency and uniformity [13]. However, problems persist, including the need for standardization in detection methodologies, particularly for food samples [14]. Multiple studies have shown that salivary immunoassays are successful at detecting norovirus infections by measuring antibody responses [15–18]. These tests are very sensitive and specific, detecting both symptomatic and asymptomatic infections [16,18]. Salivary antibody responses usually peak

approximately 14 days after infection and stay increased for many months [15,18]. Ongoing research is aimed at creating biosensors and sophisticated molecular approaches to enhance norovirus detection [19].

Recent research has focused on salivary diagnostics as a promising alternative. Studies have demonstrated that salivary antibody responses can accurately detect norovirus infections, with high sensitivity and specificity [15,16]. Multiplex immunoassays have been developed to detect multiple norovirus genotypes simultaneously, enhancing the utility of salivary testing [17,18]. These non-invasive methods offer advantages for monitoring norovirus transmission in community settings and during outbreaks [17]. However, challenges remain, including the unaffordable characteristics and complex attributes of the traditional methods, and the rapid evolution of norovirus strains [20,21].

Moreover, electrochemical point-of-care devices present a promising alternative as a biosensor for diagnosing norovirus infections in saliva due to their remarkable sensitivity, with detection limits as low as 0.84 copies/mL [22]. Saliva testing is a non-invasive alternative to stool diagnostics, allowing for better monitoring of norovirus transmission in community settings [17]. These devices offer several benefits over traditional approaches, including shorter testing durations and the potential for point-of-care applications [23]. The combination of nanomaterials, microfluidics, and smartphone technology has improved salivary biomarker detection [24], making electrochemical sensors attractive instruments for rapid viral detection and outbreak control in precision medicine.

In this context, peptidomics, the complete study of peptides in biological systems, has emerged as a potential strategy for discovering biomarkers in a variety of disorders [25,26]. Peptides have several benefits, including high purity, specificity, and simplicity of modification, making them useful for diagnostic procedures [27]. Furthermore, the combination of *in silico* prediction, sophisticated chemical synthesis, and multiplexed tests has accelerated the development of peptide-based diagnostic materials [28]. Principal component analysis, support vector machines, and artificial neural networks are examples of machine learning approaches used as promising alternatives in processing complicated biosensor data [29]. Machine learning algorithms were successfully integrated with electrochemical impedance spectroscopy [30].

Bioinformatic assays using *in silico* molecular docking enable the

screening of a huge number of candidate peptides, identifying those with high binding affinity to viral targets [31]. These techniques may predict peptide-protein interactions by taking into account hydrogen bonding and hydrophobic interactions [31,32]. Besides, molecular dynamics simulations improve predictions by determining the stability of peptide-protein complexes with target molecules [33]. Additionally, these approaches have been effectively used for many viruses, including influenza A, herpes simplex, Zika, and SARS-CoV-2 [32,34,35].

This study aims to develop a fast, portable and non-invasive point-of-care diagnostic tool for effectively managing norovirus outbreaks. We aim to evaluate the use of saliva as a diagnostic fluid for norovirus detection using bioinformatics technologies to identify salivary peptides with high affinity for norovirus surface proteins to develop a specific electrochemical biosensor supported by machine learning algorithm.

2. MATERIALS AND METHODS

Peptide Development

The framework using bioinformatic assays based on *in silico* molecular docking was used in a systematic, multi-stage approach aimed to evaluate and find salivary peptides with high binding potential. The approach began with the collection of a data set including 723 salivary peptides, each represented as a linear sequence of amino acids and their relative interaction energies with the murine norovirus VP1 protein. These molecular interactions were evaluated using the HPEPDOCK molecular coupling program, which returned affinity energy values. This framework iteratively discovered peptides, culminating in the selection of the three best-performing peptides (Table 1):

Table 1. Docking score of the salivary peptides selected by framework using bioinformatic assays based on *in silico* molecular docking.

Peptide	Peptide Sequence	Interaction Energy
NoroPep1	FLHAHGKLFTR	-233 Kcal/Mol
NoroPep2	HSHREFPFYGDYGS	-225 Kcal/Mol

Experimental Procedures

Cyclic voltammograms were recorded in 10 mL of a KCl solution (0.5M) as a supporting electrolyte and ferro/ferri-cyanide ($[\text{Fe}(\text{CN})_6]^{3-/4-}$) as the analyte (5mM) using Palmsens3 (Palmsens, Compact Electrochemical Interfaces, Houten, Utrecht, The Netherlands). In the described cyclic voltammetry (CV) experiment, the equilibration time ($t_{\text{equilibration}}$) was set to 2 seconds to stabilize the system before measurements began. The initial potential (E_{begin}) was set at -0.15V, and the scan was initiated from this potential. The voltage was then cycled to the first vertex potential (E_{vertex1}) of -0.15V, up to the second vertex potential (E_{vertex2}) of 0.55V, creating a triangular wave. The step potential (E_{step}) was 0.002V, allowing for satisfactory resolution of the potential changes. The scan rate was 0.075V/s, determining the speed at which the potential was swept from the initial to the vertex and back, ensuring detailed capture of the electrochemical behavior of the system [36].

Screen-Printed Electrode Modification

Screen-printed electrodes (DropSens DRP-110) were modified in two steps to improve detection capabilities. Gold Nanoparticles (AuNPs - Sigma Aldrich, Burlington, USA) with 50nm of diameter were initially administered to the electrodes by adding 5 μL of a 0.1mM solution to the working electrode. It was followed by five cyclic voltammetry (CV) measurements with a Ferro/Ferri-KCl solution (5 mM in 0.5M KCl) and one more CV reading under the same circumstances. The electrodes were then modified by adding 5 μL of either NoroPep1, NoroPep2 or NoroPep3 at 100 $\mu\text{g/mL}$. Five CV measurements were performed identically to assess the electrochemical behavior after modification, followed by one additional CV reading using the Ferro/Ferri-KCl solution. This step-by-step modification is intended to analyze and compare the electrochemical reactions produced by each modification agent [36].

Sample Preparation

Project was approved by the institutional Ethics Committee #H-0299.

In the first round of assays, murine norovirus 1 (MNV-1) was used. This

virus was serially diluted (1:10) in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Saint Louis, MO, USA), resulting in a range of dilutions from 1.97×10^5 to 1.04×10^3 genome copies. As a control, plain media was used.

Later, saliva samples from healthy mice were obtained and centrifuged at 3000 rpm for 15 minutes at 4°C, with the supernatant collected and pooled. MNV-1 was combined with pooled saliva to produce ten infected samples with 7.1×10^4 genome copies each and ten control samples free of the virus.

Finally, saliva was taken from 20 mice, either MNV-1 positive or negative, and centrifuged at 3000 rpm for 15 minutes at 4°C, with the supernatant collected. 5 µL of each prepared sample was used for the assays for all phases [37].

Data analysis

The classification was tested with state-of-the-art machine learning algorithms, including Support Vector Machine (SVM), AdaBoost, Random Forest, Neural Network, Gradient Boosting, and Naive Bayes. These algorithms were selected based on their performance during model training. To analyze the predictive performance of these algorithms, ten times stratified cross-validation was used. The samples were divided into ten subsets; in each iteration, nine subsets were used to train the algorithm while one subset was exclusively used to test it. This process ensured that each subset was part of the test set once. Additionally, the procedure was repeated three times with different sample configurations to achieve a closer estimate of the real performance of the models, resulting in a total of thirty executions.

To measure the results obtained, three performance metrics consolidated in the literature were used: sensitivity, specificity, and accuracy. Sensitivity, or the true positive rate, is the proportion of positive cases (e.g., MNV-1 positive) correctly classified. Specificity, or the true negative rate, is the proportion of negative cases (e.g., MNV-1 negative) correctly classified. Accuracy is defined as the total number of samples correctly classified, considering both true positives and true negatives.

3. RESULTS

Peptides bind to VP1 using molecular docking

The interaction of the peptides with the norovirus VP1 protein are represented in 3D (Figure 1 A-C), and the protein-peptide interaction is detailed in 2D interaction maps (Figure 2, A-C).

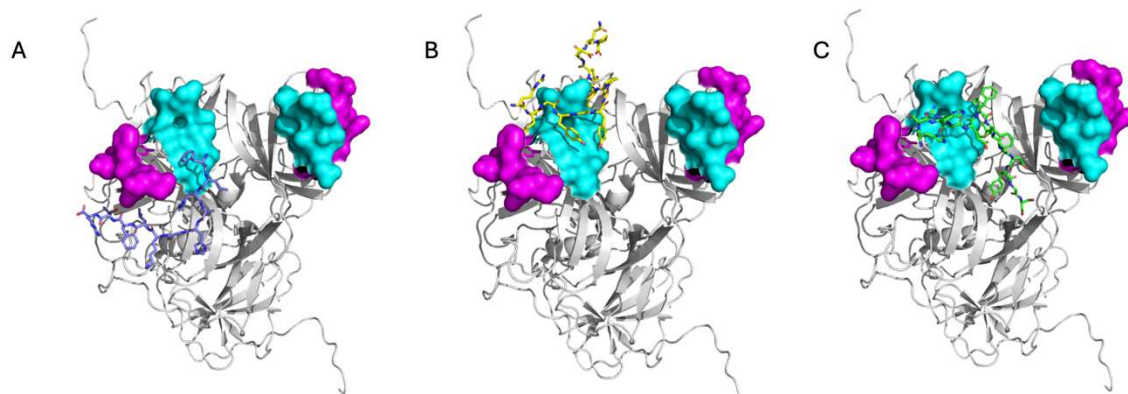


Figure 1. 3D docking representations for selected peptides interacting with norovirus VP1. The VP1 protein is represented in grey. Molecular interaction is represented as (A) for NoroPep1 in blue sticks; (B) NoroPep2 in yellow sticks; and (C) NoroPep3 in green sticks.

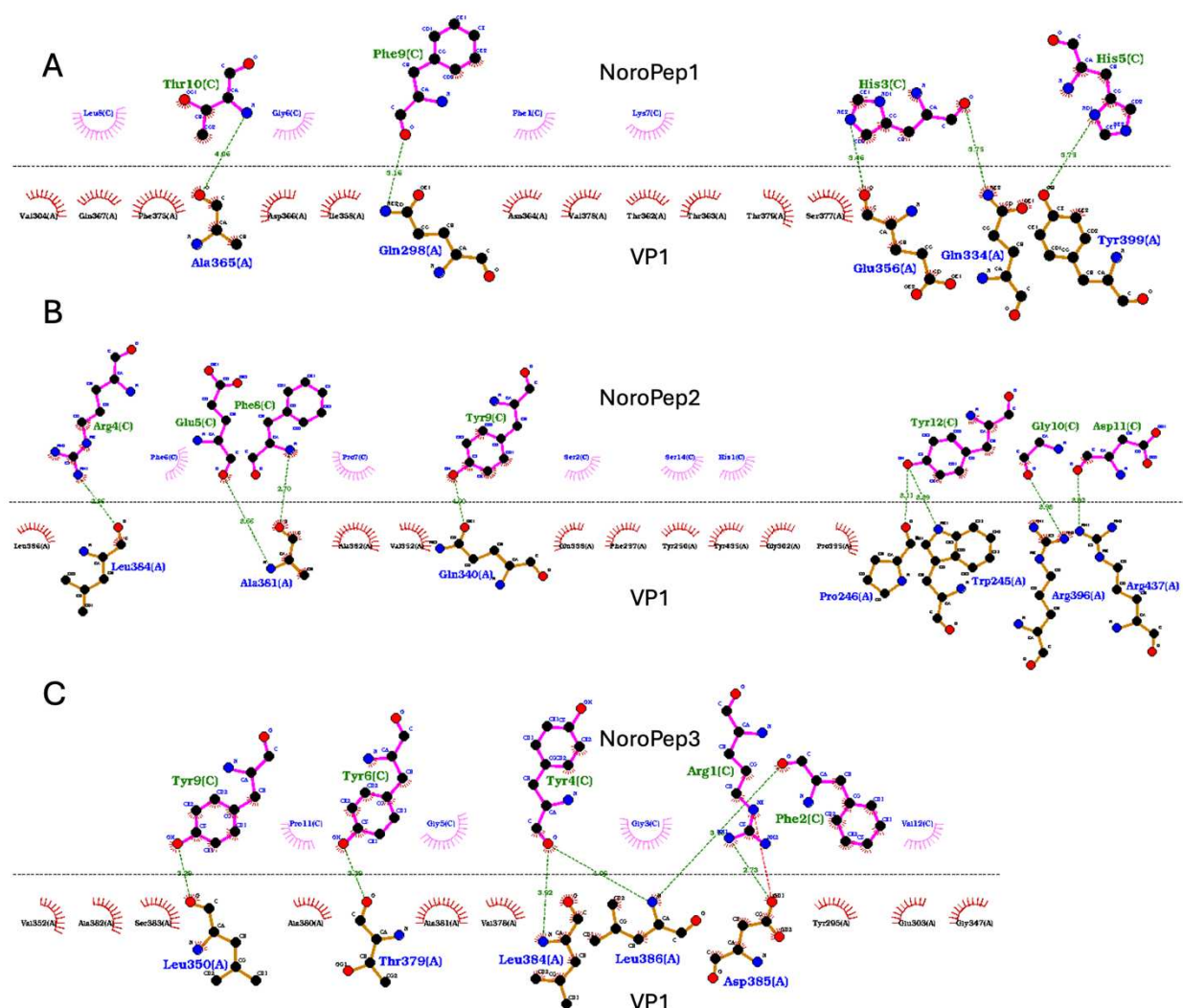


Figure 2. 2D interaction maps for NoroPep1 (A), NoroPep2 (B), and NoroPep3 (C) interacting with norovirus VP1. Above the dotted lines are the peptides, and below them is the VP1 complex. Hydrogen bonds are represented as green dotted lines between the peptides and the VP1 complex.

Modification of Screen-printed Electrodes

Screen-printed electrodes were successfully modified in two steps to improve detection capabilities. AuNPs were initially used by adding 5 μL of a 0.1mM solution to the working electrode. It was followed by the modification by adding 5 μL of NoroPep1, NoroPep2 or NoroPep3 at 100 $\mu\text{g/mL}$ (Figure 3).

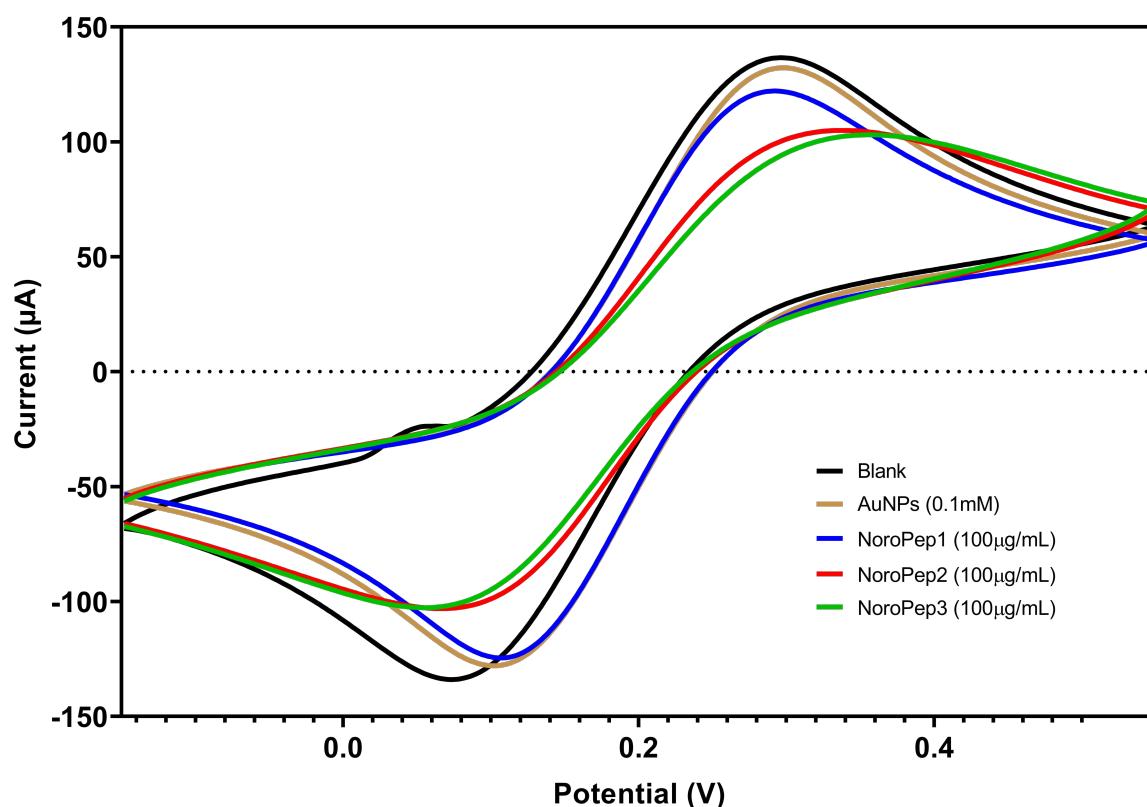


Figure 3. Cyclic voltammograms of a bare screen-printed electrode (black line), electrode modified with AuNPs at 0.1mM (golden line), and electrode modified with NoroPep1 (blue line), NoroPep2 (red line) and NoroPep3 (green line), E_{step} at 0.002V and scan rate at 0.075V/s.

Biosensor Performance

In the first phase of the experiment, successive dilutions of MNV-1 were generated in a 1:10 ratio, beginning with an initial concentration of 1.97×10^5 to 1.04×10^3 genome copies. Each dilution was examined using a biosensor equipped with AuNPs and NoroPep1, NoroPep2 or NoroPep3 to detect differences in electrochemical signals. The CV measurements revealed separate peaks for the control and the samples containing virus at all concentrations (Figure 4).

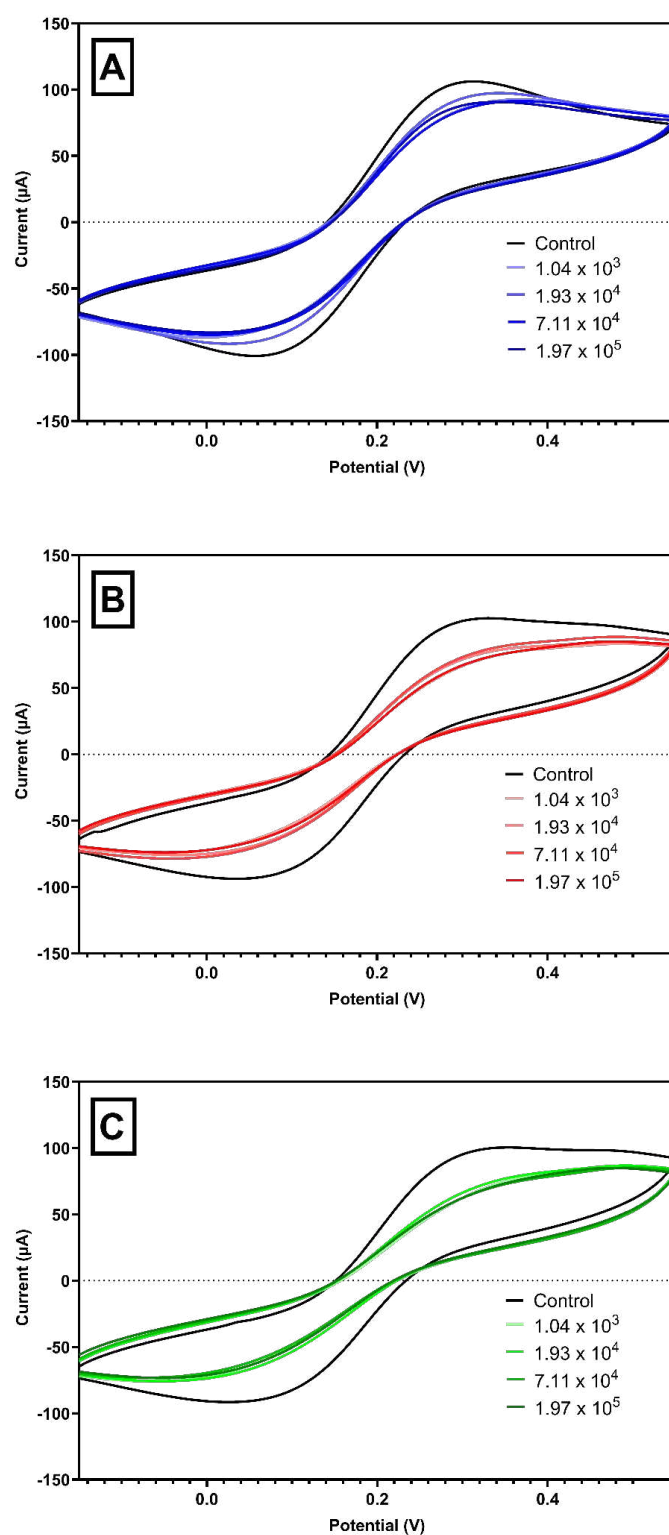


Figure 4. Voltammograms of serial dilutions stratified by viral load (genome copies per sample). The voltammograms illustrate the electrochemical responses for different concentrations of the virus. Each color-coded curve highlights the distinct peak currents corresponding to the respective viral

concentrations for NoroPep1 (A), NoroPep2 (B) and NoroPep3 (C).

In the second phase, saliva samples from healthy mice were collected, pooled, and spiked with the MNV-1 at 7.1×10^4 genome copies. Voltammograms from the healthy saliva pool, both with and without the virus, demonstrated a clear distinction in peak currents, indicating successful virus detection in saliva (Figure 5). For these samples, ROC curve analysis of the biosensor exhibited a sensitivity of 75% and a specificity of 72.2% for NoroPep1; a sensitivity of 90.91% and a specificity of 90% for NoroPep2; and a sensitivity of 75% and a specificity of 82.82% for NoroPep3 (Figure 6).

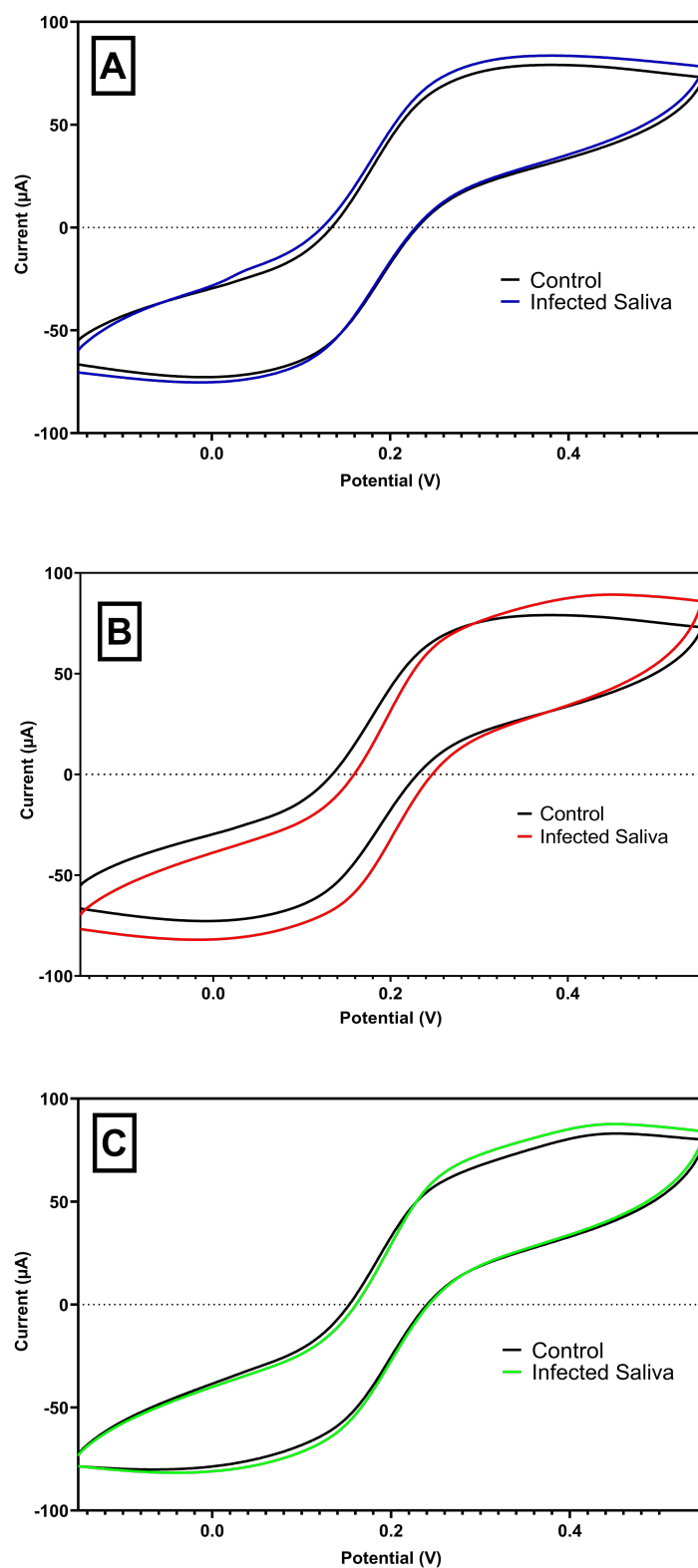
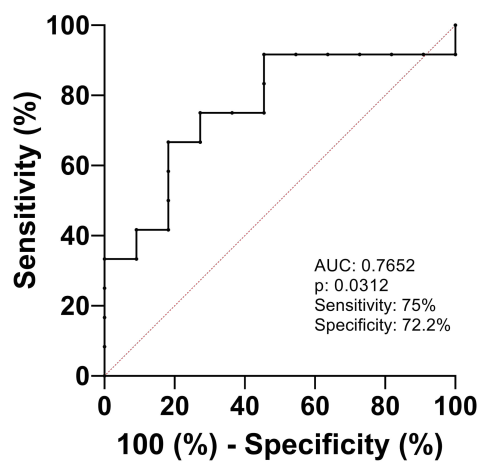
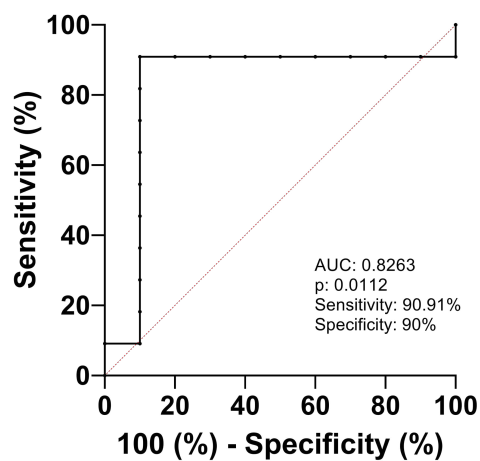


Figure 5. Voltammograms of pooled saliva samples of healthy mice. The voltammograms illustrate the electrochemical responses for two groups: control, without MNV-1, in black; Infected saliva, with 7.1×10^4 genome copies of the virus per samples for NoroPep1 (A), NoroPep2 (B) and NoroPep3 (C).

