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REVISÃO SOBRE NOVOS ANTIVIRAIS CONTRA PARVOVÍRUS, ANÁLISE GENÉTICA E DESENVOLVIMENTO *IN SILICO* DE UMA VACINA CANDIDATA CONTRA A PARVOVIROSE CANINA

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Tese apresentada ao Programa de Pós-graduação em Biotecnologia da Universidade de Caxias do Sul, como parte dos requisitos para a obtenção de grau de Doutor em Biotecnologia.

Orientador: Prof. Dr. André Felipe Streck

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Tamiris Silva Lopes

Tese submetida à banca examinadora designada pela coordenação do Programa de Pós-graduação em Biotecnologia da Universidade de Caxias do Sul, como parte dos requisitos para a obtenção de grau de Doutor em Biotecnologia.

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“A sorte favorece os audazes.”

Alexandre, o Grande.

SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS

AI - inteligência artificial (do inglês “artificial intelligence”)

B19V - parvovírus B19

CPV-2 - parvovírus canino tipo 2 (do inglês "canine parvovirus type 2")

CPV-2a - parvovírus canino tipo 2a (do inglês "canine parvovirus type 2a")

CPV-2b - parvovírus canino tipo 2b (do inglês "canine parvovirus type 2b")

CPV-2c - parvovírus canino tipo 2c (do inglês "canine parvovirus type 2c")

DNA - ácido desoxirribonucleico (do inglês “deoxyribonucleic acid”)

kb - quilobase

MAbs - anticorpos monoclonais (do inglês “monoclonal antibodies”)

mL - mililitro

mg - miligrama

µL - microlitro

MD - dinâmica molecular (do inglês “molecular dynamics”)

ng - nanograma

ORFs - fases de leitura aberta (do inglês “open reading frames”)

PPV - parvovírus suíno (do inglês “porcine parvovirus”)

qPCR - reação em cadeia da polimerase quantitativo em tempo real (do inglês “real time quantitative polymerase chain reaction”).

RNA - ácido ribonucleico (do inglês “ribonucleic acid”)

$TCID_{50}$ - Dose infectante 50% em cultura de tecidos (do inglês “50% tissue culture infectious dose”)

VN - vírus neutralização

TfR - receptor da transferrina (do inglês “transferrin receptor”)

TLR4- receptor do tipo toll 4 (do inglês “tool-like receptor 4)

LISTA DE ABREVIATURAS DE AMINOÁCIDOS

Alanina – A

Cisteína – C

Aspartato – D

Glutamato – E

Fenilalanina – F

Glicina – G

Histidina – H

Isoleucina – I

Lisina – K

Leucina – L

Metionina – M

Asparagina – N

Prolina – P

Glutamina – Q

Arginina – R

Serina – S

Treonina – T

Valina – V

Triptofano – W

Tirosina – Y

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RESUMO

A família *Parvoviridae* é composta pelos menores vírus de DNA conhecidos e se caracteriza pela ausência de envelope lipídico. Esta família é subdividida em três subfamílias, cada uma com características distintas quanto a hospedeiros e ciclos de replicação. Os parvovírus da subfamília *Parvovirinae* infectam vertebrados e causam diversas doenças, incluindo a parvovirose canina, que é provocada pelo parvovírus canino tipo 2 (CPV-2). Esta doença, altamente contagiosa, resulta em gastroenterite aguda grave em cães, especialmente filhotes. Este estudo revisou as estratégias terapêuticas existentes e avaliou compostos com potencial antiviral contra membros significativos da subfamília *Parvovirinae*, tanto na medicina humana quanto veterinária. Apesar da ausência de um tratamento específico, as pesquisas estão cada vez mais focadas em preencher essa lacuna clínica e encontrar soluções terapêuticas eficazes. Além disso, foram selecionadas e analisadas várias mutações na proteína VP2 do CPV-2, incluindo F267Y, S297A, V300G, D305Y, Y324I, N426D, N426E e T440A, devido ao seu impacto na replicação viral. Mutações como N426D, N426E e T440A mostraram potencial para aumentar a capacidade replicativa do vírus. Foi realizada uma análise das variantes do CPV-2 no Brasil, evidenciando a evolução contínua do vírus e a necessidade de vacinas atualizadas. Com isso, foi proposta uma vacina multi-epítopo *in silico* adaptada às variantes brasileiras, selecionando cinco regiões epitope dentro da proteína VP2. Nas simulações, a vacina apresentou elevada antigenicidade, ausência de toxicidade e potencial para estimular uma resposta imune robusta, com aumento da produção de anticorpos e atividade de células T auxiliares. Este estudo oferece uma compreensão mais aprofundada do parvovírus canino, fornecendo informações importantes para o desenvolvimento de futuras estratégias terapêuticas e vacinas atualizadas, assegurando proteção eficaz contra as variantes atuais do vírus no Brasil.

Palavras-chave: parvovirus canino; proteína VP2; variantes; vacinas

ABSTRACT

The family *Parvoviridae* comprises the smallest known DNA viruses and is characterized by the absence of a lipid envelope. This family is divided into three subfamilies, each with distinct characteristics regarding hosts and replication cycles. Viruses from the subfamily *Parvovirinae* infect vertebrates and cause various diseases, including canine parvovirus infection, which is caused by canine parvovirus type 2 (CPV-2). This highly contagious disease results in severe acute gastroenteritis in dogs, especially puppies. This study reviewed existing therapeutic strategies and evaluated compounds with potential antiviral activity against significant members of the subfamily *Parvovirinae*, in both human and veterinary medicine. Despite the lack of a specific treatment, research is increasingly focused on filling this clinical gap and finding effective therapeutic solutions. Additionally, several mutations in the VP2 protein of CPV-2 were selected and analyzed, including F267Y, S297A, V300G, D305Y, Y324I, N426D, N426E, and T440A, due to their impact on viral replication. Mutations such as N426D, N426E, and T440A showed potential to increase the viral fitness. An analysis of CPV-2 variants in Brazil was also conducted, highlighting the virus's ongoing evolution and the need for updated vaccines. Therefore, an *in silico* multi-epitope vaccine adapted to Brazilian variants was proposed, selecting five epitope regions within the VP2 protein. In simulations, this vaccine demonstrated high antigenicity, absence of toxicity, and potential to stimulate a robust immune response, including increased antibody production and activity of helper T cells. This study provides a deeper understanding of canine parvovirus, offering valuable information for developing future therapeutic strategies and updated vaccines, ensuring effective protection against current virus variants in Brazil.

Keywords: canine parvovirus; VP2 protein; variant; vaccine

1 INTRODUÇÃO

A família *Parvoviridae* abrange uma ampla e diversificada gama de vírus, reconhecidos por serem os menores vírus de DNA conhecidos e por não possuírem envelope lipídico (Gallinella, 2019). Esta família é dividida em três subfamílias. Na subfamília *Parvovirinae*, encontram-se os parvovírus que infectam vertebrados, sendo responsáveis por uma ampla variedade de doenças tanto em humanos quanto em animais.

A parvovirose canina é uma doença infecciosa aguda que representa uma significativa preocupação para médicos veterinários, tutores e pesquisadores na área da saúde animal. Causada pelo membro da subfamília *Parvovirinae* conhecido como parvovírus canino tipo 2 (CPV-2) (*Carnivore protoparvovirus 1*), essa enfermidade é caracterizada por uma gastroenterite hemorrágica severa, frequentemente fatal, principalmente em filhotes não vacinados (Mangia, 2018).

A infecção por CPV-2 geralmente resulta em alta morbidade e mortalidade (Tuteja et al., 2022), e até o momento não há um tratamento específico disponível, apenas terapias de suporte para aliviar os sintomas (Mazzaferro, 2020). Recentemente, avanços significativos têm sido alcançados na investigação dos mecanismos moleculares associados à replicação do CPV-2, bem como na identificação de possíveis alvos para intervenções antivirais. Apesar da ausência de medicamentos licenciados, as pesquisas estão progredindo nessa direção.

Logo após o surgimento do CPV-2 no final dos anos 1970, a prevenção da doença tem sido alcançada por meio de vacinação. Esse vírus é objeto de intensa pesquisa devido à sua rápida disseminação global e à constante evolução de suas variantes antigênicas. As falhas na imunização são uma das principais razões para a persistente circulação do CPV-2 globalmente. Essas falhas podem ser atribuídas a várias causas, incluindo a persistência da imunidade materna durante a vacinação, a falta de resposta à vacina, a presença de diferentes variantes antigênicas do vírus e a alta quantidade de animais não vacinados ou a execução inadequada dos protocolos de vacinação (Decaro et al., 2020).

A cepa original do CPV-2 ainda é utilizada em muitas vacinas comerciais. No entanto, logo após seu surgimento, foi totalmente substituída por variantes. Essa substituição levanta questionamentos sobre a eficácia das vacinas disponíveis em

proteger os animais contra as variantes (McElligott et al., 2011). Em resposta a essa preocupação, algumas vacinas baseadas na cepa CPV-2b foram licenciadas (Decaro et al., 2020). Entretanto, no Brasil, as vacinas disponíveis são majoritariamente baseadas em cepas originais de CPV-2 (Lencina et al., 2023). Embora alguns estudos indiquem que essas vacinas geram anticorpos neutralizantes que oferecem proteção cruzada contra as variantes CPV-2a, CPV-2b e CPV-2c (Wilson et al., 2013; Glover et al., 2015; Packianathan et al., 2022), outros revelam casos da doença em animais com protocolo vacinal completo (Decaro et al., 2008; Decaro et al., 2009; de Oliveira et al., 2018).

