UNIVERSIDADE FEDERAL DE ALFENAS

LEONARDO PEREIRA DE ARAÚJO

IDENTIFICAÇÃO DE CANDIDATOS VACINAIS MULTIVALENTES UTILIZANDO EPÍTOPOS CONSERVADOS DE PROTEÍNAS ENVOLVIDAS EM PROCESSOS DE ENTRADA E SAÍDA DO GÊNERO *Orthopox vírus*

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Ciências Fisiológicas pela Universidade Federal de Alfenas. Área de concentração: Fisiologia.

Orientador: Prof. Dr. Leonardo Augusto de Almeida

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O Presidente da banca examinadora abaixo assina a aprovação da Dissertação apresentada como parte dos requisitos para a obtenção do título de Mestre em Ciências Fisiológicas pela Universidade Federal de Alfenas. Área de concentração: Ciências Fisiológicas

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O ontem é história, o amanhã é um mistério, mas o hoje é uma dádiva. É por isso que se chama presente.

(Oogway, Kung Fu Panda, 2008)

RESUMO

O gênero *Orthopoxvirus* (OPXV) inclui vírus zoonóticos emergentes e reemergentes que representam uma ameaça à saúde global e impactam uma ampla variedade de animais. Entre eles, a varíola causou pandemias no século XX, o vírus *Borealpox* provocou a primeira morte registrada no Alasca em 2024, e o *Monkeypox vírus* (Mpox), foi classificado como emergência de saúde pública de importância internacional pela Organização Mundial da Saúde em 2022 e 2024, devido à emergências de uma novas variantes como Clado Ib. As limitações das vacinas de vírus atenuados utilizadas nos dias atuais, especialmente em populações imunocomprometidas, ressalta a urgência de novas estratégias vacinais. Assim, o objetivo deste trabalho foi identificar epítopos conservados proteínas envolvidas nos processos de entrada e saída viral nas células hospedeiras, abrangendo todos os vírus do gênero OPXV e desenvolver *in silico* uma vacina quimérica multiepítopo imunogênica. Para isso, proteínas envolvidas nos processos de entrada e saída viral foram extraídas do banco de dados do *National Center for Biotechnology Information* (NCBI). Um total de 160 sequências de todos os vírus descritos do gênero OPXV foram analisadas para identificar epítopos conservados, utilizando o banco de dados *Immune Epitope Database* (IEDB). Após processos de filtragem de antigenicidade, alergenicidade, toxicidade, bem como de exclusão de regiões transmembranares e sítios de N-glicosilação, os epítopos candidatos foram concatenados para construir uma proteína quimérica multiepítopo, combinada com os adjuvantes β-defensina e PADRE, visando potencializar a resposta imune. Duas proteínas quiméricas foram desenvolvidas: uma contendo oito epítopos conservados abrangendo todos os vírus do gênero OPXV e outra com epítopos específicos para Mpox. Essas proteínas foram avaliadas quanto à antigenicidade, alergenicidade e estabilidade estrutural. Além disso, as vacinas multiepítopos propostas demonstraram boa interação com o receptor do tipo Toll, bem como previsões favoráveis de indução de respostas imunológicas humorais e celulares para três doses dos candidatos vacinais. Os resultados sugerem que essas vacinas podem representar uma nova abordagem multivalente contra vírus zoonóticos do gênero OPXV. As proteínas candidatas apresentam potencial para estudos *in vitro* e *in vivo* e, caso sua eficácia seja confirmada, poderão oferecer uma solução eficaz para a prevenção de doenças causadas por esses patógenos, bem como na produção de testes de imunodiagnóstico com os epítopos identificados nestas análises.

Palavras-chave: *Orthopoxvírus;* Imunoinformática; Vacinologia reversa; Epítopos.

ABSTRACT

The *Orthopoxvirus* (OPXV) genus includes emerging and reemerging zoonotic viruses that pose a threat to global health and affect a wide range of animals. Among them, smallpox caused pandemics in the 20th century; *Borealpox* virus led to the first recorded death in Alaska in 2024; and *Monkeypox virus* (Mpox) was declared a public health emergency of international concern by the World Health Organization in 2022 and 2024 due to the emergence of new variants such as Clade Ib. The limitations of currently used live attenuated vaccines—particularly in immunocompromised populations—highlight the urgent need for novel vaccination strategies. Thus, the aim of this study was to identify conserved epitopes in proteins involved in the processes of viral entry and exit from host cells, encompassing all viruses within the OPXV genus, and to design an *in silico* chimeric multi-epitope immunogenic vaccine. For this purpose, proteins associated with viral entry and exit were retrieved from the National Center for Biotechnology Information (NCBI) database. A total of 160 sequences from all known OPXV viruses were analyzed to identify conserved epitopes using the Immune Epitope Database (IEDB). After filtering for antigenicity, allergenicity, toxicity, and excluding transmembrane regions and N-glycosylation sites, candidate epitopes were concatenated to construct a chimeric multi-epitope protein. This construct was combined with the β-defensin and PADRE adjuvants to enhance immune response. Two chimeric proteins were developed: one containing eight conserved epitopes spanning all OPXV viruses, and another with epitopes specific to Mpox. These proteins were evaluated for antigenicity, allergenicity, and structural stability. Additionally, the proposed multi-epitope vaccines demonstrated strong interaction with Toll-like receptors and showed favorable predictions for eliciting both humoral and cellular immune responses after three doses. The results suggest that these vaccines may represent a novel multivalent approach against zoonotic viruses of the OPXV genus. The candidate proteins show promise for in vitro and in vivo studies, and if proven effective, may offer a viable solution for the prevention of diseases caused by these pathogens, as well as for the development of immunodiagnostic tests using the identified epitopes.

Keywords: *Orthopoxvirus*, Immunoinformatics, Reverse Vaccinology, Epitopes.

SUMÁRIO

1 INTRODUÇÃO GERAL

Nos últimos anos, a emergência e reemergência de vírus zoonóticos têm evidenciado a fragilidade dos sistemas de saúde global (Choi, 2021). A pandemia de COVID-19, causada pelo SARS-CoV-2, demonstrou o impacto devastador que um vírus emergente pode causar, com cerca de 7 milhões de mortes registradas (De Araújo, *et al.,* 2022). Em paralelo, o ressurgimento do *Monkeypox* vírus (Mpox), pertencente ao gênero *Orthopoxvirus* (OPXV), acendeu um novo alerta global (Adepoju, 2024). O Mpox, inicialmente restrito ao continente africano, se espalhou rapidamente, culminando em uma declaração de emergência de saúde pública pela Organização Mundial da Saúde (OMS) em 2022 (Gieryńska, *et al.,* 2023). O alerta foi reforçado em 2024 com o surgimento da variante denominada Clado Ib, caracterizada por maior transmissibilidade. Essa situação sublinha a importância de uma vigilância contínua e do desenvolvimento de estratégias eficazes de controle e prevenção para o gênero OPXV (Adepoju, 2024).

O gênero OPXV, presente na família *Poxviridae*, engloba um total de dezesseis espécies virais documentadas até o momento e é um dos grupos com maior impacto na saúde humana (Babkin *et al.,* 2022; Mühlemann *et al.,* 2020). Esses vírus, que apresentam um genoma de DNA de fita dupla e estrutura complexa, possuem aproximadamente 180 proteínas caracterizadas (*Centers for Disease Control and Prevention*, 2024; Babkin *et al.,* 2022). O gênero OPXV inclui outros vírus significativos, sendo o vírus *Variola* (VARV), causador da varíola, responsável por uma das pandemias mais devastadoras do século XX (Babkin *et al.,* 2022; Mühlemann *et al.,* 2020).

Atualmente, duas vacinas estão disponíveis para proteção abrangente contra os vírus do gênero OPXV, de acordo com os Centros de Controle e Prevenção de Doenças (CDC): ACAM2000 (Emergent BioSolutions, Estados Unidos da América) e JYNNEOS (Bavarian Nordic, Dinamarca) (*Centers for Disease Control and Prevention*, 2024). Ambas são licenciadas para uso em pessoas que tiveram contato com indivíduos infectados por OPXV e estão sendo administradas para conter o aumento de casos (*Centers for Disease Control and Prevention*, 2024). No entanto, é importante destacar que a vacina ACAM2000 utiliza o vírus atenuado da varíola, sendo contraindicada para indivíduos imunocomprometidos e grupos de risco, como gestantes e idosos (Vartak; Sucheck, 2016). Por outro lado, a vacina JYNNEOS utiliza um vírus vivo não replicante, o que permite seu uso seguro nesses grupos. Contudo, sua imunogenicidade é inferior à da ACAM2000 (*Centers for Disease Control and Prevention*, 2024; Vartak, Sucheck, 2016).

Considerando que a vacinação é o método mais eficaz para prevenir a propagação de doenças infecciosas, uma solução promissora para contornar as limitações das vacinas tradicionais é o uso de técnicas computacionais na produção de vacinas de subunidades por meio da vacinologia reversa (Hong *et al.,* 2023). Esse método permite, a partir dos genomas virais, identificar regiões conservadas que podem ser utilizadas para a seleção de epítopos imunogênicos, resultando no desenvolvimento de candidatos vacinais (Moutaftsi *et al.,* 2006). Essas vacinas de subunidade oferecem várias vantagens, como imunogenicidade direcionada com o uso de adjuvantes, facilidade de produção e armazenamento, além de aplicação segura em populações vulneráveis, incluindo idosos, crianças e indivíduos imunocomprometidos, o que reforça a justificativa para seu desenvolvimento (Vartak, 2016).

Este estudo teve como objetivo identificar epítopos conservados em dez proteínas-chave dos processos de entrada e saída viral de células hospedeiras, abrangendo os 16 vírus do gênero OPXV. Com base nos epítopos identificados, foram desenvolvidas vacinas multiepítopicas: uma específica para Mpox e uma multivalente para todo o gênero OPXV, ambas avaliadas quanto às propriedades físico-químicas, imunogenicidade e segurança.

O presente trabalho está estruturado em três capítulos principais, cada um deles composto por artigos científicos previamente publicados em periódicos especializados. Esses artigos foram incorporados integralmente ao corpo deste relatório e refletem, de forma detalhada, os principais resultados e análises desenvolvidas ao longo do curso de mestrado. A organização em capítulos visa apresentar de maneira clara e sequencial os avanços obtidos nas diferentes etapas da pesquisa.

2 REVISÃO DE LITERATURA

2.1 CARACTERIZAÇÃO DO GÊNERO OPXV

O gênero *Orthopoxvírus* (OPXV), pertence à família *Poxviridae* e inclui dezesseis espécies virais documentadas até o momento, de modo, a ser o grupo viral que apresenta um dos maiores impactos na saúde humana (Babkin *et al.,* 2022; Mühlemann *et al.,* 2020). Esses vírus, que apresentam um genoma de DNA de fita dupla e estrutura complexa, possuem aproximadamente 180 proteínas caracterizadas (*Centers for Disease Control and Prevention*, 2024; Babkin *et al.,* 2022). Dentre os membros desse gênero, destacam-se os vírus *Abatino, Akhmeta, Borealpox, Buffalopox, Camelpox, Cowpox, Ectromelia, Horsepox, Monkeypox, Rabbitpox, Raccoonpox, Skunkpox, Taterapox, Vaccinia, Variola e Volepox* (*Centers for Disease Control and Prevention*, 2024, Molteni *et al.,* 2023). Embora muitos desses vírus tenham sido nomeados a partir dos animais nos quais foram identificados, acredita-se que atuem apenas como hospedeiros, não sendo necessariamente a origem dos patógenos (Babkin *et al.,* 2022).

Como resultado, os OPXV são frequentemente classificados como vírus emergentes ou reemergentes (Choi, 2021). Os vírus emergentes, como o *Borealpox* (Alaskapox) — recentemente reconhecido como parte do gênero — provocam novos casos sem conhecimento prévio, enquanto os reemergentes, como o Mpox, causam aumentos periódicos no número de infecções, com surtos documentados em 2003, 2022 e, mais recentemente, em 2024 (Li *et al.,* 2023; Devi, 2024; Adepoju, 2024).

Em 2003, o vírus Mpox (clado Ia) provocou um aumento no número de casos, apresentando sintomas semelhantes aos da varíola, como febre, dores musculares, corporais e lesões cutâneas, especialmente pústulas. Apesar do surto, o vírus permaneceu restrito ao continente africano (Karagoz *et al.,* 2023). Em 2022, duas novas variantes de Mpox, classificadas como clado IIa e IIb, surgiram, apresentando cerca de 40 mutações em relação à variante anterior, resultando em uma disseminação mais rápida (Kumar *et al.,* 2022). A Organização Mundial da Saúde (OMS) declarou essas variantes uma Emergência de Saúde Pública de Importância Internacional (PHEIC) após os casos globais ultrapassarem 100.000 infecções (Li *et al.,* 2023). Em 2024, o clado Ib emergiu, provocando outro aumento expressivo de casos. Com maior transmissibilidade e capacidade de se espalhar globalmente, essa nova variante levou a OMS a emitir outro alerta de PHEIC, destacando seu impacto mundial (Adepoju, 2024).

No mesmo ano, o novo vírus emergente do gênero OPXV, o *Alaskapox*, recentemente renomeado como *Borealpox*, foi responsável pela primeira morte documentada (Devi, 2024). A vítima, um paciente imunocomprometido, foi arranhado por seu gato e desenvolveu fadiga e erupções cutâneas, levando à hospitalização e ao óbito (Devi, 2024). Acredita-se que a origem do vírus esteja em pequenos roedores.

O gênero OPXV inclui outros vírus significativos, sendo o *Variola vírus* (VARV), causador da varíola, responsável por uma das pandemias mais devastadoras do século XX, resultando em aproximadamente 300 a 500 milhões de mortes (Babkin *et al.,* 2022; Mühlemann *et al.,* 2020). Graças a um programa global de vacinação, a varíola foi oficialmente erradicada em 1980 (Shchelkunova; Shchelkunov, 2022). Contudo, o declínio nas campanhas de imunização aumentou o risco de transmissão de outros vírus do gênero OPXV, levantando preocupações sobre a morbidade dessas infecções zoonóticas (Diaz *et al.,* 2021). Nos últimos anos, surtos de infecções por OPXV foram registrados em várias regiões do mundo, com casos de *Cowpox, Camelpox, Buffalopox* e Mpox relatados na Europa, Oriente Médio, Índia, América do Sul, África e Estados Unidos (Diaz *et al.,* 2021).

2.2 PATOLOGIA E CONTEXTO EPIDEMIOLÓGICO

O VARV, causador da varíola, foi responsável por uma das maiores tragédias de saúde pública do século XX, resultando em aproximadamente 400 milhões de mortes em um período de 80 anos (Molteni, *et al.,* 2023). A varíola apresentava uma taxa de mortalidade que variava entre 5% e 40%, dependendo da cepa e das condições de saúde da população afetada (Gieryńska, *et al.,* 2023). O sucesso na erradicação da varíola em 1980, por meio de um programa de vacinação global, foi um marco histórico na medicina (Fenner *et al.,* 1988). No entanto, a cessação da imunização em massa aumentou a vulnerabilidade das populações a outros vírus do gênero OPXV (Mühlemann, *et al.,* 2023).

O Mpox, que causa uma infecção semelhante à varíola, caracteriza-se por lesões cutâneas distintas e possui uma taxa de mortalidade que pode chegar a 10% (Shchelkunova, *et al.,* 2022). Recentemente, o vírus começou a se espalhar globalmente, com o primeiro surto significativo fora do continente africano ocorrendo nos Estados Unidos em 2003, resultando em 72 casos confirmados (Diaz, *et al.,* 2021). A situação se agravou nos últimos anos. De 2022 a 2024, quase 100 mil casos foram registrados em todo o mundo, sendo 33 mil casos nos Estados Unidos e 11 mil casos no Brasil (Walter *et al.,* 2022). A OMS declarou uma Emergência de Saúde Pública de Importância Internacional (ESPII) em 2022, e em 2024, com

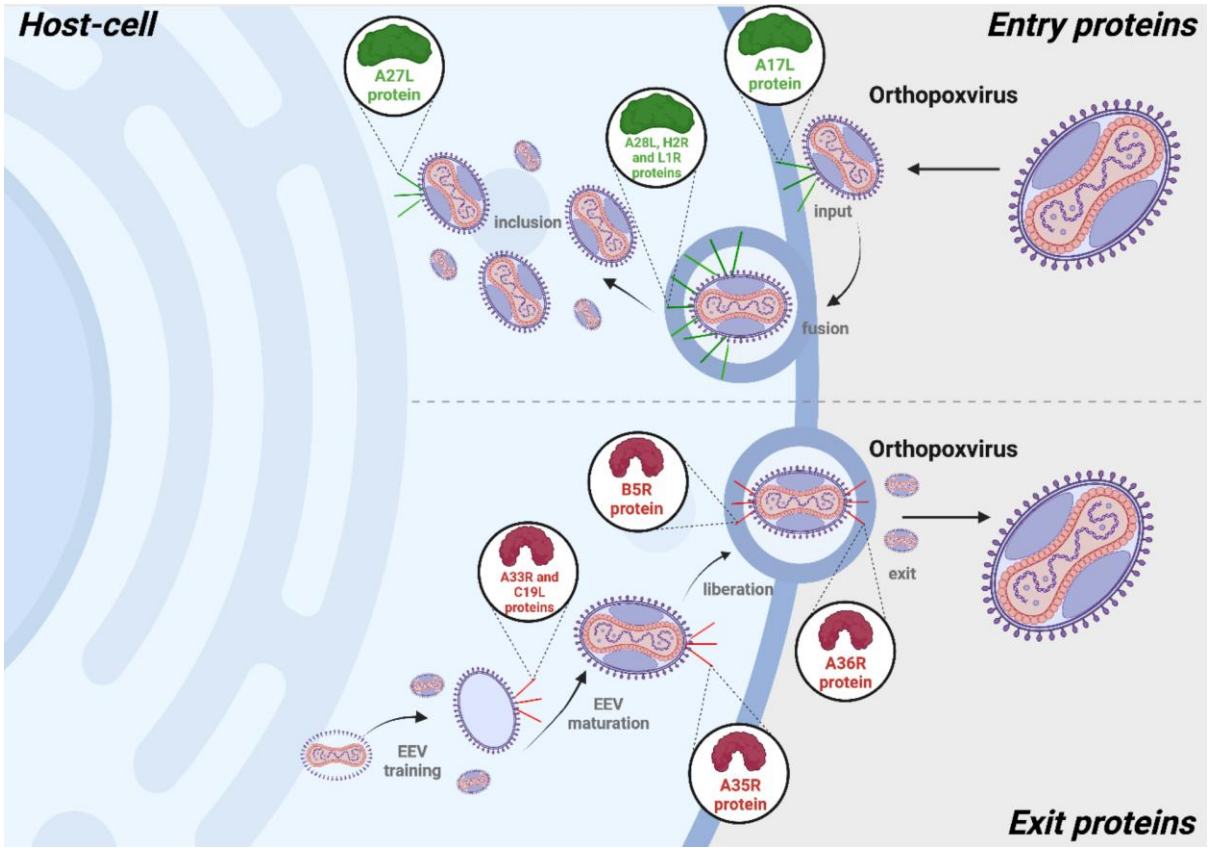
o surgimento da variante Clade IB levou a uma nova declaração de emergência devido ao aumento das infecções (Adepoju, 2024). No Brasil, foram registrados 709 casos em 2024, evidenciando a necessidade de monitoramento contínuo e de respostas rápidas (*Centers for Disease Control and Prevention*, 2024).