A ROC curve of NoroPep1



B ROC curve of NoroPep2



C ROC curve of NoroPep3

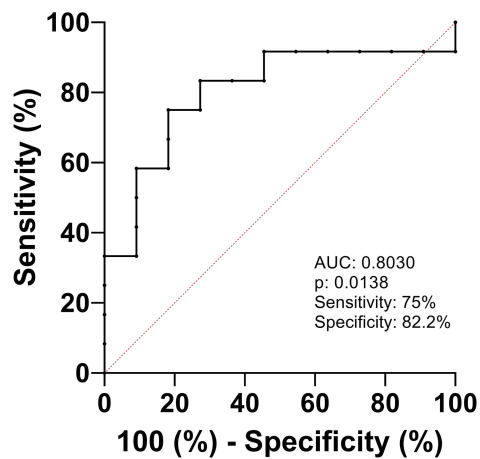


Figure 6. Graph representing the electrochemical sensor diagnostic test

effectiveness using ROC curve analysis for NoroPep1, NoroPep2, and NoroPep3.

In the third phase, saliva from infected and non-infected mice was collected and analyzed. Voltammograms showed a distinction between the two groups, suggesting that the virus was successfully detected in the saliva of infected mice (Figure 7). The ROC curve analysis for the oxidation curve yielded an AUC of 85.71% of sensitivity and 90.91% of specificity (Figure 8).

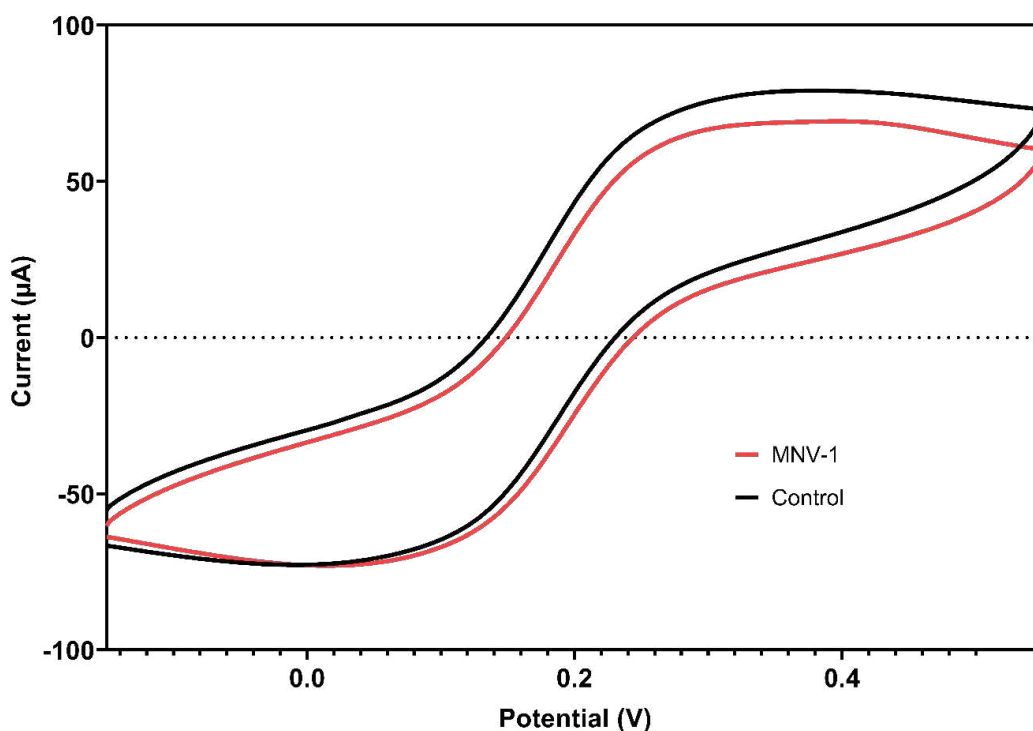


Figure 7. Voltammograms of saliva samples from infected mice tested on NoroPep2. The voltammograms represent the electrochemical responses for two groups: samples from animals not infected with MNV-1 in black and samples from animals who tested positive for MNV-1 in red.

ROC curve of MNV-1 positive saliva

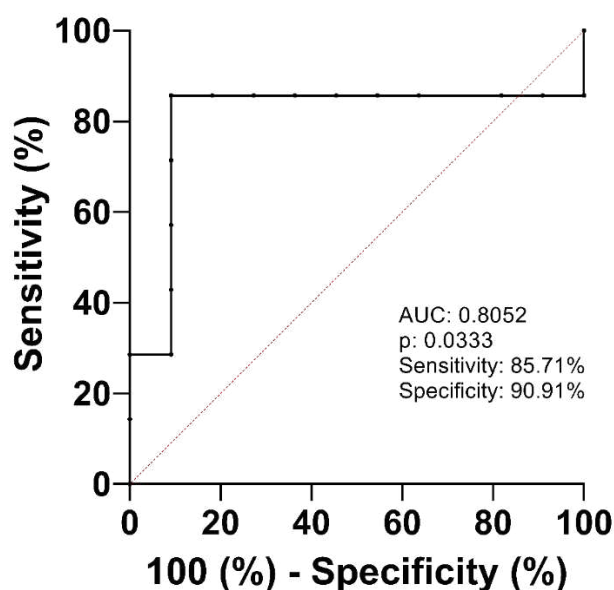


Figure 8. Graph representing the electrochemical sensor diagnostic test effectiveness using ROC curve analysis for MNV-1 using NoroPep2.

Discrimination Analysis

Artificial intelligence tools, most notably machine learning algorithms, were applied to classify and discriminate salivary voltammogram samples from positive and negative MNV-1 mice using NoroPep2 faster and with greater reliability. The classification of salivary voltammogram data was tested using Support Vector Machine (SVM), AdaBoost, Random Forest, Neural Network, Gradient Boosting, and Naive Bayes algorithms. The results obtained in these analyses indicate that the best discrimination was achieved using both oxidation and reduction curves on AdaBoost, Random Forest, Neural Network, Gradient Boosting and Naïve Bayes (Table 2).

The classification of the reduction curve of salivary voltammogram data by the Neural Network algorithm showed a sensitivity of 100%, specificity of 90.9%, and accuracy of 94.1% between positive and negative MNV-1 mice. This high level of accuracy demonstrates the potential of using electrochemical biosensors combined with advanced machine learning techniques for non-invasive diagnosis of MNV-1.

Table 2. Machine learning algorithms applied in the reduction curve of salivary voltammogram to discriminate positive MNV-1 mice from negative animals.

Data Used	Algorithm	Accuracy	Sensitivity	Specificity
Raw Data (Reduction curve)	SVM	88.2%	83.3%	90.9%
	AdaBoost	82.4%	83.3%	81.8%
	Random Forest	88.2%	83.3%	90.9%
	Neural Network	94.1%	100%	90.9%
	Gradient Boosting	88.2%	83.3%	90.9%
	Naive Bayes	82.4%	83.3%	81.8%

The Shapley Additive Explanations (SHAP) method was applied to the main points of the voltammogram that contribute significantly to the discrimination of the samples, distinguishing them as positive or negative for MNV-1 infection (Figure 8). In general, the main points of discrimination in the results are present in the peak region of the reduction curve.

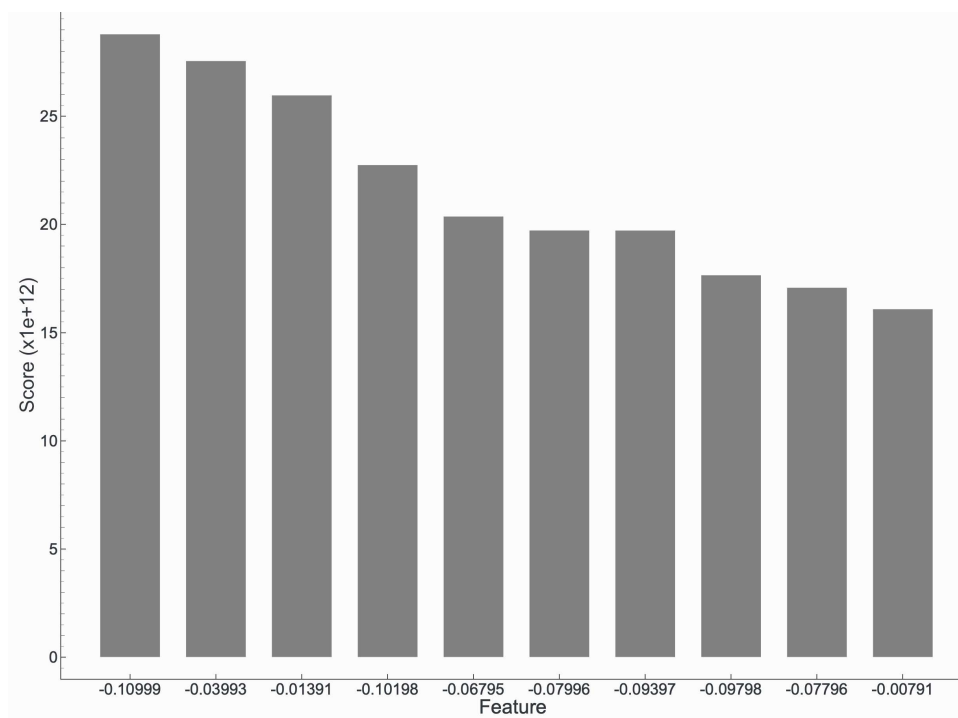


Figure 8. SHAP demonstration used by the Neural Network computational model with the raw data of the reduction curve. The potentials of -0.10999V, -0.03993V, -0.01391V and -0.10198V were the main attributes used in the applications of the algorithm. The regions of -0.06795V, -0.07996V, -0.09397V, -0.09798V, -0.07796V and -0.00791V were also used for this differentiation.

4. DISCUSSION

Our study successfully modified screen-printed electrodes with AuNPs and AI-generated peptides NoroPep1, NoroPep2, and NoroPep3 to enhance viral detection capabilities. Cyclic voltammetry indicated unique electrochemical signals for modified electrodes when compared to bare ones. In biosensor performance testing, dilutions of MNV-1 in spiked saliva samples showed distinct electrochemical responses, with high sensitivity and specificity across viral doses. Furthermore, saliva from infected and uninfected mice had unique voltammetric patterns, indicating effective viral identification. Machine learning algorithms such as AdaBoost, Random Forest, Neural Network, Gradient Boosting, and Naive Bayes were used to classify salivary voltammogram data, with 100% accuracy, sensitivity, and specificity in distinguishing between positive and negative MNV-1 samples, demonstrating the potential of combining electrochemical biosensors with AI for non-invasive norovirus diagnosis.

Current diagnostic procedures for norovirus infection differ in terms of accuracy, sensitivity, and specificity, with rRT-PCR exhibiting sensitivity up to 94.1% [38]. Enzyme immunoassays (EIA) and immunochromatographic tests (ICT) have excellent specificity but low sensitivity, ranging from 23% to 59% [39–41], and it improve when numerous epidemic samples are tested [40]. Electron microscopy (EM) has higher specificity but lesser sensitivity than PCR techniques [38]. Methods using electrochemistry also present high specificity and sensitivity [42], however, this is the first time that machine learning algorithms were used to achieve up to 100% of accuracy.

In the context of electrochemistry, aptamers, peptides, and antibodies are among the recognition components that have already been used to modify biosensors [22,43,44], with concanavalin A and gold nanoparticles being used to improve sensor performance [42,45]. Moreover, electrochemical approaches produce findings quickly, frequently within 30-60 minutes [42,43]. These biosensors have been effectively applied to a variety of sample types, including food and clinical specimens, indicating their use in point-of-care testing and outbreak management [22]. Current methods for detecting norovirus in saliva include a multiplex immunoassay measuring salivary IgG responses to common norovirus genotypes, which has shown 71% sensitivity, and 96% specificity

compared to PCR-diagnosed infections [17]. ELISA-based methods using virus-like particles (VLPs) can detect norovirus attachment to histo-blood group antigens in saliva [46]. Here we efficiently provided a fast and accurate method for norovirus detection using saliva as specimen for diagnostics and the present study was the pioneer in detecting norovirus infection in saliva using electrochemistry.

MNV-1 contacts with host cells are mediated by the VP1 capsid protein, specifically its protruding (P) domain[47]. VP1 increases viral RNA-dependent RNA polymerase activity [48], whereas certain amino acid residues in VP1, such as position 296, influence virulence and B cell infection effectiveness [49]. On the other hand, CD300lf has been discovered as a cellular receptor for MNV, with bile acids functioning as cofactors to promote infection [50]. Here, NoroPep1, NoroPep2 and NoroPep3 were selected based on their interaction with MNV-1 VP1, and our results corroborate the proposal that VP1 is a potential target for viral detection.

Artificial intelligence (AI) and machine learning (ML) are revolutionizing electrochemical sensor technology, enhancing specificity, sensitivity, and accuracy. AI algorithms improve selectivity, drift correction, and efficiency in various sensor types [51]. ML models enable virtual sensing, fault prediction, and management in electrochemical sensors [52]. These techniques address common issues like electrode fouling, poor signal-to-noise ratio, and chemical interferences [53]. Embedded AI models, such as TinyML, can discriminate between interfering species in complex samples, improving reliability in portable devices [54]. AI-assisted electrochemical multisensor systems show promise in diverse fields, including medicine, the food industry, and ecology [55]. The integration of AI with sensing technology enables rapid detection with high sensitivity, accuracy, and precision [56]. This approach is also advancing catalyst development, operational condition optimization, and battery lifespan prediction in electrochemical energy conversion and storage processes [57].

Despite their potential, electrochemical biosensors face challenges in commercialization and real-world applications [58]. Limitations include the need for specialized equipment, difficulty in detecting low virus concentrations in environmental samples, and potential interference from other biomolecules [59,60]. However, ongoing research aims to address these issues through the

integration of nanotechnology, artificial intelligence, and innovative techniques like electrochemical collision [61,62]. Also, considering the experiments here reported were developed using animals, further studies in infected saliva with human norovirus are pivotal for additional validation. Future developments may include wearable sensors, multiplexed detection, and reusable biosensors for on-site monitoring [60,63].

5. CONCLUSION

In conclusion, our study effectively modified screen-printed electrodes with AuNPs and AI-generated peptides NoroPep1, NoroPep2, and NoroPep3, which improved viral detection capabilities. Cyclic voltammetry revealed distinct electrochemical responses in changed electrodes. Biosensor performance tests demonstrated differentiation across virus loads in spiked saliva samples and non-spiked saliva. The effective distinction of saliva from infected and uninfected mice, combined with Neural Networks algorithm, resulted in 100% sensitivity, 90.9% specificity and 94.1% accuracy in the classification of salivary voltammogram data. This demonstrates the potential of electrochemical biosensors with AI for non-invasive viral detection. Compared to current approaches, our methodology is more accurate and produces faster findings, making it a viable tool for point-of-care testing and epidemic control.

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CAPÍTULO 3

*Artificial Intelligence Bioinspired Peptide for
Salivary Detection of SARS-CoV-2 in
Electrochemical Biosensor*

Artificial Intelligence Bioinspired Peptide for Salivary Detection of SARS-CoV-2 in Electrochemical Biosensor

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ABSTRACT

The development of innovative bioactive molecules for application in low-cost, accurate, and rapid portable biosensors is critical for the surveillance and early detection of SARS-CoV-2. We aimed to create a bio-inspired molecular docking peptide, remodeled by a unique artificial intelligence platform, for salivary diagnostics of COVID-19 using a portable electrochemical device coupled with machine learning algorithms. SAGAPEP artificial intelligence algorithm platform was used to model a bioactive and bio-inspired peptide through in silico molecular docking against the RBD domain of the SARS-CoV-2 Spike protein. The selected Bio-Inspired Artificial Intelligence Peptide 1 (BIAI1) was synthesized and ATR-FTIR spectroscopy was applied to exhibit molecular interactions between BIAI1 with the SARS-CoV-2. BIAI1 was functionalized to rhodamine-modified screen-printed carbon electrodes. Cyclic voltammetry (CV) measurements were performed using the Ferro/Ferri-KCL solution on the PalmSens3 potentiostat. Serial titrations of the virus were measured, followed by unstimulated human saliva samples with and without the SARS-CoV-2 virus applied to the electrochemical platform. The CV measurements of the electrochemical biosensor were validated using classical analysis and a pioneering application of support vector machine (SVM)-based machine learning algorithm in electrochemistry data derived from saliva samples. ATR-FTIR exhibit interaction of BIAI1 and SARS-CoV-2 in vibrational modes at 1620 cm^{-1} and 1204 cm^{-1} , indicating molecular coupling with Spike protein through carbonyl stretching in nucleic acids and amide III in proteins, respectively. Differences in serial titrations of the virus were detected. Our electrochemical platform detected $1,8 \times 10^4$ of the virus in infected saliva with 100% sensitivity, 80% specificity, and 90% accuracy. It was validated with saliva from COVID-19-positive and negative subjects using a Neural Network algorithm, showing 90% sensitivity, specificity, and accuracy. Our data demonstrate the potential of the BIAI1 peptide-based electrochemical biosensor coupled with machine learning as a portable, non-invasive, and scalable tool for a non-invasive screening and salivary detection of COVID-19.

Keywords: Biosensors; COVID-19; Bio-Inspired Peptides; Artificial Intelligence; Salivary Diagnostics; Electrochemical Detection

1. INTRODUCTION

SARS-CoV-2 is the virus that causes COVID-19, a disease that has resulted in over 775 million cases worldwide and about 7 million deaths [1]. The disease condition ranges from asymptomatic to severe respiratory symptoms [2]. Aside from COVID-19 symptoms, it is possible to identify long-term repercussions of SARS-CoV-2 virus infection in multiple organ systems, such as shortness of breath, cough, chest and joint pains, palpitations, myalgia, smell and taste dysfunctions, headache, cognitive and mental impairments, and gastrointestinal and cardiac issues [3]. Testing on nasopharyngeal swabs or saliva is commonly used for COVID-19 detection, with saliva representing a non-invasive alternative [4]. Although qRT-PCR offers adequate sensitivity and specificity, it has limited bedside application and is time-consuming [4,5]. Furthermore, early detection of COVID-19 is critical for reducing its spread and mortality rates [6]. To address these issues, point-of-care devices have been developed for rapid diagnosis [5]. However, resource shortages and the need for specific, sensitive, affordable, and non-invasive portable testing methods persist [7].

In this context, electrochemical biosensors provide various advantages for salivary diagnostics, including shorter reaction times, simplicity of use, enhanced affinity, and increased sensitivity [8]. These biosensors have successfully detected low levels of salivary biomarkers, providing a simple and rapid diagnostic procedure [9]. Regarding the COVID-19 pandemic, electrochemical biosensors have shown promise to detect the disease in saliva [10]. Some electrochemical biosensors have been created for the sensitive detection of SARS-CoV-2 at multiple stages of infection [11,12]. This detection is generally performed by detection based on antibody functionalization in electrochemical electrodes to detect viral proteins in saliva [10–13]. However, chemically synthesized short peptides offer competitive cost advantages and are easier to produce using recombinant bacteria compared to antibodies, which can improve the democratization of salivary diagnostics in low-income and middle-income countries with more non-invasive options in the benchmark diagnostic availability.

Considering that viral transmission can occur through saliva and SARS-CoV-2 is composed of three structural proteins: spike (S), matrix (M), and envelope (E) [14], the detection of these proteins in non-processed saliva may

represent an even faster diagnostic tool. From this perspective, the receptor-binding domain (RBD) in the S protein interacts with the host cellular receptor to permit viral entrance [15]. Thus, detecting the S-RBD motif represents a target on which an especially tailored modified electrochemical sensor can distinguish the virus in saliva and allow COVID-19 detection. Herein, selecting peptides that bind to S-RBD presents a promising field in developing accurate detection tools for SARS-CoV-2 [16]. It also poses a significant challenge considering the rise of new variants – Omicron, for instance, presents several structural alterations, 15 of which are focused on the RBD [17]. From this point of view, artificial intelligence-based tools and applications have also been proposed to improve accuracy [18].

The evaluation of molecular docking of several peptides with a specific target is time-consuming and costly to perform numerical simulations so the optimal solution is unaffordable. Therefore, the supervised machine learning (ML) algorithm based on the Surrogate-assisted optimization model is an attractive tool for multi-objective discrete optimization problems (MODOPs) for these problems [19]. Supervised ML is a effective tool for peptide discovery and design, providing faster and more efficient predictions of therapeutic peptide value [20]. Supervised ML has been used in antimicrobial peptides to quickly screen sequence space and direct tests to promising candidates with high putative activity [21]. Besides, peptides have been designed to bind to SARS-CoV-2 viral proteins by mimicking human angiotensin-converting enzyme 2 (hACE2) receptors using new computational methods [22]. Supervised ML has also been employed to anticipate de novo sequences for viral infectivity improvement, resulting in the identification of short functional self-assembling peptides [23]. Computational techniques have been created for identifying and optimizing possible peptide hits from viral envelope proteins, with a particular emphasis on preventing the fusion process, as well [24] demonstrating the potential of machine learning in peptide creation for antiviral medicine.

Considering that early detection of SARS-CoV-2 is imperative for controlling the spread of COVID-19 and traditional diagnostic methods, while effective, are often costly and time-consuming, there is a pressing need for portable, non-invasive, low-cost, and rapid diagnostic tools that can be deployed widely worldwide. Our study aims to develop an electrochemical biosensor that

utilizes bio-inspired peptides selected and redesigned using AI coupled to machine learning algorithms to detect SARS-CoV-2 in saliva.

2. MATERIALS AND METHODS

Peptide Development

The Surrogate-Assisted Genetic Algorithm for Peptide Evaluation and Prediction (SAGAPEP) framework was used in a systematic, multi-stage approach aimed to evaluate and find peptides with high binding potential. The approach began with the collection of a data set including 296 peptides, each represented as a linear sequence of amino acids and their relative interaction energies with the SARS-CoV-2 Spike protein. These interactions were evaluated using the HPEPDOCK molecular coupling program, which returned affinity energy values. Feature extraction approaches were critical in converting the linear peptide sequences into a format appropriate for model training. Three descriptors were assessed: amino acid composition (AAC), amino acid linking (AAL), and the composition of k-spaced nucleotide acid pairs (CKSNAP). These characteristics aided in the development of a surrogate model using regression, which could quickly estimate the binding energies of fresh peptide candidates. This surrogate model allowed for the rapid assessment of peptide-protein interactions without the requirement for time-consuming in vitro investigations. By integrating these features into a surrogate-assisted genetic algorithm, the SAGAPEP framework iteratively discovered and optimized peptides, culminating in the selection of Bio-Inspired Artificial Intelligence 1 peptide (BIAI1) - FIRWRFRWWRR.

ATR-FTIR Analysis

The spectra of VSV-eGFP-SARS-CoV-2, the peptide BIAI1, and BIAI1 + VSV-eGFP-SARS-CoV-2 were recorded using Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy (Agilent Cary 630 FTIR, Agilent Technologies, Santa Clara, CA, USA). For this analysis, the lipidic region (3050-2800 cm^{-1}) and the fingerprint infrared signature (1800-800 cm^{-1}) were utilized. Samples were prepared with the peptide BIAI1 and VSV-eGFP-SARS-

CoV-2, respectively. A volume of 3 μL of each sample was placed on the diamond cell, forming a thin layer on the ATR crystal. The spectra were then recorded at a resolution of 2 cm^{-1} with 64 scans. Second derivative spectra were generated from the original data, plotted in Origin Pro 9.8.0.200 (OriginLab, Northampton, MA, USA) software, and adjusted using the Savitzky-Golay algorithm with a polynomial order of 2 and a window of 20 points.

Experimental Procedures

Cyclic voltammograms were recorded in 10 mL of a KCl solution (0.5M) as a supporting electrolyte and ferro/ferri-cyanide ($[\text{Fe}(\text{CN})_6]^{3-/4-}$) as the analyte (5mM) using Palmsens3 (Palmsens, Compact Electrochemical Interfaces, Houten, Utrecht, The Netherlands). In the described cyclic voltammetry (CV) experiment, the equilibration time (*t equilibration*) was set to 2 seconds to stabilize the system before measurements began. The initial potential (*E_{begin}*) was set at -0.3V, and the scan was initiated from this potential. The voltage was then cycled to the first vertex potential (*E_{vertex1}*) of -0.3V, up to the second vertex potential (*E_{vertex2}*) of 0.3V, creating a triangular wave. The step potential (*E_{step}*) was 0.002V, allowing for satisfactory resolution of the potential changes. The scan rate was 0.075V/s, determining the speed at which the potential was swept from the initial to the vertex and back, ensuring detailed capture of the electrochemical behavior of the system.