A vacinologia reversa representa uma abordagem inovadora no desenvolvimento de vacinas, aproveitando a bioinformática e análises computacionais dos genomas dos patógenos para identificar alvos promissores (Rappuoli, 2000). Diferentemente da vacinologia tradicional, que envolve o cultivo e a inativação/atenuação do patógeno completo, essa técnica se concentra na identificação de epítopos proteicos específicos nos genomas dos patógenos. Esses epítopos são selecionados por sua capacidade de desencadear respostas imunológicas eficazes, permitindo o desenvolvimento de vacinas baseadas em múltiplos epítopos (Enayatkhani et al., 2021; Khalid e Poh, 2023). Além disso, essa estratégia possibilita uma ágil adaptação das vacinas, o que é fundamental em cenários envolvendo patógenos com maior taxa de mutação (Monterrubio-López e Delgadillo-Gutiérrez, 2021).

2 REVISÃO DE LITERATURA

2.1 Subfamília *Parvovirinae*

A subfamília *Parvovirinae* faz parte da família *Parvoviridae* (Cotmore et al., 2019). Os representantes desse grupo compartilham características importantes, como a resistência à dessecação no ambiente e a necessidade das células infectadas estarem na fase S para a replicação do DNA viral (Gallinella, 2019). A subfamília *Parvovirinae* é subdividida em onze diferentes gêneros, sendo eles: *Amdoparvovirus*, *Artiparvovirus*, *Aveparvovirus*, *Bocaparvovirus*, *Copiparvovirus*, *Dependoparvovirus*, *Erythroparvovirus*, *Loriparvovirus*, *Protoparvovirus*, *Sandeparvovirus* e *Tetraparvovirus* (Capozza et al., 2023). Cada gênero apresenta características e perfis patogênicos únicos, contribuindo para a diversidade de doenças que causam em diferentes hospedeiros.

Entre os representantes dessa subfamília, encontram-se patógenos importantes tanto para a medicina humana quanto veterinária. O parvovírus B19 (B19V), pertencente ao gênero *Erythroparvovirus*, é considerado o parvovírus mais patogênico para humanos, podendo causar uma variedade considerável de manifestações clínicas diferentes (Gallinella, 2013). A gravidade dessas manifestações depende da complexa interação entre o vírus e o estado fisiológico e imunológico do indivíduo infectado (Gallinella, 2019).

Na medicina veterinária, o parvovírus suíno (PPV), membro do gênero *Protoparvovirus*, representa uma grande preocupação econômica para a suinocultura. O PPV é majoritariamente associado a falhas reprodutivas, que podem variar desde a recorrência ao estro até morte fetal (Streck e Truyen, 2020). Outro representante significativo desse gênero é o CPV-2, amplamente conhecido por sua importância na saúde canina. O CPV-2 é altamente contagioso e causa doenças graves em cães, especialmente filhotes não vacinados.

2.2 Parvovirose canina

2.2.1 Etiologia

A parvovirose canina, doença infecciosa aguda, é causada pelo parvovírus canino tipo 2 (CPV-2). O genoma do CPV-2 é altamente compacto e consiste em uma única molécula de DNA de aproximadamente 5 kb, com capsídeo não envelopado de 25 nm de diâmetro e apresentando simetria icosaédrica (Hao et al., 2022).

De acordo com a classificação mais recente, o CPV-2 pertence à família *Parvoviridae*, subfamília *Parvovirinae*, gênero *Protoparvovirus*, formando uma espécie única juntamente com o parvovírus felino e outros parvovírus de carnívoros (*Carnivore protoparvovirus 1*) (Cotmore et al., 2019; Decaro et al., 2020). O genoma do CPV-2 possui duas regiões codificantes (*open reading frames*), com a extremidade 3' codificando as proteínas não estruturais NS1 e NS2, e a extremidade 5' codificando as proteínas estruturais VP1 e VP2.

Através da cristalografia de raios-X, foi descoberto que o capsídeo do CPV-2 é formado por 60 cópias de uma combinação de VP1, VP2 e VP3. VP1 contém a sequência completa de VP2, juntamente com um domínio adicional na região N-terminal. VP2, a proteína estrutural mais abundante, representa 90% do capsídeo, determinando a gama de hospedeiros e as interações entre o vírus e o hospedeiro (Decaro e Buonavoglia, 2012; Hao et al., 2022). A VP3 aparece como um produto de clivagem pós-traducional da VP2 (Tu et al., 2015).

Os parvovírus não conseguem realizar a síntese de DNA de forma independente. Para se replicarem, eles dependem da maquinaria de replicação do hospedeiro, que está ativa principalmente durante a fase S do ciclo celular, quando o DNA é sintetizado. Por isso, a replicação desses vírus ocorre nos núcleos celulares e requer células em rápida divisão, como as de fetos e recém-nascidos, ou os tecidos hematopoiéticos e intestinais de animais jovens e adultos. Além disso, o CPV-2, assim como outros parvovírus, é altamente estável no ambiente e extremamente resistente a mudanças de pH e temperatura (Decaro e Buonavoglia, 2012).

2.2.2 Epidemiologia

O surgimento do CPV-2 ocorreu no final dos anos 1970 e análises filogenéticas indicam uma estreita relação com o vírus da panleucopenia felina (Decaro et al., 2020).

As diferenças entre essas duas viroses envolvem apenas seis ou sete mutações nos aminoácidos da VP2, seguido por adaptação e evolução no novo hospedeiro. Nos anos seguintes à sua emergência, o CPV-2 disseminou-se pelo mundo, frequentemente associado a uma gastroenterite hemorrágica altamente fatal (Truyen, 1999; de Oliveira et al., 2018). A gravidade da doença observada nessa época foi atribuída à ausência de imunidade natural da população canina em relação ao novo vírus. Atualmente, os cães são mais resistentes ao CPV-2, provavelmente pelas medidas profiláticas e resistência natural (Morais e Costa, 2007). No entanto, a disseminação do CPV-2 permanece endêmica em populações de cães e canídeos selvagens em todo o mundo, com taxas variadas de morbidade e mortalidade (Qi et al., 2020).

O CPV-2 pode afetar cães de qualquer raça, idade e gênero, sendo as infecções graves mais comuns em filhotes com idades entre 6 semanas e 4 meses (Nandi e Kumar, 2010). A maior suscetibilidade nessa faixa etária está relacionada ao fato de que os anticorpos maternos conseguem oferecer proteção nas primeiras semanas de vida. No entanto, esses níveis de proteção começam a diminuir com o tempo, tornando-se insuficientes para proteger os filhotes, mas ainda são capazes de interferir no desenvolvimento de uma resposta imune eficaz após a vacinação. Esse período é conhecido como 'janela de suscetibilidade' e explica por que alguns animais adequadamente vacinados podem ser infectados e desenvolver a doença (Morais e Costa, 2007).

Diferentemente da maioria dos vírus de DNA, o CPV-2 apresenta uma elevada taxa de mutação em seu genoma, semelhante àquela encontrada em vírus de RNA (Gogone et al., 2019). Essa característica foi evidenciada pelo fato de o CPV-2 original ter sido globalmente substituído por duas variantes antigênicas, distinguíveis por meio de anticorpos monoclonais (MAbs), apenas alguns anos após sua emergência na década de 1980 (Decaro e Buonavoglia, 2012). A primeira variante descrita, conhecida como CPV-2a, diferenciava-se do CPV-2 original nos sítios M87L, A300G, D305Y e V555I da VP2 (Parrish et al., 1985). Em 1984, a variante denominada CPV-2b emergiu, diferenciando-se da CPV-2a apenas nos sítios N426D e I555V (Gogone et al., 2019).

Em 2001, uma nova variante, denominada CPV-2c, foi identificada na Itália. Posteriormente, foi observado que essa variante afetava tanto cães adultos quanto cães imunizados, além de acometer gatos (de Oliveira et al., 2018). A CPV-2c espalhou-se rapidamente e se estabeleceu nas populações caninas em todo o mundo, apresentando uma importante substituição no sítio D426Y da VP2 (Gogone et al., 2019). No Brasil, a

variante CPV-2c foi identificada pela primeira vez em 2009 (Streck et al., 2009) e atualmente é descrito no país a co-circulação das três variantes, sendo a CPV-2a mais prevalente, seguida da CPV-2c e CPV-2b (Lopes et al., 2024).

A presença de uma simples mudança de aminoácido no sítio 426 entre as variantes CPV-2a, CPV-2b e CPV-2c confere propriedades antigênicas distintas (Decaro et al., 2020). No entanto, alterações adicionais nos sítios V139I, F267Y, S297A, Y324I, A347T e T440A têm sido descritas como assinaturas de novas variantes (de Oliveira et al., 2018). Isso aponta para a existência de uma ampla diversidade de perfis de VP2 de CPV-2 em circulação mundial.