Além do Mpox, outros vírus do gênero OPXV continuam a representar riscos à saúde humana (Diaz, *et al.,* 2021). A varíola bovina, causada pelo *Cowpox vírus* é uma doença esporádica que pode ser transmitida a humanos, sendo particularmente perigosa para indivíduos imunocomprometidos (Shchelkunova, *et al.,* 2022) O *Vaccinia virus* (VACV), foi utilizado por décadas como vacina contra a varíola, desempenhando papel crucial na erradicação da doença (Fenner, 1988). O vírus *Alaskapox* (AKPV), recentemente renomeado como *Borealpox*, é o mais recente acréscimo ao gênero OPXV (Devi, 2024). Identificado pela primeira vez no Alasca em 2015, o AKPV foi responsável por causar a primeira morte registrada de um paciente imunocomprometido em 2024 (Devi, 2024). Esse evento destaca a necessidade urgente de vigilância e desenvolvimento de vacinas capazes de proteger populações vulneráveis contra uma ampla gama de vírus OPXV.

2.3 MECANISMOS DE INFECÇÃO E RESPOSTA IMUNOLÓGICA

No contexto da infecção viral, as proteínas de entrada são aquelas que facilitam a penetração do vírus na célula hospedeira, enquanto as proteínas de saída estão envolvidas no processo de liberação do vírus para infecção de novas células (Wang *et al.,* 2023). Na figura 1, tem-se uma representação esquemática das proteínas envolvidas neste processo.

Figura 1 - Proteínas de entrada e saída do gênero OPXV na célula hospedeira.



Fonte: Do autor

Legenda: Na parte superior da figura, a proteína A17L está envolvida na entrada do vírus na célula hospedeira; subsequentemente, as proteínas da membrana viral A28L, H2R e L1R facilitam a fusão do vírus com a célula hospedeira, com a A28L também permitindo a formação de inclusões virais, auxiliando na entrada do vírus. Na parte inferior da figura, as proteínas A33R e C19L contribuem para a formação de partículas virais extracelulares. A proteína A35R está envolvida na formação e maturação de vírions envelopados extracelulares (EEV); a proteína B5R é essencial para a formação de EEV e para a liberação do vírus; e, por fim, a proteína A36R facilita a saída do vírus e a infecção de células vizinhas.

Entre as proteínas envolvidas no processo de entrada viral, destacam-se A17L, A27L, A28L, H2R e L1R, cada uma desempenhando funções críticas na interação com a célula hospedeira (Hatmal *et al.,* 2022). A A17L é uma proteína miristoilada, cuja modificação lipídica facilita sua ancoragem na membrana viral, promovendo a fusão eficiente entre o envelope do vírus e a membrana da célula hospedeira (Wang *et al.,* 2023). A A27L, por sua vez, é uma proteína de inclusão tipo A, que desempenha um papel duplo. Ela facilita a interação do vírus com os receptores presentes na superfície da célula hospedeira, e também contribui para a formação de inclusões virais no citoplasma, favorecendo a estabilidade e organização das partículas virais (Wang *et al.,* 2023).

A A28L, conhecida como fator de inclusão truncado P4c/ATI, é essencial para garantir que o envelope viral se funda de maneira eficiente com a membrana da célula hospedeira, facilitando a entrada do material genético viral no interior da célula (Hatmal *et al.,* 2022). Já

as proteínas H2R e L1R desempenham funções complementares no processo de fusão. H2R é um componente crucial do complexo de fusão viral, necessário para a penetração do vírus na célula hospedeira, enquanto L1R, uma proteína de membrana associada ao vírus maduro intracelular (IMV), garante a estabilidade estrutural durante a fusão, permitindo que o vírus estabeleça a infecção de maneira eficiente (Hatmal *et al.,* 2022).

Já no processo de saída do vírus da célula hospedeira, as proteínas A33R, A35R, A36R, C19L e B5R desempenham papéis essenciais na constituição e liberação das partículas virais (Wang *et al.,* 2023). A proteína A33R, juntamente com a A36R, auxilia na movimentação das partículas virais recém-formadas até a superfície celular. A33R está associada às partículas virais extracelulares (EEV), enquanto a A36R é uma glicoproteína de membrana que participa tanto na infecção de novas células quanto na saída eficiente do vírus da célula hospedeira (Wang *et al.,* 2023).

A A35R, uma fosfoproteína de membrana, desempenha um papel crítico na maturação das partículas virais. Essa fosfoglicoproteína contribui para o desenvolvimento de partículas virais extracelulares maduras, fundamentais para a disseminação eficiente do vírus (Hatmal *et al.,* 2022). Por sua vez, a proteína C19L, uma proteína palmitilada associada à membrana do vírus, contribui para a estabilidade estrutural da partícula viral e facilita a ligação com a célula hospedeira durante o processo de liberação, auxiliando na saída do vírus para o ambiente extracelular (Wang *et al.,* 2023).

Por fim, a proteína B5R atua em múltiplas etapas do ciclo viral, estando envolvida tanto nos processos de entrada quanto de saída viral. Durante a fase de saída, a B5R é essencial para a formação e liberação das partículas virais, especialmente as partículas virais extracelulares, que desempenham um papel fundamental na transmissão do vírus entre células (Hatmal *et al.,* 2022)

2.4 ESTRATÉGIAS ATUAIS DE PREVENÇÃO E TRATAMENTO

Diante do quadro sintomatológico causado por infecções por vírus do gênero OPXV, torna-se necessário o uso de compostos de pequenas moléculas para o tratamento dessas infecções (Wang *et al.,* 2023). Atualmente, dois compostos aprovados pela *Food and Drug Administrion* (FDA) estão disponíveis para esse fim: Tecovirimat (ST-246) e Brincidofovir (CMX001), ambos apresentando ampla atividade antiviral contra os vírus desse gênero (Delaune; Iseni, 2020).

O Tecovirimat (ST-246) atua inibindo a proteína p37, essencial no processo de

formação de partículas virais. Esse composto demonstrou eficácia contra diversos vírus, incluindo *cowpox virus, mpox virus, variola virus,* e *vaccinia virus*, entre outros (Smith *et al.,* 2009). No entanto, a proteína p37 apresenta suscetibilidade a mutações, tanto entre diferentes vírus quanto dentro de uma mesma espécie viral, gerando novas linhagens e exigindo vigilância constante (Wang *et al.,* 2023).

Já o Brincidofovir (CMX001) age inibindo a DNA polimerase viral, o que interfere diretamente na replicação do DNA do vírus (Wang *et al.,* 2023). Esse composto mostrou eficácia contra infecções por *variola virus, vaccinia virus, mpox virus*, e outros vírus do gênero OPXV (Chan-Tack *et al.,* 2021). Apesar de haver pesquisas em andamento sobre outros compostos específicos para vírus individuais, nenhum deles foi aprovado pela FDA até o momento (Wang *et al.,* 2023).

Embora existam tratamentos disponíveis para infecções por vírus do gênero OPXV, a vacinação continua sendo a melhor estratégia preventiva (Hong *et al.,* 2023). O programa global de vacinação contra a varíola, implementado na década de 1980, foi notoriamente responsável por erradicar uma das infecções virais mais devastadoras do século passado. (Shchelkunova; Shchelkunov, 2022). Contudo, o declínio nas campanhas de imunização aumentou o risco de transmissão de outros vírus do gênero OPXV, levantando preocupações sobre a morbidade dessas infecções zoonóticas (Diaz *et al.,* 2021).

Atualmente, duas vacinas estão disponíveis para proteção abrangente contra os vírus do gênero OPXV, de acordo com os Centros de Controle e Prevenção de Doenças (CDC): ACAM2000 e JYNNEOS (*Centers for Disease Control and Prevention*, 2024). Ambas são licenciadas para uso em pessoas que tiveram contato com indivíduos infectados por OPXV e estão sendo administradas para conter o aumento de casos (*Centers for Disease Control and Prevention*, 2024). No entanto, é importante destacar que essas vacinas utilizam vírus atenuados, o que as torna contraindicadas para indivíduos imunocomprometidos e grupos de risco, como gestantes e idosos (Vartak; Sucheck, 2016).

A vacinação continua sendo uma das estratégias mais eficientes no controle de doenças infecciosas, mas limitações associadas às abordagens tradicionais têm impulsionado o uso de métodos computacionais, como a vacinologia reversa, no desenho de vacinas de subunidades (Hong *et al.,* 2023).

2.5 VACIONOLOGIA REVERSA

A vacinologia reversa é uma técnica introduzida pelo pesquisador Rino Rappuoli no

ano 2000 (Rappuoli, 2000). Durante um estudo com *Neisseria meningitidis* (meningococo B), o pesquisador demonstrou que ferramentas computacionais podem ser utilizadas para identificar e desenvolver potenciais antígenos diretamente a partir de sequências genômicas, eliminando a necessidade de isolar ou cultivar o microrganismo em laboratório (Goodswen *et al.,* 2023).

De forma geral, a vacinologia reversa utiliza informações genômicas de patógenos, amplamente disponíveis em bancos de dados públicos, para identificar proteínas antigênicas e regiões específicas capazes de induzir uma resposta imune adequada. Após essa identificação, pequenas porções dessas proteínas são selecionadas para processos de triagem (Goodswen *et al.,* 2023). Desde a introdução do termo "vacinologia reversa" por Rino Rappuoli, essas triagens têm evoluído continuamente. Atualmente, incluem análises avançadas, como predição de antigenicidade, alergenicidade, estabilidade estrutural, conservação entre cepas e modelagem tridimensional, além de estudos de interação com receptores da imunidade inata (De Araújo *et al.,* 2022; Goodswen *et al.,* 2023).

Esse processo permite o desenvolvimento de vacinas de subunidade de forma rápida e com menor custo, uma vez que elimina a necessidade de realizar inúmeros testes iniciais para a identificação de antígenos (Goodswen *et al.,* 2023). No entanto, a validação experimental dos resultados obtidos por métodos computacionais é essencial para garantir sua eficácia e segurança (De Araújo *et al.,* 2022). Considerando a importância das doenças causadas por vírus do gênero OPXV, as limitações dos tratamentos disponíveis e a existência predominante de vacinas atenuadas, que são contraindicadas para populações de risco, torna-se essencial o desenvolvimento de estratégias vacinais mais seguras e eficazes (De Araújo *et al.,* 2022; Goodswen *et al.,* 2023). Nesse contexto, a vacinologia reversa surge como uma abordagem promissora, permitindo a identificação racional de candidatos vacinais com diversas vantagens, como imunogenicidade direcionada (especialmente quando combinada a adjuvantes), facilidade de produção e armazenamento, além de segurança para aplicação em populações vulneráveis, incluindo idosos, crianças e indivíduos imunocomprometidos (Vartak; Sucheck, 2016).

3 OBJETIVOS

3.1 OBJETIVO GERAL

Identificar epítopos conservados em dez proteínas envolvidas nos processos de entrada e saída viral nas células hospedeiras, abrangendo todos os vírus do gênero OPXV, bem como, construir e desenvolver *in silico* uma vacina quimérica multiepítopo imunogênica para o gênero OPXV e uma específica para Mpox.

3.2 OBJETIVOS ESPECÍFICOS

a) Identificar proteínas envolvidas em processos de entrada e saída viral em todos os vírus pertencentes ao gênero *Orthopoxvírus*;
b) Identificar regiões conservadas entre proteínas envolvidas em processos de entrada e saída viral dos 16 vírus pertencentes ao gênero *Orthopoxvírus*;
c) Identificar epítopos imunogênicos conservados, bem como específicos para cada vírus e avaliar características de propriedades físico-químicas, de alergenicidade e antigenicidade;
d) Desenvolver novas proteínas quiméricas multi-epítopos a partir de epítopos identificados e avaliar características de propriedades físico-químicas, de alergenicidade e antigenicidade;
e) Modelar a estrutura tridimensional das proteínas multiepítopos a partir de modelos moleculares e validar as estruturas por dinâmica molecular;
f) Avaliar a capacidade das proteínas multiepítopos de interagirem com receptores da imunidade inata semelhantes a Toll (TLRs);
g) Simular a resposta imunológica humoral e celular provável da interação das proteínas multi-epítopos com hospedeiro vertebrado.

4 ARTIGO 1 – *IMMUNOINFORMATIC APPROACH FOR RATIONAL IDENTIFICATION OF IMMUNOGENIC PEPTIDES AGAINST HOST ENTRY AND/OR EXIT MONKEYPOX PROTEINS AND POTENTIAL MULTI-EPITOPE VACCINE CONSTRUCTION*

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4.1 FUNDAMENTOS TEÓRICOS E CONTEXTUALIZAÇÃO DO ESTUDO

Vírus emergentes e reemergentes representam uma preocupação constante para os órgãos de saúde mundiais, exigindo vigilância rigorosa e contínua. A compreensão aprofundada desses patógenos, especialmente dos zoonóticos, constitui um dos pilares para o avanço das práticas em saúde, controle e prevenção em escala global (Choi, 2021). Um exemplo emblemático é o vírus *Monkeypox* (Mpox), pertencente ao gênero *Orthopoxvírus*, um grande vírus de DNA fita dupla que produz aproximadamente 180 proteínas. Seu ciclo de vida ocorre no citoplasma das células do hospedeiro (Walter *et al.,* 2022). Em 2022, esse vírus apresentou o maior número de mutações já registrado, 40 mutações distintas, sugerindo uma evolução no sentido de maior disseminação viral (Gieryńska, *et al.,* 2023).

Diante desse cenário e considerando que a vacinação é o método de prevenção mais eficaz contra vírus emergentes e reemergentes, diversos estudos têm buscado superar os desafios associados à identificação precoce e intervenção em surtos de doenças infecciosas,

(De Araújo *et al.,* 2022). Embora vacinas baseadas em vírus atenuados ou mortos ofereçam alguma proteção cruzada contra o MPXV, elas apresentam limitações quanto à segurança, imunogenicidade e disponibilidade, especialmente em populações imunocomprometidas (De Araújo et al., 2024). Nesse contexto, as vacinas de subunidades, particularmente aquelas baseadas em epítopos imunogênicos conservados, representam uma alternativa promissora, podendo induzir respostas imunes direcionadas e seguras (De Araújo *et al.,* 2022).

Nesse contexto, o estudo intitulado "*Immunoinformatic Approach for Rational Identification of Immunogenic Peptides Against Host Entry and/or Exit Mpox Proteins and Potential Multiepitope Vaccine Construction*" — no qual este autor figura como primeiro autor — apresenta uma abordagem imunoinformática inovadora para a identificação e o desenvolvimento de novos epítopos capazes de enfrentar tais desafios. O principal objetivo do artigo foi investigar, a partir de dez proteínas estruturais envolvidas nos processos de entrada e saída do vírus Mpox em células hospedeiras, a identificação de epítopos estáveis e antigênicos que possam ser utilizados tanto no desenvolvimento de candidatos vacinais multiepitópicos quanto na criação de testes de imunodiagnóstico para o vírus Mpox.

The Journal of Infectious Diseases
SUPPLEMENT ARTICLE

Immunoinformatic Approach for Rational Identification of Immunogenic Peptides Against Host Entry and/or Exit Mpox Proteins and Potential Multiepitope Vaccine Construction

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COVID-19 has intensified humanity's concern about the emergence of new pandemics. Since 2018, epidemic outbreaks of the mpox virus have become worrisome. In June 2022, the World Health Organization declared the disease a global health emergency, with 14 500 cases reported by the Centers for Disease Control and Prevention in 60 countries. Therefore, the development of a vaccine based on the current virus genome is paramount in combating new cases. In view of this, we hypothesized the obtainment of rational immunogenic peptides predicted from proteins responsible for entry of the mpox virus into the host (A17L, A26L/A30L, A33R, H2R, L1R), exit (A27L, A35R, A36R, C19L), and both (B5R). To achieve this, we aligned the genome sequencing data of mpox virus isolated from an infected individual in the United States in June 2022 (ON674051.1) with the reference genome dated 2001 (NC_003310.1) for conservation analysis. The Immune Epitope Database server was used for the identification and characterization of the epitopes of each protein related to major histocompatibility complex I or II interaction and recognition by B-cell receptors, resulting in 138 epitopes for A17L, 233 for A28L, 48 for A33R, 77 for H2R, 77 for L1R, 270 for A27L, 72 for A35R, A36R, 148 for C19L, and 276 for B5R. These epitopes were tested in silico for antigenicity, physicochemical properties, and allergenicity, resulting in 51, 40, 10, 34, 38, 57, 25, 7, 47, and 53 epitopes, respectively. Additionally, to select an epitope with the highest promiscuity of binding to major histocompatibility complexes and B-cell receptor simultaneously, all epitopes of each protein were aligned, and the most repetitive and antigenic regions were identified. By classifying the results, we obtained 23 epitopes from the entry proteins, 16 from the exit proteins, and 7 from both. Subsequently, 1 epitope from each protein was selected, and all 3 were fused to construct a chimeric protein that has potential as a multiepitope vaccine. The constructed vaccine was then analyzed for its physicochemical, antigenic, and allergenic properties. Protein modeling, molecular dynamics, and molecular docking were performed on Toll-like receptors 2, 4, and 8, followed by in silico immune simulation of the vaccine. Finally, the results indicate an effective, stable, and safe vaccine that can be further tested, especially in vitro and in vivo, to validate the findings demonstrated in silico.