Screen-Printed Electrode Modification

Screen-printed electrodes (DropSens DRP-110) were modified in two steps to improve detection capabilities. Rhodamine was initially administered to the electrodes by adding 5 μL of a 0.5 mg/mL solution to the working electrode. It was followed by five cyclic voltammetry (CV) measurements with a Ferro/Ferri-KCl solution (5 mM in 0.5M KCl) and one more CV reading under the same circumstances. The electrodes were then modified by adding 5 μL of Bio-Inspired Artificial Intelligence Peptide 1 (BIAI1) at 1 mg/mL. Five CV measurements were performed identically to assess the electrochemical behavior after modification, followed by one additional CV reading using the Ferro/Ferri-KCl solution. This step-by-step modification is intended to analyze and compare the electrochemical reactions produced by each modification agent.

Sample Preparation

Research project was approved by Institutional Ethics Committee: #4.602.081. In the first round of assays, a pseudotyped vesicular stomatitis virus (VSV) with the glycoprotein gene (G) substituted with the spike protein of SARS-CoV-2 (VSV-eGFP-SARS-CoV-2-S) was used. This virus was serially diluted (1:2) in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Saint Louis, MO, USA), resulting in a range of dilutions from 1.44×10^5 ffu to 2.25×10^3 ffu.

Later, saliva samples from healthy, asymptomatic patients were obtained and centrifuged at 3000 rpm for 15 minutes at 4°C, with the supernatant collected and pooled. VSV-eGFP-SARS-CoV-2-S was combined with pooled saliva to produce ten infected samples with $1,8 \times 10^4$ ffu each and ten control samples free of the virus.

Finally, saliva was taken from 20 patients with flu-like symptoms, either COVID-19 positive (ten samples) or negative (ten samples). 5 µL of each sample was used for the assays for all phases.

Data analysis

The classification was tested with state-of-the-art machine learning algorithms, including Support Vector Machine (SVM), AdaBoost, Random Forest, Neural Network, Gradient Boosting, and Naive Bayes. These algorithms were selected based on their performance during model training. To analyze the predictive performance of these algorithms, ten times stratified cross-validation was used. The samples were divided into ten subsets; in each iteration, nine subsets were used to train the algorithm while one subset was exclusively used to test it. This process ensured that each subset was part of the test set once. Additionally, the procedure was repeated three times with different sample configurations to achieve a closer estimate of the real performance of the models, resulting in a total of thirty executions.

To measure the results obtained, three performance metrics consolidated in the literature were used: sensitivity, specificity, and accuracy. Sensitivity, or the true positive rate, is the proportion of positive cases (e.g., COVID-19 positive) correctly classified. Specificity, or the true negative rate, is the proportion of negative cases (e.g., COVID-19 negative) correctly classified. Accuracy is

defined as the total number of samples correctly classified, considering both true positives and true negatives.

3. RESULTS

Peptide-Virus Interaction

A scheme of the ATR-FTIR technology based on infrared analysis to evaluate samples BIAI1 and the VSV-eGFP-SARS-CoV-2 is represented in Fig. 1. We found different molecular changes in the virus after incubation with the compound using ATR-FTIR analyses. As an outcome, the binding interactions between VSV-eGFP-SARS-CoV-2 and BIAI1 were suggestive of 2 vibrational modes (Table 1). The vibrational mode at 1620 cm^{-1} was related to carbonyl stretching in nucleic acids (Fig. 1B). Another vibrational mode was 1204 cm^{-1} which can be related to amide III in proteins (Fig. 1C). The presence of these vibrational modes suggests a high presence of interactions between the VSV-eGFP-SARS-CoV-2 and BIAI1 and absent of these vibrational modes in VSV-eGFP-SARS-CoV-2.

Table 1. Indication of the wavenumber suggested to indicate the interaction between VSV-eGFP-SARS-CoV-2 and BIAI1 and its molecular assignment.

Wavenumber (cm^{-1})	Assignment	Reference
1204	Amide III in proteins	[25]

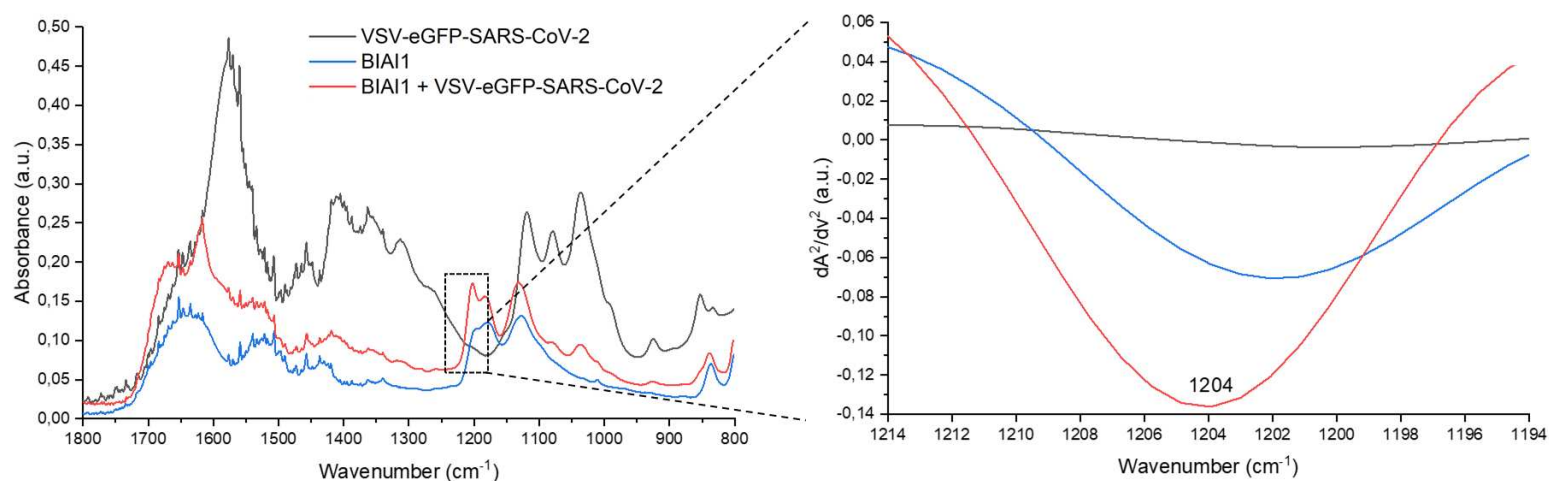


Figure 1 - Infrared spectroscopy indicates molecular interactions between VSV-eGFP-SARS-CoV-2-S plus BIAI1. The spectra were expressed in raw data or second-derivative spectra.

Modification of Screen-printed Electrodes

Screen-printed electrodes were successfully modified in two steps to improve detection capabilities. Rhodamine was initially used by adding 5 μL of a 0.5 mg/mL solution to the working electrode. It was followed by the modification by adding 5 μL of Bio-Inspired Artificial Intelligence Peptide 1 (BIAI1) at 1 mg/mL (Figure 2).

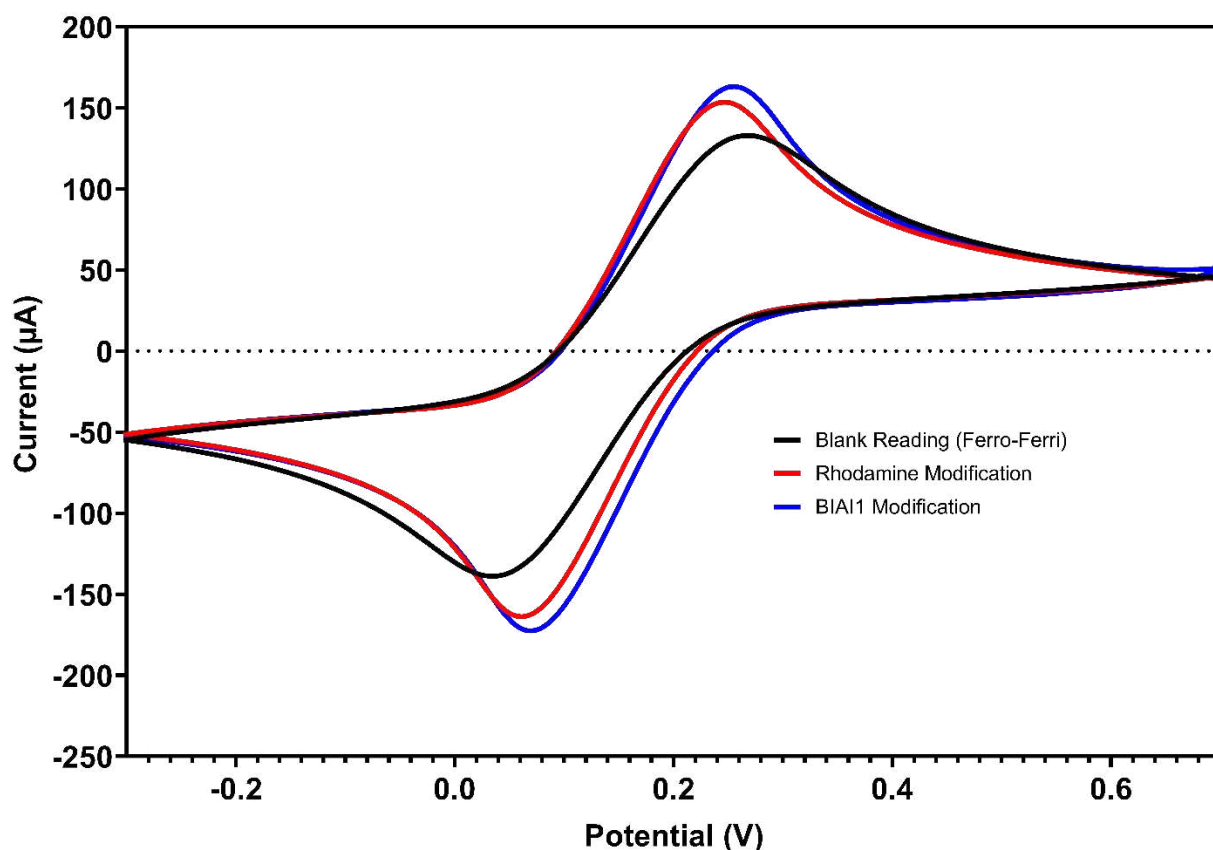


Figure 2. Cyclic voltammograms of a bare screen-printed electrode (black line), electrode modified with rhodamine at 0.5mg/mL (red line), and electrode modified with BIAI1 (blue line), E_{step} at 0.002V and scan rate at 0.075V/s.

Biosensor Performance

In the first phase of the experiment, successive dilutions of VSV-eGFP-SARS-CoV-2 were generated in a 1:2 ratio, beginning with an initial concentration of 1.44×10^5 focus-forming units down to 1.125×10^3 . Each dilution was examined using a biosensor equipped with rhodamine and BIAI1 peptide to detect differences in electrochemical signals. The CV measurements revealed separate peaks for each concentration level, with the highest signal occurring at the initial concentration and a progressive drop in peak current with consecutive dilutions (Figure 3).

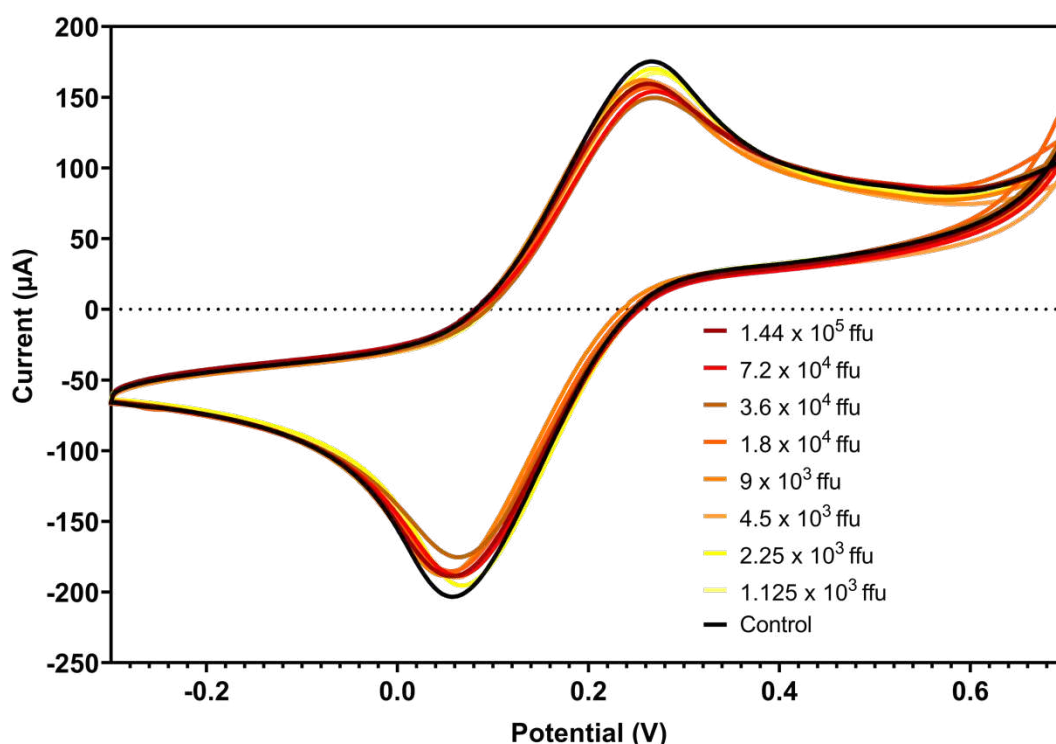


Figure 3 - Voltammograms of serial dilutions stratified by viral load. The voltammograms illustrate the electrochemical responses for different concentrations of the virus. Each color-coded curve highlights the distinct peak currents corresponding to the respective viral concentrations, demonstrating the sensitivity of the biosensor to varying levels of VSV-eGFP-SARS-CoV-2.

In the second phase, saliva samples from healthy individuals were collected, pooled, and spiked with the VSV-eGFP-SARS-CoV-2-S virus at various concentrations. Voltammograms from the healthy saliva pool, both with and without the virus, demonstrated a clear distinction in peak currents, indicating successful virus detection in saliva (Figure 4). For these samples, the biosensor exhibited a sensitivity of 100% and a specificity of 80%, resulting in an accuracy of 90%. The ROC curve analysis for this phase provided an area under the curve (AUC) of 0.9532, with a standard error of 0.01785 and a p-value of <0.0001. These assays demonstrate an interesting efficiency in detecting real positives while minimizing false positives at higher thresholds (Figure 5).

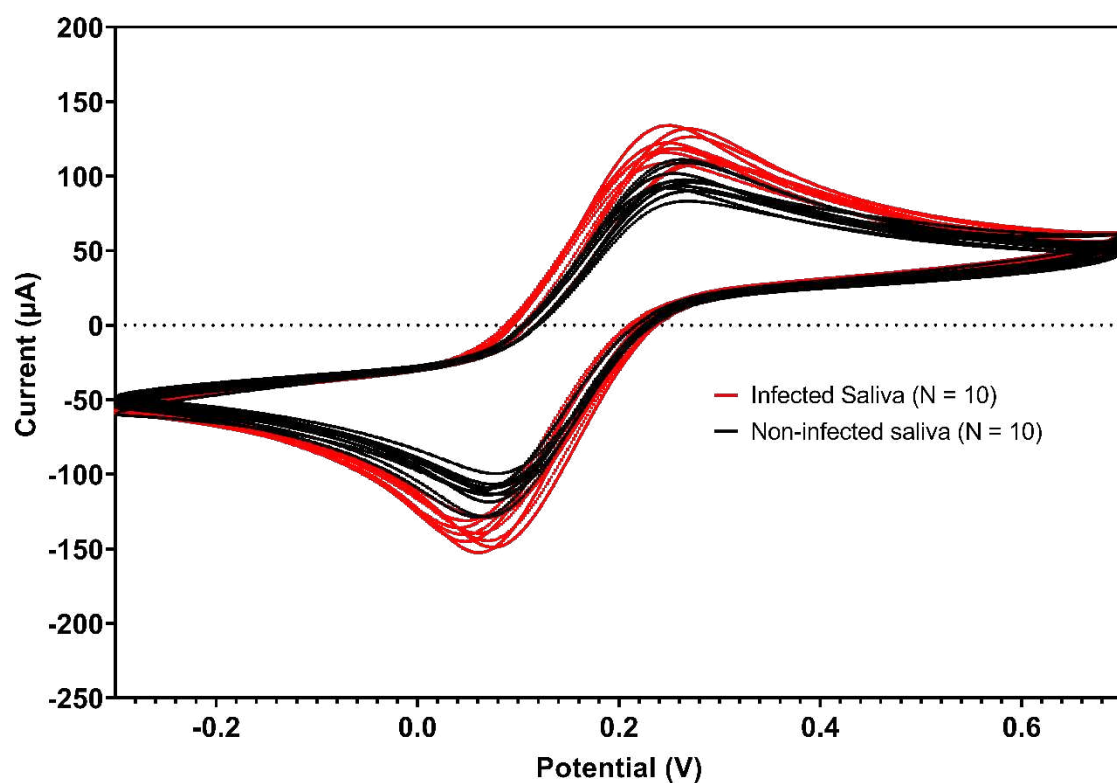


Figure 4 - Voltammograms of pooled saliva samples of healthy asymptomatic individuals. The voltammograms illustrate the electrochemical responses for two groups: Non-infected saliva, without VSV-eGFP-SARS-CoV-2, in black; Infected saliva, with $1,8 \times 10^4$ of the virus, in red.

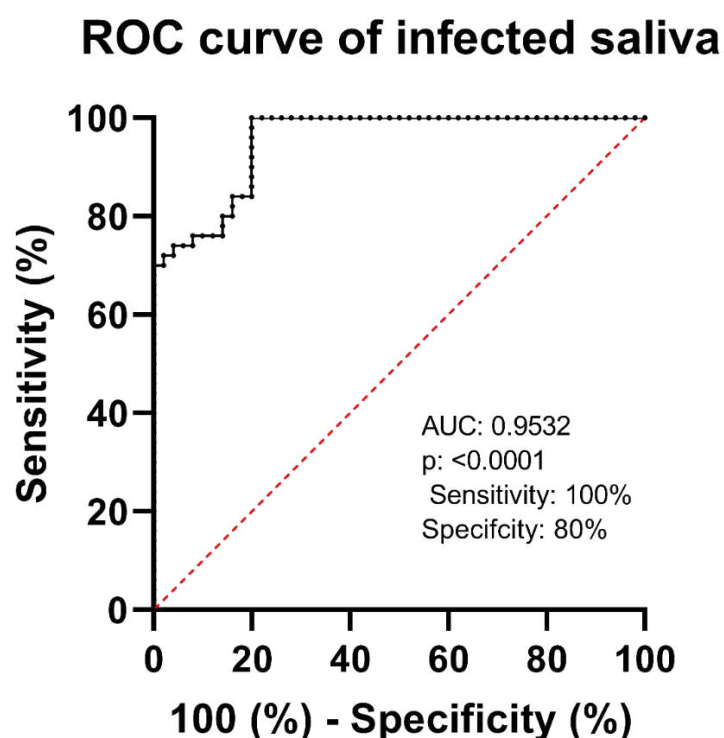


Figure 5 – Graph representing the electrochemical sensor diagnostic test effectiveness using ROC curve analysis, producing an AUC of 0.9532 (95% CI: 0.9182 to 0.9882) and a p-value <0.0001.

In the third phase, saliva from patients with flu-like symptoms and negative RT-PCR test to detect COVID-19 and COVID-19 patients were analyzed. Voltammograms from the negative and positive patients showed a distinction between the two groups, suggesting that the virus was successfully detected in the saliva of patients (Figure 6). The ROC curve analysis for oxidation yielded an AUC of 0.69 with a standard error of 0.1213, sensitivity and specificity of 70% and a p-value of 0.1509, indicating moderate discriminatory power that is not statistically significant (Figure 7).

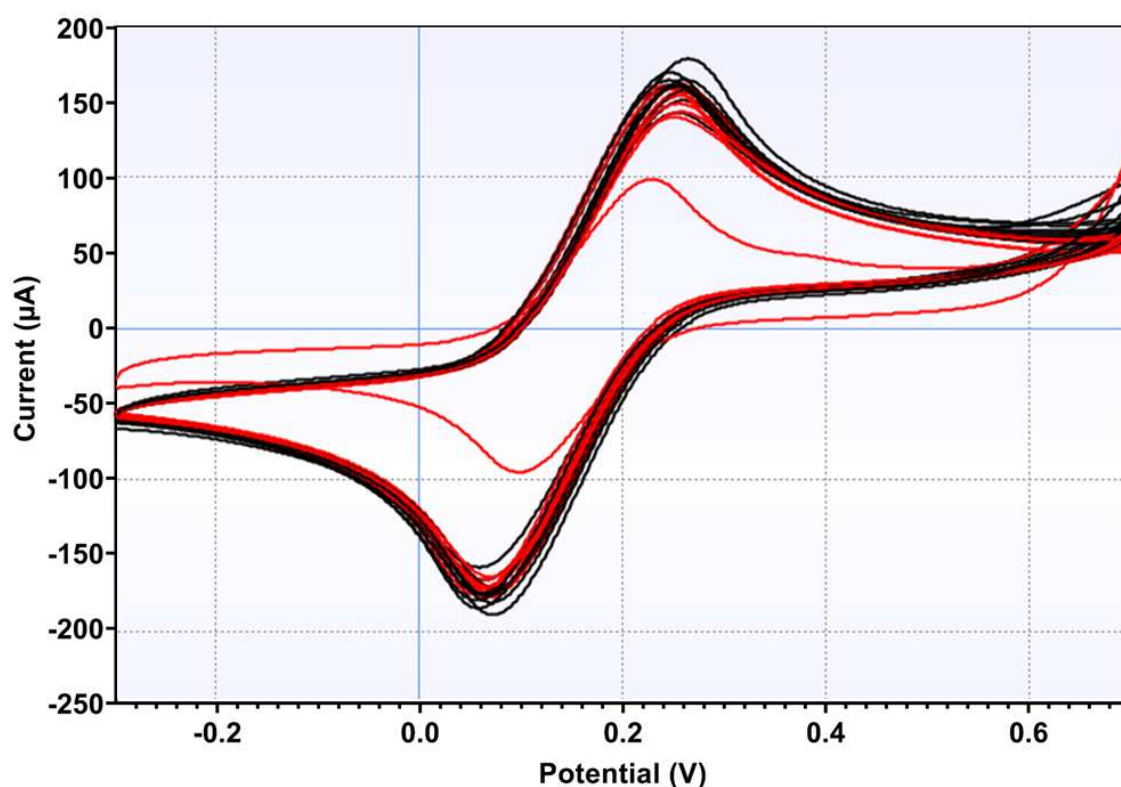


Figure 6 - Voltammograms of saliva samples from patients with flu-like symptoms and negative RT-PCR test to detect SARS-CoV-2 (black) and COVID-19 patients (black).

ROC curve of saliva from patients

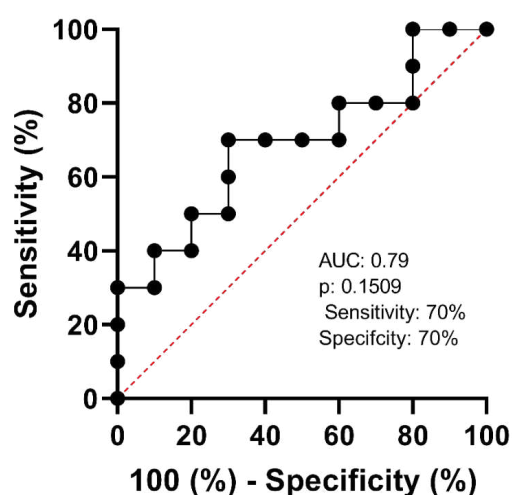


Figure 7 – Graph representing the electrochemical sensor diagnostic test effectiveness using ROC curve analysis, producing an AUC of 0.79 with sensitivity and specificity of 70%, a standard error of 0.1213, and a p-value of 0.1509.

Discrimination Analysis

To classify and discriminate salivary voltammogram samples from positive COVID-19 symptomatic patients and negative COVID-19 symptomatic patients more quickly and with greater reliability, artificial intelligence tools were applied, most notably machine learning algorithms. The classification of salivary voltammogram data was tested using Support Vector Machine (SVM), AdaBoost, Random Forest, Neural Network, Gradient Boosting, and Naive Bayes algorithms. The results obtained in these analyses indicate that the best discrimination was achieved using a Neural Network (Table 2).

The classification of salivary voltammogram data by the Neural Network algorithm showed a sensitivity of 90%, specificity of 90%, and accuracy of 90% between positive COVID-19 symptomatic patients and negative COVID-19 symptomatic patients. This high level of accuracy demonstrates the potential of using electrochemical biosensors combined with advanced machine learning techniques for non-invasive diagnosis of COVID-19.

Table 2. Machine learning algorithms applied in salivary voltammograms to discriminate positive COVID-19 symptomatic patients from negative COVID-19 symptomatic patients.