A transmissão do CPV-2 ocorre principalmente pela via fecal-oral (Behdenna et al., 2019). A necessidade de uma baixa carga viral para a infecção e a alta resistência do CPV-2 no ambiente, resultante de sua estrutura não envelopada, são fatores cruciais que contribuem para a disseminação eficaz deste vírus. As pessoas, equipamentos veterinários, insetos e roedores também podem atuar como veículos para propagação (Morais e Costa, 2007). O vírus é excretado principalmente pelas fezes, mas também pode ser encontrado na urina, vômito, saliva e sangue. Estudos indicam que que as novas variantes antigênicas do CPV-2 são excretadas nas fezes por até 20 dias, antes mesmo do surgimento dos sintomas clínicos, em concentrações consideravelmente superiores às do CPV-2 original, o que as torna mais contagiosas (Decaro e Buonavoglia, 2012; Sykes et al., 2013).

2.2.3 Patogenia e sinais clínicos

Após a exposição oronasal, o vírus replica-se inicialmente nos tecidos linfóides da orofaringe antes de estabelecer a viremia (Sykes, 2013). Durante a disseminação, o vírus encontra-se preferencialmente em tecidos com rápida divisão celular, como a medula óssea, órgãos linfopoiéticos e criptas do jejuno e íleo (Morais e Costa, 2007). O período de incubação do CPV-2 varia de 7 a 14 dias, embora períodos mais curtos já tenham sido descritos. A gravidade dos sinais clínicos depende de fatores como a cepa do vírus, a imunidade do hospedeiro, a presença de anticorpos maternos e a ocorrência de infecções concomitantes, como outras infecções virais entéricas e parasitárias (Sykes, 2013). Os animais com imunidade parcial apresentam infecção subclínica ou formas clínicas mais brandas (Morais e Costa, 2007).

Nos animais que desenvolvem a doença, os primeiros sintomas, como anorexia, prostração, febre, vômito e desidratação, geralmente se manifestam de 6 a 10 dias após a contaminação (Tilley e Smith, 2014). Durante a infecção intestinal, o CPV-2 se replica nas células epiteliais das criptas da mucosa intestinal, levando ao achatamento das vilosidades, o colapso e a necrose epitelial. Como consequência desse processo, a diarreia que se manifesta costuma ser hemorrágica, devido ao sangramento dos capilares subjacentes ao revestimento do epitélio da mucosa (Morais e Costa, 2007). Assim, a gastroenterite hemorrágica se destaca como a manifestação clínica mais característica da parvovirose canina. Além disso, os danos no epitélio intestinal podem favorecer a translocação de bactérias e outros agentes para a corrente sanguínea, resultando em bacteremia e endotoxemia. Essa bacteremia secundária tem o potencial de desencadear falência múltipla dos órgãos e choque séptico, culminando em morte (Sykes, 2013).

Outra manifestação clínica significativa da parvovirose canina é a miocardite, que pode ocorrer em cães recém-nascidos que contraíram a infecção intrauterina ou nas primeiras semanas de vida. Esses animais podem apresentar morte súbita ou sintomas inespecíficos, que posteriormente evoluem para insuficiência cardíaca congestiva. Atualmente, essa condição é considerada rara, possivelmente devido à alta prevalência de anticorpos contra o CPV-2 na população canina (Morais e Costa, 2007; Nandi e Kumar, 2010).

No hemograma, os animais afetados pela parvovirose canina apresentam alterações significativas, tais como leucopenia, neutropenia e linfopenia, frequentemente acompanhadas de anemia, hipoproteinemia, redução nos níveis de potássio sérico e aumento nos níveis séricos de uréia e creatinina (Morais e Costa, 2007).

2.3 Diagnóstico

Os exames laboratoriais possuem um papel crucial no diagnóstico definitivo da parvovirose canina, uma vez que vários outros patógenos virais podem causar sinais clínicos parecidos nos cães. Dessa forma, vários métodos foram desenvolvidos para a detecção do CPV-2, incluindo: métodos tradicionais, métodos imunológicos e métodos moleculares (Nandi e Kumar, 2010).

Os métodos tradicionais para identificação do CPV-2 envolvem o isolamento do vírus utilizando linhagem de células mamíferas, seguido de visualização por microscopia eletrônica. No entanto, embora sejam padrão ouro para o diagnóstico definitivo do vírus, essas abordagens são demoradas, laboriosas, dispendiosas e requerem profissionais experientes, limitando a aplicabilidade (Tuteja et al., 2022).

A respeito dos métodos imunológicos, pode-se citar como principais a aglutinação em látex, teste de inibição - aglutinação em lâmina (SIT-SAT), teste de anticorpos fluorescentes, ensaios imunoenzimáticos (ELISA), hemaglutinação e inibição da hemaglutinação. A maioria desses métodos é capaz de detectar antígenos ou anticorpos específicos do parvovírus canino tipo 2 (CPV-2). Dentre eles, o ELISA destaca-se como o teste mais amplamente utilizado por clínicos e profissionais da área, sendo considerado um diagnóstico confirmatório (Tuteja et al., 2022). Isso se deve principalmente à sua rapidez, ao custo relativamente baixo e à praticidade de realização em qualquer clínica veterinária (Nandi e Kumar, 2010). A hemaglutinação e a inibição da hemaglutinação representam abordagens imunológicas de diagnóstico mais antigas. Na hemaglutinação, aproveita-se a capacidade do CPV-2 de aglutinar hemácias, sendo a presença de aglutinação interpretada como positiva para a detecção do vírus na amostra testada. Por outro lado, na inibição da hemaglutinação, adicionam-se anticorpos específicos contra o CPV-2 à amostra, os quais, se presentes, irão impedir a aglutinação das hemácias (Cubel Garcia et al., 2000). Apesar de demonstrarem alta especificidade para o diagnóstico do CPV-2, a sensibilidade desses métodos pode variar devido a possíveis flutuações na quantidade de vírus presentes nas fezes dos animais (Bergmann et al., 2021).

Com os avanços na biologia molecular, as técnicas de detecção do material genético viral começaram a ser cada vez mais utilizadas como ferramentas para o diagnóstico da parvovirose canina. Entre essas técnicas, a PCR e a PCR em tempo real se destacam, possibilitando a diferenciação de variantes e a quantificação relativa da carga viral por meio do valor de Ct, especialmente no caso da PCR em tempo real (Tuteja et al., 2022). Essas abordagens são conhecidas por fornecer diagnóstico rápido, sensível e preciso da doença, sendo capazes de detectar menos partículas de CPV-2 do que outros testes como hemaglutinação e ELISA (Nandi e Kumar, 2010). Entretanto, a disseminação dos testes moleculares é dificultada pelo alto investimento exigido em termos de equipamentos, reagentes e expertise operacional (Decaro et al., 2005).

2.4 Tratamento

2.4.1 Tratamentos convencionais

Não existe tratamento antiviral específico para a parvovirose canina. O manejo é predominantemente de suporte e inclui a administração de fluidos intravenosos para combater a desidratação, juntamente com o uso de medicações para controlar sintomas como dor, náuseas e diarreia (Mylonakis et al., 2016). A fluidoterapia e a manutenção de concentrações adequadas de glicose no sangue são os aspectos mais críticos do tratamento (Sykes, 2013). Adicionalmente, é recomendado o uso de agentes antimicrobianos de amplo espectro para prevenir e controlar infecções bacterianas secundárias (Morais e Costa, 2007).

Antivirais específicos contra o CPV-2 vêm sendo prospectados a fim de fornecer novas abordagens terapêuticas. Alguns medicamentos já foram avaliados *in vivo* no combate à infecção pelo vírus, com destaque o uso de interferon-omega felino (Zhou et al., 2015). No entanto, embora o tratamento com interferon tenha levado a uma recuperação mais rápida dos sintomas clínicos, seu alto custo limita seu uso (Gerlach et al., 2020).

2.4.2 Novas perspectivas de tratamento

À medida que a necessidade de tratamentos antivirais eficazes contra o CPV-2 se torna cada vez mais evidente, avanços contínuos estão sendo feitos na pesquisa médica para atender a essa demanda. Diante desse cenário, estudos recentes têm se dedicado à investigação de novas abordagens terapêuticas visando uma intervenção mais precisa e eficaz, com o propósito de melhorar o prognóstico dos animais que apresentam sintomas graves da doença. As estratégias mais comuns na busca por tratamentos antiparvovírus se desdobram em três direções principais: i) reposicionamento de medicamentos já conhecidos; ii) identificação ao acaso de compostos com potencial promissor contra o CPV-2; iii) triagem de bibliotecas químicas, desde simulações computacionais até testes *in vitro* (Zakrzewska et al., 2023).