Keywords. Reverse vaccinology; mpox virus; immune response prediction; multiepitope vaccine; in silico vaccine design.

Zoonotic viral pathogens represent a major concern for global public health, as the transmission of these pathogens can result in severe diseases and even the emergence of pandemics such as COVID-19. For instance, during the recent SARS-CoV-2 pandemic, the world faced the additional challenge of a reemerging outbreak of mpox caused by the mpox virus (MPXV).

Prior to May 2022, MPXV infections in individuals living outside of Africa were mainly associated with travel to regions where MPXV was endemic or exposure to animals infected with the virus [1]. This virus belongs to the *Orthopoxvirus* genus of the Poxviridae family and is a large double-stranded DNA virus, with an average size of 200 to 250 nm. Its entire life cycle occurs within the cytoplasm of infected cells, where various proteins necessary for replication machinery are encoded by open reading frames of the MPXV genome [2–4].

The outbreak of the virus in 2022 exhibited a higher number of mutations, indicating an evolution toward greater efficiency in dissemination, with 40 distinct mutations shared by the isolates from this outbreak as compared with its closest variant [5]. The best way to prevent the spread of infectious diseases such as mpox is through vaccination [6]. Therefore, the development of a vaccine based on the current virus genome is paramount in combating new cases. Despite the effectiveness of current vaccines for mpox that employ the attenuated virus methodology, a subunit vaccine may increase

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their safety due to (1) nonuse of the whole virus; (2) targeted immunogenicity through the use of adjuvants; (3) ease of production and storage; and (4) their application in vulnerable populations, such as the elderly, children, and individuals who are immunocompromised [7].

In view of this, the objective of this study was the identification and construction of a chimeric protein, in which the epitopes were obtained through an immunoinformatics approach by using proteins responsible for the entry and exit of MPXV in the host, as well as adjuvants and ligands. The constructed vaccine was analyzed for its physicochemical, antigenic, and allergenic properties. Protein modeling, molecular dynamics, and molecular docking were also performed on Toll-like receptors 2, 4, and 8 (TLR-2, TLR-4, and TLR-8). Finally, an in silico immune simulation of the vaccine was conducted. Overall, the vaccine showed satisfactory indices for all analyses, but further tests are necessary, especially in vitro and in vivo.

METHODS

Obtaining Mpox Virus Sequences

The reference genome and the genome of an infected individual in Florida in June 2022 were acquired from the National Center for Biotechnology Information database with the identification "taxid:10244" (https://www.ncbi.nlm.nih.gov/datasets/taxonomy/10244/). The proteins involved in the virus entry and exit mechanisms for each genome were obtained in FASTA format. The conservation of the proteins was evaluated with the Immune Epitope Database (IEDB) server (http://tools.iedb.org/conservancy/). The pipeline followed the chart indicated in Figure 1*A*.

Prediction of Major Histocompatibility Complex I or II or B-Cell Receptor Epitopes

Algorithms from the IEDB Analysis Resource (https://www.iedb.org/) were used, with only the methods being modified for each prediction. To identify potential major histocompatibility complex I and II (MHC-I and MHC-II) epitopes, the NetMHCpan EL 4.1 prediction method was used (http://tools.iedb.org/mhci/ and http://tools.iedb.org/mhcii/), with the option to obtain epitopes that interact with the 27 reference alleles simulating broader global population coverage, representing 97% to 99%. For MHC-I, a cutoff of 1% of the total quantity of each structure was established [8, 9]. For MHC-II, a consensus percentile threshold < 20.0 and interaction with >50% of the reference alleles were established [10]. To identify potential B-cell epitopes, we used the BepiPred Linear Epitope Prediction 2.0 server (http://tools.iedb.org/bcell/), with a cutoff limit of 0.5 [11]. All cutoff values were recommended by the IEDB platform.

Evaluation of Epitope Properties: Antigenic, Allergenic, and Physicochemical

The VaxiJen 2.0 server was used for antigenicity testing (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html), with the target organism set as virus, and epitopes with a cutoff threshold < 0.5 and labeled "likely nonantigen" were removed. The server has 89% accuracy [12]. For allergenicity testing, we used the AllerCatPro 2.0 server (https://allercatpro.bii.a-star.edu.sg/), with a sensitivity of 93.2%, specificity of 98.8%, and accuracy of 96% [13]. The standard method provided by the server was utilized, and epitopes predicted as allergenic were removed. The ProtParam tool from the Expasy server (https://web.expasy.org/protparam/) was used for physicochemical property testing, and epitopes with structure stability values > 40 were removed [14].

Alignment, Consensus Epitope Determination, and Validation of Epitope Regions

The approved epitopes from all previous steps were aligned with the MultiAlign server (http://multalin.toulouse.inra.fr/multalin/), and manual alignment corrections were performed when necessary for improved accuracy [15]. From the alignment, for each protein, epitope regions were selected with the highest repetition rate and the highest antigenicity value according to the Vaxijen server. Epitope regions in transmembrane regions were removed with the SOSUI server (http://harrier.nagahama-i-bio.ac.jp/sosui/sosuisubmit.html) [16]. Epitope regions were subjected to the MHC-I, MHC-II, and B-cell receptor (BCR) steps of the IEDB server again for validation of interaction with their receptors.

Construction of Multiepitope Chimeric Protein and Evaluation of Similarity, Antigenicity, Allergenicity, Solubility, and Physicochemical Properties

Next, the epitopes were classified according to their interactions with the receptors. For the construction of the chimeric protein, 1 epitope from each protein was selected per the following criteria: (1) interaction with BCR, MHC-I, and MHC-II receptors; (2) highest antigenicity value; and (3) lowest stability value. The selected epitopes were sequentially conjugated with the adjuvants PADRE and β-defensin via the EAAAK linker between the adjuvants and the GGGGGG linker between the epitopes. The chimeric protein was subjected to the same servers and parameters as the epitopes, including Vaxijen for antigenicity, AllerCatPro 2.0 for allergenicity, and Protparam for stability. The solubility of the chimeric protein was evaluated with the SOLpro server (https://scratch.proteomics.ics.uci.edu/index.html) [17]. Finally, an analysis of the chimeric protein was performed on the BLAST server to assess similarity [18].

Modeling, Molecular Dynamics, and Molecular Docking

The AlphaFold Colab tool (https://colab.research.google.com/github/deepmind/alphafold/blob/main/notebooks/AlphaFold.ipynb) was used for 3-dimensional structure prediction, with default options selected [19]. Molecular dynamics simulation was performed with GROMACS 2019.3 software, with the OPLS-AA/L force field. The protein was placed in a 1-nm cubic

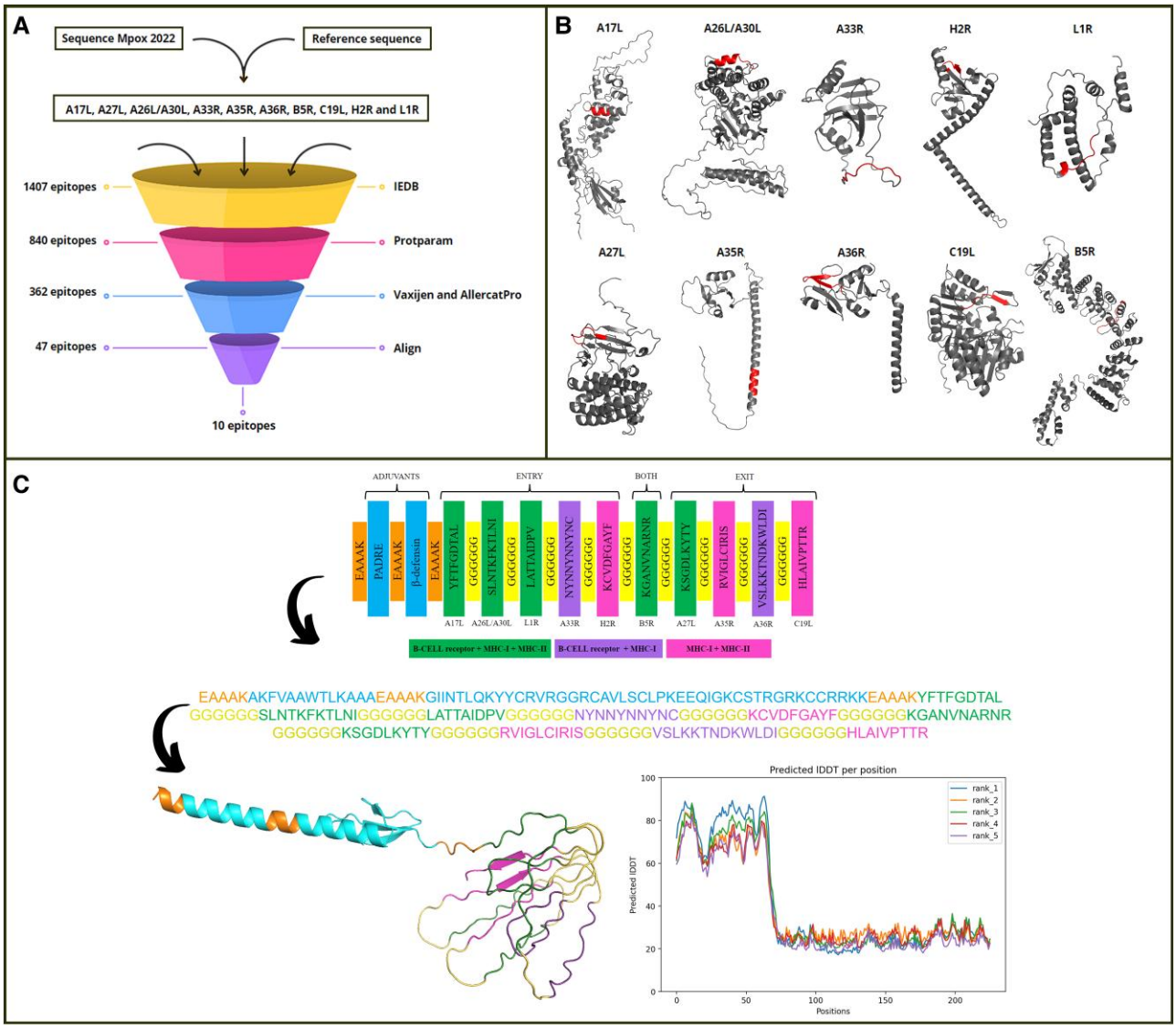

Figure 1. Identification of epitopes from entry and/or exit proteins from mpox virus into the host. *A*, Flowchart of the processes carried out to obtain the epitopes, illustrating at each step the software used and the total quantity of epitopes obtained. *B*, Three-dimensional diagram of the input and output proteins of the mpox virus; in red are the portions of the rationally obtained epitopes. *C*, The 3-dimensional structure prediction of the chimeric protein shows high values for the prediction of adjuvants and low values for the epitope region. IEDB, Immune Epitope Database; IDDT, local distance difference test.

unit cell and solvated with water, and ions (Na and Cl) were added to neutralize the system based on their charges. Water equilibrium under NVT and NPT conditions was performed for 100 picoseconds, with a temperature of 300 K and a pressure of 1 bar. A 70-nanosecond simulation was then conducted, and root mean square deviation values were generated by GROMACS [20]. For molecular docking, the final position (70 nanoseconds) from the molecular dynamic simulation was selected, and water molecules and ions were removed by Pymol software. The protein was then subjected to molecular docking with the receptors of TLR-2 (PDB:2Z7X), TLR-4 (PDB:3FXI), and TLR-8 (PDB:5AWA) via the ClusPro server (https://cluspro.bu.edu) [21].

In Silico Immune Simulation of the Vaccine

The chimeric protein was subjected to the C-ImmSim server (https://kraken.iac.rm.cnr.it/C-IMMSIM/) for predicting the immune response profile. The parameters were as follows: single and 3 doses; random seed = 12345; injection without lipopolysaccharide; simulation volume of 10; 300 simulation steps; and dosing interval of 1, 100, and 200 steps. Humoral and cellular responses were analyzed [22].

RESULTS

Entry and Exit Proteins of MPXV Remain Conserved After 20 Years

The viral protein sequence in this study was retrieved as a FASTA file from the National Center for Biotechnology

Information database via the sequencing data of the MPXV genome isolated from an infected individual in the United States in June 2022 (ON674051.1), with the reference genome dated to 2001 (NC_003310.1) for conservation analysis. The proteins responsible for the entry of MPXV into the host (A17L, A28L, A33R, H2R, L1R), the exit (A27L, A35R, A36R, C19L), and both (B5R) showed a high conservation rate among the proteins (Supplementary Table 1), at an average of 98.70%, and were therefore selected for obtaining rational immunogenic peptides. The predicted tridimensional structure from each protein is shown in Figure 1*B*.

Epitope Identification Indicates Greater Interactions With MHC-I and MHC-II Receptors and Fewer Interactions With BCR

The identification and characterization of epitopes for each protein demonstrated that a greater number of them are capable of interacting with MHC-I or MHC-II molecules while fewer interact with BCR. Subsequently, the epitopes were tested in silico for their antigenicity, allergenicity, and physicochemical properties, and those that did not meet the cutoff thresholds were removed. The number of epitopes related to each step is described in Supplementary Table 2.

The selected epitopes were aligned to obtain the region with the highest repetition, and the regions were again tested for antigenicity to obtain the most promiscuous peptide. In some cases, the number of amino acids in the epitopes originally provided by the IEDB was altered to enhance antigenicity, and for this purpose, the analyses were repeated in the IEDB to confirm that the interaction with MHC-I, MHC-II, and BCRs still occurred. The epitopes were selected by their interaction with MHC-I, MHC-II, and BCR or MHC-I and BCR or MHC-I and MHC-II. The final amino acid sequences of each epitope and their antigenicity values are presented in Supplementary Table 1.

It is important to mention that transmembrane regions were identified by the SOSUI server, and epitopes present in these regions were removed. However, only a few regions were found, with 1 in the A17L protein (341–363), 1 in H2R (30–52), 3 in A27L (92–114, 138–160, and 195–271), and 1 in A35R (38–60). The remaining proteins were identified as soluble.

Filtering Epitopes for a Chimeric Protein Designed With Promiscuous Epitopes Shows No Similarity, High Antigenicity, Low Allergenicity, and Good Stability

For the construction of a chimeric protein, it was necessary to select 1 epitope from each protein, requiring a standardized selection process. The first selection prioritized epitopes that interact with BCRs, MHC-I, and MHC-II simultaneously. Since the A27L protein had 4 epitopes, all 4 underwent VaxiJen and ProtParam analyses to choose the best among them, and only 1 was obtained, as defined by its antigenicity and stability. However, not all proteins had epitopes that interacted with BCRs, MHC-I, and MHC-II simultaneously, so a second selection was performed. The other proteins that have epitopes that interact only with BCRs and MHC-I or MHC-I and MHC-II also underwent VaxiJen and ProtParam to select the best epitopes for each protein within these interactions.

In addition, for the construction of the chimeric protein, 2 adjuvants were used: β-defensin to enhance vaccine immunogenicity and PADRE to enhance vaccine efficacy by favoring CD4+ T-cell activation [23]. These adjuvants were combined with the EAAAK ligand, known for its rigid binding; then, the preselected epitopes were linked with the GGGGGG ligand, known for its flexible binding. In total, the chimeric protein had 226 amino acids, and the design of the vaccine construct can be seen in Figure 1*C*. The BLAST analysis indicated that the chimeric protein exhibits similarity only in the adjuvant portion, whereas the epitope region showed no similarity with any other protein deposited in the databases.

The chimeric protein had a good antigenicity value of 0.9442. The molecular weight of the chimeric protein was 22 230.11 KDa, and the theoretical isoelectric point was 9.85, suggesting a basic nature. The stability index was 37.56, indicating that the protein is stable, and the aliphatic index was 61.41, demonstrating broad thermostability. The hydrophobicity (GRAVY) was −0.315, showing that the molecule is hydrophilic. For the prediction of allergenicity, the AllerCatPro 2.0 server was used, and the chimeric protein showed no evidence of allergenicity. For solubility prediction, we used the SOLpro server and obtained a value of 0.884415, specifying that the protein is soluble.

Chimeric Protein Exhibits Good Stability, Low Fluctuation, and TLR Interaction in Adjuvants and Epitopes

The 3-dimensional structure of the chimeric protein corresponding to rank 1 was used in the molecular dynamics step because it had the best predicted local distance difference test result. However, it is important to mention that the structure showed high prediction values for the adjuvants and low values for the epitope regions. The reason is that the crystallographic forms of the adjuvant proteins can be found in databases such as the Protein Data Bank, while the entry and exit proteins of the virus do not have well-defined crystallographic forms.

The modeled protein was then subjected to molecular dynamics simulation (Figure 2*A*). The stability of the structure over time was evaluated by generating the root mean square deviation graph, showing that the protein achieved stability after 30 nanoseconds and remained stable until the end of the simulation (70 nanoseconds), with a structure variation ranging from 1.11 to 1.53 nm (Figure 2*A*). The root mean square fluctuation graph indicated higher variation in the β-defensin adjuvant as compared with the rest of the protein.