Data Used	Algorithm	Accuracy	Sensitivity	Specificity
Raw Data (Oxidation and Reduction curves)	SVM	0.75	0.6	0.9
	Random Forest	0.75	0.7	0.8
	AdaBoost	0.7	0.6	0.8
	Neural Network	0.75	0.8	0.7
	Gradient Boosting	0.7	0.7	0.7
	Naive Bayes	0.75	0.8	0.7
Raw Data (Oxidation curve)	SVM	0.5	0.5	0.5
	AdaBoost	0.65	0.7	0.6
	Random Forest	0.5	0.5	0.5
	Neural Network	0.5	0.6	0.4
	Gradient Boosting	0.55	0.6	0.5
	Naive Bayes	0.35	0.4	0.3
Raw Data (Reduction curve)	SVM	0.6	0.2	1.0
	AdaBoost	0.7	0.7	0.7

Random Forest	0.75	0.7	0.8
Neural Network	0.9	0.9	0.9
Gradient Boosting	0.7	0.7	0.7
Naive Bayes	0.75	0.8	0.7

The Shapley Additive Explanations (SHAP) method was applied to the main points of the voltammogram that contribute significantly to the discrimination of the samples, distinguishing them as positive or negative for COVID-19 (Figure 8). In general, the main points of discrimination in the results are present in the peak region of the reduction curve.

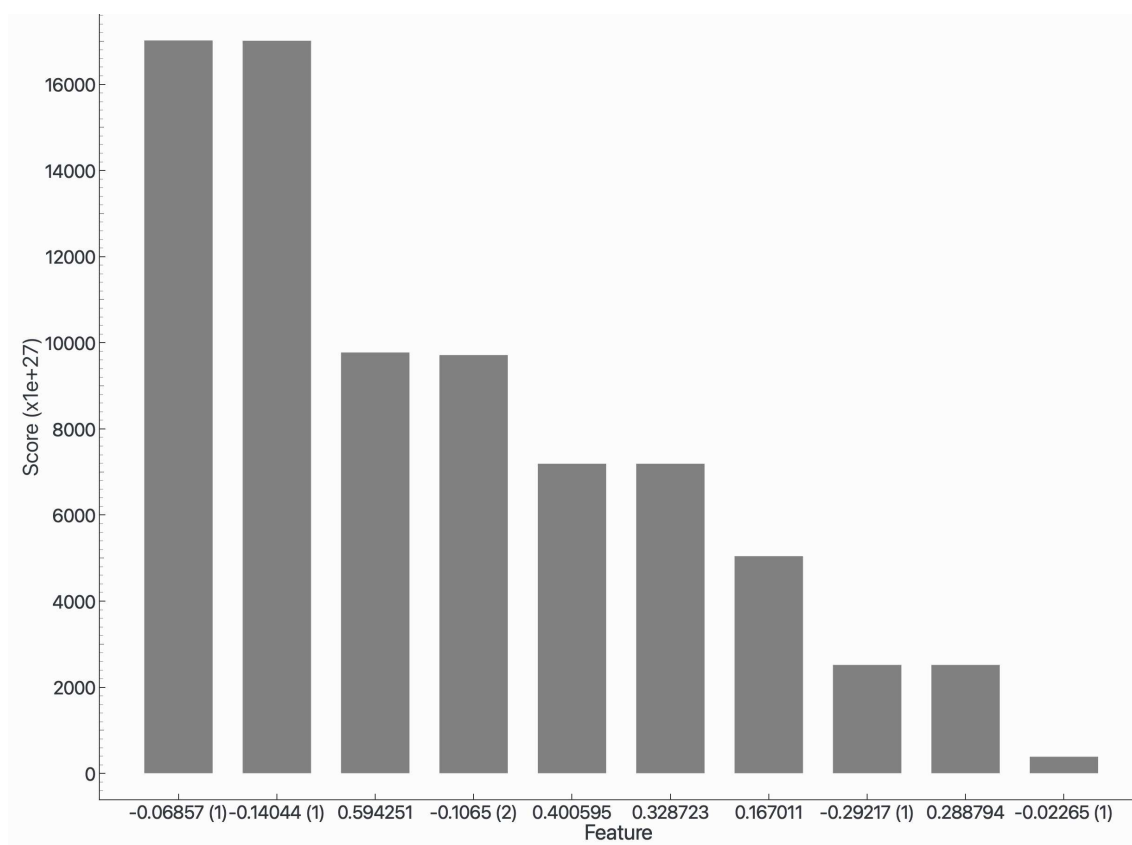


Figure 8 – SHAP demonstration used by the Neural Network computational model with the raw data of the reduction curve. The potentials of -0.06857V, -0.14044V, 0.594251V, and -0.1065V were the main attributes used in the applications of the algorithm. The regions of 0.400595V, 0.328723V, 0.167011V, -0.29217V, 0.288794V, and -0.02265V were also used for this differentiation.

4. DISCUSSION

We presented one bio-inspired peptide selected and redesigned using AI, explicitly designed to strongly interact with the S-RBD motif of SARS-CoV-2, to be used in an electrochemical biosensor to detect COVID-19 when coupled with machine learning algorithms. Due to their excellent specificity and sensitivity, peptides are increasingly being employed in electrochemical devices to detect viruses. For example, electrochemical peptide-based sensors for HIV and West Nile Virus can detect an array of clinically relevant viral concentrations [26,27]. Peptides have also been used for specific viral antigens, such as influenza virus antigen and avian influenza virus [28,29]. Even more complex approaches have been tested, utilizing antibody-coated peptide nanotubes for herpes simplex virus type 2 [30]. These findings show that peptides can improve the effectiveness of electrochemical sensors to detect viruses. Still, our study reports the first recorded application of an electrochemical biosensor technology based on screen-printed carbon electrodes functionalized with an AI-generated synthetic peptide to detect the SARS-CoV-2 Spike protein, presenting a viable approach to quick and inexpensive, point-of-care diagnostics.

The biosensor displayed good sensitivity by detecting different concentrations of VSV-eGFP-SARS-CoV-2 in affinity assays. Serial dilutions were made in a 1:2 ratio, starting from an initial concentration of 1.44×10^5 ffu and decreasing to 2.23×10^3 . The CV measurements indicated separate peaks for each concentration level, with the highest signal detected at the starting concentration. The peak currents reduced with each consecutive dilution, demonstrating the capability of this biosensor to differentiate between different virus loads. Several electrochemical technologies have been developed to detect viral concentrations, with benefits including label-free detection, quick on-site analysis, and high sensitivity [31,32]. An electrochemical sensor based on gold nanoparticles was created to detect HIV-1 virus-like particles label-free, with a detection range of 600 fg/mL to 375 pg/mL, allowing direct electron flow between the virus and the electrode surface [31].

Similarly, a sponge-based electrochemical sensor that can detect the H1N1 virus with a limit of detection of 0.4 TCID₅₀/mL and a practical concentration range of 1-10⁶ TCID₅₀/mL has been presented before [32].

Regarding SARS-CoV-2 detection, a simple electrochemical biosensor device coupled with screen-printed gold electrodes functionalized with a particular synthetic peptide to detect the interaction between the peptide and the SARS-CoV-2 Spike protein using electrochemical impedance spectroscopy [33]. This last biosensor was tested using commercial Spike protein solutions and lysed SARS-CoV-2 particles, in opposition to our study, where we used entire viral particles. In this context, our study represents an advancement in the detection of the virus, considering that interaction with other viral proteins might affect the virus's binding ability to the sensor [34].

Pooled saliva samples from healthy individuals injected with VSV-eGFP-SARS-CoV-2-S produced unique voltammogram peaks, suggesting successful viral identification in the saliva matrix. The biosensor achieved 100% sensitivity and 80% specificity, yielding 90% accuracy. ROC curve research revealed an AUC of 0.9532, demonstrating the sensor's excellent efficacy in detecting real positives while limiting false positives, making it a dependable instrument for viral detection in saliva samples. Several investigations have shown either pooled or artificial saliva may be used as a matrix to identify SARS-CoV-2. It has been investigated using immunochromatographic and automated molecular tests, which yielded encouraging findings but did not offer comprehensive sensitivity, specificity, or accuracy values [35,36]. Studies have also looked at the stability of SARS-CoV-2 RNA in saliva and used an artificially intelligent nanopore for fast testing [37,38]. Overall, our biosensor performance with pooled saliva spiked with VSV-eGFP-SARS-CoV-2-S provided clear and quantifiable results, demonstrating high sensitivity and specificity compared to the literature.

Saliva samples from symptomatic individuals were tested using our AI-generated peptide-based electrochemical sensor. The ROC curve analysis produced an AUC of 0.69, showing moderate discriminating power; however, the p-value of 0.1509 indicates this discrimination is not statistically significant. Sensitivity and specificity were 70%, emphasizing the potential of saliva analysis for COVID-19 identification and the need for additional diagnostic refinement and validation. In this context, the composition of saliva in infected individuals has a considerable influence on the outcomes of salivary diagnostic techniques. Saliva includes a variety of biomolecules, such as DNA, RNA, proteins, and metabolites, which can change in the presence of illness [39]. However, this changed

composition of saliva in infected individuals might impair the accuracy of salivary diagnostic techniques, potentially resulting in false positive or false negative findings [39].

Electrochemical sensors have attractive diagnostic applications, but they are limited by electrode fouling, a low signal-to-noise ratio, and chemical interference [40]. These challenges may be addressed using ML, which improves sensitivity, repeatability, and accuracy in data analysis. ML offers simultaneous measurement of several toxicants [41] and improves cancer biomarker identification [42]. Detection of ischemic heart disease was demonstrated over ten years ago, which attained performance levels equivalent to doctors while significantly boosting sensitivity and specificity [43,44]. Deep learning and machine learning algorithms have also been used for illness prediction and medical diagnostics [45,46]. However, ML applications need big datasets and meticulous picture capture [47]. In our study, we used artificial intelligence algorithms to analyze the spectra of the positive and negative samples, increasing sensitivity, specificity, and accuracy to 90%. Previous research has highlighted the potential of these algorithms in analyzing multi-component and Raman spectra, for instance [48,49].

Further emphasis has been placed on using algorithms in analyzing electromagnetic emission spectrum data [50]. Nevertheless, the application of machine learning is still embryonic, and our approach represents a novel mechanism for analyzing the spectra generated by cyclic voltammetry. Algorithms have demonstrated tremendous promise in assessing electrode reaction processes and interpreting data from polarography, linear scanning voltammetry, and electrochemical impedance spectroscopy [51,52]. It has also been used to model smartphone-based electrochemiluminescence sensor data and pinpoint accurate signs of localized corrosion [53,54]. Despite advances, commercialization of point-of-care devices remains difficult due to the requirement for expensive reagents, equipment, and skilled staff [55]. Although, further studies with a higher number of samples in multicentric cohorts are pivotal for additional validation, this present study emphasize the importance of AI in improving electrochemical analysis, and our research contributes to further its application to a different branch of analytic electrochemistry for salivary diagnostics.

5. CONCLUSION

The functionalization of the electrode with BIAI1, a bio-inspired peptide selected and redesigned using AI, to perform an electrochemical biosensor supported by the Neural Network machine learning algorithm offers a promising tool for portable, non-invasive, and scalable COVID-19 diagnostics using saliva. This technology could significantly enhance the capacity for rapid screening and early detection of SARS-CoV-2.

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CAPÍTULO 4

*Synthetic Mimotopes of Salivary Peptides
Against SARS-CoV-2 in vitro*

Synthetic Mimotopes of Salivary Peptides Against SARS-CoV-2 in vitro

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ABSTRACT

SARS-CoV-2 requires its RBD motif in the Spike protein to interact with specific cellular receptors, such as ACE2, in human cells to replicate. Hence, we aimed to select high-affinity naturally expressed salivary peptides by bioinformatics to assess their potential to block Spike-RBD/ACE2 interaction in vitro and to evaluate their SARS-CoV-2 antiviral performance. We designed a pipeline with 2193 salivary peptides and performed molecular docking with 298 peptides using BLASTp. In silico molecular docking was evaluated using HPEPDOCK and validated by molecular dynamics with GROMACS-2020.3 against crystallographed Wuhan wild-type SARS-CoV-2 (SARS-CoV-2_{WT}) Spike-RBD and the four best-performing peptides were re-docked against Gamma, Delta, and Omicron Spike-RBD. The four selected peptides were synthesized, and antiviral activity was evaluated using VSV-eGFP-SARS-CoV-2 pseudotyped virus and SARS-CoV-2_{WT}. Finally, we evaluated the inhibition of cell death in SARS-CoV-2_{WT} and viral replication inhibition of Gamma and Omicron variants by synthetic mimotopes of salivary peptides by quantifying viral titers using real-time PCR. Salivary peptides SalivaPep1, SalivaPep2, SalivaPep3, and SalivaPep4 had higher binding energy to SARS-CoV-2_{WT}-Spike-RBD, and their effects were maintained against variants, suggesting salivary peptide interactions with SARS-CoV-2. The VSV-eGFP-SARS-CoV-2 infection inhibition rates of selected peptides ranged from 67.5% to 37.6%, respectively, at about 100% cell viability. SalivaPep1, SalivaPep2, and SalivaPep3 reduced cell death from viral replication against SARS-CoV-2_{WT}. Lastly, real-time PCR showed up to 75% decrease in viral copies against the Gamma variant and 62.5% against the Omicron variant by the treatment with SalivaPep3 and SalivaPep2, derived from salivary Ataxin-1 and Statherin, respectively. The presented bioinformatics workflow was profitable to select naturally occurring salivary peptides on-demand with confirmed antiviral properties in SARS-CoV-2_{WT}, Gamma, and Omicron variants by synthetic mimotopes of salivary peptides, with potential application to prophylactic, diagnostic and therapeutic strategies towards COVID-19.

Keywords: Antivirals; Salivary peptides; SARS-COV-2; Infection Control; Bioinformatics.

1. INTRODUCTION

SARS-CoV-2, the causative virus of COVID-19, has led to over 37.5 million cases in Brazil and more than 775 million globally, with almost 7 million deaths (Mallah et al. 2021; Singh et al. 2021; World Health Organization, 2023). The disease varies from asymptomatic to severe respiratory syndromes. (Karlinsky and Kobak, 2021). Aside from the symptoms occurring during COVID-19, it is possible to recognize long-term consequences after SARS-CoV-2 viral infection, such as fatigue, sleep difficulties, anxiety, depression, impaired pulmonary capacities, and Long Covid-19 (Andrade et al. 2021; Huang et al. 2021; Moneer et al. 2022 May 10). Detection is typically via PCR tests on nasopharyngeal swabs; however, a recent meta-analysis indicates that saliva presents similarity in SARS-CoV-2 RNA detection, providing a non-invasive detection of COVID-19 (Caixeta et al. 2023). Preventing infection by hindering viral replication remains the best strategy against new outbreaks (Sharma, Ahmad Farouk and Lal, 2021). Currently, there are very few antiviral drugs licensed for COVID-19, however they are constrained and expensive, which hinders their application, particularly in low- and middle-income countries (LMICs) (Pan et al. 2022; Butler et al. 2023; Liu et al. 2023; Rezapour et al. 2023).

SARS-CoV-2, a member of the Coronaviridae family, is composed of the following structural proteins: spike protein (S), matrix protein (M), and envelope protein (E) (Lu et al. 2020). For virus entry into the host cells, the S receptor-binding domain (RBD) interacts with the host cellular receptor (Zawilska et al. 2021). The most well-characterized SARS-CoV-2 human receptor is the angiotensin-converting enzyme 2 (ACE2), although transmembrane serine protease 2 (TMPRSS2), CD147 and NRP1 have also been identified as viral entry receptors (Cantuti-Castelvetri et al. 2020; Wang et al. 2020; Zhang et al. 2021). ACE2 receptors are highly expressed on oral mucosae, characterizing it as a favorable site for SARS-CoV-2 infection (Surboy et al. 2021).

Human saliva is an attractive alternative diagnostic tool due to its non-invasive self-collection method and low cost (Caixeta et al., 2023). It includes both glandular and serum components that may be utilized to detect systemic and oral disorders, and its collection also presents a low risk of nosocomial infection (Caixeta et al., 2023). Human saliva has antiviral and antibacterial

activity, working through innate or acquired immune pathways to enhance oral health (Fábián et al. 2012). The immune activity of saliva against viruses stems from specific antibodies and naturally occurring peptides interacting with viral surfaces to block replication (Malamud et al. 2011). Saliva also contains natural defense mechanisms against certain viruses, such as Zika; however, it has not been shown to prevent SARS-CoV-2 infection (Conzelmann et al., 2020).

In this context, bioinformatic approaches such as molecular docking and molecular dynamics have all been practical in finding antiviral compounds. These approaches were utilized to identify inhibitors of the Nipah virus glycoprotein and to screen broad-spectrum inhibitors of coronavirus 3CLpro (Berry et al. 2015; Kalbhor et al. 2021). These techniques were also used to identify therapeutic compounds for HCV and SARS-CoV-2 (Anwar et al. 2020; Peele et al. 2020).

We hypothesize that specific salivary peptides may prevent SARS-CoV-2 cell entry to the host cells by interacting with RBD, potentially preventing COVID-19. Hence, we aimed to identify salivary peptides capable of interacting with SARS-CoV-2 and to validate their antiviral activity targeting viral entry blockage.

2. MATERIALS AND METHODS

Salivary peptides, peptide homology, molecular docking, and molecular dynamics

Based on 2193 salivary peptides (Caseiro et al. 2013), We used an in-house previously developed pipeline (Palmeira and Sena 2021) for modeling and docking 298 sequences. This pipeline uses the BLASTp algorithm (Altschul 1997) to filter homologous reference crystallographic 3D protein structures from the Protein Data Bank (Berman 2000) and then perform peptide-protein molecular docking using the HPEPDOCK Server (Zhou et al. 2018). The four best affinity energy peptide-SARS-CoV-2 Spike-RBD complexes from Wuhan, Gamma, Delta, and Omicron variants were manually selected and submitted to a molecular dynamics simulation (MD) of stability of 100 nanoseconds using GROMACS 2020.3 software (Berendsen et al. 1995). Stable peptides validated by MD calculations were selected for *in vitro* experiments.

Cell culture

Vero E6 cells (kidney tissue derived from an average adult African green monkey, ATCC E6) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich) supplemented with 1% penicillin (Gibco Life Technologies), 1% streptomycin (Gibco Life Technologies), 1% non-essential amino acids (Gibco Life Technologies) and 10% fetal bovine serum (FBS; Hyclone) at 37 °C in a humidified 5% CO₂ incubator (Ammerman et al. 2008; Grosche et al. 2023).

Cell viability

Cell viability was measured using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (Sigma-Aldrich) method, as previously described (Kamiloglu et al. 2020; Grosche et al. 2023). Briefly, Vero E6 cells were seeded and incubated with peptide-containing medium in different concentrations. After 24h, the supernatant was removed, and 1 mg/mL MTT solution was added to the plates and incubated for 30 min. DMSO replaced the MTT solution to dissolve formazan crystals. Absorbance was measured at 560 nm, and cell viability was calculated using the $(T/C) \times 100\%$ equation.

Antiviral Assays with VSV-eGFP-SARS-CoV-2-S (VSV-S) and SARS-CoV-2_{WT}

A pseudotyped vesicular stomatitis virus (VSV) expressing eGFP as a marker of infection, in which the glycoprotein gene (G) was replaced by the spike protein of SARS-CoV-2 (VSV-eGFP-SARS-CoV-2-S), was used for trial infection assays (Case et al. 2020). VSV-S and peptides at the determined concentration were incubated for 1h before the naïve Vero E6 cells were infected. After 2h, the inoculum was removed, and cells were washed with PBS and filled with DMEM 2%. 24 hours post-infection (h.p.i.), assays were analyzed via fluorescence microscopy and foci of infection were counted. Antiviral activity was calculated using the $(T/C) \times 100\%$ equation (Case et al. 2020; Grosche et al. 2023).

Antiviral assays with SARS-CoV-2 Wuhan-Hu-1, wild-type (WT), were conducted in three scenarios: (i) general infection assay, where Vero E6 cells were infected in the presence or absence of peptides for 48h; (ii) pre-treatment assay, where cells were treated with peptides for 1h before infection; and (iii)

virucidal assay where the virus and peptides were incubated together before infection. The infection rates were determined at 48 h.p.i. by measuring cell death due to the infection using a cellular viability assay (Song et al. 2014).

Antiviral Assays with SARS-CoV-2_{WT} and Variants Gamma and Omicron Measured by qPCR

Antiviral assays were performed by infecting Vero E6 cells with SARS-CoV-2 Variants Gamma and Omicron and treated with each peptide at 50 µg/mL. 24 h.p.i., the supernatants were collected, viral RNA was extracted, complementary DNA was synthesized, and a real-time PCR for viral titer quantification was performed (Grosche et al., 2023).

Statistical analysis

Individual experiments were performed in triplicate, and all assays were performed a minimum of three times to confirm the reproducibility of the results. GraphPad Prism 8 software was used to assess statistical differences in means of readings using the Ordinary One-Way ANOVA test in multiple comparisons. P values < 0.05 were statistically significant.

3. RESULTS

Peptides bind to Spike-RBD using molecular docking

Using a pipeline for modeling and docking sequences, we filtered 2193 protein structures with BLASTp and performed molecular docking, then selected and simulated stable peptide-SARS-CoV-2 Spike-RBD complexes for in vitro experiments. Four peptides (SalivaPep1 - PGPRYQPFRLT, SalivaPep2 - GRFGYGYGPY, SalivaPep3 - PAAAPPTLPPYFMKGSIIQLANGELKKV, and SalivaPep4 - RIGRFGYGYGPY) were selected for their high scores and MD stability. Peptides SalivaPep2 and SalivaPep4 are derived from Statherin, while SalivaPep3 and SalivaPep1 are derived from Ataxin-1 and Period circadian protein 1, respectively. Their interaction with the SARS-CoV-2 spike RBD protein is represented 3D (**Figure 1 A-D**), as well as protein-peptide interaction is detailed as 2D interaction maps (**Figure 2, A-D**) in which is possible to see that

SalivaPep1 formed the highest number of hydrogen bonds (9), and in second place SalivaPep2 and 3 with four, while SalivaPep2 made only one hydrogen bond with the RBD region. On the other hand, all peptides formed electrostatic interactions with the protein receptor, which is crucial for their stability inside the RBD.

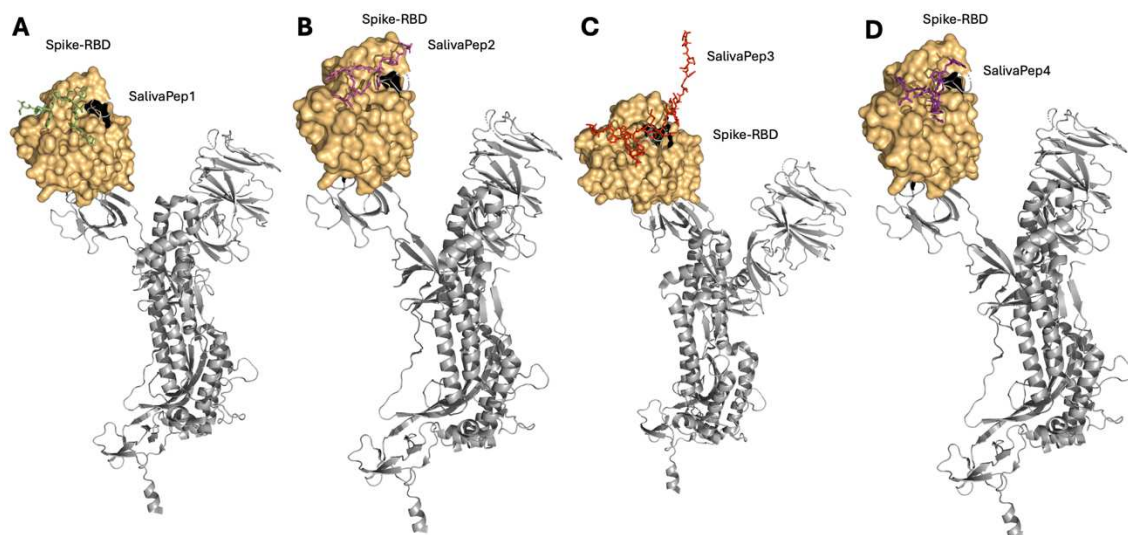


Figure 1. 3D docking representations for selected salivary peptides interacting with SARS-CoV-2 Spike-RBD. The Spike protein is represented in grey and RBD in gold. Molecular interaction is represented as (A) for SalivaPep1 in green sticks; (B) SalivaPep2 in pink sticks; (C) SalivaPep3 in red sticks; (D) and SalivaPep4 in purple sticks.



Figure 2. 2D interaction maps for SalivaPep1 (A), SalivaPep2 (B), SalivaPep3 (C), and SalivaPep4 (D) which interact with SARS-CoV-2 Spike-RBD. Above the dotted lines are the peptides, and below them is the Spike-RBD complex. Hydrogen bonds are presented as green dotted lines between the peptides and the Spike-RBD complex.

Table 1 analyzes the docking affinity energies of four selected salivary peptides with the SARS-CoV-2 spike receptor-binding domain (RBD) of Gamma, Delta, and Omicron variants. The interaction was similar across all variants, with less than 10% energy fluctuation for Omicron compared to Wuhan. Delta and Omicron showed over 10% variation on average. Peptide SalivaPep3 had higher interaction energy with Wuhan and Gamma, while SalivaPep4 performed best with Delta and Omicron. Compared to Wuhan, SalivaPep3 and SalivaPep4 adapted better to Gamma, but SalivaPep4 had the slightest change with Delta.

Table 1. The affinity energy of selected peptides and SARS-CoV-2 S protein based on a *silico* bioinformatic molecular docking assay.