O reposicionamento de medicamentos é uma estratégia que utiliza medicamentos já testados para novas indicações, oferecendo vantagens como menor risco de falha, cronogramas de desenvolvimento mais rápido, investimento reduzido e a possibilidade de descobrir novos alvos terapêuticos (Pushpakom et al., 2019; Begley et al., 2021). No entanto, enfrenta desafios como estabelecer a concentração adequada, estratégias para alcançar corretamente o alvo e garantir segurança para a nova indicação (Begley et al., 2021). Utilizando essa abordagem, foi observado que o cloreto de lítio, usado para tratar doenças não infecciosas, inibiu a infecção por CPV-2 em células F81 (Zhou et al., 2015). Ele pareceu impedir a entrada do vírus nas células, afetando as vesículas revestidas por clatrina. Outras drogas antiparasitárias, como nitazoxanida, closantel sódico e closante, também mostraram potencial anti-CPV-2 (Zhou et al., 2019). O mecanismo exato de ação dessas drogas ainda não é conhecido.

As descobertas fortuitas de novos medicamentos antivirais muitas vezes estão associadas à triagem de bibliotecas químicas. Essas bibliotecas são compostas por moléculas com propriedades bem definidas de bioatividade, segurança e biodisponibilidade, podendo ser inspiradas por compostos naturais ou fragmentos químicos. Utilizando algoritmos computacionais, essas bibliotecas são exploradas para identificar compostos biologicamente ativos que possam inibir alvos virais, como as proteínas reguladoras envolvidas na síntese de DNA (Schlueter e Peterson, 2009). Nesse contexto, estudos destacaram o potencial antiviral da quercetina e dos polissacarídeos fosforilados de *Radix Cyathulae officinalis* (pRCPS) contra o CPV-2 (Carvalho et al., 2013; Feng et al., 2017). Enquanto a quercetina interferiu na infectividade viral ao prejudicar a ligação aos receptores celulares e o processo de internalização (Carvalho et al., 2013), os pRCPS dificultaram a adsorção do CPV-2 e sua entrada nas células ao formarem um complexo com a cápside viral (Feng et al., 2017).

2.5 Profilaxia

2.5.1 Vacinação

A vacinação é a maneira mais eficaz de controlar a propagação do CPV-2 e prevenir infecções clínicas, sendo considerada uma vacina essencial para os cães (Behdenna et al., 2019). Recomenda-se que os filhotes recebam a vacinação primária

contra CPV-2 com 6-8 semanas de idade, seguido de um protocolo de revacinação a cada 2-4 semanas até completar 16 semanas, com doses de reforço ao longo da vida para assegurar a proteção (Decaro et al., 2020). No entanto, apesar dos programas intensivos de vacinação adotados em todo o mundo, a infecção por CPV-2 representa uma das doenças infecciosas mais frequentes e causa de morte em cães jovens, inclusive em países desenvolvidos (Decaro e Buonavoglia, 2012).

2.5.2 Tipos de vacinas disponíveis contra o CPV-2

Existem dois principais tipos de vacinas disponíveis contra o CPV-2: as vacinas inativadas e as vacinas de vírus vivo modificado. As opções de vacinas inativadas são limitadas no mercado devido à sua baixa imunogenicidade, o que requer administração repetida durante o ciclo primário e reforços anuais. Elas são sugeridas principalmente para animais exóticos e cadelas gestantes (Day et al., 2016). Por outro lado, as vacinas contendo vírus vivo modificado são amplamente utilizadas devido à sua capacidade de induzir imunidade forte e duradoura, sendo capazes de replicar no hospedeiro sem causar sinais clínicos graves (Decaro et al., 2020).

Na maioria dos países, apenas a cepa original CPV-2 e sua variante CPV-2b estão presentes nas formulações das vacinas (Yip et al., 2020). Isso levanta questões sobre a eficácia das vacinas baseadas na cepa antiga (CPV-2) contra as variantes emergentes (Decaro et al., 2020). A presença de novas mutações em aminoácidos específicos da VP2, localizados em regiões de alta antigenicidade e na área de ligação ao receptor, tem sido descrita, aumentando a possibilidade de escape imunológico do CPV-2 (Alexis et al., 2021). Outros fatores associados às falhas vacinais incluem a presença de anticorpos maternos (títulos de anticorpos no HI acima de 20), que podem levar à neutralização do antígeno viral após a administração de vacinas, a existência de não-respondedores e possíveis reversões à virulência (Decaro et al., 2020).

2.6 Novas ferramentas para o desenvolvimento de vacinas

2.6.1 Vacinologia reversa

Tradicionalmente, o desenvolvimento de vacinas depende do cultivo e da inativação ou atenuação de um patógeno completo para estimular uma resposta imunológica (Khalid e Poh, 2023). No entanto, a vacinologia reversa oferece uma abordagem alternativa, aproveitando a bioinformática e a análise computacional dos genomas dos patógenos para identificar potenciais alvos de vacinas sem a necessidade de técnicas tradicionais de cultivo (Rappuoli, 2000; Monterrubio-López e Delgadillo-Gutiérrez, 2021). Essas ferramentas computacionais prospectam antígenos capazes de induzir respostas protetoras, incluindo regiões de epítopos reconhecidos pelo sistema imunológico (Moxon et al., 2019).

A vantagem das abordagens da vacinologia reversa é a agilidade na identificação de vacinas candidatas, resultando em economia de esforços e custos. Esse método é particularmente valioso no desenvolvimento de novas vacinas contra agentes patogênicos altamente mutáveis, acelerando a criação de imunizantes que conferem proteção precisa contra as cepas prevalentes no campo (Monterrubio-López e Delgadillo-Gutiérrez, 2021). Além disso, a vacinologia reversa permite a produção de vacinas com menor dependência de modelos animais, o que pode reduzir o impacto ambiental associado à criação e manejo desses animais de laboratório.

2.6.1.1 Vacinas de subunidade

As vacinas proteicas de subunidade são constituídas apenas por fragmentos específicos do patógeno e constituem o principal ramo da vacinologia moderna (Bayani et al., 2023). Para produzir vacinas de subunidade recombinantes é empregada uma abordagem de engenharia genética para expressar genes específicos do patógeno em uma célula hospedeira adequada, como *Escherichia coli*, levedura, células de insetos ou células de mamíferos (Li et al., 2023).

O processo de prospecção do melhor imunógeno geralmente começa com análises comparativas de múltiplas sequências de material genético, visando a identificação de antígenos conservados em uma população heterogênea de patógenos (Del Tordello et al., 2017). Em seguida, essa metodologia é complementada com dados provenientes do sequenciamento de epítopos peptídicos obtidos por meio da espectrometria de massas (Backert e Kohlbacher, 2015). Posteriormente, são realizadas

análises de predição de antigenicidade, toxicidade, solubilidade, alergenicidade, estabilidade e outros parâmetros, visando o desenho final da vacina por meio de modelagem computacional, que posteriormente poderá ser sintetizada para passar por testes *in vitro* e *in vivo*.

Essas vacinas podem ser compostas por sequências integrais de proteínas ou apenas por subunidades de epítopos imunogênicos ligados entre si, sendo facilmente sintetizadas em larga escala e na forma pura. Além disso, são consideradas mais seguras, pois diminuem os riscos associados ao manejo de patógenos, à recuperação da virulência e à indução de respostas imunológicas prejudiciais (Bayani et al., 2023). No entanto, como as proteínas exógenas geralmente induzem respostas do MHC classe II e geralmente não produzem respostas fortes de células T, é necessária a estimulação do adjuvante e múltiplas doses para aumentar a imunogenicidade (Li et al., 2023).

Na medicina humana, diversas vacinas de subunidade se destacam, como as desenvolvidas contra o coronavírus SARS-CoV-2, o vírus da hepatite B e o papilomavírus humano. Todas essas vacinas são compostas por proteínas de superfície viral (Moni et al., 2023; Wei et al., 2023). Na medicina veterinária, também existem vacinas de subunidade disponíveis, contendo fragmentos específicos do vírus que estimulam o sistema imunológico. Por exemplo, as vacinas contra os patógenos responsáveis pela gripe aviária em aves e pela leucose felina em gatos (Tartaglia et al., 1993; Dey et al., 2023). No caso dos cães, um exemplo comum é a vacina contra o vírus da cinomose, na qual um canarypox recombinante expressa as glicoproteínas virais HA e F (Thibault et al., 2022).

3 OBJETIVOS

3.1 Objetivo geral

O objetivo deste estudo foi discorrer sobre as estratégias terapêuticas para o tratamento de infecções humanas e animais por parvovírus, compreender a antigenicidade de CPV-2 e projetar uma vacina *in silico* visando o futuro desenvolvimento de uma vacina atualizada contra esse patógeno.