Molecular docking indicated good interactions between the Toll-like receptors and the chimeric protein (Figure 2*B*). With TLR-2, the chimeric protein achieved a binding energy of −955.5 kcal/mol with 25 members. It formed 4 hydrogen bonds, 2 salt bridges, and 62 hydrophobic interactions. With TLR-4, the

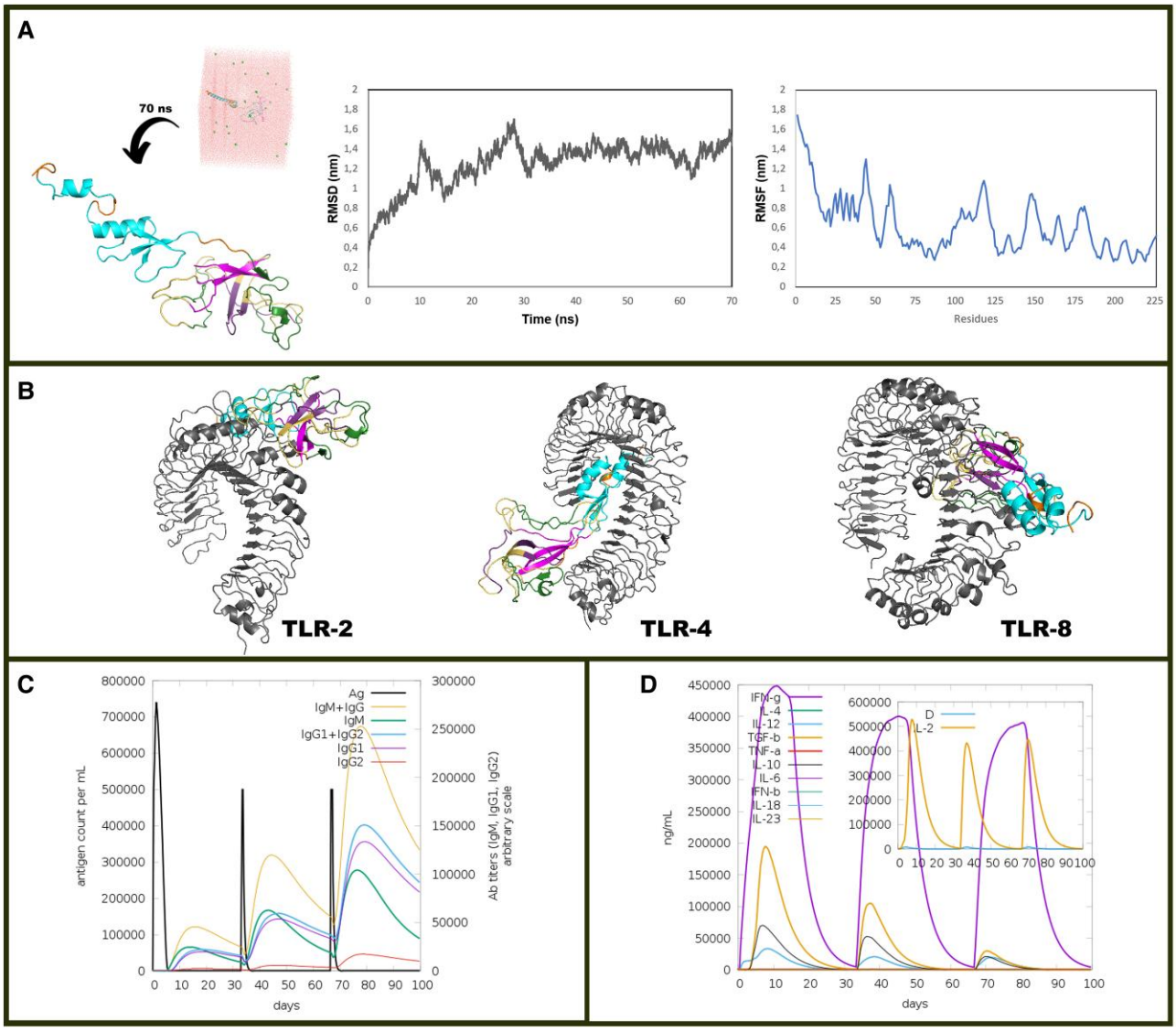

Figure 2. The constructed chimeric protein demonstrated stability after 30 nanoseconds with good interaction with Toll-like receptors (TLRs) and promising results for the immune response. *A*, Chimeric protein after molecular dynamics and RMSD and RMSF results. *B*, Molecular docking of the chimeric protein with TLR-2, TLR-4, or TLR-8. *C*, Results in silico of immunoglobulin concentrations relative to antigen concentration. *D*, Cytokine and interleukin levels for 3-dose application. The "D" in the inserted graph represents a potential danger signal and potential response from interleukin 2 as a leukocyte growth factor. RMSD, root mean square deviation; RMSF, root mean square fluctuation.

protein obtained a binding energy of −1164.8 kcal/mol with 34 members, 19 hydrogen bonds, and 38 hydrophobic interactions. With TLR-8, the protein had a binding energy of −1456.2 kcal/mol with 71 members, 44 hydrogen bonds, 4 salt bridges, and 60 hydrophobic interactions. Note that the vaccine interacted with amino acids involving epitopes and adjuvants in all TLRs.

In Silico Immune Response Simulation Indicates Good Humoral and Cellular Response

In silico immune response simulation was performed. It showed that although the graph for a single dose reveals a high peak, the lateral scale demonstrates that the response is small when compared with that from 3 doses in terms of IgM + IgG, IgG1, and IgM. The IgM + IgG antibodies showed an increase from 45 000 at 30 days to 250 000 at 90 days (Figure 2*C*).

Regarding cytokines, the in silico results demonstrated that the production of interferon γ occurs similarly with 1 or 3 doses, as the response reaches similar values of milligram per milliliter in the first dose, with a slight decrease in the following 2 doses. The values of transforming growth factor β, interleukin 10, and interleukin 12 decrease from the first dose to the subsequent ones. Finally, the use of 1 or 3 doses does not generate alterations in potential danger levels, as identified by the letter "D" in Figure 2*D*.

DISCUSSION

Emerging and reemerging MPXV infections have threatened the health care sector due to the evolution of genomic characteristics and the lack of specific targeted therapy [24]. The treatments for mpox consist of medications such as tecovirimat, brincidofovir, and cidofovir, with proven results for the smallpox treatment, but there are still no data regarding their effectiveness for MPXV [25]. There are a number of historic or more recently developed smallpox vaccines, using replication competent vaccinia virus or replication impaired vaccinia virus, which may be anticipated to protect against mpox. In the U.S., [26] the historic Aventis Pasteur Vaccine was potency tested and stored in multi dose vials and may be used under emergency use auspices for smallpox. ACAM2000, a clonal derivative of the historic Dryvax smallpox vaccine, was licensed for smallpox and JYNNEOS, a replication deficient vaccinia virus was licensed for smallpox and mpox considerations in 2019. JYNNEOS was developed as a vaccine produced to be with fewer adverse reactions related to virus replication; it requires two doses for immunogenicity equivalent to that of replication competent vaccinia virus smallpox vaccines. Vaccines used during the smallpox era and JYNNEOS have demonstrated protection against mpox in humans at risk of infection [27, 28].

In this study, we observed that the proteins responsible for the entry and exit of MPXV in host cells exhibit high conservation values, averaging 98.70%. This can be justified by the fact that MPXV is a double-stranded DNA virus, which has fewer chances of mutation. Therefore, the mutations mentioned by Kumar et al [5] are likely to occur in regions outside the proteins responsible for viral entry and exit from the host. According to our findings, Shantier et al [29], who used the MPXV binding protein (E8L), and Sanami et al [30], who used the A28L protein, showed a higher conservation in those proteins. In the present study, these proteins also had conservation values from 98% to 100%.

According to Hatmal et al [3], viral proteins are essential for MPXV and can be grouped into viral entry proteins that facilitate virus entry into host cells, proteins involved in virus release from cells, and proteins that modulate the immune response and host cell. Since viral entry and release proteins could be targets for neutralizing antibodies or induce a T cell–mediated cellular response, epitopes interacting with BCRs, MHC-I, and MHC-II were selected in this study to design a potential vaccine against MPXV. Computational vaccine design offers thermodynamically stable, effective, specific, and cost-effective solutions as compared with traditional vaccine development [31]. Epitope-based vaccines can help overcome challenges such as genetic and antigenic variations [32]. Here, using a stringent selection protocol, we rationally obtained 10 antigenic epitopes.

Furthermore, the combination of protein antigens with adjuvants triggers local immune responses and the production of proinflammatory cytokines by macrophages [31]. This response is extremely necessary for activated dendritic cells or macrophages: it allows T cells to recognize the MHC-antigen complex through their T-cell receptors, leading to the production of memory T cells and the development of adaptive immunity [31]. To trigger a humoral immune response that activates B cells to produce antibodies, B-cell epitopes are required [33]. Therefore, a multiepitope vaccine that interacts simultaneously with T-cell receptors and BCRs significantly stimulates humoral and cellular immune responses [34]. Here, from 10 selected epitopes that interact with not only MHC molecules but also BCRs, a new protein was designed that is linked to adjuvants performing as 226 amino acids. Additionally, the chimeric protein exhibits unprecedented potential due to its dissimilarity to existing proteins.

The molecular dynamics of the chimeric protein were performed owing to the possibility of conformational changes occurring in the protein's structure in environments with variables such as pressure, pH, and temperature. The results indicated that the new protein obtains a stable conformational structure and could thus be subjected to molecular docking studies.

Based on the molecular docking analyses, high binding energy values can be observed in Toll-like receptors. For example, the binding energy between the vaccine and TLR-2 was −955.5 kcal/mol; for TLR-4, −1164.8 kcal/mol; and for TLR-8, −1456.2 kcal/mol. These results suggest good affinity to the receptors: the binding energy is similar to that of studies in the literature that used a methodology like ours [34] and even in methodology involving other microorganisms [35, 36].

Finally, immune simulation analysis was performed, which monitors the immune system response in terms of antibody production after the injection of constructed vaccines [31]. According to our analyses, the use of 3 doses of the vaccine shows potential, given the high levels of immune response. In the work of Shantier et al [29] and Aiman et al [37], levels of interferons and interleukin 2 increased after the initial injection and remained at elevated levels after repeated doses of antigen, a situation similar to our study. According to the authors, this implies a high number of T-helper cells and, as a result, efficient generation of antibodies, which supports a humoral response. Therefore, all these data point to the potential of the chimeric vaccine developed here to induce a long-lasting cellular and humoral immune response.

CONCLUSIONS

The emerging increase in the number of MPXV infection cases has been notoriously causing alarm to global public health organizations, mainly due to the lack of effective treatments against the virus's pathogenesis. The vaccine is considered a more efficient preventive method. Thus, this study carried

out the development of a stable, antigenic, and nonallergenic chimeric protein with epitopes capable of simultaneous interactions with immune receptors. Furthermore, the protein exhibited strong binding to TLR-2, TLR-4, and TLR-8, and in immune response simulations, it showed an increased cellular adaptative response and the production of IgM and IgG antibodies without demonstrating any danger indicators. Finally, further tests, particularly in vitro and in vivo, are necessary to validate the findings and results demonstrated in silico.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Author contributions. L. P. A.: conceptualization, data curation, formal analysis, investigation, methodology, validation, writing–original draft. N. C. M.: conceptualization, data curation, formal analysis, investigation, methodology, validation, writing–original draft. L. A. A. and P. P. C.: conceptualization, data curation, formal analysis, financial support, acquisition, investigation, resources, supervision, validation, visualization, writing–original draft.

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Supplementary Table 1 – Epitopes from entry and/or exit proteins from MPXV into the host. The amino acid sequences of the epitopes found for each input and output protein, along with the values of antigenicity and conservation, provided in parentheses. Epitopes that do not have a conservation value represent 100% similarity between the current protein and the reference protein.

Protein	BCR, MHC-I and MHC-II	BCR and MHC-I	MHC-I and MHC-II
A17L (99,2%)	YFTFGDTAL (1,4845)	GIADIRDKY (2,4687) IKTNDINVR (1,4509)	SFKFRPGSL (2,3612)
A27L (99,56%)	FSVFSTRYD (1,1579) KSGDLKYTY (1,6991) RTAIGIADYQ (1,6240 / 90%) FAILNLSID (1,1056)	TTDKKSKCF (1,2718) YNPLEDPNYV (0,6197) SLDMEREMR (1,0977) RNVEWELSRL (1,1341)	-
A26L/A30L (96,15%)	SLNTKFKTLNI (1,5044)	EITSNDKNL (1,2933) DYNPKPTPI (2,4516/ 88,88%)	LKFTYLGES (2,2078) LVYIRTNNR (1,3956) VILARKINM (1,3848)
A33R (95,86%)	-	NYNNYNNYNC (1,1425/ 70%)	VTINDLKMM (1,0433)
A35R (98,34%)	-	AAASSTHRK (1,0499)	RVIGLCIRIS (1,9609)
A36R (99,4%)	-	VSLKKTNDKWLDI (1,4608)	GIGTFLHYR (0,8904)
B5R (99,46%)	KGANVNARNR (1,6768)	GSDVDKKDT (1,1887) NIDSVDFNG (0,7223) RGVIDINYR (2,1594)	-
C19L (99,46%)	-	ETLPENMDFR (1,1189)	ITLAKKYIYIASFCC (0,8549) TRGALIFDK (0,9014) HLAIVPTTR (2,0033) EYVHITSANF (1,5553)
H2R (100%)	-	DWKSLTDSK (1,2045) VKKAAKVDP (1,2427)	KCVDFGAYF (2,2962) LIKHKSNNV (0,5899) LNELGYSGY (0,8633)
L1R (99,64%)	LATTAIDPV (1,1260)	VPIVTDGRVK (0,6989)	QYLDFLLLLLIQSKN (1,3973) FDFVISLMRFK (1,2387) RDIAFSNVMDI (1,5376)

Supplementary Table 2 – Number of epitopes obtained at each filtering step**.**

Protein	BCELL	MHC-I	MHC-II	Total	Protparam	Vaxijen	Align
A17L	12	93	33	138	86	51	4
A27L	9	182	79	270	166	57	8
A26L/A30L	11	137	75	223	109	40	7
A33R	3	38	7	48	36	10	2
A35R	5	44	23	72	46	25	2
A36R	5	50	18	78	36	7	2
B5R	17	152	107	276	139	53	7
C19L	6	107	35	148	102	47	5
H2R	4	50	23	77	66	34	5
L1R	4	45	28	77	54	38	5

5 ARTIGO 2 – *SHARED IMMUNOGENIC EPITOPES BETWEEN HOST ENTRY AND/OR EXIT PROTEINS FROM MONKEYPOX AND ALASKAPOX*

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5.1 FUNDAMENTOS TEÓRICOS E CONTEXTUALIZAÇÃO DO ESTUDO

Após a identificação de epítopos imunogênicos para o vírus *Monkeypox* (Mpox) — cujo estudo foi publicado no início de 2024 — (De Araújo *et al.,* 2024), um novo evento de importância epidemiológica ocorreu: a primeira morte causada por um vírus emergente em um paciente imunocomprometido no Alasca. Posteriormente, confirmou-se que o agente etiológico pertencia ao gênero *Orthopoxvirus*, sendo inicialmente denominado *Alaskapox* vírus (Devi, 2024). O caso envolveu um paciente arranhado por seu gato doméstico, embora se acredite que o felino tenha atuado apenas como vetor, sendo os roedores silvestres os prováveis hospedeiros naturais do vírus (Devi, 2024).

Diante desse cenário, formulamos a hipótese de que os epítopos imunogênicos previamente identificados no Mpox poderiam estar conservados no novo vírus, posteriormente renomeado como *Borealpox*. A confirmação dessa hipótese destacaria não apenas a importância da vigilância molecular constante, mas também a viabilidade de estratégias imunológicas mais amplas e integrativas frente aos vírus do gênero OPXV.

Nesse contexto, o artigo intitulado "*Shared immunogenic epitopes between host entry and exit proteins from monkeypox and Alaskapox viruses*", publicado na *The Lancet Microbe*, apresenta uma análise comparativa entre os epítopos identificados anteriormente no Mpox

(Artigo 1) e suas proteínas homólogas no *Alaskapox virus*. Para isso e por meio de ferramentas de imunoinformática, foram realizados alinhamentos e análises dos epítopos quanto às propriedades antigênicas, alergenicidade e estabilidade estrutural. A identificação de epítopos altamente conservados entre os dois vírus reforça sua aplicabilidade em estratégias vacinais multivalentes, já os epítopos com diferenças podem abrir caminho para o desenvolvimento de testes diagnósticos capazes de diferenciar infecções por Mpox e *Borealpox*.

Este capítulo apresenta o artigo na integra com os achados desta investigação, que discute a relevância da conservação antigênica interespécies como base racional para o desenvolvimento de vacinas de subunidade e ferramentas diagnósticas inovadoras. A incorporação desses resultados contribui para o avanço da imunologia comparada entre vírus emergentes e reemergentes do gênero OPXV, oferecendo soluções promissoras para o enfrentamento de futuras emergências virais.

Shared immunogenic epitopes between host entry and exit proteins from monkeypox and Alaskapox viruses

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Studies on emerging and re-emerging infectious diseases are crucial because of the constant threat of epidemics owing to unknown microorganisms or those already identified, with outbreak cases being reported annually.[1] In 2015, a new virus called Alaskapox virus (AKPV) was isolated from an Alaska resident, and on the basis of sequencing, its taxonomy was assigned to the genus Orthopoxvirus (OPXV).[2] In 2024, in *The Lancet Infectious Diseases*, Sharmila Devi reported the first death caused by AKPV in an immunocompromised patient, which exacerbated disease severity, thus illustrating the importance of a healthy immune system.[3]

Microorganisms belonging to the OPXV genus are a global threat as they trigger various infectious diseases worldwide.[4] In 2022, an outbreak of infections caused by the monkeypox virus (MPXV) was induced by a new variant with mutations that favoured its spread.[5] In 2024, in *The Lancet Microbe*, Raianna Fantin and Camila Coelho commented on the need for effective vaccines against MPXV considering the low antibody neutralisation levels of currently available vaccines against MPXV.[6] Because vaccination is a quick and effective strategy and subunit vaccines can be used to discover new vaccine agents, we focused efforts on developing a multiepitope immunogenic vaccine derived from proteins involved in viral entry and exit in host cells.[7] Thus, we aimed to investigate the presence of previously identified epitopes, from an earlier study on MPXV, in AKPV proteins and assess the effect of any mutations.