Salivary Peptide	Variants						
	Wuhan	Gamma		Delta		Omicron	
	Kcal/Mol	Kcal/Mol	%*	Kcal/Mol	%*	Kcal/Mol	%*
SalivaPep1	-218.227	-213.746	-2.1	-194.251	-12.3	-216.522	-0.8
SalivaPep2	-209.840	-214.670	2.2	-192.561	-9.0	-218.531	4.0
SalivaPep3	-227.293	-272.072	16.5	-216.101	-5.2	-213.107	-6.7
SalivaPep4	-218.979	-257.878	15.1	-218.512	-0.2	-227.982	3.9

* Percentage of variation when compared to the Wuhan variant

Salivary peptides inhibit VSV-eGFP-SARS-Cov-2 infection.

Cell viability for the selected salivary peptides was above 95%. Salivary peptide SalivaPep3 showed a higher infection inhibition rate at 67.5%. The infection inhibition rate for salivary peptides SalivaPep1 and SalivaPep4 ranged from 44.2% to 49.8%, respectively. Besides, the salivary peptide SalivaPep2 provided 37.6% of infection inhibition (**Table 2**).

Table 2. Effect of salivary peptides on VERO cell viability and VSV-eGFP-SARS-CoV-2 infectivity.

Salivary Peptides	Concentration (µg/mL)	Cell Viability (%)	Inhibition of VSV Infection (%)
SalivaPep1	50	100.2	44.2
SalivaPep2	50	108.5	37.6
SalivaPep3	50	95.1	67.5
SalivaPep4	50	101.6	49.8

Salivary peptide antiviral assays using SARS-CoV-2_{WT}.

In the general infection assay, peptides SalivaPep1 and SalivaPep2 inhibited 32.5% and 31.0% of cell death caused by SARS-CoV-2 infection, respectively. SalivaPep3 inhibited 21.5% of deaths caused by viral infection. In the pre-treatment assay, SalivaPep3 performed best, inhibiting 34.6% of infection, followed by SalivaPep1 (25.3%) and SalivaPep2 (22.2%). In the virucidal assay, SalivaPep2 had the best antiviral effect, inhibiting 28.4% of

infection, followed by SalivaPep3 (24.7%) and SalivaPep1 (21.0%) (**Figure 3**).

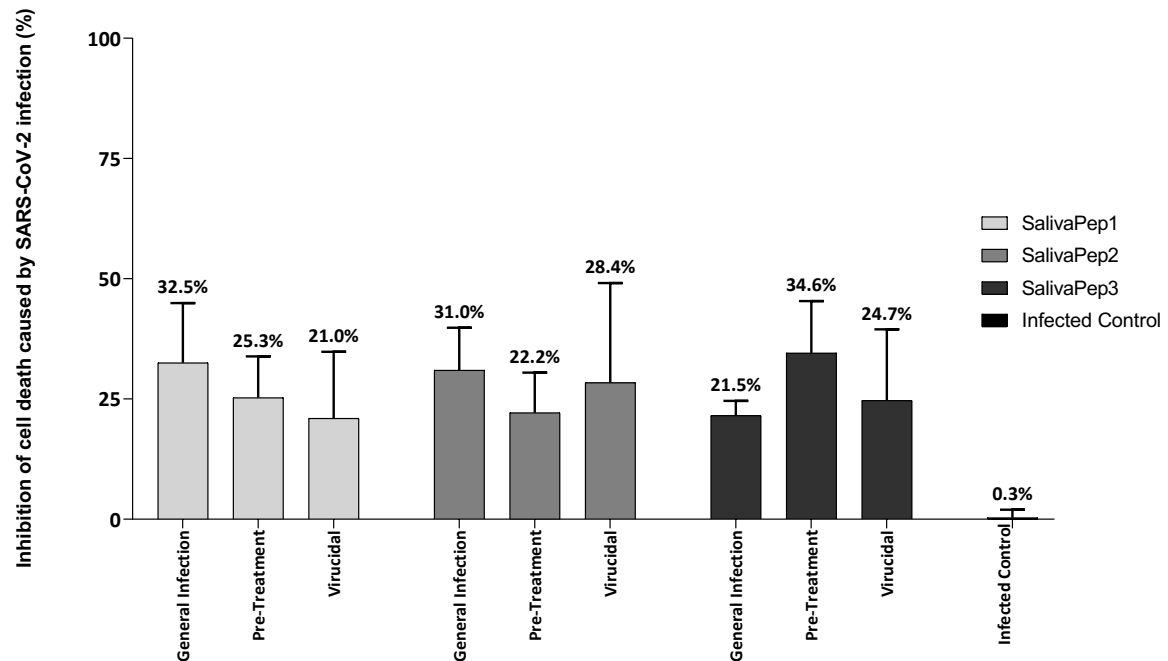


Figure 3. Effect of isolated salivary peptides SalivaPep1, SalivaPep2, and SalivaPep3 in inhibiting cell death caused by SARS-CoV-2_{wt} infection. The number of RNA viral yields at 0,01 MOI on SARS-CoV-2_{wt} Wuhan-Hu-1.

Salivary peptides inhibit infection by Omicron and Gamma variants.

Considering that Omicron and its subvariants have ranked currently as the predominant SARS-CoV-2 strains worldwide and the elevated likelihoods of hospitalization/ admission to ICU among Gamma cases in Brazil, the antiviral effect of salivary peptides was evaluated by titrating viral yields in the presence or absence of treatment using qRT-PCR. On the Omicron variant, the reduction in the RNA viral yields ranged from 40% to 50% on the evaluated peptides compared to the infected control (**Figure 4A**). Finally, for the Gamma variant, SalivaPep1, SalivaPep2, and SalivaPep3 showed up to 70% reduction in RNA viral yields compared to the infected control (**Figure 4B**).

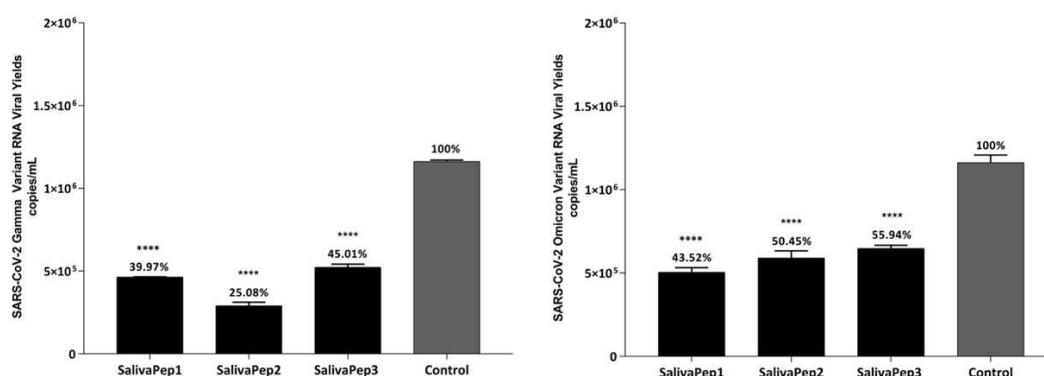


Figure 4A. Effect of isolated salivary peptides SalivaPep1, SalivaPep2, and SalivaPep3 at 50 $\mu\text{g/mL}$ the RNA viral yields at 0,01 MOI on SARS-CoV-2wt Omicron variant. **Figure 4B.** Effect of isolated salivary peptides SalivaPep1, SalivaPep2, and SalivaPep3 at 50 $\mu\text{g/mL}$ the RNA viral yields at 0,01 MOI on SARS-CoV-2_{WT} Gamma variant.

4. DISCUSSION

Our results point to a promising new approach against SARS-CoV-2 infection, demonstrating that naturally occurring salivary peptides can reduce cell death caused by viral infection and SARS-CoV-2 viral replication by up to 34.6% and 74.92%, respectively. The main goal of identifying the salivary peptides that could interact with SARS-CoV-2 spike protein was to evaluate if they could be used as a prophylactic approach to prevent viral infection. By blocking the interaction of SARS-CoV-2 with ACE2 receptors, it is possible to prevent viral infection and replication. Human recombinant soluble ACE2 (hrsACE2) has already been presented as an alternative for preventing viral replication, but it is dose-dependent and more helpful in preventing infection by already infected patients in the early stages than protecting healthy individuals against infection. However, human saliva is a resourceful supply of thousands of peptides that can be used for prophylactic and therapeutic strategies for oral and systemic diseases. Saliva also includes inherent defensive mechanisms against some viruses, including Zika, but failed to inhibit SARS-CoV-2 infection. This has resulted in the development of novel ways to reduce viral transmission and infection in the oral cavity, such as virus-trapping chewing gum and virucidal oral rinses. We also tested the antiviral ability of lyophilized salivary compounds from

healthy individuals' saliva pools in inhibiting viral replication. Nonetheless, this scenario had no antiviral activity (Appendix Figure 1). Together with what has been reported in the literature, these findings underscore the potential for saliva-based therapies to prevent and manage viral infections. We are pioneers in showing that specific naturally occurring salivary peptides were a feasible alternative against SARS-CoV-2.

Many studies have explored the potential of peptides and host defense peptides as antiviral agents. (Barh et al. 2020). Synthetic peptides derived from the variable region of an anti-idiotypic Ab representing the functional internal image of a yeast killer toxin showed significant antiviral activity against the herpes simplex virus. (Sala et al., 2018). Similarly, ovotransferrin-derived peptides exhibited antiviral activity against Marek's disease virus (Giansanti et al. 2005). Also, the broad-spectrum antiviral activity of human host defense peptides, including alpha- and beta-defensins and LL-37, has been reported (Brice and Diamond 2020). Regarding the antiviral activity of peptides targeting SARS-CoV-2, ACE2 analogs and cyclic peptides targeting the main protease of the virus have presented promising results (Johansen-Leete et al. 2022; Quagliata et al. 2023). In this context, peptide-based antivirals are characterized by low toxicity and can potentially overcome resistance issues associated with conventional medications (Vilas Boas et al. 2019; Zakaryan et al. 2021). Nevertheless, to our knowledge, our research is the first to evaluate the antiviral activity of naturally occurring salivary peptides present in saliva against coronaviruses.

There are several potential mechanisms through which salivary peptides inhibit the interaction between SARS-CoV-2 spike protein and ACE2 receptors. Computational approaches have found peptide-based drugs that can bind to the spike protein-interacting surface of ACE2 (Baig et al. 2020; Egieyeh et al. 2021). Also, synthetic peptides targeted to ACE2 interaction hotspots have shown considerable effectiveness in inhibiting the spike-ACE2 interaction, and peptide inhibitors have been shown to cause structural changes in the spike protein, which disrupts its interaction with ACE2 (Souza et al. 2020; Sadremontaz et al. 2022). Moreover, structure-activity relationship studies have shown the important significance of amino acid residues in peptide-spike protein binding considering that short decoy peptides produced from the spike protein's receptor-binding domain successfully prevented viral attachment to host cells (Chowdhury et al.

2020; Loi et al. 2023). Thus, peptide-based techniques have presented great potential for developing treatments against SARS-CoV-2 (Dërmaku-Sopjani and Sopjani 2021).

Since synthesizing and evaluating 2193 salivary peptides to evaluate their ability to bind to the Spike-RBD motif would be expensive and time-consuming. In this context, molecular docking and dynamics assays present a feasible alternative to ranking and selecting the best-performing peptides (Mahmud, Paul, et al. 2021). We used batch homology search of proteins based on the possibility of interactions, considering it is a well-established procedure in bioinformatics and aids in decision-making (Altschul 1997). Similar approaches have previously presented efficiency between 69.4% and 79.4% using HADDOCK (Trellet et al. 2013), representing a bottleneck of only 30% of our peptide database, with more than 1500 peptides to be synthesized and tested. Here, we showed that using HPEPDOCK (Zhou et al. 2018), we were capable of ranking peptides that interacted with our target domain in SARS-CoV-2 and selecting suitable salivary peptides with high docking interaction and persistent interaction in molecular dynamics with the variants of the virus (Appendix Figure 2 and 3). The salivary peptides interact with Spike-RBD at different regions through several hydrogen bonds. Hydrogen bonding plays a crucial role in the antiviral activity of various compounds, including arabinosyladenine, 2'-deoxyuridines, acyclic nucleoside analogs, and nucleoside reverse transcriptase inhibitors (De Clercq, 2023). This is further supported by the correlation between hydrogen bonds in benzaldehyde derivatives and their antiviral properties (Tolstorozhev et al. 2012). The importance of hydrogen bonding in antiviral activity is also highlighted in the design, synthesis, and evaluation of thiourea derivatives, which exhibit significantly higher anti-TMV activity (Lu et al. 2015). Thoroughly, considering the results of the in-silico assays with SalivaPep3, SalivaPep4, SalivaPep1, and SalivaPep2, these salivary peptides were chosen as the best-performing peptides, so we proceeded with peptide synthesis and in vitro assays. Considering the higher number of amino acids, the salivary peptide SalivaPep3 was commercially synthesized. The other salivary peptides, SalivaPep4, SalivaPep1, and SalivaPep2, were synthesized in-house according to the methodology presented before (Appendix Figures 4 and 5).

Furthermore, molecular docking and molecular dynamics simulations have

exhibited promise in predicting peptide antiviral activity against a variety of viruses, including SARS-CoV-2, Zika, and dengue. These approaches have been used to screen a large number of peptides, identify interesting candidates, and better understand binding processes (Hossain et al. 2023; Chaurasiya et al. 2023 Feb 27). A strong agreement between computational predictions and experimental findings has also been reported (Jesús et al. 2012; Isa et al. 2019). Thus, integrating various approaches, such as docking and molecular dynamics simulations, can enhance dependability by overcoming some of the constraints of individual methods (Mahmud, Biswas, et al. 2021; Biswas et al. 2022).

SARS-CoV-2 is a virus with high morbidity, mortality, and transmission rates, so the use of pseudotyped viruses is an alternative for reducing the risks concerning infection (Grosche et al. 2023). Based on that, we performed the first in vitro assays using an infectious molecular clone of vesicular stomatitis virus (VSV) expressing eGFP as a marker of infection, and we replaced its glycoprotein gene with the Spike protein from SARS-CoV-2, ensuring that the assays could be performed at a biosafety level 2 laboratory (Case et al. 2020). Our infection inhibition and cell viability results concerning the four selected peptides showed high cell viability and low toxicity for all salivary peptides, even at high concentrations. SalivaPep3 was the peptide with the highest infection inhibition rate - up to 67.5% - in agreement with the results from our *in-silico* assays, where it presented the lowest interaction energy necessary and the best molecular interaction and dynamics results. On the other hand, SalivaPep2 had a high cell viability rate and, therefore, presented lower toxicity and safety, focusing on virus inhibition. Peptides SalivaPep1 and SalivaPep4 presented similar cell viability (100.2% and 101.6%, respectively) and similar infection inhibition rates (44.2% and 49.8%, respectively). Other studies have already tested SARS-CoV-2 infection inhibition using peptides with high viability and efficient infection inhibition (Curreli et al. 2020; Kandeel et al. 2021; Souza et al. 2022); however, our peptides were used at a 50 µg/mL concentration, which is a high concentration compared to the natural occurrence of these peptides in saliva. Hence, our results on cell viability are incredibly positive, and, considering that they are naturally occurring salivary peptides, their infection inhibition rates are also satisfactory for prophylactic and therapeutic strategies, which led to the second round of in vitro assays using Wuhan-Hu-1 infectious virus at a biosafety

level 3 laboratory.

For the assays against the Wuhan-Hu-1 infectious virus, three peptides were selected to validate the results from VSV-eGFP-SARS-CoV-2 assays: SalivaPep3, since it was the best-performing peptide on the in-silico assays and showed the highest infection inhibition on the first in vitro assay. SalivaPep1 and SalivaPep2 were selected for their antiviral potential and ease of synthesis. For these new analyses, three scenarios were set: general infection assay, pre-treatment assay, and virucidal assay. In the pre-treatment assay, peptide SalivaPep3 inhibited infection by 34.5%, indicating that it might be used prophylactically. In the general infection assay, the peptides SalivaPep2 and SalivaPep1 inhibited infection by 31% and 32.5%, respectively. Based on these findings, the tested salivary peptides exhibited reasonable infection inhibition rates, averaging 27.16% across the three situations, indicating its potential application against SARS-CoV-2 infection. These positive findings suggest that all three peptides have comparable efficacy and promise in treating SARS-CoV-2 infection. Also, the peptide SalivaPep3 presented a promising infection inhibition rate on the pre-treatment assay, with satisfactory overall performance. Our results indicate that each peptide presents its peak performance at different infection scenarios, suggesting that their combined use represents an effective alternative for preventing viral infection. Several peptides have been identified as potential virucidal agents against SARS-CoV-2, among cyclic peptides that inhibit the main protease of SARS-CoV-2, with some demonstrating antiviral activity in vitro and designed stapled peptides based on the ACE2 receptor, which showed potent antiviral activity in cell assays (Curreli et al. 2020; Johansen-Leete et al. 2022). Also, rationally designed ACE2-derived peptides inhibited SARS-CoV-2 infection, with some also inhibiting the replication of a common cold-causing coronavirus (Larue et al. 2021). These studies collectively suggest the potential of various peptides as virucidal agents against SARS-CoV-2. However, our peptides not only reinforce the findings of the previously published data but also present new perspectives on their use in different stages of the virus's replicative cycle.

When evaluating the impact of the peptides on the number of RNA viral yields on the *Omicron* and *Gamma* variants, the reduction varied from 40% to 70% compared to the infected control. Recent studies have identified several

broad-spectrum antiviral peptides, such as P9, derived from mouse β -defensin-4, which binds to viral glycoproteins and prevents endosomal acidification, thereby blocking membrane fusion and viral RNA release (Zhao et al. 2016). Similarly, a peptide derived from the cowpox virus protein CPXV012 acts as a broad-spectrum antiviral that inhibits infection by interacting with phosphatidylserine in the viral envelope (Luteijn et al., 2020). Despite several studies presenting peptides as broad-spectrum antivirals, including their effect on variants of SARS-CoV-2 (Johansen-Leete et al. 2022; Quagliata et al. 2023), our salivary peptides represent a novel approach to inhibition and reduction of viral load for both *Omicron* and *Gamma* variants using naturally occurred salivary peptides.

As previously reported, our study also presents limitations, such as test condition variability and the possibility of underestimating virus infectivity (Hu and Hsiung 1989). Monitoring viral susceptibility also presents challenges, particularly with novel antiviral drugs and variations (Tisdale 2000). The literature also reports cell cultures and antigen tests for identifying respiratory syncytial virus to be less sensitive (Kellogg 1991). Alternative approaches have been developed to overcome these difficulties, including sensitive viral yield tests, but these techniques still need further improvement (Goebel et al. 2016). Physiologically based pharmacokinetic (PBPK) methods, for instance, have been identified as a viable foundation for quantitative in vitro to in vivo extrapolation (Wetmore 2015; Honda et al. 2019). Moreover, when evaluating in vitro data, it is important to consider pharmacokinetic parameters, protein binding, and bioavailability (Yoon et al. 2012; Quignot et al. 2014). The need of using suitable dosage measurements in in vitro tests is underlined in order to increase extrapolation accuracy considering SARS-CoV-2 research has unique hurdles, including genetic alterations during serial replication in Vero cells (Funnell et al. 2021).

Finally, different studies have explored the potential of various antiviral agents to prevent SARS-CoV-2 infection in the oral cavity. It has been shown that mouth rinses and antiseptics, such as povidone-iodine, chlorhexidine, and essential oils, can reduce salivary viral load and cut the viral load of SARS-CoV-2 (Carrouel et al. 2021; Stathis et al. 2021). Moreover, oral antivirals, such as molnupiravir and nirmatrelvir, have presented significant importance in preventing and treating SARS-CoV-2 infection, particularly in the context of

waning immunity and emerging variants (Soriano et al. 2022). Peptides are viable alternatives to existing antibiotics, with broad-spectrum action against bacteria, viruses, and fungi (Vilas Boas et al. 2019; Vanzolini et al. 2022). Promising outcomes have been demonstrated for treating viral infections in aquaculture and reducing antibiotic resistance (Dutta and Das 2015; Cárdenas et al. 2020). Future research should concentrate on improving stability, lowering toxicity, optimizing administration, and performing appropriate bioassays to confirm and increase effectiveness (Eckert 2011; Browne et al. 2020). Thus, considering the effect of these selected salivary peptides in preventing viral cellular entry and replication, they present the potential to be used as non-toxic antivirals against COVID-19.

5. CONCLUSION

The molecular docking energy, the interaction maps and dynamics of peptides SalivaPep1, SalivaPep2, and SalivaPep3 with the SARS-CoV-2 Spike-RBD suggest they could prevent viral replication by blocking ACE2 receptor interaction. The *in vitro* performance of these salivary peptides indicates potential for use in oral antiviral drug delivery systems, possibly aiding in COVID-19 prevention. They also show promise as a safe, effective, and selective anti-SARS-CoV-2 agent.

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Fernando Rodrigues Goulart Bergamini contributed to the validation.

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Robinson Sabino-Silva contributed to Conceptualization, Methodology, Resources, Data curation, Writing—review and editing, Supervision, and Funding acquisition.

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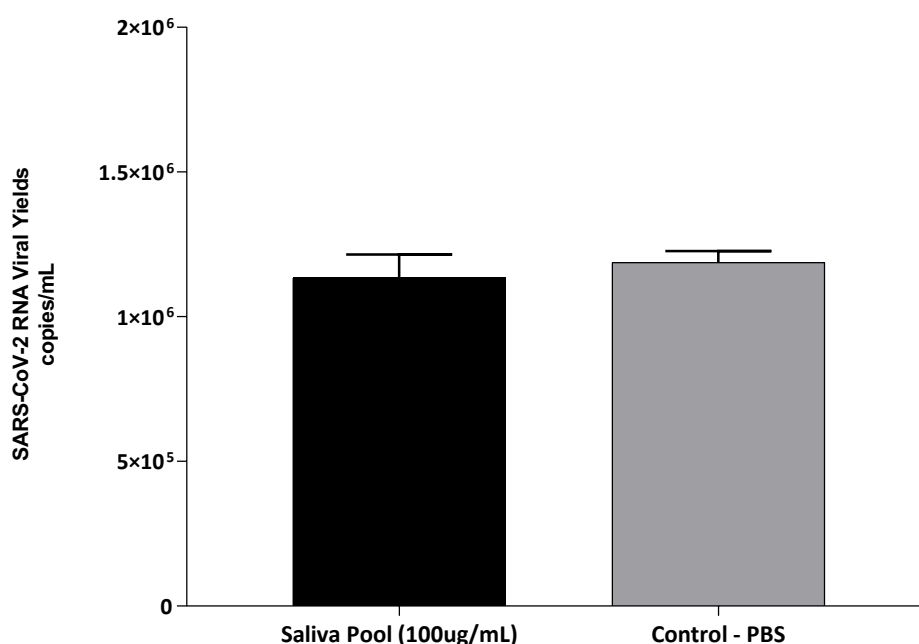
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SUPPLEMENTARY MATERIAL

Salivary Pool and Nasal Wash Assay with SARS-CoV-2_{WT}

SARS-CoV-2 Wuhan-Hu-1 virus and salivary pool were previously incubated for 1h. Vero E6 cells were infected with the inoculum at 37°C for 2h. The supernatant was removed, the cell monolayers were gently washed with PBS, and the wells were completed with DMEM 2 %. 24 hours post-infection (h.p.i.), the supernatant was collected; viral RNA was extracted; complementary DNA was synthesized; and a real-time PCR for viral titer quantification was performed. The mean values of three independent experiments measured in triplicate, including the standard deviation, are shown. P values < 0.01 were considered significant (**Appendix Figure 1**).



Appendix Figure 1. SARS-CoV-2 RNA Viral Yields in Vero E6 Cells After Incubation with Saliva Pool and Control. The graph shows the mean values of three independent experiments, each measured in triplicate, with standard deviation indicated.

Molecular Modelling by Peptide Homology

A molecular homology modeling pipeline was developed using the Python 3.8 programming language (<https://www.python.org/>), as well as the Biopython library (<http://biopython.org/>) and the Modeller 9.21 program (Šali and Blundell 1993). The peptide consists of data in sequences2.org via the BLASTp algorithm.

From the search, only peptides with identity above 70% concerning their homolog were modeled, giving 298 modeled peptides.

Molecular Docking

Among the 298 peptides, a molecular coupling with the RBD-Receptor Binding-Domain) of the Spike Glycoprotein of SARS-CoV-2 (PDB: 6LZG) (Wang et al. 2020). The connection server through molecular (docking) was made by the web server HPEPDOCK Server (Zhou et al. 2018). Fitting results were tabulated and ranked by energy score from highest to lowest. **Table 1** lists the selected salivary peptides with high docking scores with the SARS-CoV-2 spike receptor-binding domain (RBD).