3.2 Objetivos específicos

- Realizar uma revisão bibliográfica sobre alternativas terapêuticas promissoras contra importantes membros da subfamília *Parvovirinae*, com foco particular nos vírus B19V, CPV-2 e PPV.
- Avaliar o impacto de mutações pontuais na proteína VP2 na capacidade replicativa do CPV-2, visando compreender melhor a relação entre a variabilidade genética do vírus e sua antigenicidade.
- Analisar as sequências genômicas de cepas de CPV-2 isoladas no território brasileiro, assim como as sequências presentes em vacinas disponíveis no país para combater a doença, a fim de identificar variações genéticas.
- Desenvolver uma sequência consenso da proteína VP2 contendo as alterações de aminoácidos mais comuns presentes nas cepas de CPV-2 encontradas no Brasil.
- Utilizar programas de predição do sistema imunológico para determinar as regiões mais imunogênicas na sequência consenso da proteína VP2, visando otimizar a composição da vacina.
- Submeter a sequência final da vacina candidata a programas de predição para avaliar sua antigenicidade, toxicidade e alergenicidade, bem como prever sua estrutura.
- Fazer a docagem da vacina candidata com um receptor Toll-like canino.
- Simular *in silico* a resposta do sistema imune frente à vacina candidata.

4 RESULTADOS

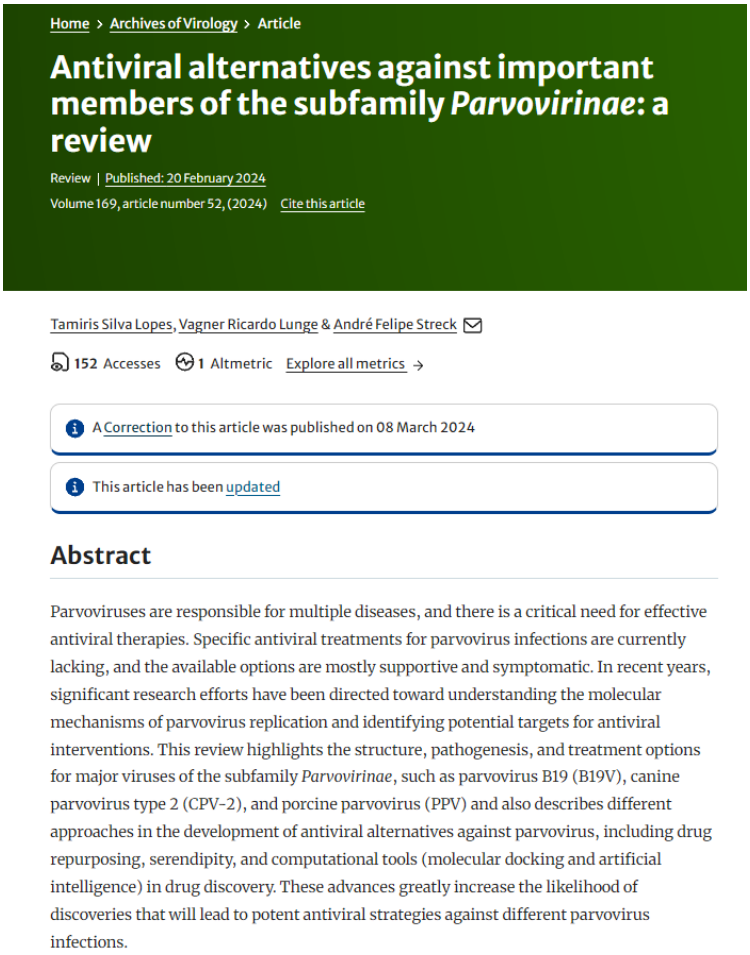
Os resultados obtidos são então apresentados em três capítulos distintos. O Capítulo I traz o artigo publicado na revista "Archives of Virology". Esta revisão de literatura concentrou-se em trabalhos que exploraram novas estratégias terapêuticas contra representantes relevantes da subfamília *Parvovirinae*. A análise desses estudos oferece uma visão abrangente das pesquisas em andamento destinadas a preencher as lacunas nesta área.

O Capítulo II refere-se ao artigo que será submetido à revista “Brazilian Journal of Microbiology”. Este trabalho teve como objetivo a construção de mutantes contendo mutações pontuais na proteína VP2 do parvovírus canino, visando compreender seus impactos na capacidade replicativa do vírus. Além disso, um mutante foi selecionado para teste de neutralização viral com anticorpos policlonais, cujos resultados foram comparados com os de uma cepa padrão.

O capítulo III traz o artigo já publicado na revista "Brazilian Journal of Microbiology", que detalha o processo de construção *in silico* de uma vacina multi-epitopo contra o CPV-2. Em seguida, é explicado o procedimento de simulação da resposta imunológica desencadeada por esse imunizante.

4.1 Capítulo I

Artigo publicado eletronicamente na revista "Archives of Virology".



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Antiviral alternatives against important members of the subfamily *Parvovirinae*: a review

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Tamiris Silva Lopes, Vagner Ricardo Lunge & André Felipe Streck

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Abstract

Parvoviruses are responsible for multiple diseases, and there is a critical need for effective antiviral therapies. Specific antiviral treatments for parvovirus infections are currently lacking, and the available options are mostly supportive and symptomatic. In recent years, significant research efforts have been directed toward understanding the molecular mechanisms of parvovirus replication and identifying potential targets for antiviral interventions. This review highlights the structure, pathogenesis, and treatment options for major viruses of the subfamily *Parvovirinae*, such as parvovirus B19 (B19V), canine parvovirus type 2 (CPV-2), and porcine parvovirus (PPV) and also describes different approaches in the development of antiviral alternatives against parvovirus, including drug repurposing, serendipity, and computational tools (molecular docking and artificial intelligence) in drug discovery. These advances greatly increase the likelihood of discoveries that will lead to potent antiviral strategies against different parvovirus infections.

Antiviral alternatives against important members of the subfamily *Parvovirinae* - a review

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Abstract

Parvoviruses are responsible for several diseases and there is a critical need for effective antiviral therapies. Specific antiviral treatments for parvovirus infections are currently lacking and the available options are mostly supportive and symptomatic. In recent years, significant research efforts have been directed toward understanding the molecular mechanisms of parvovirus replication and identifying potential targets for antiviral interventions. This review highlights the structure, pathogenesis and treatment options for major viruses of the subfamily *Parvovirinae*, such as parvovirus B19 (B19V), canine parvovirus type 2 (CPV-2) and porcine parvovirus (PPV). Furthermore, this review describes different approaches in the development of antiviral alternatives against parvovirus, including drug repurposing, serendipity and computational tools (molecular docking and artificial intelligence) in drug discovery. These advances offer great expectancy in the discovery of potent antiviral strategies against different parvovirus infections.

Keywords Parvovirus B19; Canine parvovirus; Porcine parvovirus; Pathogenesis; Treatments

Introduction

The family *Parvoviridae* encompasses a broad and highly diverse assortment of viruses with linear single-stranded DNA genomes spanning 4-6 kb in length [1]. The capsid is icosahedral with a diameter of 20-25 nm, composed by 60 identical protein subunits [2, 3]. Parvoviruses host a wide range of animals and share important characteristics such as resistance to desiccation in the environment and the need for the S-phase of infected cells for viral DNA replication.

Parvoviridae is divided into three subfamilies: *Parvovirinae*, *Densovirinae* and *Hamaparvovirinae* [4,5]. *Parvovirinae* members infect vertebrate hosts, while viruses belonging to the subfamily *Densovirinae* and *Hamaparvovirinae* infect insects or shrimp [5, 6]. In the subfamily *Parvovirinae*, there are significant human and veterinary pathogens, contributing to an array of diseases. In humans, the most significant is parvovirus B19 (B19V), which is particularly concerning for children and individuals with compromised immune systems. Among non-human parvoviruses, canine parvovirus type 2 (CPV-2) and porcine parvovirus (PPV) are particularly health concerns, commonly causing hemorrhagic enteritis and reproductive disorders, respectively.

At present, there are no specific antiviral therapies for parvovirus infections, and available treatments are primarily supportive, based on symptoms, and often have limited effectiveness [7, 8]. The development of new antiviral strategies against significant pathogens in the subfamily *Parvovirinae* is a critical area of research worldwide. This review aims to discuss the subfamily *Parvovirinae*, focusing on new and promising antiviral alternatives against B19V, CPV-2 and PPV.

Subfamily *Parvovirinae*

Taxonomy and Structure

The subfamily *Parvovirinae* is subdivided into eleven genera: *Amdoparvovirus*, *Artiparvovirus*, *Aveparvovirus*, *Bocaparvovirus*, *Copiparvovirus*, *Dependoparvovirus*, *Erythroparvovirus*, *Loriparvovirus*, *Protoparvovirus*, *Sandeparvovirus* and *Tetraparvovirus* [5]. Furthermore, *Parvovirinae* is also categorized as autonomous

parvoviruses and dependoparvoviruses, which don't or do require a helper virus, respectively, for successful replication within cells [9]. As dependoparvoviruses are not linked with disease in humans or animals, they will not be the focus of this review.

The capsid cores are structurally similar across all parvoviruses, consisting of a conserved eight-stranded antiparallel β-barrel motif and an α-helix [10]. In contrast, capsid surface morphologies exhibit low sequence similarity (e.g., 14% to 36% between genera) and are characterized by depressions at the 2-fold axes (dimple) and surrounding a cylindrical channel at the 5-fold axes (canyon), and protrusions at or surrounding the 3-fold axes [11]. Viral capsids are engineered to reorganize according to specific cellular cues and gradually adopt a series of structural configurations. These configurations facilitate intracellular transport of the capsids and release of the genome into the appropriate cellular compartment for replication [12].