Available genomes of AKPV were obtained from the NCBI database. Alignments between MPXV epitopes previously identified in AKPV proteins were performed. Epitopes with mutations identified in AKPV proteins underwent analyses of antigenicity, allergenicity, and physicochemical properties, as previously described.[7]

Nine of ten MPXV proteins exhibited identity and similarity values with AKPV proteins, with only the A-type inclusion protein showing low identity and similarity values (table). Three of the ten epitopes showed 100% conservation among the proteins, and of the remaining seven epitopes, six exhibited missense substitution mutations and only one was completely deleted in AKPV. Of the six epitopes with mutations, two showed antigenicity and physicochemical property values beyond the cutoff threshold (table).

An in-silico evaluation showed that the multiepitope protein was effective in inducing an immune response by producing antibodies capable of neutralising viral proteins before infection and leaving traces in infected cells.[2] These proteins, present on infected cell membranes, can serve as targets for cellular immune response, such as cytolytic CD8 cells.[8] The presence of conserved proteins or epitopes among different species of microorganisms, with multieffector characteristics in both humoral and cellular

MPXV Protein	Identification of MPXV protein	Corresponding protein in AKPV	Identity (%)	Similarity (%)	Gaps (%)	Epitope in MPXV	Epitope in AKPV	Mutation	Mutation type	VaxiJen	Protparam
A17L	URP85069.1	QED21147.1	91·2	95	0	YFTFGDTAL	YFTFGDTAL	..	..	1·5	5·9
A26L/A30L	URP85080.1	QED21129.1	80·1	89·3	4	SLNTKFKTLNI	SLNTKFKTLNI	..	..	1·5	27·7
L1R	URP85026.1	QED21242.1	90·8	93·5	0·7	LATTAIDPV	LATATIEPV	T → A A → T D → E	Missense substitution	1·0	100·5*
A33R	URP85087.1	QED21259.1	73·8	77·9	16·6	NYNNYNNYNC	..	..	Deletion	..	..
H2R	URP85034.1	QED21222.1	96·8	98·4	0	KCVDFGAYF	RCVDFGAYF	K → R	Missense substitution	2·4	–34·0
B5R	URP85107.1	QED21126.1	80·9	86·9	2·7	KGANVNARNR	KGANVNARNK	R → K	Missense substitution	1·7	28·0
A27L	URP85079.1	QED21098.1	45·9	53·5	39·9	KSGDLKYTY	KSGDIKYTY	L → I	Missense substitution	1·8	–10·0
A35R	URP85095.1	QED21225.1	77·3	86·5	2·7	RVIGLCIRIS	HVIGLCIRIS	R → H	Missense substitution	1·5	9·0
A36R	URP85097.1	QED21233.1	86·9	95·2	0	VSLKKTNDKWLDI	VSLKKNSDKWVDI	T → N N → S L → V	Missense substitution	0·5†	–22·0
C19L	URP84978.1	QED21148.1	88·8	94·1	1·1	HLAIVPTTR	HLAIVPTTR	..	..	2·0	20·9

MPXV=monkeypox virus. AKPV=Alaskapox virus. *Stability value outside the cutoff threshold. †Antigenicity value less than the cutoff threshold.

Table: **Comparison of MPXV and AKPV proteins and analysis of antigenicity and structural stability by AKPV proteins**

responses, has great potential to be widely used. The current report showed the conservation between entry and exit proteins from MPXV and AKPV, including epitopes present in the abovementioned multiepitope construct. Because of the urgency to develop potential vaccines and diagnostic tools, the versatility of the immunoinformatics project assumes some rationality in its use by obtaining fast and promising results to control emerging and re-emerging diseases. In addition to the application of the studied peptide as a vaccine, the peptide can be used in diagnostic tests, including ELISA, for the rapid detection of antibodies present in patients' serum.[9] The results showed the presence of conservation sequences and differences that could be used to differentiate between infections caused by MPXV and AKPV. This approach, while not specifically targeted in all cases, is effective and plays an important role in early infection identification.[9]

The observation of peptide conservation in proteins similar to those in APKV reinforces the relevance of the study, highlighting the potential applicability of the study findings in different contexts. Furthermore, the ability to adapt the study findings to address issues related to MPXV control extends the usefulness of the study, demonstrating a multi-efficient approach that can benefit various public health scenarios.

The conserved and non-conserved epitopes between the entry and exit proteins from MPXV and AKPV have potential for use in rapid identification and characterisation tests for diseases caused by the viruses. Further analyses are recommended to assess the conservation of the epitopes in OPXV proteins to ascertain if the conservation occurs across the entire genus. Additionally, the epitopes show promising predictions of antigenicity, allergenicity, and structural stability, suggesting that the epitopes can be effectively used in the prevention and control of diseases caused by APKV and MPXV.

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6 ARTIGO 3 – *RATIONAL IDENTIFICATION OF A MULTIVALENT VACCINE CANDIDATE FROM CONSERVED IMMUNOGENIC PEPTIDES IN ENTRY AND EXIT PROTEINS OF THE ORTHOPOXVÍRUS GENUS*

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6.1 FUNDAMENTOS TEÓRICOS E CONTEXTUALIZAÇÃO DO ESTUDO

Ao final do artigo publicado na *The Lancet Microbe* (apresentado no Capítulo 2), surgiu uma nova hipótese a partir dos achados obtidos: seriam os três epítopos 100% conservados entre os vírus Mpox e *Alaskapox* — ou mesmo outros epítopos identificados — também conservados entre todas as 16 espécies descritas do gênero *Orthopoxvirus* (OPXV)? A confirmação dessa hipótese implicaria não apenas em um avanço na compreensão da conservação antigênica dentro do gênero, como também abriria caminho para o desenvolvimento de vacinas com amplo espectro de proteção.

Considerando o potencial pandêmico de diversos vírus do gênero OPXV e as limitações das vacinas atualmente disponíveis, como ACAM2000 e JYNNEOS — especialmente contraindicadas para indivíduos imunocomprometidos —, torna-se urgente a busca por alternativas mais seguras e eficazes. Nesse contexto, a vacinologia reversa aliada à imunoinformática destaca-se como uma estratégia promissora para o desenho racional de

vacinas de subunidade multiepítopo, com alto grau de especificidade, segurança e aplicabilidade populacional ampliada.

O artigo apresentado neste capítulo, publicado na revista *Scientific Reports*, descreve o desenvolvimento de um candidato vacinal multivalente, construído a partir de epítopos imunogênicos conservados em dez proteínas-chave envolvidas nos processos de entrada e saída viral dos 16 vírus do gênero OPXV — respondendo diretamente à hipótese levantada anteriormente. O estudo englobou a análise de 160 sequências proteicas, com posterior triagem e validação dos epítopos quanto à antigenicidade, ausência de alergenicidade e estabilidade estrutural. Esses epítopos foram concatenados em uma proteína quimérica, associada aos adjuvantes β-defensina e PADRE, e submetida à avaliação in silico de interação com o receptor TLR2 e potencial imunogênico.

Este capítulo apresenta os principais resultados dessa estratégia inovadora, que representa um avanço significativo no desenvolvimento de vacinas multivalentes com potencial de cobertura contra múltiplas espécies virais do gênero OPXV. Os achados reforçam a relevância do uso de plataformas computacionais na aceleração do desenvolvimento de imunógenos de nova geração, com aplicação tanto em estratégias profiláticas quanto em testes diagnósticos de alta especificidade.

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Multivalent vaccine candidate from conserved immunogenic peptides in entry or exit proteins of *Orthopoxvirus* genus

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Orthopoxvirus **(OPXV) genus includes emerging and re-emerging zoonotic viruses that pose threats to global health. Smallpox caused pandemics in the 20th century. Borealpox was responsible for a death in Alaska in 2024. Mpox, declared a Public Health Emergency by the WHO in 2022, with an alert reclassification in 2024. The lack of effective therapies and the limitations of attenuated virus vaccines, especially for immunocompromised individuals, reinforce the urgent need for new strategies to prevent diseases caused by pathogens of the OPXV genus. This study aimed to identify conserved epitopes in proteins essential for the entry and exit of these viruses and, based on this identification, develop a promising multivalent vaccine candidate. Viral protein sequences were extracted from the NCBI Virus database, and 160 sequences were analyzed to identify conserved epitopes using the Immune Epitope Database. After filtering the data, epitopes were concatenated to create a chimeric multi-epitope protein combined with β-defensin and PADRE adjuvants. The resulting protein, with eight conserved epitopes covering all OPXV viruses (including Mpox Clade Ib), was evaluated for antigenicity, allergenicity, and structural stability. It showed strong interaction with the TLR2 receptor, along with good predictions for immune responses after three doses. This proposed multivalent vaccine represents a potential approach against these zoonotic viruses, with promising results for in vitro and in vivo studies.**

Keywords *Orthopoxvirus*, Reverse vaccinology, Multi-epitope, Immunoinformatics

Emerging viruses, such as SARS-CoV-2 and re-emerging ones, such as *Monkeypox* (Mpox), pose constant challenges to global health[1]. While the former emerges unexpectedly and can cause sudden and devastating epidemics, the latter, although previously documented, resurface significantly, requiring continuous surveillance[2]. Recently, the Mpox virus, belonging to the genus *Orthopoxvirus* (OPXV), has once again become an international concern[3].

OPXV, part of the *Poxviridae* family, is one of the most significant viral genera for human health[4]. These viruses, characterized by their double-stranded DNA and complex structure, have a broad host spectrum, including various animal species, classifying them as zoonotic[2,4]. Although many are named after the animals with which they are associated, it is believed that these animals primarily act as vectors rather than the original sources of the viruses[4].

Currently, 16 species are recognized under the OPXV genus, including *Abatino*, *Akhmeta*, *Borealpox*, *Buffalopox*, *Camelpox*, *Cowpox*, *Ectromelia*, *Horsepox*, *Monkeypox*, *Rabbitpox*, *Raccoonpox*, *Skunkpox*, *Taterapox*, *Vaccinia*, *Variola*, and *Volepox*[2,5]. In particular, the *Variola* virus (VARV), the causative agent of smallpox, was responsible for one of the most devastating pandemics of the 20th century, resulting in approximately 300 to 500 million deaths[4,6]. Smallpox was officially eradicated in 1980, due to a global vaccination program[2,7]. However, with the decline in immunization, the risk of OPXV transmission has grown, raising concerns about the morbidity of these zoonotic infections[8].

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In recent years, outbreaks of OPXV infections have been recorded in various animals around the world. Cases of *Cowpox*, *Camelpox*, *Buffalopox*, and Mpox have been reported in Europe, the Middle East, India, South America, Africa, and the United States[8]. Moreover, new emerging viruses from this genus continue to be identified globally, such as the *Alaskapox virus* (recently renamed *Borealpox*), which caused the first death of an immunocompromised patient earlier this year[9].

Among the viruses of the OPXV genus, Mpox has stood out due to its recent global impact[3]. Although it shares symptoms similar to smallpox, Mpox is distinguished by characteristic skin lesions[10]. Initially restricted to the African continent, Mpox was declared a Public Health Emergency of International Concern (PHEIC) by the World Health Organization (WHO) in 2022, following a significant increase in cases, including around 100,000 reported infections worldwide[11]. In 2024, the WHO reaffirmed this global emergency due to the evolution of Clade Ib, which demonstrated higher transmissibility and the ability to spread to other continents[3].

According to the Centers for Disease Control and Prevention (CDC), two vaccines are available for the prevention of infections caused by OPXV: *ACAM2000* and *JYNNEOS*[2]. Both are licensed for use in at-risk individuals who have had contact with Mpox-infected persons and are being administered to contain current outbreaks[2]. However, as they use attenuated viruses, these vaccines are contraindicated for immunocompromised individuals and other at-risk groups[2].

In this context, our research group previously developed an in silico multi-epitope vaccine candidate for Mpox, focusing on the viral entry and exit proteins in the host cell[12]. Additionally, we identified three Mpox epitopes that are fully conserved in proteins similar to those of the *Borealpox virus*[13]. These findings led us to suggest that conserved epitopes might be present across the entire OPXV genus[13].

Based on this hypothesis, the presence of conserved epitopes in the ten key proteins involved in OPXV entry and exit would enable the development of a next-generation vaccine candidate capable of targeting different regions of the viruses. This would potentially trigger a broad and specific immune response against viruses of the OPXV genus, even simultaneously. This approach represents a novelty that has not yet been reported in the literature for this genus.

The aim of this study is to identify conserved epitopes in ten key proteins involved in the viral entry and exit process across all 16 viruses belonging to the OPXV genus, with particular attention to the emerging Mpox Clade Ib sub-lineage. If these epitopes are identified, we intend to construct, develop, and validate an immunogenic multi-epitope chimeric vaccine for the OPXV genus, providing a broader and more effective approach to combating these viruses.

Materials and methods

Obtaining protein sequences from viruses of the OPXV genus

The proteins involved in the viral entry mechanisms into the host cell (A17L, A28L, A33R, H2R, L1R), exit (A27L, A35R, A36R, C19L), and in both processes (B5R) of all viruses of the OPXV genus were obtained from the National Center for Biotechnology Information Virus (NCBI Virus) database using the identifier "*taxid:10242*"[12,14]. The Mpox virus Clade Ib sub-lineage sequences were obtained from the *PQ220056.1* sequence, deposited in the NCBI. To ensure the acquisition of proteins for all organisms, the selection followed this order: I—Selection of reference proteins (RefSeq); II—Selection of other proteins and generation of a consensus sequence when RefSeq were not found; III—BLAST for sequence similarity; IV—Proteins from the *PQ220056.1* sequence. If the protein was not found by these methods, it was determined to be absent.

Determination of consensus sequences and identification of epitopes for BCR, MHC-I, and MHC-II receptors

Consensus sequences for each protein, when necessary, were obtained using the Jalview software[15]. The RefSeq and consensus sequences were submitted to the Immune Epitope Database and Analysis Resource (IEDB) server for epitope identification[16]. For BCR epitopes, the BepiPred Linear Epitope Prediction 2.0 tool was used with a cutoff of 0.5. For MHC-I epitopes, the NetMHCpan EL 4.1 tool was used, selecting peptide options with sizes ranging from 8 to 14 amino acids and a reference set of 27 alleles, representing 97–99% of the global population. For MHC-II epitopes, the same tool was used, with peptides of 15 amino acids and a reference set of 7 HLA alleles. After obtaining the epitopes, the following exclusion criteria were applied: for MHC-I, a cutoff limit of 1% of epitopes interacting with each allele was established, ensuring an equal number of epitopes for each allele. For MHC-II, a consensus percentile cutoff of $<= 10.0$ was defined. For BCR, all found epitopes were selected. All cutoff thresholds followed the recommendations of the platform[16].

Analysis of antigenic, allergenic, and physicochemical properties

For the antigenicity analysis, the VaxiJen 2.0 server was used, configured to recognize the target organism as a virus with a cutoff threshold of 0.5[17]. For allergenicity assessment, the AllerCatPro 2.0 server was used, following the default settings[18]. For the analysis of physicochemical properties, the ProtParam tool from the Biopython library was used, with a structural stability parameter set to < 40.

Alignment, consensus epitope determination, and validation of epitope regions

The epitopes approved in all previous stages were aligned with the sequences of all viruses for each specific protein using the Jalview software, through the ClustalW plugin. Conserved regions for each protein were identified, defined as those with at least eight conserved amino acids in all sequences and containing at least one epitope aligned with the region. Conserved regions located in transmembrane areas and containing N-glycosylation sites were identified using the SOSUI[19] server and NetNGlyc 1.0 server[20], respectively, and they were removed from the analysis.

Construction of a multi-epitope chimeric protein with conserved epitopes

Four multi-epitope chimeric proteins were created: I - containing the epitopes with the highest antigenicity value from each protein; II - with all conserved epitopes from the A17L protein; III - with all conserved epitopes from the H2R protein; and IV - with all conserved epitopes from the C19L protein. In the construction of chimeric protein I, the epitopes were arranged in sequence (entry, both, and exit). The other chimeric proteins were designed based on the increasing antigenicity value of the epitopes. To link the epitopes, the AAY linker was used to provide flexibility to the proteins[12,13]. At the beginning of the proteins, two adjuvants, PADRE and beta-defensin, were added using the EAAAK linker to provide a more rigid characteristic to these adjuvants[12,13]. At the end of the protein, a HIS-tag (HIS, 6x) was added.

Evaluation of the multi-epitope chimeric protein properties

Allergenicity, toxicity, and physicochemical properties were evaluated using the servers Vaxijen, AllerCatPro 2.0, and ProParam, respectively. The search for potential autoimmune responses was conducted by aligning the sequence of the multi-epitope chimeric protein with human and murine genomes using the BLASTp tool. The solubility analysis of the proteins was performed on the SoluProt 1.0 server, where proteins with values > 0.5 are considered soluble[21]. The chimeric protein was submitted to the C-ImmSim server for immune response profile prediction using the following parameters: single injection, random seed = 12,345, injection without LPS, simulation volume of 10, and 300 simulation steps, with doses set at 1, 100, and 200 steps, representing days 0, 30, and 60 respectively, where both humoral and cellular responses were analyzed[22].

Modeling, molecular docking, and molecular dynamics

For molecular modeling by homology and molecular docking, the AlphaFold Server was used, powered by the new AlphaFold3 and AlphaFold2 prediction model[23]. The amino acid sequences of the multi-epitope chimeric proteins and the TLR2 receptor, available in the PDB under code 2Z7X, were provided to the tool. Additionally, ClusPro 2.0 was used to perform molecular docking between the multi-epitope protein and TLR2 receptor for comparative analysis. Molecular dynamics simulation was performed using GROMACS 2019.3 software with the OPLS-AA/L force field[24]. The water equilibrium under NVT (Number of particles, Volume, and Temperature constant) and NPT (Number of particles, Pressure, and Temperature constant) conditions was maintained for 100 ps, at a temperature of 300 K and pressure of 1 bar. Subsequently, a 100 ns simulation was conducted, and RMSD values were generated by GROMACS[24].