Appendix Table 1. Docking score and interaction position of the selected peptides on in silico assay

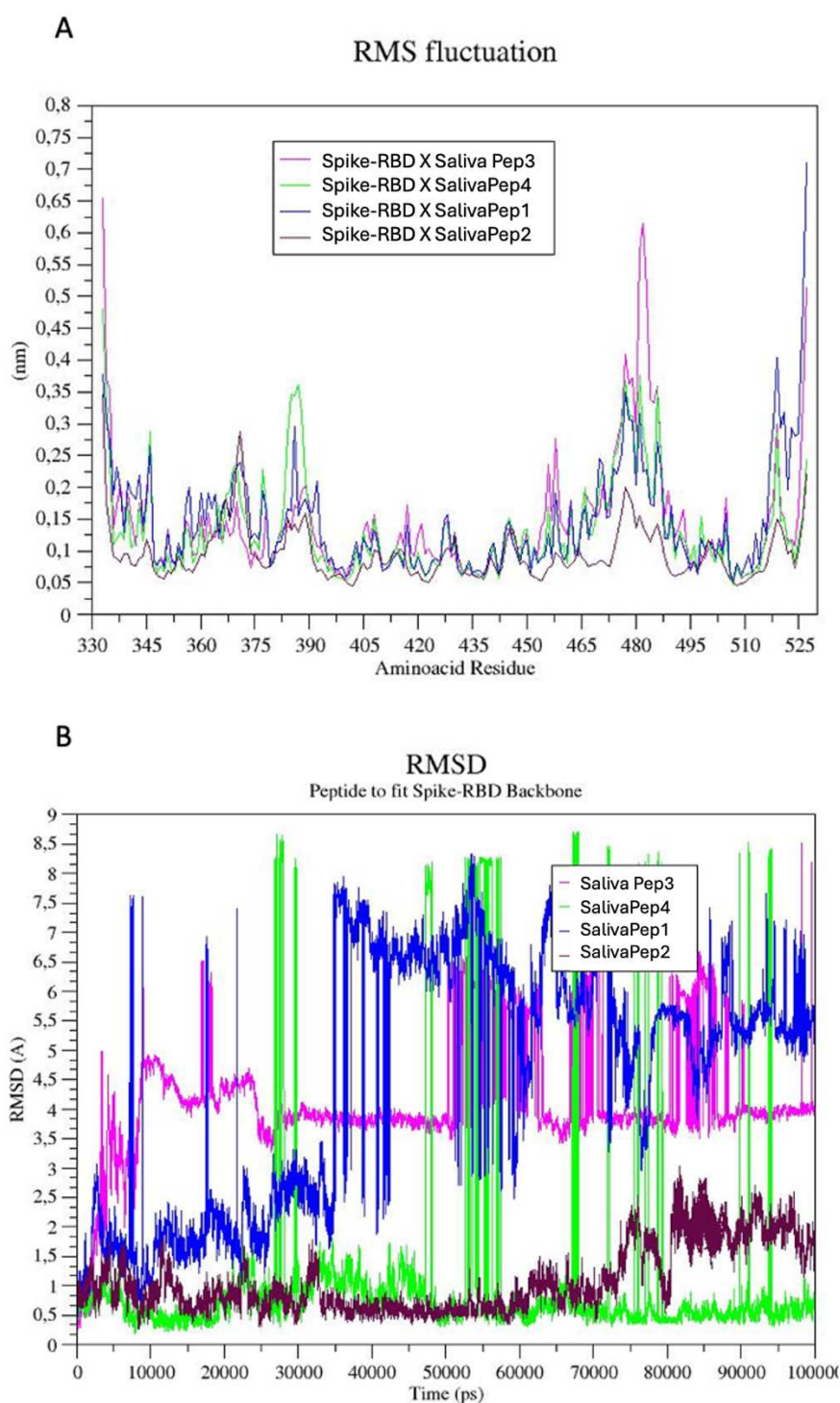
Peptide	Peptide Sequence	Docking Score	Interaction Position
SalivaPep3	PAAAPPTLPPYFMKGSIIQLANGELKKV	-227.293	good
SalivaPep4	RIGRFGYGYGPY	-218.979	good
SalivaPep1	PGPRYQPFRLT	-218.227	good
SalivaPep2	GRFGYGYGPY	-209.840	good

After that, we ranked the peptides based on their interaction level with the Wuhan variant and synthesized the compounds. They were produced using fluorenyl-9-methoxycarbonyl (Fmoc) methodology (Albericio 2004) and Poly-Prep Chromatography Columns (Bio-Rad).

Molecular Dynamics RBD-Peptides

Molecular dynamics calculations were performed on the protein-peptide complexes using the program Gromacs 2020.3 (Berendsen et al. 1995). The OPLS-AA force field (Kaminski et al. 2001) was used considering a simulation time of 100 ns, where the RMSD, RMSF, and short-ranged interaction energy coulombic and Lenard Jones graphs were generated and evaluated. The aminoacidic residue fluctuation graph (RMS) for the Spike-RBD bound to best docking salivary peptides showed that all selected peptides interacted with the

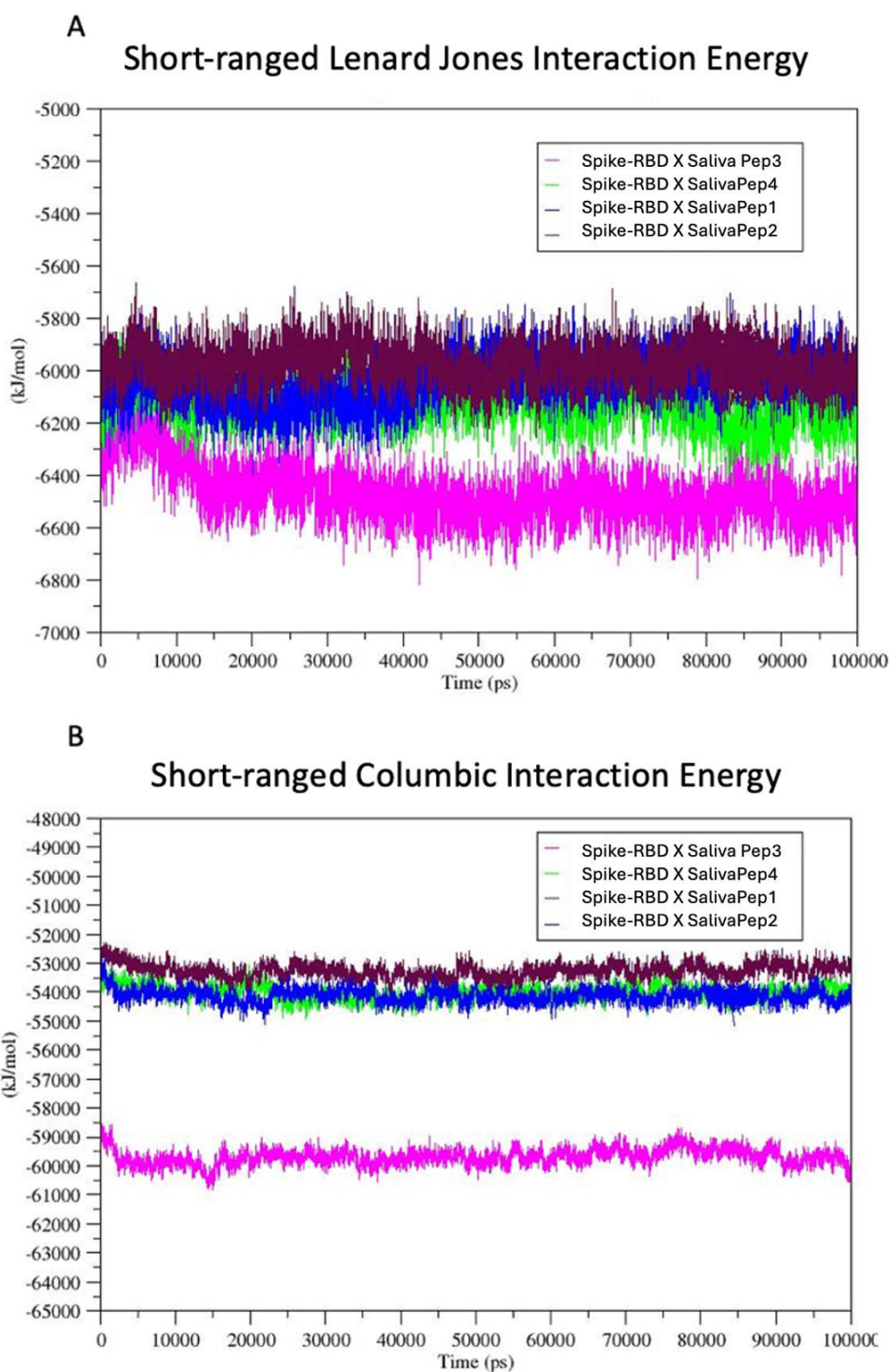
active site and maintained aminoacidic fluctuations below 0.2 nm (2 Å) (**Appendix Figure 2, A-B**). The peptide deviation RMSD graph showed that peptides SalivaPep3, SalivaPep1, SalivaPep4, and SalivaPep2 deviated from the RBD active site during the MD simulation, while peptide SalivaPep1 presented values below 2 Å during almost all simulation time.



Appendix Figure 2. Spike-RBD amino acid fluctuation graph (RMS) for each

protein-peptide complex with the peptides SalivaPep3, SalivaPep4, SalivaPep1, and SalivaPep2 during 100 ns MD simulation (A). Peptide deviation from the Spike-RBD backbone (RMSD) for each peptide – SalivaPep3, SalivaPep4, SalivaPep1, and SalivaPep2 during 100 ns simulation time (B).

Lenard Jones and Coulombic interaction energies result in the interaction between Spike-RBD, and all selected peptides showed negative values for all complexes during all 100 ns MD time, indicating a persistent interaction in this region for all the time. Lenard Jones energies ranged from –6,800 to –5,850 Kcal/Mol, where the complex between Spike-RBD and peptide SalivaPep3 present the best energy values, followed by peptide SalivaPep4, SalivaPep1, and SalivaPep2, respectively. The same pattern was identified for the Coulombic interaction energies calculations, where the graph ranged from –60,500 to –52,500 Kcal/Mol, with peptide SalivaPep3 with the best interaction energy result (**Appendix Figure 3, A-B**).



Appendix Figure 3. *Lenard Jones interaction energy graph for the Spike-RBD complexes with the peptides SalivaPep3, SalivaPep4, SalivaPep1, and SalivaPep2 (A). Coulombic interaction energy graph for the Spike-RBD complexes with the peptides SalivaPep3, SalivaPep4, SalivaPep1, and SalivaPep2 (B).*

Peptide Synthesis

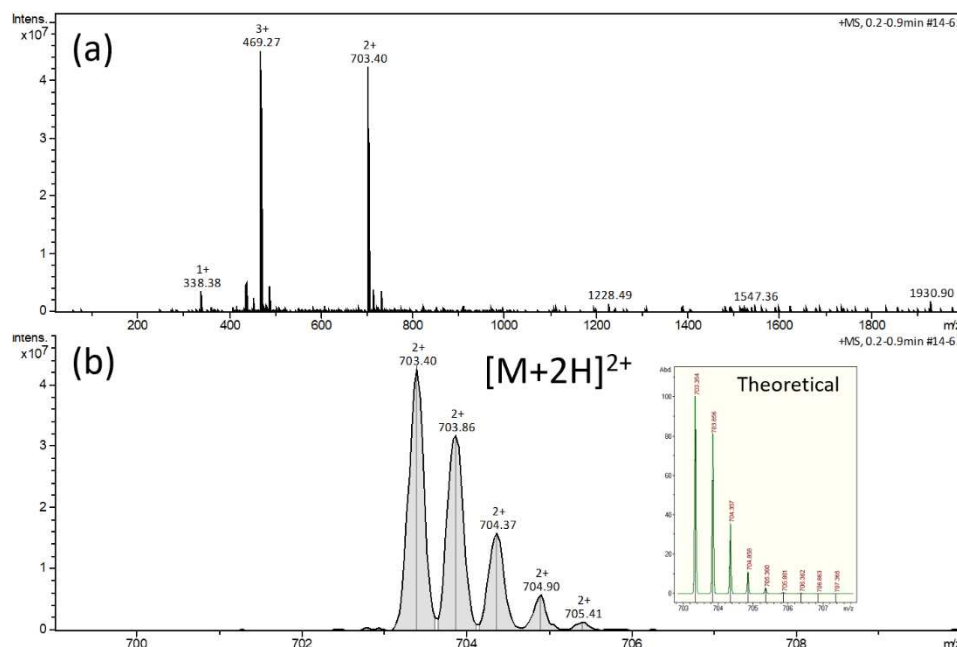
Among the peptides with the highest scores of molecular dockings against SARS-CoV-2 Spike, the peptides SalivaPep1, SalivaPep4, and SalivaPep2 were selected to be synthesized due to their relative sequence homology. The peptide syntheses were carried out according to standard solid-phase procedure employing fluorenyl-7-methoxycarbonyl (fmoc) - protected amino acids (Guy and Fields 1997; Merrifield 1997; Albericio 2004) in Bio-Rad columns. 2-Chlorotrityl chloride resin was used as the solid phase, and 4-methylpiperidine was used as the deprotecting base. The synthesized peptides were then cleaved from the solid phase employing a 2% TFA in DCM (10 mL) solution and deprotected with an acidic solution containing 9mL TFA, 0.5mL H₂O and 0.5mL triisopropylsilane. The peptides were further purified employing preparative HPLC using an ÄKTA purifier chromatograph with a Jupiter C-18 column (250 mm length, 10 mm inner diameter, 10 µm particle size, Phenomenex, Torrance, CA, USA). The samples were dissolved in water and acetonitrile (3:1) and eluted with a 0% to 70% gradient of solution B, where solution A is 0.1% TFA in water. Solution B is 0.1% TFA in 95% acetonitrile for 70 min at RT and 3 mL/min. Peak signals were detected at 215 nm, 254 nm, and 280 nm; these fractions were collected and analyzed by ESI-MS, and those containing the target molecule were selected. The fractions were then gathered and lyophilized to obtain the purified peptide.

Peptide Characterization

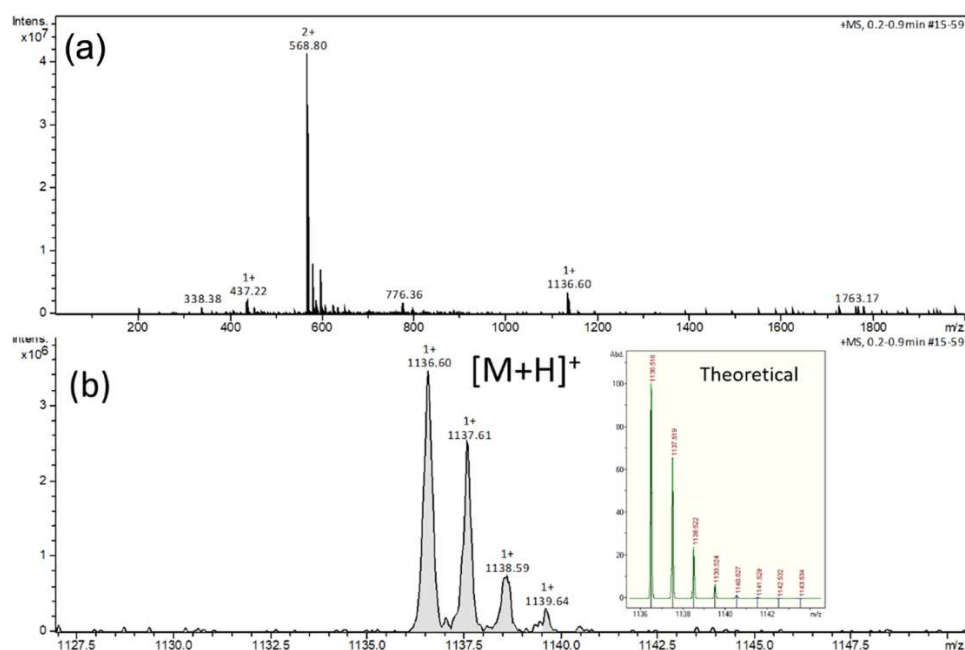
The successful syntheses of the peptides were confirmed by mass spectrometry. The peptide samples were dissolved in diluted in 0.1% TFA in CAN and analyzed in ESI+ mode using the instrument Amazon Speed ETD (Bruker) with the following acquisition parameters: nebulizer:12 Psi, drying gas: 5 L/min, temp: 250°C, HV: 4500V.

For the peptide SalivaPep4 spectrum (**Appendix Figure 4**) (RIGRFGYGYGPY), two signals corresponding to the molecular ion in different charges due to the binding of H⁺ were observed. They were observed at m/z 703.4 and 469.3 and assigned as [RIGRFGYGYGPY + 2H]²⁺ and [RIGRFGYGYGPY + 3H]³⁺ respectively. For peptide SalivaPep2 spectra

(Appendix Figure 5) (GRFGYGYGPY), signals with m/z 1136.60 and 568.80 were assigned to $[\text{GRFGYGYGPY} + \text{H}]^+$ and $[\text{GRFGYGYGPY} + 2\text{H}]^{2+}$ respectively. The calculated isotopic patterns confirm the respective assignment.



Appendix Figure 4. (a) The Mass spectra of peptide SalivaPep4 in MeCN is 0.1% formic acid. (b) The expanded region from m/z 700 to m/z 708 with an insert of the calculated isotopic pattern to ion $[\text{RIGRFGYGYGPY} + 2\text{H}]^{2+}$ (inbox).

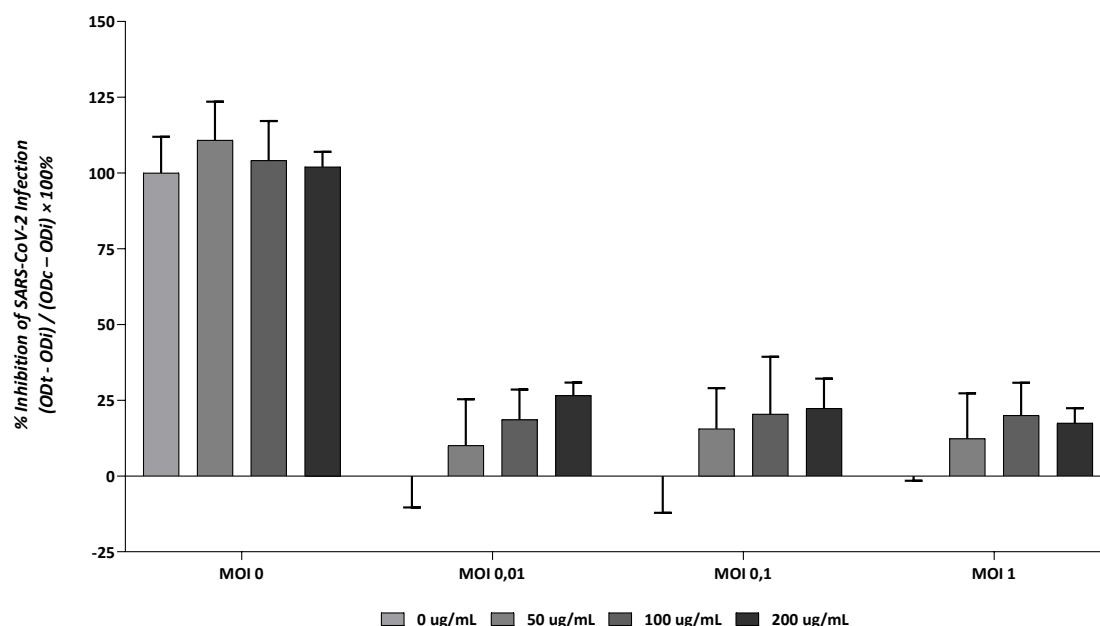


Appendix Figure 5. (a) Mass spectra of peptide SalivaPep2 in MeCN, 0.1% formic acid. (b) The expanded region from m/z 1130 to m/z 1147.5 with the calculated isotopic pattern to ion $[\text{GRFGYGYGPY} + \text{H}]^+$ (inbox).

Salivary peptide SalivaPep3 antiviral assays using SARS-CoV-2wt at different MOIs.

For the antiviral assay with different MOIs, SARS-CoV-2wt, at MOIs of 1, 0,1, 0,01, and 0, and isolated peptide SalivaPep3 at concentrations of 200, 100, 50, and 0 µg/mL were incubated previously for 1h at room temperature. Then, cells were infected with the inoculum at 37 °C. The supernatant was removed 48 hours post-infection (h.p.i.), and the assay was analyzed according to the peptide's cell viability and antiviral effect.

Considering that SalivaPep3 showed the lowest cell viability on the previous assays, this compound was investigated at different concentrations and under infection of SARS-CoV-2 Wuhan-Hu-1 at a range of MOIs (1; 0,1; 0,01, and 0). The results demonstrated that peptide SalivaPep3 presented antiviral activity against SARS-CoV-2 in all MOIs. Even against the highest viral MOI, the 100 µg/mL peptide inhibited over 20% of the infection. On the following MOIs, the highest concentration tested, 200 µg/mL, had a better effect against the SARS-CoV-2, inhibiting 22,3% of the infection at MOI of 0,1, and 26,6%, at MOI of 0,01. All the concentrations tested had cell viability > 100%, meaning the peptide SalivaPep3, even in high concentrations, is non-cytotoxic in *vitro* assays (**Appendix Figure 6**).



Appendix Figure 6. Effect of isolated salivary peptide SalivaPep3 at different concentrations on SARS-CoV-2 Wuhan-Hu-1 infectivity at different MOIs.

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CAPÍTULO 5

*Artificial Intelligence-Generated Peptides
Against SARS-CoV-2 in vitro*

Artificial Intelligence-Generated Peptides Against SARS-CoV-2 in vitro

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ABSTRACT

Background: SARS-CoV-2 replicates in human cells via interacting with certain cellular receptors, such as ACE2. Here, we used bioinformatics coupled to the artificial intelligence algorithms platform SAGAPEP to generate high-affinity peptides and aimed to examine their ability to disrupt viral Spike-RBD/ACE2 interaction in vitro, measuring their antiviral effectiveness against SARS-CoV-2 and their cell viability. **Methods:** The SAGAPEP platform was used to model a bioactive and bio-inspired peptide through in silico molecular docking against the RBD domain of the SARS-CoV-2 Spike-RBD. The three best-performing peptides (BIAI1, BIAI2, and BIAI3) were synthesized, and their antiviral efficacy was tested against VSV-eGFP-SARS-CoV-2 pseudotyped virus and SARS-CoV-2_{WT}. Finally, we examined the prevention of cell death in SARS-CoV-2_{WT} and viral replication inhibition of Gamma and Omicron variants by synthetic mimotopes of salivary peptides, measuring viral titers by real-time PCR. **Results:** The VSV-eGFP-SARS-CoV-2 infection inhibition rates for BIAI1, BIAI2, and BIAI3 ranged from 65.9% to 90.1%, presenting cell viability up to 100%. Lastly, real-time PCR showed up to 83.67% decrease in viral copies against the Wuhan strain and 72.69% against the Gamma variant when treated with BIAI2; whereas a reduction of up to 67.84% against the Omicron variant was achieved in the presence of BIAI1. **Conclusions:** BIAI1, BIAI2, and BIAI3 presented antiviral properties in SARS-CoV-2_{WT}, Gamma, and Omicron variants, with potential application to prophylactic, diagnostic and therapeutic strategies towards COVID-19.

Keywords: Antivirals; Peptides; SARS-COV-2; Artificial Intelligence; Bioinformatics.

1. INTRODUCTION

The SARS-CoV-2 spike protein's receptor-binding domain (RBD) interacts with the human ACE2 receptor to begin viral entry into host cells via a network of salt bridges, hydrophobic and electrostatic contacts, and hydrogen bonding [1]. The SARS-CoV-2 RBD-ACE2 binding has a greater affinity than SARS-CoV-1, which is owing in part to interactions between the SARS-CoV-2 RBD and the N-linked glycan on ACE2's Asn90, with important residues such as K417 playing critical roles in binding [2,3]. Also, the S1 monomer of the RBD has changes that improve ACE2 binding, thus understanding these interactions is critical for developing treatment methods that include neutralizing antibodies and peptide inhibitors of the RBD-ACE2 interface [4,5]. Current COVID-19 diagnostic approaches, including as nucleic acid amplification tests like RT-PCR, serological antibody tests, and imaging techniques, have drawbacks, including false-positive and false-negative findings, especially early in the infection cycle [6]. Antiviral medications, such as remdesivir and favipiravir, are used in combination with supportive care measures, while new treatments, such as PF07321332 and molnupiravir, are being tested in clinical trials [7,8].

In this context, peptides are attractive antiviral therapeutics due to their specificity, chemical variety, and powerful molecular interventions [9]. Synthetic peptides outperform small molecule therapeutics in target affinity and protein-based therapies in cellular penetrability and stability [10]. Cationic antimicrobial peptides, in particular, can damage viral envelopes, preventing virus entrance and reproduction [11]. These peptides can be extracted from natural sources or synthesized using Boc and Fmoc protecting groups, native chemical ligation with chemoselective reactions of unprotected peptides, click reaction, ligation of peptide hydrazides and insertion of plasmid vectors by electroporation to peptide expression [PMID: 23584057] often being more selective and effective [11]. Their self-assembling characteristics enable the development of nanostructures for better medication delivery [12] and have demonstrated antiviral effectiveness against several viruses, including HIV-1, influenza, and coronaviruses [13–15]. Their small size and specific bioactive binding properties make them ideal for interfering with protein-protein interactions [16], positioning peptides as promising therapeutic options for viral infections.

A variety of computational approaches, such as molecular dynamics simulations, molecular docking, and peptide design algorithms, have been used to identify key residues involved in the interaction and design potential inhibitory peptides [17–19]. These strategies have resulted in the creation of promising peptide candidates such as DDFEDY, DEYEDY, and DFVEDY [17], as well as chimeric peptides such as cnCoV-4 to block the SARS-CoV-2 surface domains [19]. Other investigations have focused on creating peptides from ACE2 fragments [20] or utilizing discontinuous peptide libraries [21]. Computational techniques have also been used to analyze peptide characteristics, including binding affinity, allergenicity, toxicity, and [17,22]. Furthermore, AI-driven techniques, such as generative adversarial networks (GANs) and language models, may produce unique peptide sequences with desirable attributes fastly [23,24]. These approaches allow for the synthesis of peptides with higher binding affinity and effectiveness against particular targets, such as viral proteins [25]. The incorporation of deep learning approaches into standard drug development pipelines has sped the identification of potential peptide binders while decreasing the time and expense associated with experimental validation [24].