The single-stranded *Parvovirinae* genomes contain palindromic sequences, which can create a hairpin structure, providing most of the cis-acting information needed for both viral DNA replication and encapsidation [13]. The size, sequence, and predicted structures of the hairpins exhibit notable variations among different parvoviruses, and can either be identical, forming inverted terminal repeats (e.g., B19V and AAV), or different between the two ends of an individual genome (e.g, CPV, PPV) [14]. The coding region of the genome contains two major expression cassettes, with open reading frames (ORFs) on the left-hand side leading to the production of highly conserved non-structural (NS/Rep) proteins. In contrast, mRNA populations responsible for translating structural proteins (VPs) are transcribed from the right- hand cassette [3, 15]. In some viruses, additional ORFs are detected, enabling the expression of smaller regulatory proteins [3, 13].

Owing to their small size and limited number of proteins, nonstructural and structural proteins in parvoviruses fulfill many different functions [9]. Nonstructural protein 1 (NS1) performs key functions, including binding to the 5' end of viral DNA during replication, acting as the helicase for replication and DNA packaging, serving as a site-specific nickase, facilitating cell cycle arrest in the G1 phase, and playing a role as a transcription factor [16, 17]. Nonstructural protein 2 (NS2) participates in viral DNA replication, viral mRNA translation, capsid assembly, and parvoviral cytotoxicity [18]. Meanwhile, structural proteins play a crucial role in host receptor binding, antigenic properties, and environmental stability. Viral proteins 1 and 2 (VP1 and VP2) are formed by alternative splicing of the same mRNA. The VP1-specific regions contain the

enzymatic core of a phospholipase A2 (PLA2), along with nuclear localization signal (NLS) sequences [13]. VP2, on the other hand, constitutes the primary component of the capsid protein, and in some cases (only in DNA-containing capsids), VP3 appears as a post-translational cleavage product of VP2 [19].

Many parvoviruses are highly specialized for infecting specific host cells, binding glycosylated cell surface molecules and getting internalized by receptor-mediated endocytosis [4]. During the entry process, viral particles are exposed to progressively lower pH levels inside the endosomes, inducing changes in the conformation of capsid proteins, such as exposure of the amino-terminal region of VP1 and cleavage of the amino-terminal region of VP2, followed by denudation of the genome [20]. The PLA2 lipolytic activity and the nuclear localization signals of VP1 are essential for efficient penetration of virions through the cellular membrane and for the transfer of the viral genome from late endosomes/lysosomes to the nucleus to initiate replication [13]. In the nucleus, virus replication occurs and appears to require the cell to go through S-phase. Due to their high dependence on host cellular factors, parvoviruses have adopted strategies to modulate the cellular environment, including exerting control over the cell cycle and manipulating different cell signaling pathways to their advantage. Examples include hijacking of the cellular DNA replication machinery and activation of DNA damage response pathways [9].

Incoming viral particles contain a single copy of the linear DNA genome which is initially converted to a duplex replication intermediate by cellular DNA polymerases and auxiliary factors [21]. An early phase of transcription primarily produces mRNAs coding for the NS protein [7]. Amplification continues via a unidirectional single-strand displacement mechanism through a series of monomeric and concatemeric duplex replicative-form intermediates. The viral initiator protein, NS1, acts both as a site- and strand-specific nickase and as a 3′-to-5′ helicase, exerting resolution of terminal hairpin structures and unwinding of double-stranded replicative intermediates, allowing replication through a rolling hairpin mechanism [6, 14].

Lastly, the translated structural proteins are transported to the nucleus, where they assemble into the capsid. This capsid is composed of a few copies of VP1 and 50 to 55 copies of VP2 [3, 22]. As preassembled empty viral particles accumulate, viral genomes are packaged by the NS1 3′-to-5′ helicase [4, 23]. Thereafter, these progeny virions may be exported from cells or accumulate in the nucleus until released through cell lysis or apoptosis [24] (Fig. 1).

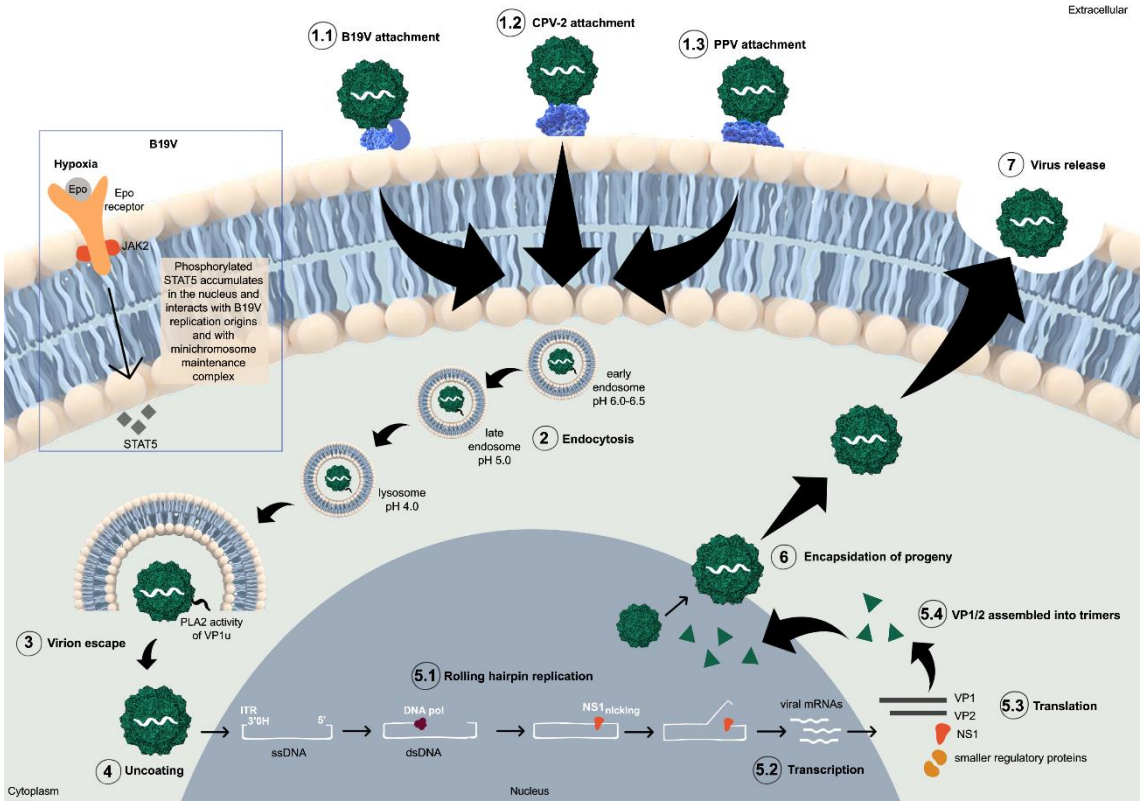

Fig. 1 General schematic representation of B19V, CPV-2 and PPV life cycles and virus-host cell interactions. 1.1. B19V first binds to the globoside receptors; then occurs the extrusion of VP1 unique (VP1u) region and binding to an erythroid specific receptor, in erythroid progenitor cells. 1.2. CPV-2 binds to the transferrin receptor type 1. 1.3. PPV binds to sialic acid receptors. 2. Thereupon, the virus enters the host cells through clathrin-mediated endocytosis. Virions are enclosed within endosomal vesicles. Early endosomes form at pH 6.0-6.5, turning into late endosomes at pH 5. They then transform into lysosomes, activating the viral VP1u-associated phospholipase A2 (PLA2) at pH 4.0. 3. The activation of PLA2 compromises the integrity of the lysosomal membrane and the virions are released into the cytoplasm. 4. The virions are transported towards the nucleus and target on the karyotheca with assistance from the VP1 nuclear localization signal. In the nucleus, complete release of viral ssDNA occurs. 5.1. Beginning of the second strand synthesis by rolling hairpin mechanisms, via self-primed single-strand and formation of dsDNA replicative intermediate. After dsDNA conversion, NS1 binds and nicks one of the strands. This event creates a new 3′ OH to lead DNA synthesis. 5.2. Transcription produces different mRNAs. In the initial phase of transcription, the focus is primarily on encoding mRNAs for the NS protein. During the late phase of transcription, the emphasis shifts mainly to encoding mRNAs for VP and smaller proteins. 5.3. mRNAs are exported to the cytoplasm where proteins are then translated. 5.4. VP1/2 assemble into trimers to form capsids, which are transported back to the nucleus. 6. Using strand displacement, ssDNA is packaged into capsids, a process likely dependent on NS1. 7. Matured virions release via cell lysis or apoptosis after accumulation. The presence of the NS1 protein along with smaller regulatory proteins (e.g., 11 kDa of B19V) in the cytoplasm has the potential to induce apoptosis. Within the rectangle is represented the activation of the erythropoietin (Epo) pathway and the presence of a hypoxic condition, similar to that of the bone marrow, necessary to establish a permissive intracellular environment for B19V replication. Erythropoietin (EPO) and EPO receptor signaling is required. Janus signal transducer kinase 2 (JAK2) and activator of transcription 5 (STAT5) signaling play a key role in B19V replication. Phosphorylated STAT5 interacts with B19V origins of replication and the minichromosome maintenance complex in human endothelial progenitor cells, and functions as a scaffold protein to bring MCM to the viral replication.