Results

Protein profile of selected OPXV genus viruses

The proteins responsible for entry (A17L, A28L, A33R, H2R, L1R), exit (A27L, A35R, A36R, C19L), and both (B5R) of viruses from the OPXV genus were obtained in FASTA format from the NCBI Virus database. The "*taxid:10242*", which covers all organisms of this genus, was selected. A total of 16 virus species were identified, including *Orthopoxvirus Abatino*, *Akhmeta virus*, *Borealpox virus*, *Buffalopox virus*, *Camelpox virus*, *Cowpox virus*, *Ectromelia virus*, *Horsepox virus*, *Monkeypox virus*, *Rabbitpox virus*, *Racoonpox virus*, *Skunkpox virus*, *Taterapox virus*, *Vaccinia virus*, *Variola virus*, and *Volepox virus*.

Among these, nine have RefSeq for all studied proteins, four have RefSeq for nine of the ten proteins, necessitating the generation of a consensus for one protein. Three viruses do not have reference sequences; thus, the available proteins were collected, and a consensus was generated. It is important to note that *Rabbitpox virus* was the only one with a protein (A27L) found by BLAST, and Mpox virus had two RefSeqs for each protein, corresponding to clades I and II, except for A27L, for which a consensus sequence was generated.

For sequencing of the Mpox clade Ib virus, the PQ220056.1 sequencing was used, with the respective proteins aligned and identified. The A27L protein was not found and thus was marked as absent. All data are detailed in Table 1.

Antigenic, non-allergenic, and stable epitopes identified in conserved regions of eight proteins

The IEDB server was used to find epitopes that can bind to MHC-I, MHC-II, and BCR, with all parameters set by the server. A global consensus sequence was generated for each of the ten proteins from each virus. The epitopes were identified, collected, and subjected to antigenicity analysis on the VaxiJen server, and their stability was assessed using the ProtParam tool from the Biopython library. It was noted that there were more epitopes for MHC-I and MHC-II receptors compared to BCR (Table 2).

The obtained epitopes were aligned using Jalview software and the ClustalW tool with the proteins from the sixteen viruses to identify conserved epitopes across all proteins, i.e., conservation regions that had at least one epitope were selected (Table 2).

Proteins A28L and B5R, although having conserved regions, did not show the presence of epitopes in these regions or, when present, the epitopes did not fully occupy these regions, failing to meet the minimum length of 8 amino acids. A total of 26 epitopes were identified and none of them presented evidence of allergenicity using the AllerCatPro 2.0. The amino acid sequences of each conserved epitope along with their respective antigenicity values are presented in Table 3. It is worth noting that the identified conserved epitopes are also present in the new Mpox variant, clade Ib. Although proteins A17L, A36R, C19L, and L1R have mutations in their protein composition, the identified epitopes remain conserved in this new variant (Fig. 1).

The new Mpox sublineage, declared a global emergency by the WHO due to the evolution of Clade Ib, has demonstrated increased transmissibility and the ability to spread to other continents[3]. Therefore, it is crucial to verify if conserved epitopes remain preserved in this new variant. Sequencing conducted in 2024 on patients from the Democratic Republic of Congo regarding the new Clade Ib lineage is available on NCBI under code

	Entry					Both	Exit			
Vírus	A17L	A28L	A33R	H2R	L1R	B5R	A27L	A35R	A36R	C19L
Abatino	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq
Akhmeta	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq
Borealpox	1	1	1	1	1	1	1	1	1	1
Buffalopox	Cons (4)	Cons (4)	Cons (6)	Cons (2)	1	Cons (5)	Cons (6)	Cons (6)	1	1
Camelpox	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq
Cowpox	Refseq	Cons (52)	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq
Ectromelia	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq
Horsepox	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Cons (3)	Refseq	Refseq	Refseq
Monkeypox*	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Cons (18)	Refseq	Refseq	Refseq
Mpox Ib	1	1	1	1	1	1	X	1	1	1
Rabbitpox	1	1	1	1	1	1	Blast	1	1	1
Racoonpox	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq
Skunkpox	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq
Taterapox	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq
Vaccinia	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq
Variola	Refseq	Cons (19)	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq
Volepox	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq	Refseq

Table 1. Information on collecting protein sequences involved in the entry and exit mechanisms of viruses belonging to the OPXV genus. *Refseq* Reference sequence available in NCBI Virus, *Cons* Consensus sequence generated from the number of sequences (indicated in parentheses) present in NCBI Virus but not reference sequences, *X* No sequences found, *number* number of sequences available for a given protein. *Two Refseq were obtained for this virus.

Protein	BCR	MHC-I	MHC-II	Total	ProtParam	Vaxijen	Align
A17L	10	305	112	427	271	139	8
A28L	8	509	211	728	365	136	0
A33R	2	107	75	184	142	55	1
H2R	3	118	64	185	150	62	6
L1R	4	106	72	182	118	60	2
B5R	18	509	229	756	401	142	0
A27L	7	1.228	488	1.723	881	316	1
A35R	4	100	52	156	92	41	1
A36R	3	129	52	184	72	20	1
C19L	7	322	194	523	379	169	6

Table 2. Quantities of epitopes obtained at each filtering step.

PQ220056.1[25], and it was used for analysis. The A27L protein was not found, which is consistent with the results in Table 1, where A27L is the only analyzed Mpox protein without a RefSeq. Additionally, among the eight proteins with conserved epitopes, A33R, A35R, and H2R are 100% conserved, ensuring epitope conservation (Fig. 1A). The A17L, A36R, C19L, and L1R proteins show 99.20%, 99.40%, 99.46%, and 99.34% conservation, respectively, compared to Mpox Clade I proteins; however, mutations do not occur in the identified epitopes (Fig. 1B–D).

Characterization and evaluation of the constructed multi-epitope chimeric proteins indicate adequate physicochemical properties, absence of allergenicity, good antigenicity, and positive humoral and cellular immune responses

After identifying the epitopes for each protein, four multi-epitope chimeric proteins were constructed. Two adjuvants were selected and added to the beginning of the chimeric protein: PADRE, a synthetic epitope capable of binding to MHC-II, which enhances the immune response by increasing the activation and proliferation of helper T cells, and β-defensin, which stimulates both the innate and adaptive immune responses[12]. It is worth noting that the chimeric proteins related to A17L, C19L, and H2R were created individually as they were the only ones presenting more than one conserved epitope.

The chimeric protein constructs range from 146 to 173 amino acids, molecular weights varying from 16 to 19 kDa, and isoelectric points of approximately 9, indicating a basic nature for all proteins. Furthermore,

	Protein	Conserved region	Epitope	Vaxijen	Protparam
Entry	A17L	GIADIRDKYM	GIADIRDK	2.81	– 15.69
		YYYETSPGEIKPKFCLID	PGEIKPKF	1.97	3.36
		GEPCSSFKFRPG	CSSFKFRP	1.82	38.11
		RCHFIKKDYLLGDSDSVA	CHFIKKDY	1.06	25.93
		YKTEHCDDFMTGFC	KTEHCDDF	0.85	38.05
		DPGNPNCLEW	GNPNCLEW	1.50	-3.77
		RPDYFTFGDTAL	FTFGDTAL	1.67	5.39
		GDKYLGPRVCWLHECTDESRDRKWLYYNQDVQR	RDRKWLYY	1.27	30.18
	A28L	–	–	–	–
	A33R	SILNTLRFLEKTSFYNC	LRFLEKTSF	1.07	30.29
	H2R	MDKTTLSVNACNLEYVREKAI	NLEYVREK	1.11	8.75
		YMRIKNTV	YMRIKNTV	1.04	33.02
		DWKSLTDSKTKLESDRG	DWKSLTDSK	1.20	30.29
		DFGAYFIAMRLD	DFGAYFIAMR	0.75	– 14.57
		KKAAKVDPSAQQFCQYLIKHKS	KKAAKVDP	1.41	– 20.65
		ITCGNEMLNELGYSGYF	LNELGYSGY	1.14	– 9.98
	L1R	SLSDILQITQYLDFLLLLLIQSKNKLEA	QYLDFLLL	1.61	– 10.10
		NKGYLFDFV	NKGYLFDFV	0.90	21.67
Both	B5R	–	–	–	–
Exit	A27L	DRYRNRVLLLTPE	RYRNRVLLL	1.28	– 4.57
	A35R	SLLSMITMSAFLIV	SLLSMITMSAFLIV	0.50	34.61
	A36R	PDTRHLRV	PDTRHLRV	1.04	28.69
	C19L	ETLPENMDFRSD	ETLPENMDFR	1.11	38.10
		NEIITLAKKYIY	NEIITLAKKYIY	1.28	32.83
		LLDERGKRNL	LLDERGKR	0.88	28.28
		SIDIEHLAIVPTTR	LAIVPTTR	2.04	22.21
		IYNSIIEAAINRGVKIRLLV	GVKIRLLV	0.61	– 5.46
		YVHITSANFDGTHYQNHGFVSFNS	YQNHGFVSFN	1.44	– 1.37

Table 3. Epitopes derived from conserved regions of entry and exit proteins: antigenicity and stability.

all proteins meet the cut-off thresholds for stability, antigenicity, allergenicity, solubility, and autoimmunity, indicating that they are suitable for vaccination (Table 4).

Subsequently, the simulation of the possible immune response to the four vaccine candidates was conducted on the C-ImmSim server. The multi-epitope protein containing epitopes from different proteins exhibited lower IgM + IgG titers compared to the vaccine candidates based on individual proteins (Fig. 2A). Additionally, the population of memory B cells was lower in the vaccine candidate containing epitopes from different proteins during the first 30 days, with an increase observed after the second dose administration. Comparing the cellular response, IFN-γ cytokine production was similar among the four vaccine candidates, while variations were observed in the levels of IL-2, TGF-β, IL-10, and IL-12 (Fig. 2A–D). All four vaccine candidates showed nearly null values for the "Danger" variable (Fig. 2A–D).

Structural stability and interaction with the TLR-2 receptor of the multivalent chimeric protein: modeling and molecular dynamics reveal good stability and fluctuation patterns

To determine the three-dimensional structure of the vaccine candidates and assess their interaction capability with the TLR-2 receptor, the AlphaFold 3 server was used. The predicted template modeling (pTM) score, interface predicted template modeling (ipTM) score, and per-residue measure of local confidence (pLDDT) were evaluated for each vaccine candidate. The modeling showed similarities, with TLR-2 exhibiting high pLDDT values, while the vaccine candidates showing low values. It was observed that the adjuvant region is responsible for anchoring to TLR-2 (Fig. 3A–D). Additionally, the pTM values ranged between 0.70 and 0.73, suggesting that the overall folds are close to the true structure, as values above 0.5 indicate good correspondence. However, ipTM values ranged from 0.20 to 0.22, indicating a potential failure in predicting the interaction. Therefore, validation of the structures through molecular dynamics was necessary.

Molecular dynamics was performed exclusively with the chimeric protein containing epitopes from different proteins due to its potential representativeness and comprehensiveness for immunization against the OPXV genus. Structural stability over time was assessed using the Root Mean Square Deviation (RMSD) graph, and the protein's residual fluctuation was analyzed with the Root Mean Square Fluctuation (RMSF) graph. The RMSD graph showed that the protein reached stability after 20 ns and remained stable until the end of the simulation (100 ns), with a structural variation from 0.4 nm to 0.5 nm (Fig. 3E). The RMSF graph indicated low variation in the initial part of the chimeric protein, with an increase in fluctuation occurring in the last amino acids of

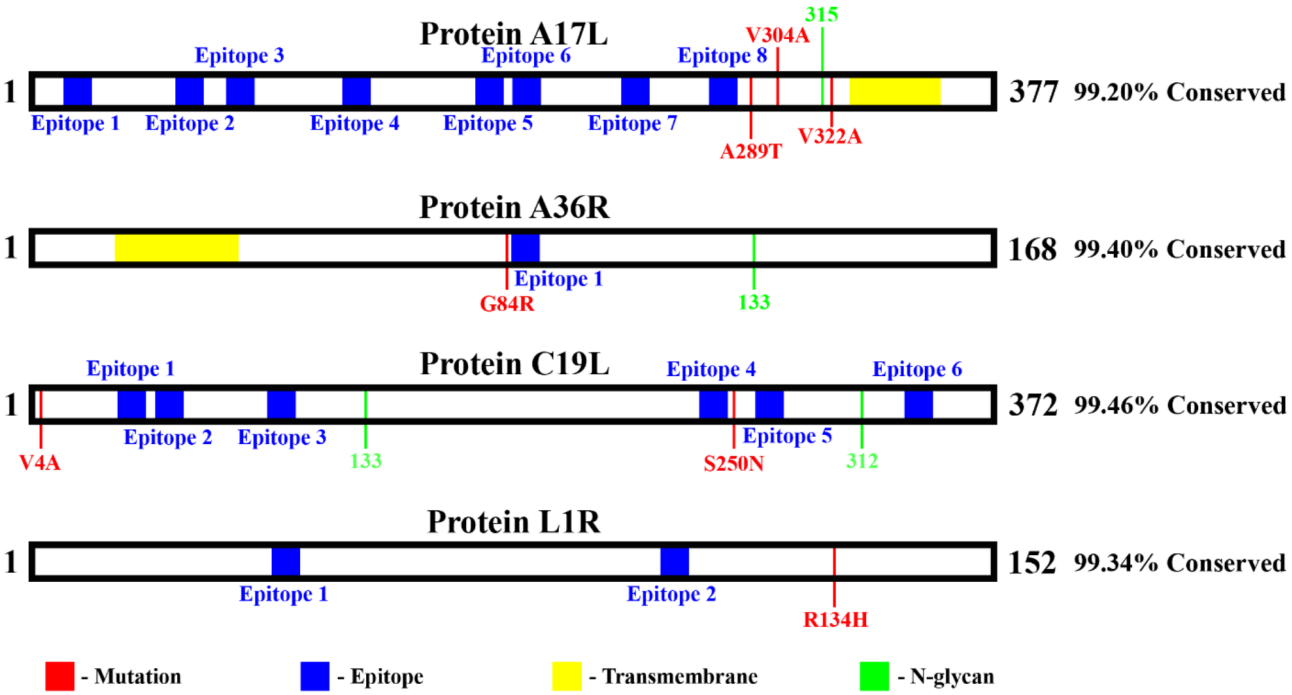

Fig. 1. Analysis of the A17L, A36R, C19L, and L1R proteins from the new Mpox virus lineage (Clade Ib), sequenced in 2024. The conservation percentages of these proteins compared to Clade I are indicated to the right of each protein. Conserved epitopes are highlighted in blue, mutations in red, transmembrane regions in yellow, and N-glycosylation sites in green. (**A**) Protein A17L with 99.20% conservation, displaying multiple conserved epitopes but with mutations outside these regions. (**B**) Protein A36R with 99.40% conservation, showing one mutation and one conserved epitope. (**C**) Protein C19L with 99.46% conservation, revealing several mutations and conserved epitopes. (**D**) Protein L1R with 99.34% conservation, having two conserved epitopes and one mutation outside these regions. The identified mutations do not affect the conserved epitopes, suggesting that they remain preserved in this new virus lineage.

Software	Info	Multi-epitope different proteins	Multi-epitope A17L	Multi-epitope C19L	Multi-epitope H2R
Protparam	Length (aa)	173	164	150	146
	Molecular weight (Da)	19217.64	18541.33	16829.60	16358.91
	Theoretical pI	9.99	9.44	9.81	9.70
	Instability index	21.21	23.87	25.11	27.71
	GRAVY	− 0.052	− 0.493	− 0.299	− 0.475
Vaxijen	Antigenicity	0.73	0.78	0.66	0.71
AllerCarPro	Allergenicity	No evidence	No evidence	No evidence	No evidence
SoluProt	Solubility	0.831	0.932	0.906	0.850
BLASTp	Autoimmunity	No evidence	No evidence	No evidence	No evidence

Table 4. Multi-epitope proteins properties.

the protein (Fig. 3F). In contrast, the TLR-2 receptor exhibited a characteristic fluctuation pattern, with two fluctuation peaks occurring between amino acids 200 and 300 (Fig. 3G).

Additionally, analyses of hydrogen bonds (H-bonds) and the radius of gyration (Rg) were conducted, as presented in Supplementary Fig. S1. The data show that the interaction complex between the TLR-2 receptor and the multi-epitope protein, composed of epitopes from different proteins, maintained between 450 and 550 hydrogen bonds throughout the simulation (Supplementary Fig. S1A). The radius of gyration ranged from 3.00 to 3.14 nm (Supplementary Fig. S1B), corroborating the RMSD values presented in Fig. 3E. These results indicate the preservation of structural integrity and stability of amino acid interactions during the 100 ns simulation, demonstrating the structural viability of the complex.