From this perspective study aims to assess the antiviral efficacy and cell viability of AI-generated synthetic peptides against SARS-CoV-2, investigating their capacity to disrupt the Spike-RBD/ACE2 connection, measuring cell survival after treatment with the peptides, and evaluating their antiviral activity against SARS-CoV-2. Our findings might help to create new prophylactic, diagnostic, and therapeutic techniques for COVID-19.

2. MATERIALS AND METHODS

Peptide development

The Surrogate-Assisted Genetic Algorithm for Peptide Evaluation and Prediction (SAGAPEP) framework was employed in a systematic, multi-stage method to evaluate and identify peptides with high binding potential. The method begins with the acquisition of a data set including 296 peptides, each represented as a linear sequence of amino acids and their respective interaction energies with the SARS-CoV-2 Spike protein. These interactions were assessed using the HPEPDOCK molecular coupling tool, which provided affinity energy values.

Feature extraction methods were crucial in transforming the linear peptide sequences into a format suitable for model training. Three descriptors were considered: amino acid composition (AAC), amino acid linking (AAL), and the composition of k-spaced nucleotide acid pairs (CKSNAP). These properties contributed in the construction of a regression-based surrogate model capable of rapidly estimating the binding energies of new peptide candidates. This surrogate model enabled fast assessment of peptide-protein interactions without the need for time-consuming in vitro experiments. The SAGAPEP framework repeatedly identified and refined peptides by incorporating these properties into a surrogate-assisted genetic algorithm, resulting in the selection of Bio-Inspired Artificial Intelligence 1, 2 and 3 (BIAI1, BIAI2, and BIAI3) peptides (Table 1).

Table 1. Docking score of the peptides generated by SAGAPEP on in silico assay

Peptide	Peptide Sequence	Docking Score
BIAI1	FIRWRFRWWRR	-245,355 Kcal/Mol
BIAI2	RRRWWRRWWRR	-243,513 Kcal/Mol
BIAI3	RWRWWRR	-238,372 Kcal/Mol

Peptide and Spike-RBD interaction validation using infrared spectroscopy

The spectra of VSV-eGFP-SARS-CoV-2; the peptides BIAI1, BIAI2 and BIAI3; and their interaction with VSV-eGFP-SARS-CoV-2 were recorded using Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy (Agilent Cary 630 FTIR, Agilent Technologies, Santa Clara, CA, USA). For this analysis, the lipidic region (3050-2800 cm⁻¹) and the fingerprint infrared signature (1800-800 cm⁻¹) were utilized. Samples were prepared with the peptides and VSV-eGFP-SARS-CoV-2, respectively. A volume of 3 µL of each sample was placed on the diamond cell, forming a thin layer on the ATR crystal. The spectra were then recorded at a resolution of 2 cm⁻¹ with 64 scans. Second derivative spectra were generated from the original data, plotted in Origin Pro 9.8.0.200 (OriginLab, Northampton, MA, USA) software, and adjusted using the Savitzky-Golay algorithm with a polynomial order of 2 and a window of 20 points.

Cell Culture

Vero E6 cells (from an adult African green monkey, ATCC E6) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 1% penicillin (Gibco Life Technologies), 1% streptomycin (Gibco Life Technologies), 1% non-essential amino acids (Gibco Life Technologies), and 10% fetal bovine serum (FBS; Hyclone) at 37 °C in a humidified 5% CO₂ incubator [26,27].

Virus titration

A pseudotyped vesicular stomatitis virus (VSV) expressing eGFP as a marker of infection, in which the glycoprotein gene (G) was replaced by the spike protein of SARS-CoV-2 (VSV-eGFP-SARS-CoV-2), was used for infection assays [28]. The virus was amplified employing Vero cells in 175 cm² flask. To determine viral titers, 1 x 10⁴ Vero cells were seeded in each of 96 well plates 48 hours prior to the infection. Cells were infected with 10-fold serially dilutions of VSV-eGFP-SARS-CoV-2 and supplemented with medium containing 1% penicillin, 1% streptomycin, 2% FBS and 1% carboxymethyl cellulose (CMC). Infected cells were incubated for 48h in a humidified 5% CO₂ incubator at 37°C. The viral foci were counted using EVOs cell imaging systems fluorescence microscopy (Thermo Fisher Scientific), by detecting eGFP expression, to determine viral titer which was expressed in focus formation unit per milliliters (FFU/mL).

SARS-CoV-2 Wuhan-Hu-1 (SARS-CoV-2WT) and variants Gamma and Omicron were amplified employing Vero E6 cells in 175 cm² flask. To determine viral titres, 1 x 10⁵ Vero E6 cells were seeded in each of 24 wells plate 24 hours prior to the infection. Cells were infected with 10-fold serially dilutions of SARS-CoV-2 or variants Gamma and Omicron, and supplemented with medium containing 1 % penicillin, 1 % streptomycin, 1 % non-essential amino acids, and 2 % FBS. Infected cells were incubated for 48h in a humidified 5 % CO₂ incubator at 37°C. After incubation, the medium was removed, and the cells were fixed and stained with 4 % paraformaldehyde and 0,4 % crystal violet staining solution to visualize the formation of foci resulting from the cytopathic effect due to the release of the viral particle. From the foci number, it was possible to determine

the viral titres of the supernatant in pfu/mL [26,28,29].

Cell Viability

Cell viability was measured by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (Sigma–Aldrich) method. Vero cells were seeded in a 96-well plate at a density of 1×10^4 cells per well and incubated overnight at 37 °C in a humidified 5% CO₂ incubator. Drug-containing medium at concentrations of 50 and 10 µg/mL was added to the cell culture. After 24 h at 37 °C, the media was removed and a solution containing MTT at the final concentration of 1 mg/mL was added to each well, incubated for 30 min at 37 °C in a humidified 5% CO₂ incubator after which media was replaced with 100 µL of DMSO to solubilize the formazan crystals. Absorbance was measured by optical density (OD) of each well at 560 nm, using a spectrophotometer. Cell viability was calculated according to the equation $(T/C) \times 100\%$, where T and C represent the mean optical density of the treated group and vehicle control group, respectively [26].

Antiviral Assays with VSV-eGFP-SARS-CoV-2-S (VSV-S), SARS-CoV-2_{WT}, and Variants

Vero cells were seeded at density of 1×10^4 cells per well into 96-well plates 24 h prior to the infection. VSV-eGFP-SARS-CoV-2 at a multiplicity of infection (MOI) of 0.005 and the substance at non-toxic concentration pre-determined on cell viability assays (50, 10, and 2 µmol·L⁻¹) were previously incubated for 1 h at room temperature. Cells were infected with VSV-S and Amazonian substances at 37 °C for 2 h. The supernatant was removed, the cell monolayers were gently washed with 100 µL of ice-cold PBS, and the wells were completed with DMEM 2 %. 24 hours post-infection (h.p.i.), the assays were analyzed at EVOs cell imaging systems fluorescence microscopy (Thermo Fisher Scientific) and the focus of infection was counted. The antiviral activity was calculated according to the equation $(T/C) \times 100\%$, where T and C represent the mean of the treated group and the mean of the last concentration, respectively.

A general assay was performed with SARS-CoV-2_{WT}, and its variants Omicron (HIAE-W.A; GISAID: EPI_ISL_6901961) and Gamma (IMT-MAN87209; GISAID: EPI_ISL_1060981). To this, Vero E6 cells were seeded at a density of 1

× 10⁵ cells per well into 24-well plates before the infection with the virus at a multiplicity of infection (MOI) of 0.01, in the presence or absence of lectins at non-toxic concentrations for 24 h. The supernatant of all assays were collected and frozen for carrying out the following steps of extraction of viral RNA, cDNA synthesis, and real-time PCR for viral titers quantification [26,29,30].

RNA extraction and cDNA synthesis

For the volume of supernatant harvested from each well, a correspondent volume of Trizol (Invitrogen) was added and homogenized. Then, chloroform (Merck KGaA) was incubated with the samples for 3 minutes, and centrifugated at 13,200 rpm for 15 minutes at 4°C. The RNA-containing upper aqueous phase was collected, added of isopropanol (Merck KGaA) for 10 minutes at room temperature, and centrifugated at 13,200 rpm for 15 minutes at 4°C. The supernatant was kindly removed. The pellet was resuspended with ice-cold ethanol-depc 75 % (Merck KGaA), centrifugated at 10,000 rpm for 15 minutes at 4°C, and the supernatant was discarded. The pellet was resuspended in a final volume of 20 µL of nuclease-free water. After the quantification of RNA extracted, the samples were frozen at -80°C immediately [31].

Complementary DNA (cDNA) synthesis was performed using the High-Capacity cDNA Archive® kit (Applied Biosystems) according to the manufacturer's instructions. The prepared reaction was composed of 10X RT Buffer, 25X dNTP Mix (100 mM), 10X RT Random Primers, MultiScribe™ Reverse Transcriptase, RNase Inhibitor and Nuclease-free water and the corresponding volume of viral RNA extracted. The microtubes were submitted to the Veriti Thermal Cycler (Applied Biosystems®), incubated at 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes to generate complementary DNA, in a reverse transcriptase-polymerase chain reaction.

Determination of viral load by real-time PCR

The RT-qPCR reactions were performed using a Taqman Universal PCR master mix kit (Thermo Fisher Scientific, USA). The reactions consisted of 0.96 µM of each primer and 0.48 µM of probe specifics for the viral nucleocapsid (N) gene (2019- nCoV_N1-F: 5'-GAC CCC AAA ATC AGC GAA AT-3'; 2019- nCoV_N1-R: 5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'; and 2019-

nCoV_N1-P: 5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3') (Integrated DNA Technologies, Belgium) [54], and 5 µL of 2X Taqman Universal master mix (Thermo Fisher Scientific, USA) and 1.5 µL of cDNA in DEPEC ultrapure water. Reactions were performed using the following conditions: 94 °C for 10 minutes for enzyme activation; 45 cycles of denaturation at 95 °C for 15 seconds, an-nealing at 60 °C for 1 minute. All amplifications were conducted on a QuantStudio 12 K Flex instrument (Applied Biosystems, Foster City, CA, USA). Serial tenfold dilutions of the standard plasmid of SARS-CoV-2 (2019-nCoV_N_Positive Control, 2×10^5 genome copies/µL (gc/µL) (105, 104, 103, 102, 101 and 100), obtained from IDT (Integrated DNA Technologies, Leuven, Belgium), were used to produce standard curves to quantify the SARS-CoV-2 RNA copies. The limit of detection (LOD) parameters were similar to SARS-CoV-2WT and the variants. Every RT-qPCR assay was performed in triplicate and included negative (nuclease-free water) and positive controls.

3. RESULTS

Peptides bind to Spike-RBD using molecular docking

The interaction of the peptides with the SARS-CoV-2 spike RBD protein is represented in 3D (Figure 1 A-C), as well as protein-peptide interaction map is detailed as 2D interaction maps (Figure 2, A-C).

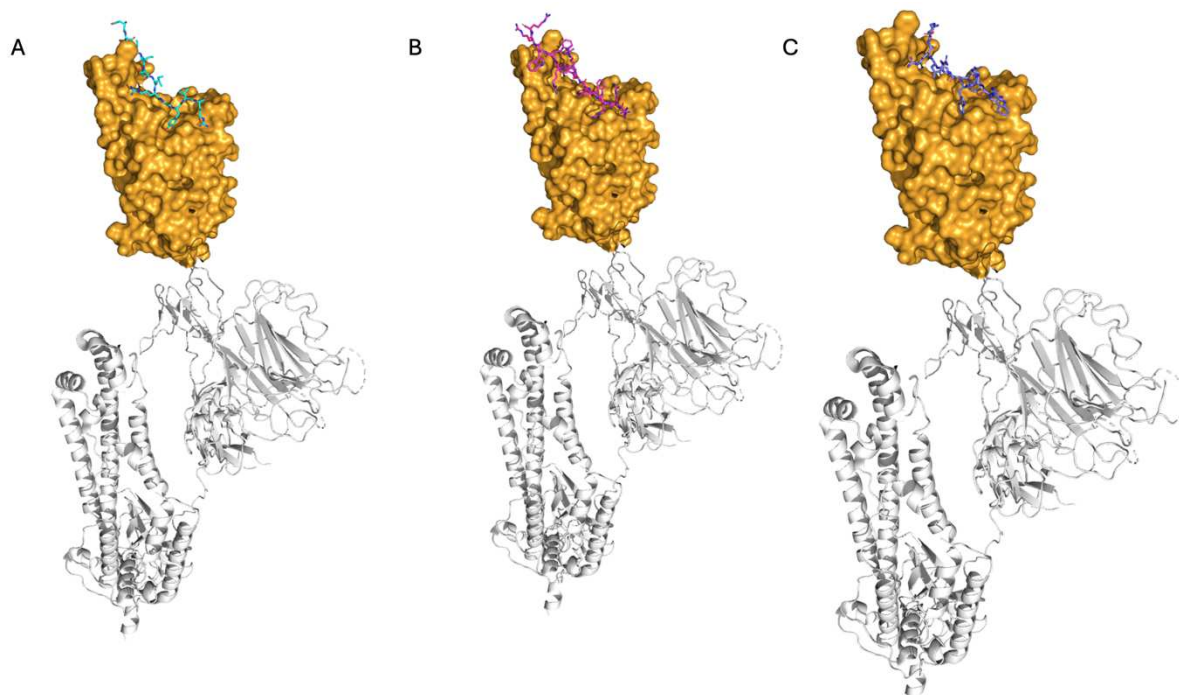


Figure 1. 3D docking representations for AI-generated peptides interacting with SARS-CoV-2 Spike-RBD. The Spike protein is represented in grey and RBD in gold. Molecular interaction is represented as (A) for BIAI1 in green sticks; (B) BIAI22 in pink sticks; and (C) BIAI3 in blue sticks.

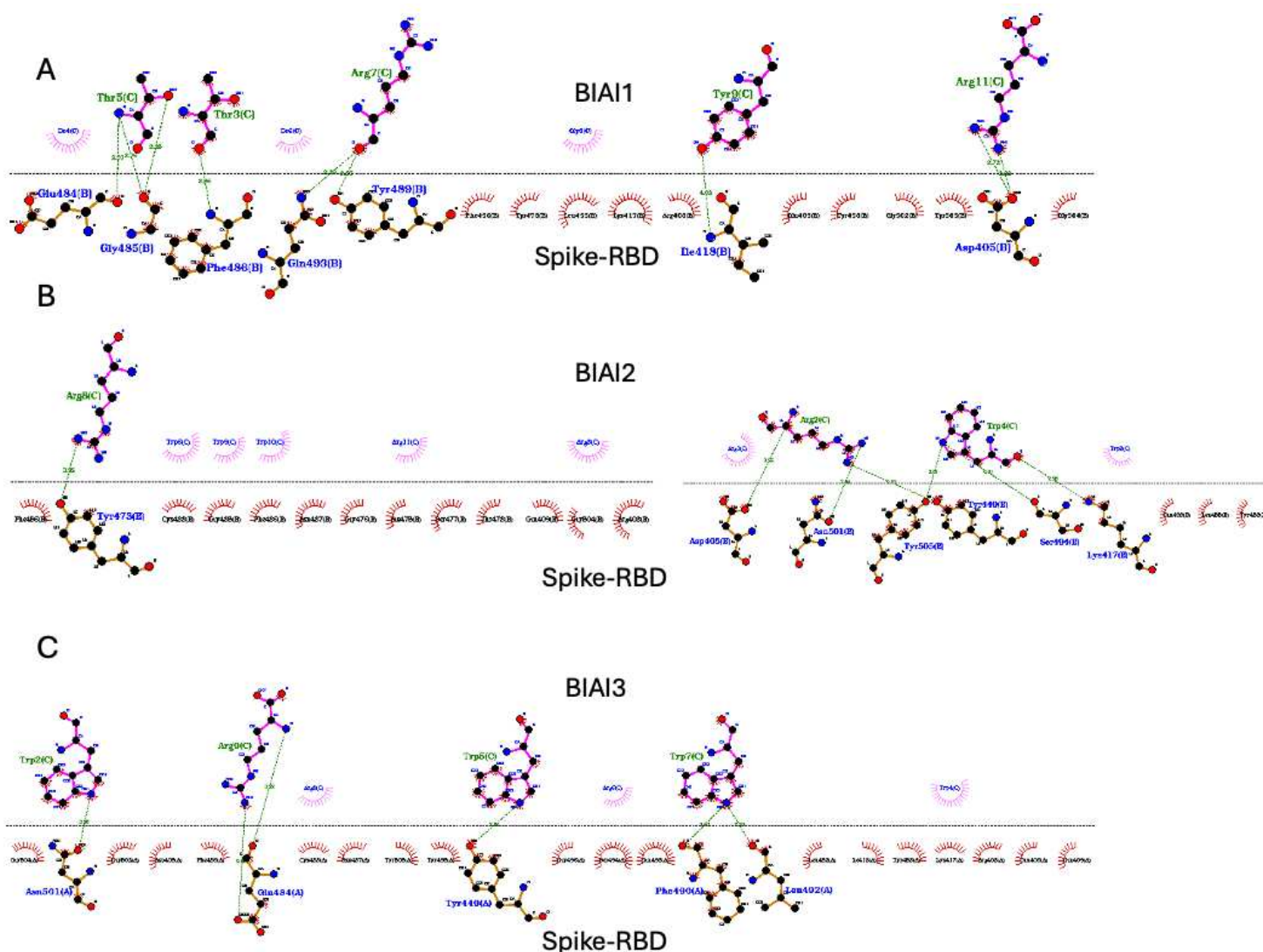


Figure 2. 2D interaction maps for BIAI1 (A), BIAI2 (B), and BIAI3 (C), which interact with SARS-CoV-2 Spike-RBD. Above the dotted lines are the peptides, and below them is the Spike-RBD complex. Hydrogen bonds are presented as green dotted lines between the peptides and the Spike-RBD complex.

Peptide-Virus Interaction

The infrared spectra of VSV-eGFP-SARS-CoV-2; BIAI1, BIAI2 or BIAI3; and VSV-eGFP-SARS-CoV-2 plus AI-peptides are represented in Figure 3, Figure 4 and Figure 5, respectively. We found unique molecular changes in VSV-eGFP-SARS-CoV-2-S incubated with BIAI1, BIAI2 or BIAI3 (Figure 3.A, Figure 4.A and Figure 5.A, respectively). These changes in functional groups occurred in the biofingerprint region between 1618 cm^{-1} and 1009 cm^{-1} suggesting interactions between VSV-eGFP-SARS-CoV-2-S with these peptides (Figure 3.B

and 3.C, Figure 4.B and 4.C, and Figure 5.B and 5.C) As an outcome, the binding interactions of 1204 cm^{-1} occurred in all peptides between VSV-eGFP-SARS-CoV-2-S with BIAI1, BIAI2 or BIAI3, suggesting interactions in amide III derived from proteins. We also noticed that the mode vibrational at 1618 cm^{-1} , 1235 cm^{-1} and 1009 cm^{-1} (Figure 3.C, Figure 4.C and Figure 5.B) were detected only after incubation of the virus with BIAI1, BIAI2 or BIAI3, respectively, suggesting interactions with functional groups of in both lectins, suggesting interactions in the Amide I region (Table 1) carbonyl stretching in nucleic acids, another vibrational mode related to amide III and stretching C-O deoxyribose in glycoproteins.

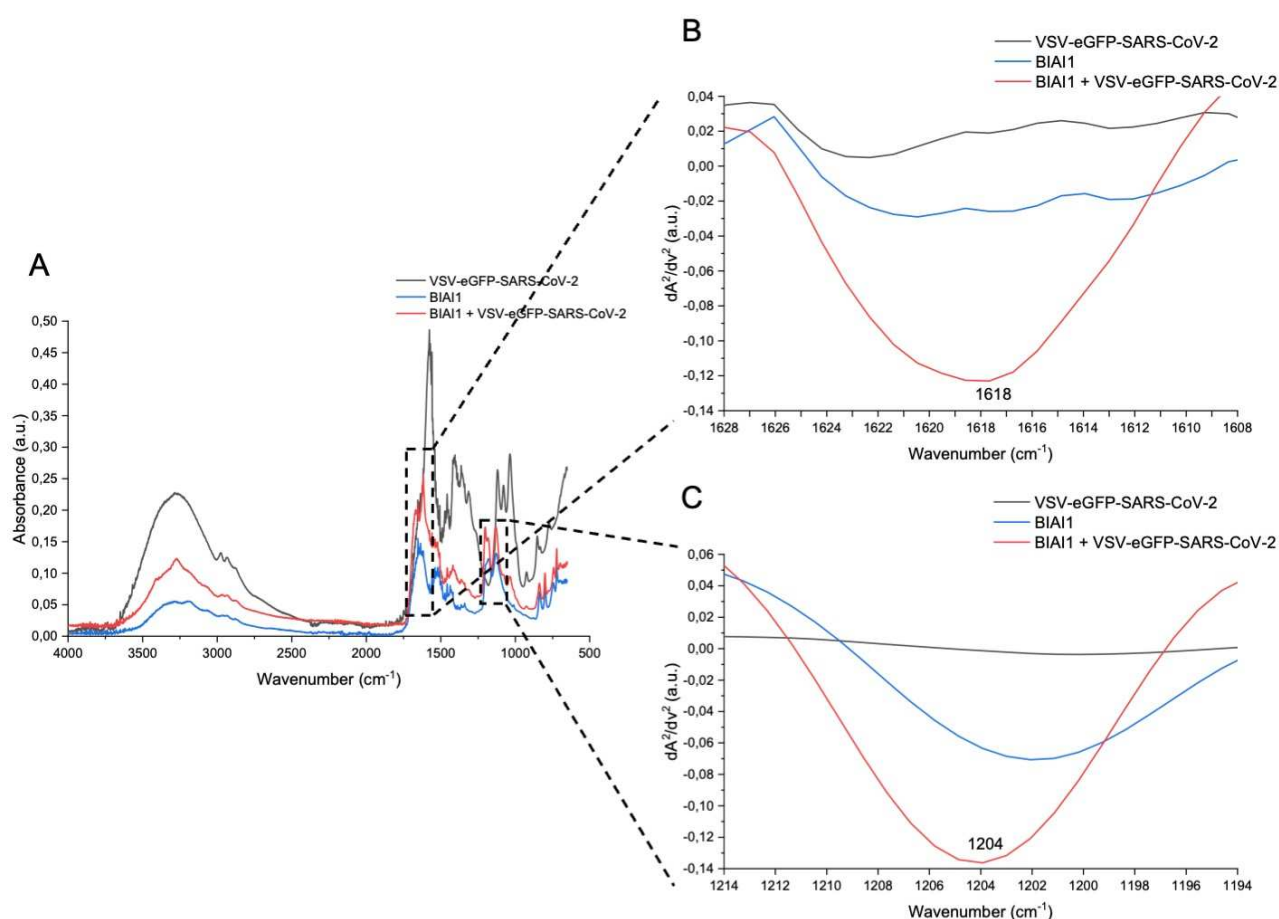


Figure 3 - Infrared spectroscopy indicates molecular interactions between VSV-eGFP-SARS-CoV-2-S plus BIAI1.

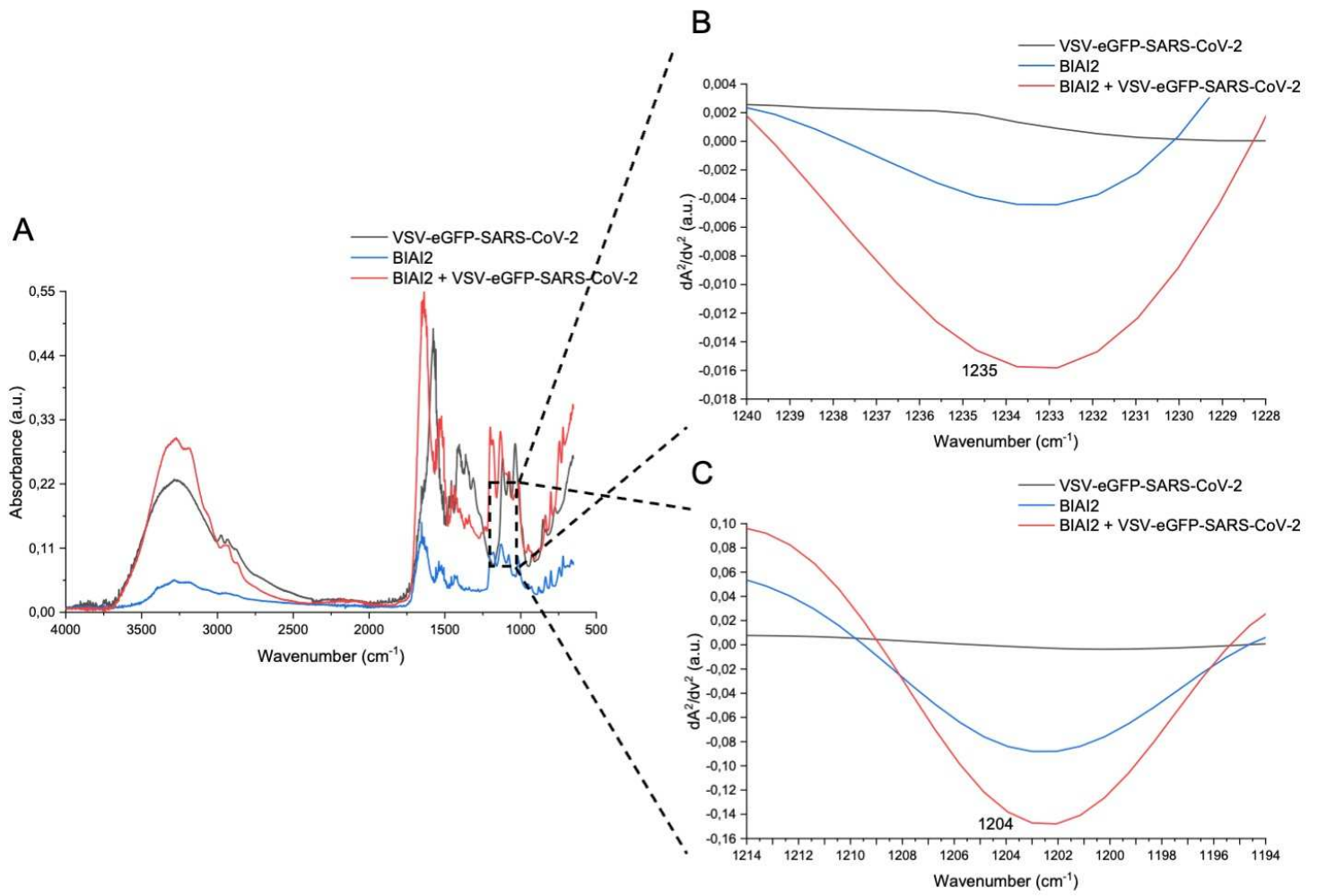


Figure 4 - Infrared spectroscopy indicates molecular interactions between VSV-eGFP-SARS-CoV-2-S plus BIAI2.