Pathogenesis and current treatments

Parvoviruses are the cause of various diseases in humans and animals. The pathogenesis of parvovirus infection is complex, involving a combination of several pathogenic mechanisms during a single infection [25]. A contributing factor is the need for S-phase cells to access DNA replication machinery and cofactors, thereby facilitating infection of young or unborn individuals or tissues in older individuals that contain proliferating cells [26, 27]. Furthermore, specific factors, such as cell surface receptor recognition, vary significantly within the parvovirus group, being a crucial determinant of tropism and contributing to pathogenesis.

In most infections, cell death promotes viral spread and incurs tissue damage, often resulting in disease [28]. The severity of symptoms ranges from subclinical to lethal, depending on host-specific factors. Among the representatives of parvoviruses, B19V poses the greatest challenge in human medicine, while in veterinary medicine, the two most significant parvoviruses are CPV-2 and PPV.

Parvovirus B19

B19V is a human pathogen belonging to the genus *Erythroparvovirus* [2]. It was discovered incidentally in 1974 and is now considered the most pathogenic parvovirus to humans, responsible for a broad spectrum of clinical manifestations. The severity of these manifestations depends on the intricate interaction between the virus and the physiological and immunological condition of the infected individual [1].

B19V primarily enters through the upper respiratory tract and then spreads through the blood circulation to the bone marrow, where it replicates in erythroid precursor cells [10]. The virus takes advantage of the naturally acidic environment of the nasal mucosa and the presence of globoside in the ciliated cell population of the airway to breach the respiratory epithelium via transcytosis [29]. The pH-dependent modulation of affinity between B19V and globoside directly influences virus tropism and pathogenesis [29]. Moreover, the virus exhibits multiple modes of transmission, including through blood or blood products, the placenta, and potentially even via tattooing. Due to its relative ease of transmission, B19V is widespread and most often causes asymptomatic or unnoticed infections [2]. However, this is not always the case. Its tropism for erythroid progenitor cells in the bone marrow can cause a partial block in erythropoiesis, which may manifest as transient or persistent erythroid aplasia [30]. Other clinical manifestations of B19V include erythema infectiosum (fifth disease),

hydrops fetalis, arthropathy, hepatitis, and cardiomyopathy [31]. B19V infection has also been linked to autoimmune destruction of granulocytes and thrombocytes [9].

In mild cases, the clinical approach to infection is conservative, as the infection is self-limiting and the development of a specific immune response is effective in controlling the virus [7]. However, in acute cases, therapeutic options are limited [2]. Currently, a vaccine against B19V infection is not available, and the therapies used are merely supportive, symptomatic, or non-specific [7, 32].

In cases of B19V infection, blood transfusions may be necessary to manage hematological complications. Additionally, intravenous immunoglobulins serve as a therapeutic intervention against B19V, playing a crucial role in neutralizing the virus and controlling infection, particularly in immunocompromised individuals [33]. Nonsteroidal anti-inflammatory drugs are also commonly used to alleviate inflammatory symptoms in arthritis and arthralgias, and sporadic reports suggest the potential usefulness of corticosteroids in managing atypical inflammation [7]. However, the beneficial effects of supportive treatments are limited until the patient's antiviral immune response develops and becomes effective [33]. Consequently, it is clear that there is a need for specific treatments to manage hematological complications occurring during the acute or chronic phase of infection, or to mitigate the inflammatory or systemic aspects in atypical cases [34].

Canine parvovirus

CPV-2 belongs to the genus *Protoparvovirus* [3]. First identified in the late 1970s following reports of an unfamiliar contagious enteric disease in the United States, CPV-2 rapidly spread worldwide among domestic and wild carnivores [35]. Within a few years, the original strain of CPV-2 was replaced by three main variants: CPV-2a, CPV-2b, and CPV-2c [8, 36].

Transmission of CPV-2 primarily occurs via the fecal-oral route [35]. Most infections result from exposure of susceptible dogs to feces contaminated with viruses, as CPV-2 is highly contagious and persistent in the environment. CPV-2 is a principal agent of acute gastroenteritis, leukopenia, and myocarditis in neonatal puppies [8]. After an incubation period of 3-7 days, clinical signs may include vomiting, hemorrhagic diarrhea, depression, lymphopenia, loss of appetite, fever, and dehydration [27]. Although CPV-2 can affect dogs of any age, severe infection is more frequent in puppies between 6 and 16 weeks of age [37].

Prevention of CPV-2 is achieved through vaccinating pregnant bitches and puppies older than six weeks [38]. Despite the common use of inactivated or live attenuated vaccines, there are many reports of vaccine failures, primarily due to the presence of maternal antibodies and the emergence of antigenic variants [8]. There is no specific antiviral treatment for CPV-2; the main approach is symptom-based supportive care.

Disease management typically involves the use of intravenous fluids, synthetic and natural colloids, correction of hypoglycemia and electrolyte imbalances, a combination of antimicrobials, antiemetics, analgesics, enteral nutrition, and anthelmintics [35]. There are other alternatives for treating CPV-2, including passive immunotherapy with CPV-immune plasma and the use of feline interferon-omega [39]. However, no significantly positive effects have been demonstrated using anti-parvovirus antibodies [40, 41]. Interferon treatment has resulted in a more rapid improvement in clinical symptoms, but its high cost limits its use [42]. For these reasons, drug therapy against CPV-2 infection requires further exploration.

Porcine parvovirus

PPV is a member of the genus *Protoparvovirus* [43]. Since its first description in pigs in 1967, PPV has been identified as the leading agent of recurrent oestrus, abortion, and the delivery of mummified or stillborn fetuses, as denoted by the acronym SMEDI (stillbirth, mummification, embryonic death, and infertility) [44]. Older pigs typically exhibit only mild or subclinical disease following infection. Reproductive failures associated with virus-induced tissue damage and cell apoptosis result in significant economic losses in the breeding industry [45, 46].

PPV primarily spreads between herds through fomites, with speculations suggesting that infected boars could introduce the virus into new herds [44]. One of the unanswered questions about PPV-induced reproductive failure is how the virus passes the placental barrier [47]. Fetuses become infected and die at different stages of pregnancy, suggesting fetus-to-fetus transmission within the uterus [48]. Pathological sequelae is related mainly to the gestational period in which infection occurs [44]. Microscopically, fetuses infected after the development of immunocompetence present disseminated lymphocytes and plasma cells in the liver, lungs, kidneys and cerebellum [48].

Once established, the virus can remain infectious in the environment for prolonged periods and is resistant to most disinfectants [45]. Immunization of sows is currently the primary preventive measure against PPV infection. However, the efficacy remains unsatisfactory due to the emergence of new strains [46]. Hence, despite vaccination, PPV remains prevalent in swine herds [43].

At present, there is no specific treatment for PPV, and management measures aim to maintain good herd health status [44]. Therefore, the development of systemic therapies for PPV infection is an urgent need, and research into new antiviral strategies has become increasingly important over the years.

Approaches used in anti-parvovirus drug research

In recent years, the search for new antivirals against parvoviruses has advanced mainly in three directions: i) reutilization of known drugs; ii) serendipity tests of compounds with promising potential for antiviral activity; iii) screening of chemical libraries [49].

Drug repurposing, a strategy redirecting extensively tested drugs for additional or unrelated indications, presents significant advantages such as lower risk of failure, shortened development timelines, reduced investment requirements, and the potential to uncover new targets and therapeutic pathways [50, 51]. The methodical strategy can be broadly divided into computational and experimental approaches, although these approaches should be used synergistically to achieve the best outcome. Challenges along the drug repurposing are substantial and include establishing adequate potency, target engagement, differentiation from standard of care, and a robust PK/PD ratio and safety profile for the new indication, all of which can be very different from the original indication [50]. Further preclinical studies of safety and efficacy may be necessary.

Chemical libraries for drug repurposing often comprise approved drugs that have well-characterized bioactivities, safety, and bioavailability properties. These libraries can also be built inspired by natural compounds or based on a set of chemical fragments. This method employs computational algorithms to discover biologically active compounds that can, for example, inhibit viral targets such as regulatory proteins involved in DNA synthesis. Compounds in chemical libraries are predominantly based on previously successful drug structures. Ideally, a chemical library should contain diverse structural classes capable of modulating a variety of therapeutic targets [52]. Improving virtual screening methods remains a key challenge in screening chemical

libraries, particularly in the face of the increasing demand to search larger libraries with higher performance levels and identify novel drug leads [53]. Given the dependence of viruses on host cellular machinery, cell-based assays play a crucial role in high-throughput screening of antiviral compounds, aiming to better predict the response of a lead compound *in vivo*. By utilizing an intracellular viral target to identify chemical inhibitors, the potency of a compound becomes intrinsically linked to its membrane permeability, especially under conditions where cytotoxicity serves as a relevant assay parameter [54]. The demanding cell culture conditions and the lack of suitable animal models for certain parvoviruses restrict the feasibility of implementing high-throughput screening against available chemical libraries and validating novel treatments.