Discussion

The OPXV genus, with its 16 documented species and new emerging threats, continues to pose an increasing concern for global health[2,3,5]. Vaccination is a crucial strategy to prevent these potentially fatal diseases, but current

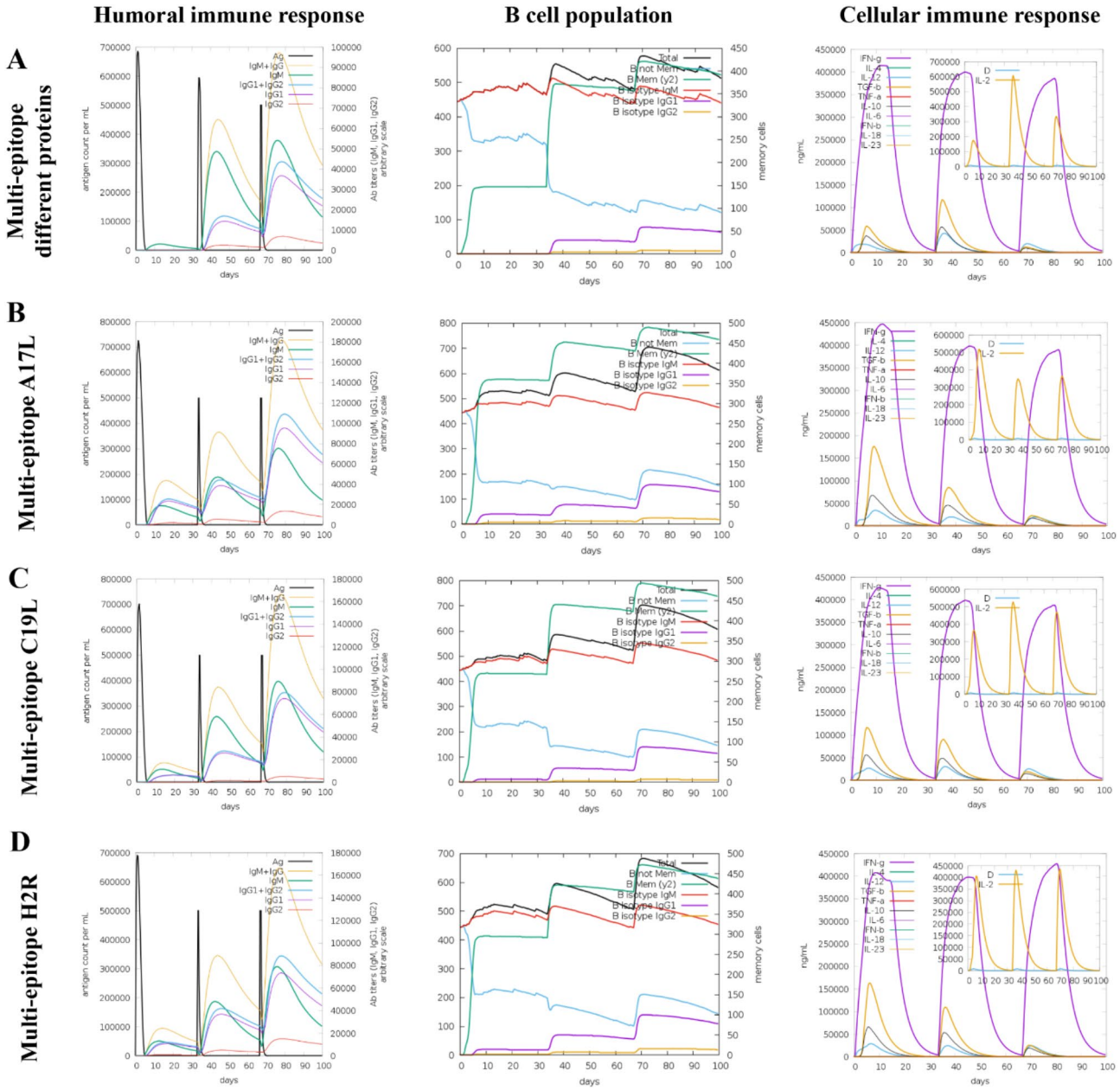

Fig. 2. The constructed multiepitope chimeric proteins show satisfactory predictions for cellular and humoral responses after three doses. Cytokine and interleukin levels after 3 doses are displayed, with "D" in the graph indicating a potential danger signal. (**A**) Results for the chimeric protein containing epitopes from different proteins. (**B**) Protein with epitopes from A17L. (**C**) Protein with epitopes from C19L. (**D**) Protein with epitopes from H2R.

vaccines like *ACAM2000* and *JYNNEOS*, which use attenuated viruses, are unsuitable for immunocompromised patients, as reported by the CDC[2]. Therefore, subunit vaccines, designed with computational techniques, offer a promising alternative to overcome these limitations and address genetic and antigenic variations[12].

In 2024, our research group reported the presence of immunogenic epitopes in the Mpox virus, developing an in silico multiepitope vaccine candidate targeting viral entry and exit processes proteins[12]. We also observed the conservation of these epitopes in *Borealpox virus* proteins, suggesting that conserved epitopes may exist throughout the OPXV genus[13]. In this study, we identified 26 conserved epitopes in eight proteins related to viral entry and release processes across the 16 OPXV species. These conserved epitopes can act as targets for neutralizing antibodies or induce a T-cell mediated cellular response[12,13].

OPXV viruses share similarities in viral entry and exit processes, making their proteins ideal targets for vaccine development[26]. The A17L, a myristoylated protein, and A27L, an inclusion body type A protein, facilitate viral entry, while A28L, the membrane protein L1R, and the fusion complex H2R are essential for virus penetration into the cytoplasm[27]. Being exposed during viral entry, these proteins are more susceptible to

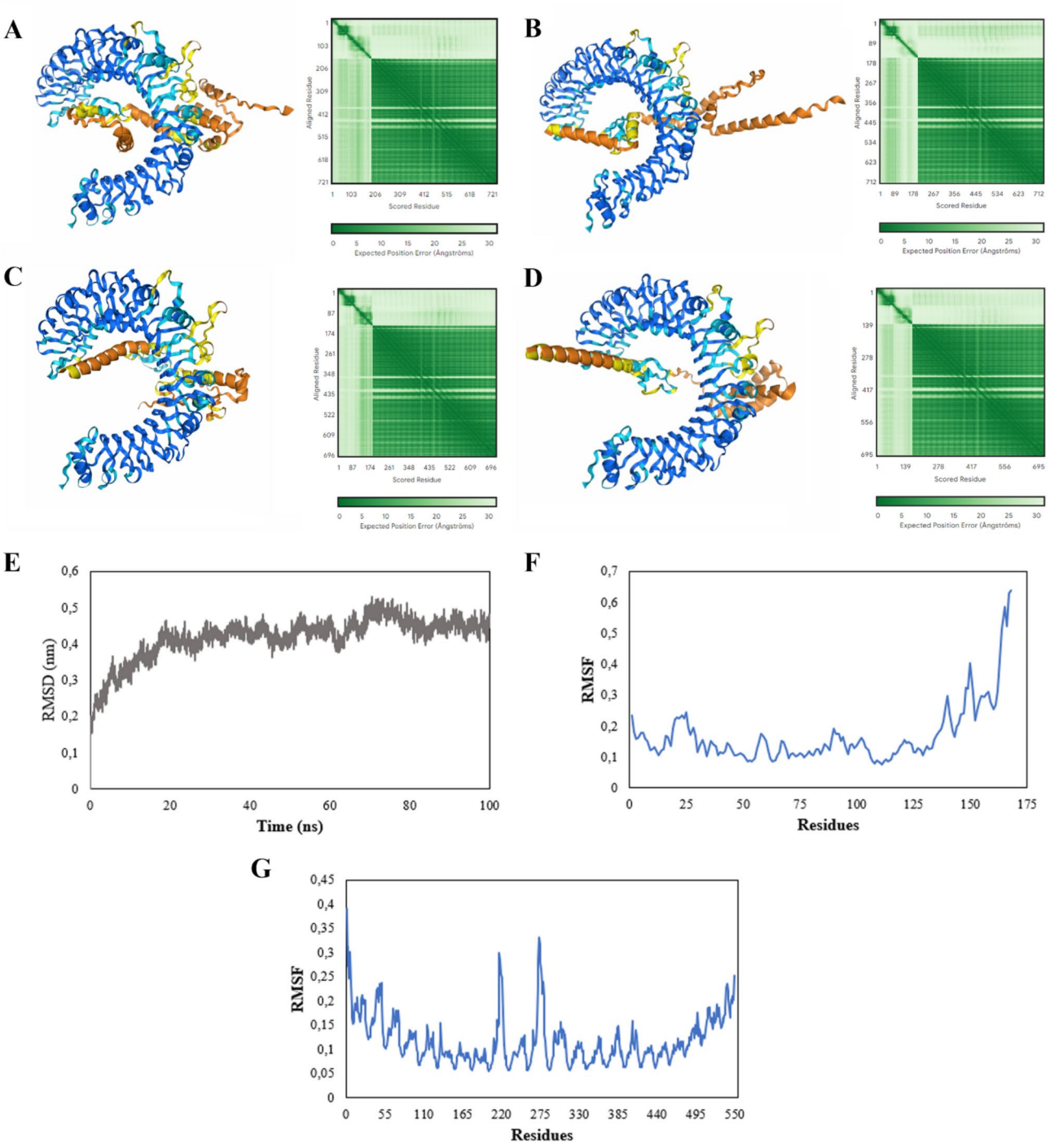

Fig. 3. The constructed chimeric proteins interact with the Toll-like receptor 2 (TLR-2), with modeling indicating good results for the receptor. However, the multiepitope proteins show low reliability. The multiepitope chimeric protein from different proteins demonstrates stability after 20 nanoseconds, maintaining a good interaction with TLR-2. (**A**) Modeling and binding of the multiepitope protein with TLR-2. (**B**) Binding of the A17L epitope protein with TLR-2. (**C**) Multiepitope from C19L and TLR-2. (**D**) Multiepitope from H2R and TLR-2. (**E**) RMSD obtained after 100 ns of molecular dynamics of the TLR-2 complex with the multiepitope protein. (**F**) RMSF of the multiepitope protein. (**G**) RMSF of TLR-2.

immune system recognition[26]. Similarly, A33R, A35R, A36R, B5R, and C19L proteins, involved in the formation and maturation of extracellular viral particles (EEV), play a crucial role in virus dissemination[27] (Fig. 4).

The use of conserved epitopes, as well as their validation in in vitro and in vivo tests for other viruses like SARS-CoV-2 and hepatitis C, has been reported in the literature[28–30].

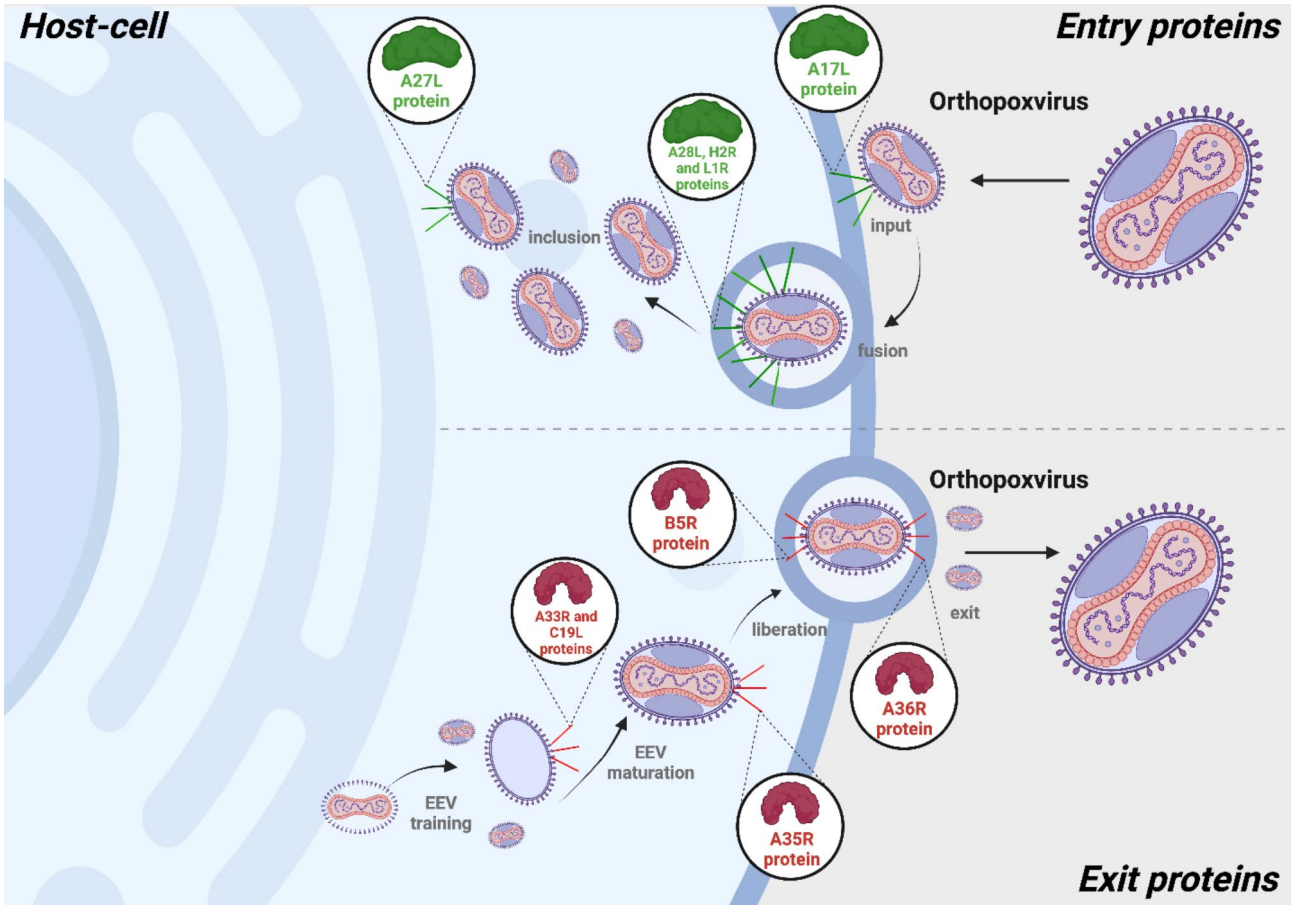

Fig. 4. Entry and exit proteins of *Orthopoxvirus* in the host cell. In the upper part of the figure, the A17L protein is involved in the virus's entry into the host cell; subsequently, the viral membrane proteins A28L, H2R, and L1R facilitate the fusion of the virus with the host cell, with A28L also enabling the formation of viral inclusions, assisting in the virus entry. In the lower part of the figure, the A33R and C19L proteins contribute to the formation of extracellular viral particles. The A35R protein is involved in the formation and maturation of extracellular enveloped virions (EEV); the B5R protein is essential for the formation of EEV and for the release of the virus; and finally, the A36R protein facilitates the virus's exit and the infection of neighboring cells. *A17L* Myristylate protein for entry, *A27L* A-type inclusion protein, *A28L* Truncated P4c or P4c/ATI inclusion factor, *A33R* CPXV166 protein, *A35R* EEV membrane phosphoglycoprotein, *A36R* IEV and EEV membrane glycoprotein, *B5R* Ankyrin-containing protein, *C19L* Palmytilated EEV membrane protein, *H2R* Entry-fusion complex essential component, *L1R* IMV membrane protein J1.

The OPXV conserved epitopes were combined with β-defensins and PADRE adjuvants into four vaccine candidates: one containing epitopes from multiple proteins, and others specific to A17L, C19L, and H2R. The use of the pipeline presented in this study reinforces the accuracy and reliability of the proposed approach, as it was also compared with a traditional workflow involving molecular modeling, molecular dynamics simulation, and molecular docking using previous-generation tools, such as AlphaFold2 and ClusPro 2.0[12]. As shown in Supplementary Fig. 2, the AlphaFold3-based strategy not only reduces the number of steps required for multi-epitope vaccine development but also provides superior structural modeling quality when compared to AlphaFold2. Furthermore, AlphaFold3 accurately predicted the interaction between the vaccine construct and the TLR2 receptor, clearly illustrating the interaction of adjuvants with the receptor.

These chimeric proteins aim to induce local immune responses, including the production of inflammatory cytokines by macrophages[12,13]. Captured by dendritic cells or macrophages, these antigens are presented on the surface by MHC molecules, allowing recognition by T-cell receptors and resulting in the generation of memory T-cells and the development of adaptive immunity[7,8]. Additionally, the presence of B-cell specific epitopes is essential for antibody production, and a multiepitope vaccine that interacts with both T-cell and B-cell receptors can effectively stimulate both humoral and cellular responses[7,8,11].

Immunological simulation revealed a decrease in IgM + IgG titration for the candidate with multiple protein epitopes compared to the multiepitope protein developed for Mpox and individual protein candidates[12]. This variation in humoral response may be attributed to differences in protein immunogenicity, as the focus of this study is on conserved epitopes rather than the most immunogenic of a protein. However, the cellular immune response, mediated by cytokines such as IFN-γ, TGF-β, IL-2, IL-10, and IL-12, remained similar, indicating a

consistent Th1 and regulatory profile. Furthermore, the robust cellular response suggests that both vaccines are effective in activating T-cells.

The results, introduce a new approach to vaccine development against OPXV genus viruses. The identification of conserved epitopes across different viruses demonstrates potential to induce robust humoral and cellular immune responses, even against the genetic diversity of these pathogens. We report the presence of conserved epitopes in eight OPXV genus proteins related to viral entry and exit processes in host cell.

As this study is based exclusively on in silico analyses, additional in vitro and in vivo studies are required to confirm the structural stability, immunogenic potential, and safety of the proposed multi-epitope vaccine candidate. Nevertheless, the structural, dynamic, and immunological data obtained strongly support the biological plausibility of the construct and provide a solid foundation for its future application not only as a prophylactic vaccine but also as a potential immunodiagnostic tool for all 16 *Orthopoxvirus* species.

Conclusion

It is evident that emerging and re-emerging viruses of the OPXV genus pose a persistent concern. Currently, vaccination is the most effective method against infections caused by this viral genus. In this study, we reported 26 conserved epitopes in key proteins and developed, as well as validated in silico, four multiepitope vaccine candidates. Analyses of antigenicity, allergenicity, structural stability, prediction of humoral and cellular immune responses, as well as the three-dimensional evaluation of interaction with the innate immune receptor TLR-2, indicated that the vaccine candidate with conserved epitopes from eight different proteins showed promising results.

This approach, combined with in vitro and in vivo testing, will be crucial for validating and refining the proposed vaccine candidates, strengthening the development of effective vaccines against the OPXV genus.