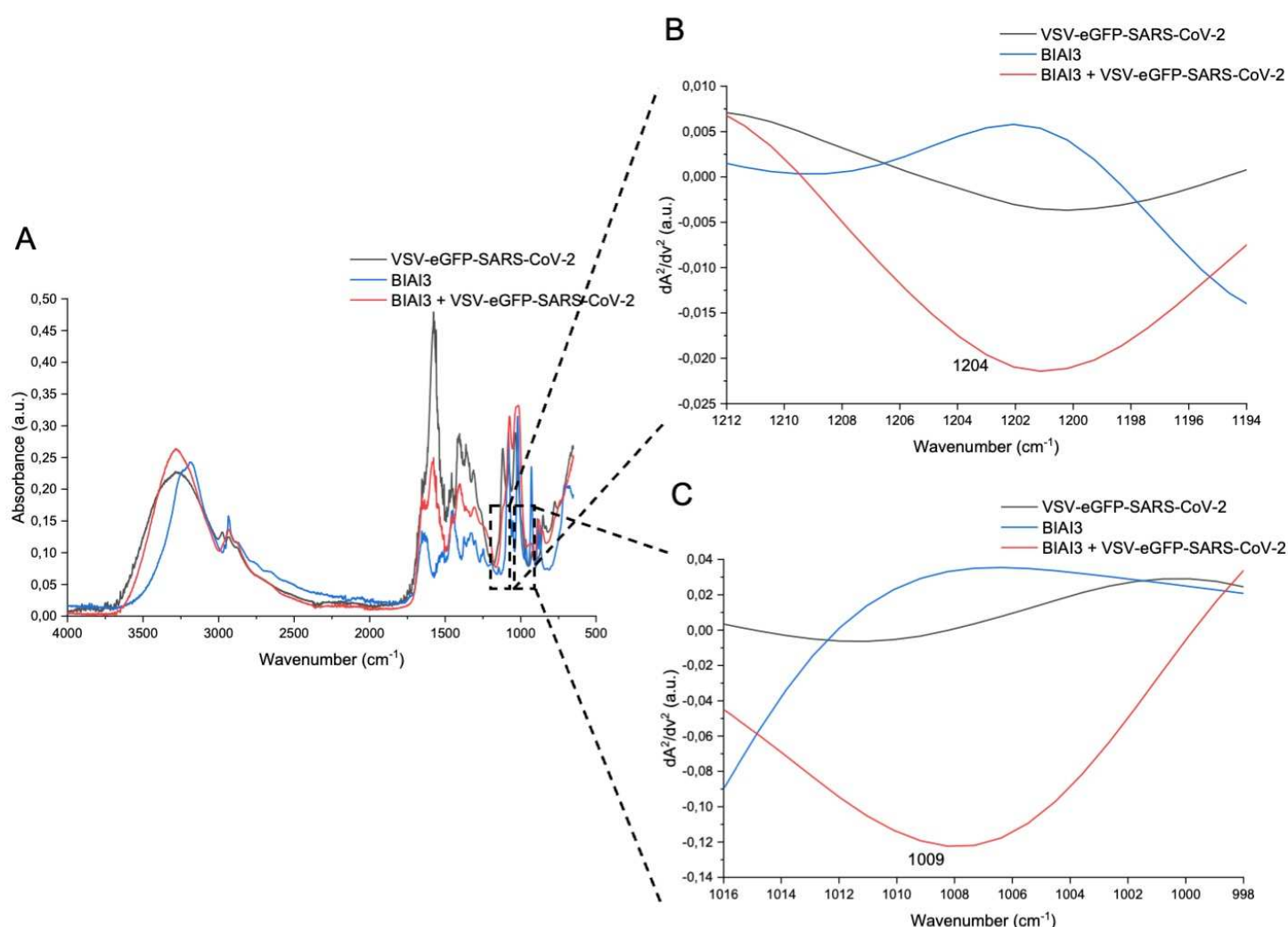


Figure 5 - Infrared spectroscopy indicates molecular interactions between VSV-eGFP-SARS-CoV-2-S plus BIAI3.

AI-generated peptides inhibit VSV-eGFP-SARS-Cov-2 infection.

Aiming to assess the cytotoxicity of the compounds, VERO cells were treated with each isolated peptide at the concentrations of 50 and 10 $\mu\text{g/mL}$. Then, cell viability was assessed by MTT assay, 24h later. PBS was used as an untreated control. Afterwards, the highest non-cytotoxic concentration of each peptide was selected to analyze their effects on VSV-eGFP-SARS-CoV-2 pseudotyped virus entrance assay. To this, cells were infected with VSV-eGFP-SARS-CoV-2 at a MOI of 0.005 in the presence or absence of each peptide. BIAI2 had the best antiviral activity against the pseudovirus model, inhibiting 90.1% of VSV-S infection. BIAI1 and BIAI3, at non-cytotoxic concentrations, inhibited, respectively, 77 and 65.9% of VSV-S infection (Table 2 and Table 3).

Table 2. *Effect of salivary peptides on VERO cells viability in 50 and 10 µg/mL concentrations*

Peptide	Cell Viability (%)	
	50 µg/mL	10 µg/mL
BIAI1	36.7	104.8
BIAI2	85.1	115.3
BIAI3	75.6	115.8

Table 3. *Effect of AI-generated peptides on VSV-eGFP-SARS-CoV-2 infectivity*

Peptide	Concentration Tested (µg/mL)	Infection Inhibition (%)
BIAI1	10	77.0
BIAI2	50	90.1
BIAI3	10	65.9

AI-generated peptide antiviral assays using SARS-CoV-2_{WT} and variants

BIAI2 had its better performance against the Wuhan-Hu-1 strain, inhibiting 83.1% of SARS-CoV-2 infection, followed by 72.7% of Gamma infection, and 62.8% of Omicron infection. BIAI1 and BIAI3 also had the best antiviral activity against Wuhan-Hu-1 strain, inhibiting the infection in 78.1% and 64%, respectively (Figure 6).

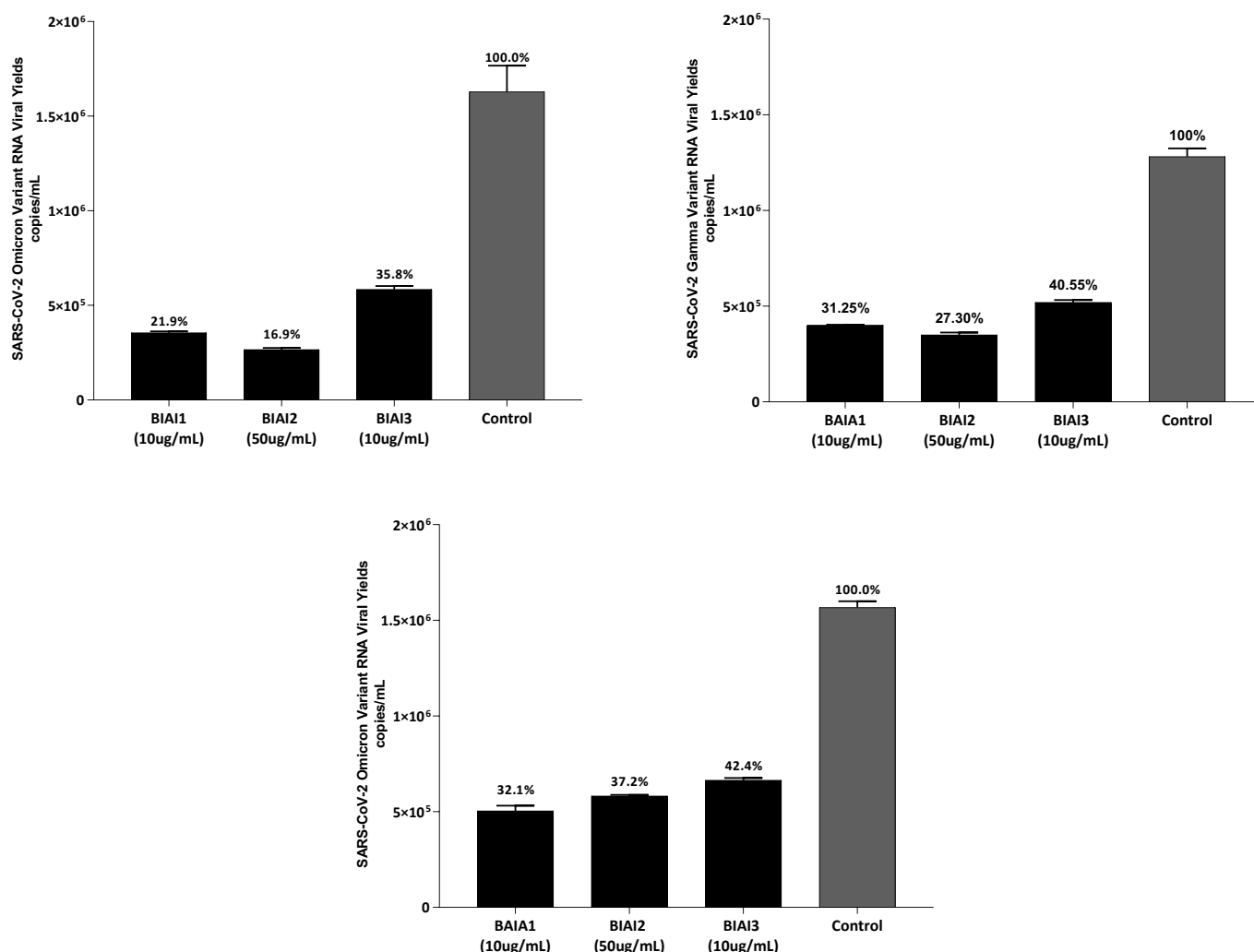


Figure 6. Effect of isolated peptides at concentrations of 50 $\mu\text{g/mL}$ or 10 $\mu\text{g/mL}$ on SARS-CoV-2_{WT} and variants Gamma and Omicron infectivity. Vero E6 cells were infected with SARS-CoV-2 Wuhan-Hu-1 virus and immediately treated with peptides BIAI1 at concentration of 10 $\mu\text{g/mL}$, BIAI2 at 50 $\mu\text{g/mL}$, and BIAI3 at 10 $\mu\text{g/mL}$ in a general infection assay. The supernatants were collected 24 h.p.i.; viral were quantified by p-RT-PCR.

4. DISCUSSION

Our study investigated the antiviral activity and cell viability of synthetic peptides BIAI1, BIAI2, and BIAI3, all generated using a surrogate-assisted artificial intelligence platform coupled with bioinformatics, against SARS-CoV-2 and its variations. BIAI2 specifically suppressed VSV-eGFP-SARS-CoV-2

infection by 90.1%, surpassing BIAI1 and BIAI2, which inhibited 77% and 65.9%, respectively. BIAI2 also demonstrated excellent inhibition at 83.1% against the wild-type SARS-CoV-2 (Wuhan-Hu-1), with lower but still significant activity against the Gamma and Omicron variants, inhibiting 72.7% and 62.8%, respectively. Cell viability assays showed that all peptides maintained excellent viability at 10 µg/mL, with BIAI2 also showing high viability at 50 µg/mL, whereas BIAI1 and BIAI3 had considerable cytotoxicity, suggesting that lower dosages are preferred.

The performance of BIAI2 is consistent with comparable peptides discovered in antiviral research, highlighting the importance of peptide design in improving antiviral efficacy. Peptide-based techniques have emerged as viable antiviral therapies for SARS-CoV-2 and other coronaviruses. These peptides can target several phases of viral infection, such as entrance, replication, and fusion [32,33]. Following our results, peptides intended to block the interaction of the SARS-CoV-2 spike protein and the ACE2 receptor have demonstrated effectiveness in vitro [34]. Antiviral peptides (AVPs) have several benefits, including excellent specificity, tunability, and biocompatibility. However, their effectiveness is restricted by poor bioavailability, which can be addressed by nanoformulation [35]. In a strategy similar to our approach, synthetic peptides that imitate viral binding domains have also been investigated as possible treatments [36,37].

AI-generated peptides may be designed using a variety of machine-learning approaches, including generative adversarial networks (GANs) [23,38] and variational autoencoders [39]. The antiviral mechanisms used by these peptides may include disruption of viral envelopes, preventing viral entrance, and limiting viral replication [11,40]. Cationic antimicrobial peptides also have been shown to target viral lipid envelopes, preventing virus entrance and reproduction [11]. In this context, deep learning techniques have been used to provide reliable predictions for antiviral peptides [41]. Here we hypothesize that our peptides present their antiviral activity due to their interaction with the Spike-RBD motif, thus blocking the interaction with ACE2 receptors and hindering viral replication.

Using a VSV-eGFP-SARS-CoV-2 2, we were able to achieve up to 90% of infection inhibition using AI-generated peptides, while maintaining high cell

viability (Table 2, Table 3). For in vitro SARS-CoV-2 screening experiments, pseudotyped viruses present several advantages: they can be safely handled in BSL-2 facilities, which allows for greater accessibility and throughput [42,43]; they have a high correlation with live virus neutralization tests, making them viable options for assessing neutralizing antibodies and prospective therapies [43–45]; and, they allow researchers to investigate viral entrance processes and screen for neutralizing drugs [46]. However, these systems may need to be optimized and standardized, therefore the need for further analysis using wild-type viruses [43].

When evaluating the effect of the peptides in wild-type viruses, we were able to reduce viral replication consistently across the original Wuhan-1 strain and variants (Figure 6). The use of these wild-type viruses enable precise evaluation of neutralizing antibodies and antiviral drugs against the real virus [43,47]. In this context, antiviral effectiveness may be tested using several methods, such as plaque reduction, tissue culture infectious dose, and RT-PCR assays [47]. These methods can be used to evaluate the efficacy of therapies for developing variations of concern [48]. Thus, our results indicate the potential of BIA1, BIA2 and BIA3 as antiviral agents for not the original strain and also against variants.

Several studies have shown that peptides can impair the interaction between the virus's spike protein and the human ACE2 receptors [21,49,50]. Some peptides, including RcAlb-Pepl, PepGAT, and PepKAA, can allosterically modify the architecture of the SARS-CoV-2 primary protease, potentially influencing viral replication [51]. In a broader sense, antimicrobial peptides have presented the potential to reduce viral load by inhibiting cellular surface receptors or breaking the cell membrane of viral structure [36]. Peptide-based treatments provide benefits including reduced toxicity to human cells and the possibility for rapid development [52,53]. Furthermore, our AI-generated peptides present promising potential to be applied in clinical set-ups to reduce viral load and hinder viral infection and replication.

Peptides are promising antiviral therapeutics because of their specificity, chemical variety, ability to damage viral envelopes, and potential to form nanostructures for effective drug delivery [9–11]. They outperform small molecule therapeutics and protein-based therapies in terms of target affinity,

cellular penetrability, and stability, and were effective against a variety of viruses, including HIV-1, influenza, and coronaviruses [12–14,16]. However, in vitro studies of antiviral activity of peptides may present limitations and challenges. The bioavailability and stability of the peptides in vivo is a limitation and a challenge that should be further studied [54]. Thus, further studies, including in vivo investigations and clinical trials, are needed to validate the present findings and fully explore their therapeutic relevance in multicentric cohorts. The impact of the peptides on the immune system also requires further in vitro investigation, considering that they are being tested in a high dosage despite the low cytotoxicity [55]. Thus, Further study, including in vivo investigations and clinical trials, is needed to verify our findings and fully explore their therapeutic relevance.

5. CONCLUSION

Finally, our findings show that the AI-generated peptides BIAI1, BIAI2, and BIAI3 can act as effective antiviral agents against SARS-CoV-2 and its variations. These peptides demonstrated significant infection suppression rates while maintaining cell viability, showing their safety and effectiveness. The considerable reduction in viral copies, notably against the Wuhan strain, Gamma, and Omicron variants, demonstrates their broad-spectrum antiviral activity. These findings indicate that BIAI1, BIAI2, and BIAI3 may be useful in developing preventative, diagnostic, and therapeutic techniques for COVID-19, providing a viable strategy for combating present and future coronavirus epidemics.

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CONSIDERAÇÕES FINAIS E CONCLUSÃO

CONSIDERAÇÕES FINAIS

O surgimento da saliva como uma ferramenta de diagnóstico não invasiva tem ganhado considerável atenção no contexto de infecções virais como o vírus da mpox (MPXV), norovírus e SARS-CoV-2. Esta tese destaca a interseção promissora entre diagnósticos salivares, tecnologias ômicas, bioinformática e plataformas de inteligência artificial (IA) no desenvolvimento de métodos de detecção precisos, de baixo custo e rápidos para esses patógenos. Além disso, a utilização de peptídeos salivares e seus mimotopos sintéticos gerados por plataformas de inteligência artificial também foi explorada, demonstrando o potencial antiviral desses compostos contra SARS-CoV-2. Ao explorar as relações intrincadas entre a patogênese viral e os diagnósticos moleculares de MPXV, norovírus e SARS-CoV-2, ressaltamos o papel central da saliva no avanço de estratégias tanto diagnósticas quanto terapêuticas.

Saliva como Meio de Diagnóstico para o MPXV

O MPXV, o agente causador da mpox, depende da maquinaria da célula hospedeira, como o retículo endoplasmático rugoso e os ribossomos, para replicação. Uma das primeiras manifestações clínicas da infecção por MPXV é o aparecimento de lesões na mucosa oral. No entanto, detectar o vírus na saliva antes do surgimento das erupções cutâneas continua sendo um desafio com os métodos atuais. O MPXV é transmitido por contato direto com lesões e fluidos corporais, incluindo a saliva, posicionando esse fluido como um meio valioso para diagnósticos.

As técnicas diagnósticas convencionais, como RT-qPCR e ELISA, embora eficazes, são invasivas e apresentam limitações. Interessantemente, ensaios clínicos com pacientes infectados pelo MPXV mostraram que o DNA do vírus pode ser detectado na saliva com sensibilidades variando de 85% a 100%. Isso destaca o potencial da saliva para diagnóstico precoce e não invasivo do MPXV. Ao empregar tecnologias ômicas—como transcriptômica, proteômica, lipidômica e metabolômica—a saliva pode revelar novos biomarcadores que aprimoram o diagnóstico precoce. Por exemplo, a proteômica baseada em espectrometria de massa (MS) revelou diferenças de expressão de proteínas em pacientes infectados por MPXV, sugerindo que essas proteínas, detectáveis na

saliva, podem desempenhar um papel importante em futuras plataformas diagnósticas.

Plataformas Eletroquímicas para Detecção de Norovírus

Norovírus, que infectam centenas de milhões de pessoas anualmente, são transmitidos pela via oral-oral, com a saliva servindo como um potencial reservatório para o vírus. Dada essa via de transmissão, a saliva representa um candidato ideal para a detecção não invasiva do norovírus. Nesta tese, foi utilizada uma abordagem orientada por bioinformática para selecionar peptídeos da saliva, que foram incorporados em um biossensor eletroquímico destinado ao diagnóstico de infecções por norovírus.

Os peptídeos selecionados por bioinformática—NoroPep1, NoroPep2 e NoroPep3—foram sintetizados e aplicados a eletrodos revestidos com nanopartículas de ouro. A plataforma eletroquímica, testada usando voltametria cíclica (VC), demonstrou capacidade de detectar norovírus com alta sensibilidade (até 90%), especificidade e precisão. Além disso, o biossensor foi validado em amostras de saliva de camundongos infectados e não infectados, com resultados mostrando sensibilidade de 85%, especificidade de 90% e acurácia de 87,5%. Algoritmos de aprendizado de máquina melhoraram ainda mais o desempenho diagnóstico, alcançando sensibilidade e especificidade quase perfeitas. Esses achados sugerem que biossensores baseados em peptídeos, assistidos por IA, têm o potencial de revolucionar os diagnósticos salivares para norovírus, oferecendo uma ferramenta diagnóstica portátil, em tempo real e não invasiva.

Diagnósticos Salivares para SARS-CoV-2

Avanços semelhantes foram feitos no diagnóstico salivar do SARS-CoV-2. O vírus usa o domínio de ligação ao receptor (RBD) de sua proteína Spike para interagir com o receptor ACE2 nas células humanas, facilitando a entrada e replicação viral. Para desenvolver uma ferramenta de diagnóstico rápida e escalável, plataformas de bioinformática e IA, como o SAGAPEP, foram utilizadas para criar peptídeos bioinspirados projetados para inibir essa interação. Os peptídeos resultantes—BIAI1, BIAI2 e BIAI3—foram sintetizados e aplicados a um biossensor eletroquímico portátil, que detectou SARS-CoV-2 na

saliva com acurácia impressionante.

O biossensor, que utilizou voltametria cíclica, apresentou sensibilidade de 100%, especificidade de 80% e acurácia geral de 90%. Esses resultados foram validados em amostras de saliva de indivíduos positivos e negativos para COVID-19, com algoritmos de aprendizado de máquina refinando ainda mais os resultados diagnósticos. Além do diagnóstico, os peptídeos também demonstraram propriedades antivirais. Experimentos *in vitro* mostraram que esses peptídeos poderiam inibir a replicação viral e reduzir a morte celular, especialmente para variantes do SARS-CoV-2, como Gamma e Omicron. Essa funcionalidade dupla—combinando diagnóstico e potencial terapêutico—destaca a versatilidade dos peptídeos salivares no combate às infecções virais.

Peptídeos Salivares como Agentes Antivirais

Além de sua utilidade diagnóstica, os peptídeos salivares demonstraram promissoras propriedades antivirais. Para o SARS-CoV-2, peptídeos como SalivaPep1, SalivaPep2 e SalivaPep3 mostraram efeitos inibitórios significativos na replicação viral. Esses peptídeos, derivados de proteínas naturalmente presentes na saliva, bloquearam efetivamente a interação Spike-RBD/ACE2, um passo crítico na entrada viral. Estudos *in vitro* usando o pseudovírus VSV-eGFP-SARS-CoV-2 e o SARS-CoV-2 selvagem (SARS-CoV-2WT) mostraram taxas de inibição da infecção de até 67,5% quando esses peptídeos são aplicados; enquanto esse valores podem chegar a 90,1% quando utiliza-se peptídeos gerados por IA inspirados nesses peptídeos naturais.

Além disso, análises de PCR em tempo real revelaram uma redução substancial nas cargas virais quando as variantes Gamma e Omicron foram tratadas com esses peptídeos. SalivaPep2 e SalivaPep3, derivados de Ataxina-1 e Statherina, respectivamente, foram particularmente eficazes, reduzindo as cópias virais em até 75% na variante Gamma e 62,5% na variante Omicron. Esses resultados destacam o potencial terapêutico dos peptídeos salivares, não apenas em diagnósticos, mas também em aplicações antivirais, abrindo novas vias para o desenvolvimento de estratégias profiláticas e terapêuticas contra a COVID-19.

CONCLUSÃO

Esta tese explora soluções inovadoras para o manejo de infecções virais usando a saliva como ferramenta diagnóstica e terapêutica. Demonstramos que:

- A saliva tem potencial como ferramenta diagnóstica para mpox, aproveitando sua presença em lesões da mucosa oral e fluidos corporais, sugerindo a integração de tecnologias ômicas para uma detecção não invasiva com alta sensibilidade.
- Peptídeos salivares selecionados por IA (NVp1, NVp2, NVp3) são capazes de realizar a detecção eletroquímica de norovírus, alcançando 100% de acurácia, sensibilidade e especificidade.
- Peptídeos modelados por IA, usados em um biossensor eletroquímico, podem oferecer uma solução não invasiva e portátil para a rápida detecção da COVID-19, validados por algoritmos de aprendizado de máquina, com sensibilidade, especificidade e acurácia notáveis.
- Peptídeos salivares de ocorrência natural podem bloquear a interação da proteína Spike do SARS-CoV-2 com os receptores ACE2, demonstrando atividade antiviral substancial e reduzindo a replicação viral em várias variantes.
- Peptídeos gerados por IA (BIAI1, BIAI2, BIAI3) exibem alta eficácia antiviral contra variantes do SARS-CoV-2, reduzindo significativamente os títulos virais e prevenindo a morte celular, destacando seu potencial para aplicações profiláticas, diagnósticas e terapêuticas.

Juntos, esses estudos avançam nos campos do diagnóstico e da terapêutica baseados em saliva, oferecendo novos caminhos para o manejo eficaz de doenças.

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