Data availability

The data supporting the findings of this study are available in the NCBI database under Taxid 10242 (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/virus?SeqType_s=Nucleotide&VirusLineage_ss=Orthopoxvirus,%20taxid:10242) and in the PDB database under the code 2Z7X (https://www.rcsb.org/structure/2Z7X). Additionally, the following protein sequences were utilized in this study: Mpox virus Clade Ib sub-lineage: PQ220056.1.Protein A17L: YP_010085594.1, QED21147.1, NP_570524.1, NP_619932.1, NP_671638.1, YP_010509346.1, YP_010377125.1, NP_536554.1, YP_010085803.1, AAS49838.1, YP_009143444.1, YP_009282829.1, YP_717445.1, YP_233018.1, NP_042164.1, YP_009281883.1.Protein A27L: YP_010085604.1, QED21098.1, NP_570534.1, NP_619942.1, NP_671647.1, YP_010085812.1, YP_009282838.1, YP_717456.1, YP_233030.1, NP_042176.1, YP_009281892.1.Protein A28L: YP_010085605.1, QED21129.1, NP_570535.1, AUO16291.1, YP_010509355.1, YP_010377134.1, NP_536565.1, YP_010085813.1, AAS49847.1, YP_009143454.1, YP_009282839.1, YP_717457.1, YP_233031.1, YP_009281893.1.Protein A33R: YP_010085610.1, QED21259.1, NP_570540.1, NP_619950.1, NP_671652.1, YP_010509361.1, YP_010377140.1, NP_536570.1, YP_010085820.1, AAS49852.1, YP_009143460.1, YP_009282845.1, YP_717462.1, YP_233036.1, NP_042182.1, YP_009281899.1.Protein A35R: YP_010085610.1, QED21259.1, NP_570540.1, NP_619950.1, NP_671652.1, YP_010509361.1, YP_010377140.1, NP_536570.1, YP_010085820.1, AAS49852.1, YP_009143460.1, YP_009282845.1, YP_717462.1, YP_233036.1, NP_042182.1, YP_009281899.1.Protein A36R: YP_010085613.1, QED21233.1, AVO21161.1, NP_570543.1, NP_619953.1, NP_671655.1, YP_010509364.1, YP_010377143.1, NP_536573.1, YP_010085823.1, AAS49855.1, YP_009143463.1, YP_009282848.1, YP_717465.1, YP_233039.1, NP_042185.1, YP_009281902.1.Protein B5R: YP_010085641.1, QED21126.1, NP_570570.1, NP_619979.1, NP_671673.1, YP_010509386.1, YP_010377161.1, NP_536593.1, YP_010085850.1, AAS49879.1, YP_009143489.1, YP_009282875.1, YP_717494.1, YP_233068.1, NP_042218.1, YP_009281929.1.Protein C19L: YP_010085505.1, QED21148.1, NP_570438.1, NP_619848.1, NP_671554.1, YP_010509261.1, YP_010377040.1, NP_536472.1, YP_010085720.1, AAS49754.1, YP_009143359.1, YP_009282743.1, YP_717360.1, YP_232934.1, NP_042081.1, YP_009281797.1.Protein H2R: YP_010085556.1, QED21222.1, AVO21113.1, NP_570488.1, NP_619896.1, NP_671602.1, YP_010509310.1, YP_010377089.1, NP_536519.1, YP_010085768.1, AAS49802.1, YP_009143408.1, YP_009282793.1, YP_717409.1, YP_232982.1, NP_042129.1, YP_009281847.1.Protein L1R: YP_010085549.1, QED21242.1, NP_570481.1, NP_619889.1, NP_671595.1, YP_010509303.1, YP_010377082.1, NP_536512.1, YP_010085761.1, AAS49795.1, YP_009143401.1, YP_009282786.1, YP_717403.1, YP_232975.1, NP_042122.1, YP_009281840.1.

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Author contributions

L.P.D.A. conceptualization, data curation, formal analysis, investigation, methodology, validation and writing—original draft. E.N.S, S.M.D.A. and P.P.C. data curation, formal analysis, investigation and methodology. L.A.D.A. conceptualization, data curation, formal analysis, investigation, resources, supervision, validation, visualization and writing—original draft.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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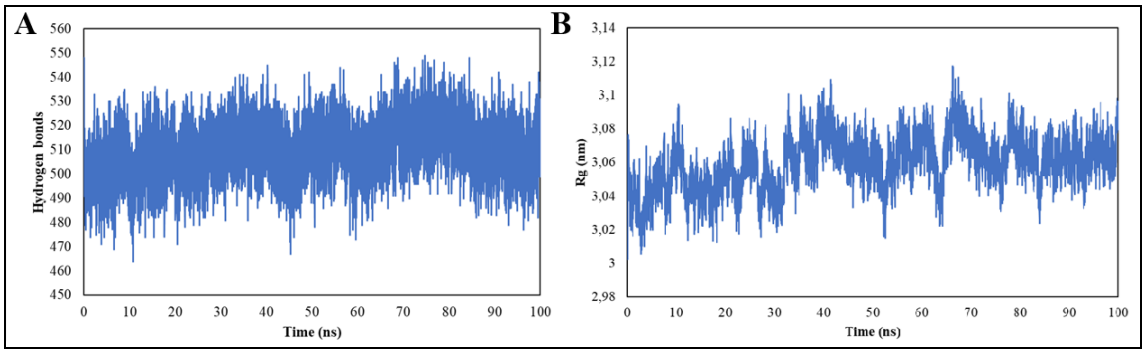
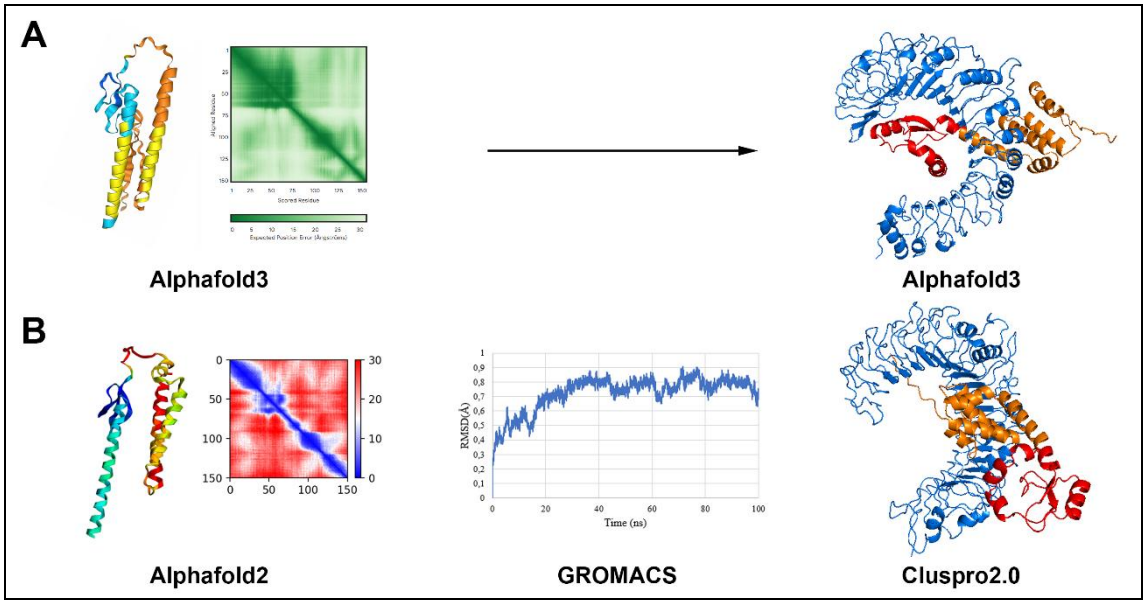


Supplementary Figure S1. Structural stability analysis of the receptor-ligand complex during a 100 ns molecular dynamics simulation. (A) Variation in the number of hydrogen bonds (H-bonds) over time, showing stability within the range of 450 to 550 bonds. (B) Radius of gyration (Rg) over time, fluctuating between 3.00 and 3.14 nm, indicating the maintenance of the complex's structural integrity throughout the simulation.



Supplementary Figure S2. Comparison between the integrated approach using AlphaFold3 versus the traditional structural modeling and docking pipeline (AlphaFold2 + GROMACS + ClusPro 2.0). In **A**, shows the structure of the isolated multi-epitope protein modeled by AlphaFold3 and its docking with the TLR2 receptor (blue). The multi-epitope protein includes adjuvants (in red) and epitopes (in orange). In **B**, (left to right) presents the structure modeled using AlphaFold2, the RMSD plot obtained from molecular dynamics simulation of the isolated peptide, and the molecular docking results of the multi-epitope protein with TLR2 (blue) using ClusPro 2.0. The structure includes adjuvants (red) and epitopes (orange).

7 CONCLUSÃO

Os resultados apresentados nesta dissertação representam, pela primeira vez na literatura, uma abordagem integrada e progressiva para o desenvolvimento de vacinas contra os vírus do gênero *Orthopoxvírus* (OPXV). O estudo inicial (Capítulo 4) estabeleceu a base ao desenvolver uma vacina multiepítopo contra o vírus *Monkeypox* (Mpox), identificando epítopos imunogênicos e racionalmente filtrados localizados em proteínas envolvidas nos processos de entrada e saída celular, com potencial para induzir respostas imunológicas humorais e celulares robustas.

Dando sequência, o segundo estudo (Capítulo 5) ampliou essa investigação ao formular a hipótese de que os epítopos imunogêncicos previamente identificados no Mpox estão conservados no novo vírus emergente, posteriormente definido como pertencente ao gênero OPXV e nomeado *Borealpox*. A análise confirmou essa conservação, reforçando a possibilidade de um candidato vacinal bivalente que pode ser eficaz contra ambos os vírus, bem como ser utilizado no desenvolvimento de testes de imunodiagnóstico específicos.

Por fim, o terceiro estudo (Capítulo 3) expandiu a abordagem para abranger todo o gênero OPXV, identificando epítopos conservados em oito das dez proteínas-chave envolvidas nos processos de entrada e saída viral nos dezesseis vírus conhecidos do grupo. Por meio de modelagem in silico, foram construídos e validados quatro candidatos a vacinas multiepítopos, incluindo um candidato promissor contendo epítopos de oito proteínas diferentes. As avaliações de antigenicidade, alergenicidade, estabilidade estrutural e capacidade de interação com receptores imunes inatos indicaram o potencial imunogênico desses candidatos.

Essa progressão lógica e incremental, desde o foco inicial no Mpox até a abrangência pan-orthopoxviral, demonstra uma estratégia robusta para o desenvolvimento de vacinas multiepítopos. Aliada a testes experimentais futuros, essa abordagem pode impulsionar significativamente o desenvolvimento de vacinas eficazes para o controle dos vírus emergentes e reemergentes do gênero OPXV, que representam uma ameaça contínua à saúde pública global. Além disso, esses epítopos podem ser utilizados no desenvolvimento de testes de imunodiagnóstico, bem como, o pipeline descrito aqui, ser utilizado na busca e conservação de outros gêneros virais.

8 PERSPECTIVAS FUTURAS

Os resultados obtidos e descritos na dissertação deste mestrado representam um importante avanço no desenvolvimento racional de um candidato vacinal multiepítopo voltada à prevenção de infecções causadas por múltiplos vírus do gênero *Orthopoxvirus*. A identificação e análise de epítopos imunogênicos conservados em proteínas-chave desses vírus, por meio de abordagens *in silico*, permitiram o delineamento de uma estratégia vacinal inovadora, com potencial para oferecer proteção cruzada contra diferentes espécies virais, inclusive em indivíduos imunocomprometidos.

Como continuidade natural deste estudo, propõe-se, como perspectiva futura, a realização da produção e validação experimental do candidato vacinal desenvolvido. Esta fase poderá ser conduzida durante um possível doutorado, permitindo aprofundar e expandir os achados aqui apresentados. A expectativa é que essa próxima etapa viabilize a confirmação da segurança, da imunogenicidade e da eficácia da formulação proposta, consolidando o conceito de uma vacina multivalente baseada em epítopos conservados.

A execução dessa fase experimental será fundamental não apenas para validar os resultados computacionais obtidos, mas também para estabelecer as bases para um possível avanço rumo à aplicação clínica. Além disso, permitirá a geração de dados relevantes que poderão contribuir para o fortalecimento da resposta nacional a emergências de saúde pública causadas por vírus emergentes e reemergentes, bem como para a construção de soluções tecnológicas alinhadas às necessidades do Sistema Único de Saúde (SUS).

Assim, a continuidade do projeto no âmbito de pesquisa de um doutorado configura-se como uma oportunidade estratégica para consolidar uma linha de pesquisa promissora, com forte potencial de impacto científico, social e sanitário, reafirmando o compromisso com a inovação, a pesquisa translacional e a soberania científica nacional.

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APÊNDICE A – DEMAIS ATIVIDADES DESENVOLVIDAS DURANTE O MESTRADO

Além dos artigos científicos apresentados nos Capítulos 1, 2 e 3 desta dissertação, diversas outras atividades acadêmicas, técnicas e formativas foram desenvolvidas ao longo dos dois anos de mestrado, contribuindo significativamente para minha formação como pesquisador. Abaixo, apresento uma lista das atividades desenvolvidas:

Quadro 1 – Demais atividades acadêmicas e científicas desenvolvidas durante o mestrado.

Atividade	Quantidade	Descrição
Artigos publicados relacionados ao Laboratório de Biologia Molecular de Microrganismos (LaBioMol)	6	• DE ARAÚJO, Leonardo Pereira et al. Immunoinformatic approach for rational identification of immunogenic peptides against host entry and/or exit Mpox proteins and potential multiepitope vaccine construction. **The Journal of infectious diseases**, v. 229, n. Supplement_2, p. S285-S292, 2024. https://doi.org/10.1093/infdis/jiad443 • DE ARAÚJO, Leonardo Pereira et al. Shared immunogenic epitopes between host entry and exit proteins from monkeypox and Alaskapox viruses. **The Lancet Microbe**, v. 5, n. 7, p. 624-625, 2024. https://doi.org/10.1016/S2666-5247(24)00095-8 • DE ARAÚJO, Leonardo Pereira et al. Multivalent vaccine candidate from conserved immunogenic peptides in entry or exit proteins of Orthopoxvirus genus. **Scientific Reports**, v. 15, n. 1, p. 12503, 2025. https://doi.org/10.1038/s41598-025-96755-4 • BRANCAGLION, Gustavo Andrade et al. Sequential macrophage DENV and ZIKV infection shows differential expression of CD86, IFN-β, and regulation of TNF-α and IL-1β depending on DENV serotype. **Brazilian Journal of Microbiology**, p. 1-12, 2025. https://doi.org/10.1007/s42770-025-01639-4 • SILVA, Letícia Barbosa et al. A computational approach for MHC-restricted multi-epitope vaccine design targeting Oropouche virus structural proteins. **Acta Tropica**, v. 263, p. 107575, 2025. https://doi.org/10.1016/j.actatropica.2025.107575 • DE ARAÚJO, Leonardo Pereira et al. Targeting conserved epitopes in structural proteins: a next-generation vaccine strategy against the newly identified HKU5-CoV-2 virus. **Signal Transduction and Targeted Therapy**, v. 10, n. 1, p. 1-2, 2025.

		https://doi.org/10.1038/s41392-025-02257-0
Artigos publicados relacionados a outros laboratórios	4	• BORBA, João Ricardo Bueno de Morais et al. Applying the bioisosterism strategy to obtain lead compounds against SARS-CoV-2 cysteine proteases: An in-silico approach. Journal of Computational Chemistry, v. 45, n. 1, p. 35-46, 2024. https://doi.org/10.1002/jcc.27217 • BRESEGHELLO, Isadora et al. Phthalocyanine derivative attenuates TNF-α production in macrophage culture and prevents alveolar bone loss in experimental periodontitis. **Journal of Periodontal Research**, 2024. https://doi.org/10.1111/jre.13341 • DE SOUZA, Amanda Bubula et al. In silico analysis for the proposal of new drugs against the phosphoprotein nucleocapsid of the severe acute respiratory syndrome coronavirus 2 virus. **Innovative Medicines & Omics**, v. 1, n. 1, p. 115-124, 2024. https://doi.org/10.36922/imo.3731 • SANTOS, Leandro Marcos et al. Medicinal Chemistry behind Capivasertib Discovery: Seventh Magic Bullet of the Fragment-based Drug Design Approved for Oncology. **Current medicinal chemistry**. https://doi.org/10.2174/0109298673331253241004110953
Coorientações de Trabalho de Conclusão de Curso	1	2024 - Amanda Bubula de Souza e Bruna de Sousa Godinho. *Virtual screening* de produtos naturais na identificação de novos *leads* antivirais contra a proteína VP39 do vírus Mpox.
Participação em eventos internacionais	4	• *14th Latin American and Caribbean Immunology Congress*. 2024. (Buenos Aires) • *Annual Meeting of the Brazilian Society of Immunology*, 2023. (Ouro Preto) • Congresso Internacional *Bioexperience* 2023. (Viçosa) • *I International Workshop on Pharmaceutical Research of* UNIFAL-MG (Alfenas)

Participação em eventos nacionais	3	• IX Workshop do Programa de Pós-Graduação em Ciências Biológicas (Alfenas) • V Workshop do Programa de Pós-Graduação Multicêntrico em Fisiologia (Alfenas) • XXII Congresso Brasileiro de Biologia Celular (São Paulo)
Resumos publicados em anais de congresso	10	3 resumos com primeira autoria do autor desta dissertação.
Apresentações de trabalhos	28	12 apresentações realizadas pelo autor
Premiações	5	• 2024 - 1º Lugar na categoria mestrado na forma de apresentação online no IV Encontro do PPGMCF, Programa de Pós Graduação Multicêntrico em Ciências Fisiológicas. • 2024 -Trabalho destaque na modalidade pôster "*Identification of MHC class I and II restricted epitopes against structural Oropouche proteins and potential multiepitope vaccine construction*", Universidade Federal de Alfenas. • 2024 - Trabalho destaque na modalidade oral "*Multi-epitope vaccine developed from Mpox entry/exit proteins shows conservation in Alaskapox virus proteins*", Universidade Federal de Alfenas. • 2024 - Trabalho destaque na modalidade oral "*OM-85 (Broncho-Vaxom®) decreases susceptibility to lung Pseudomonas aeruginosa infection in a preclinical model of cystic fibrosis but increases tissue damage*", Universidade Federal de Alfenas. • 2024 - Trabalho destaque na modalidade pôster "*Ivermectin impairs macrophage microbicidal response via MD2/TLR4 binding*" no IX Workshop do PPGCB, Universidade Federal de Alfenas.
Organização de eventos	2	• VI Curso de Inverno de Fisiologia. 2024. • IX Workshop do Programa de Pós-Graduação em Ciências Biológicas. 2024.
Minicursos ministrados	2	• Vacinologia Reversa. 2024. IX Workshop do PPGCB, Universidade Federal de Alfenas • Vacinologia Reversa. 2023. Bioexperience (Viçosa)
Palestras ministradas	2	• 2024 - Bioinformática e Biomedicina: Visões gerais e perspectivas futuras. • 2024 - Construção de proteínas multiepitopos no combate às doenças emergentes e reemergentes: desenvolvimento de vacinas para Mpox e COVID-19.