Parvovirus B19

Research on B19V has focused on acquiring a detailed understanding of the viral life cycle and the molecular machinery involved to identify critical targets. Inhibition of these targets is aimed at preventing viral replication or, at the very least, mitigating its cytotoxic effects [7]. Substantial progress has been achieved, highlighting the non-structural protein NS1 as a promising target for the development of new antivirals. The multidomain nature of B19V NS1, coupled with its fundamental role in viral replication and predicted nuclease, helicase, and gene transactivation activities, establishes it as a crucial focal point [31]. The endonuclease activity of NS1, executed through the N-terminal origin-binding domain, plays a key role in terminal resolution and the continuation of rolling hairpin replication [7]. In the pursuit of direct antiviral agents, Xu et al. [32] conducted screening in cell-based assays (CD36+ EPCs and UT7/EpoS1 cells) of a chemical library, identifying a subset of compounds with substantial *in vitro* endonuclease inhibitory activity. Notably, three compounds with a flavonoid-like structure demonstrated inhibition of B19V DNA replication in cellular assays, suggesting potential suppression of nicking at the origin of replication. Similarly, Ning et al. [55] examined a substantial number of compounds (17.040) and identified 84 with the potential to inhibit cutting in a dose-dependent manner. Of these, four were investigated and revealed significant inhibition of B19V replication in CD36+ EPCs, with the purine analog P7 outperforming the other three, with 92% inhibition of B19V infection at a concentration of 3.32 μM.

Two other strategies still using cell-based assays were applied to find potential alternatives against B19V. The first strategy, rooted in drug repositioning, identified the cell proliferation inhibitor hydroxyurea as a potential anti-B19V drug. Hydroxyurea is also used in the treatment of sickle cell disease and showed inhibitory activity against B19V *in vitro* at concentrations below those that affect cellular viability [34]. Its activity could be linked to the reduction of deoxyribonucleotide levels in cells and the induction of an increase in the percentage of cells arrested in G1/S phase with 2N DNA content. This suggests a potential reduction in the cells' ability to engage in viral replication. The second approach, centered on the study of known antiviral compounds, aimed to identify those exerting inhibitory effects on B19V. Cidofovir, an acyclic nucleoside phosphonate, emerged as the first compound with inhibitory effects on B19V replication, and its potential was increased by extending the exposure time after infection [56, 57]. Subsequently, brincidofovir, a lipid-conjugated prodrug of cidofovir, exhibited enhanced effectiveness [33]. The antiviral activity of both agents is directly related to cidofovir diphosphate, which serves as an alternative substrate for viral DNA synthesis. Telbivudine, an antiviral employed in chronic hepatitis B treatment, demonstrated endothelial-protective effects on B19V-infected endothelial cells *in vitro* and improved chronic myocarditis associated with viral transcriptional activity *in vivo* [58]. This thymidine analogue elicits an indirect effect by boosting the production of antiviral IFN-β in infected cells, although additional research remains imperative to assess its suitability for clinical application. The intricacies of its mode of action and potential side effects necessitate a critical examination of its practicality and safety profile for widespread use against B19V.

Although researchers often use cutting-edge technologies to intentionally explore compounds with antiviral activities, the scenario of unexpected discoveries presents an opportunity for navigation and exploration. In this scenario, the serendipity approach was used, defined as the accidental or unexpected discovery of a new medicine during the investigation of a different objective. A small chemical library of compounds with potential antiviral activity was screened, leading to the identification of coumarin derivatives (3-(Imidazo[2,1-b]thiazol-6-yl)-2H-chromen-2-one) that exhibited inhibition of B19V [30]. Several other coumarin core compounds are recognized for their antiviral activity in several model systems, but their precise mechanisms of action remain unclear [7, 59]. This lack of clarity raises concerns about the broader applicability of these conclusions in the context of B19V. For a comprehensive

understanding, Table 1 provides additional research results on new antiviral alternatives against B19V.

Canine parvovirus

One strategy for discovering antiviral drugs against CPV-2 is drug repurposing by screening existing drugs. Lithium chloride, conventionally used in the treatment of several non-infectious diseases, demonstrated a dose-dependent inhibition of CPV-2 infection in F81 cells [39]. Lithium chloride appears to prevent CPV-2 from entering cells, suggesting that clathrin-coated vesicles may be harmed by the treatment. The same approach, also using cell-based assay, identified the remarkable anti-CPV-2 potential of antiparasitic drugs such as nitazoxanide, closantel sodium and closante [8]. The antiviral effect of nitazoxanide has already been observed against DNA and RNA viruses [64-66] and although the exact mechanism of action remains unclear, some results suggest virus-specific effects. For closantel and closantel sodium, neither their antiviral spectrum nor their mechanisms of action are yet known [8].

Turning attention to plant extracts and isolated compounds, extensive studies have examined their potential antiviral activities. For example, quercetin and phosphorylated *Radix Cyathulae officinalis* polysaccharides (pRCPS) have been tested and shown promising anti-CPV-2 effects [38, 67]. Quercetin interfered with viral infectivity, reducing the sensitivity of CRFK cells to CPV-2 by impairing viral binding to cellular receptors and, consequently, the internalization process [38]. pRCPS exhibits a comparable antiviral mechanism, hindering CPV-2 adsorption and entry into F81 cells in a dose-dependent manner. This effect is likely attributed to the formation of a complex with the capsid, preventing CPV-2 from docking to the host cell surface [67]. Other research on antiviral alternatives against CPV-2 is summarized in Table 1.

Porcine parvovirus

There is currently no effective treatment for reproductive issues caused by PPV [70], revealing a significant gap in addressing this problem. The exploration of alternatives has led to the investigation of bioactive compounds derived from natural sources as a potential avenue for combating PPV. Diammonium glycyrrhizinate [45] and germacrone [43] exerted inhibitory effects on virus replication and propagation in cell-based assays (PK15 and ST cell lines). Notably, the antiviral mechanism of diammonium glycyrrhizinate remains elusive [45]. In contrast, research indicates that

germacrone targets the initial phase of PPV replication, specifically inhibiting the synthesis of RNA and viral proteins [43]. Promising antiviral activities have also been observed in bioactive constituents isolated from propolis. Nanometer propolis flavone, ferulic acid, chrysin, kaempferol, and galangin exhibited significant inhibitory effects on PPV infection induced in PK-15 cells [46, 71, 72]. Among them, nanometer propolis flavone and ferulic acid produced the best outcomes. Nanometer propolis flavone exhibits a higher protective effect, likely attributed to its small and homogeneous particle size, facilitating cellular penetration and absorption, along with an increase in the *in vivo* antibody titer [71]. On the other hand, ferulic acid inhibits PPV-induced apoptosis by influencing the expression of key members of the mitochondria-mediated apoptosis pathway, including Bid, caspase-3, 7, and 9 [46].

Nitric oxide, an essential molecule for intercellular signal transmission, is synthesized from L-arginine by nitric oxide synthases. Wei et al. [73] demonstrated that two nitric oxide-forming compounds, S-nitroso-L-acetylpenicillamine and L-arginine, could reduce PPV replication in PK-15 cells in a dose-dependent manner by disrupting viral DNA and protein synthesis. Some osmolytes, natural compounds found in cells of many organisms, have also undergone testing against PPV. Among them, trimethylamine N-oxide and glycine were able to stabilize viral capsid proteins and prevent them from assembling into viable virus particles in PK-13 cells [74]. Regarding glycine, its antiviral effect against PPV may be linked to alterations in membrane dielectric properties of cells [75]. Furthermore, lithium chloride has also shown promise as an anti-PPV drug. This salt was effective in the early stages of viral replication in ST cells, and treatment with concentrations up to 30 mM showed no significant toxicity [76]. Further studies on antiviral strategies against PPV are in Table 1.

Table 1 Other research on new antiviral alternatives against important members of the subfamily *Parvovirinae*

Target	Antiviral agent	Mechanism of action	Cell type	Reference
B19V	Small Interfering RNAs in adenoviral vector	B19V-VP2-specific short hairpin RNAs silenced not only the targeted VP2 but also the level of NS1-mRNA was repressed.	UT7/EpoS1	[60]
Indirectly B19V	Pimozide	Efficient B19V replication also requires hypoxic conditions, which upregulate the STAT5 pathway, whose phosphorylated form is essential for virus replication. Pimozide is an inhibitor of STAT5 phosphorylation.	UT7/Epo-S1 and EPCs	[61]
Indirectly B19V	Telbivudine	A thymidine analogue, which reverses B19V-induced dysregulation of BIRC3, intervening in the apoptosis pathway and protecting susceptible cells from cell death.	EPCs	[62]
Indirectly B19V	Celastrol	Markedly reduced B19V NS1-induced inflammation in human macrophages, decreasing cell migration, MMP-9 activity, phagocytosis, inflammatory cytokines, and inflammasome signaling, though the exact mechanisms remain unknown.	U937 and and THP-1	[63]
CPV-2	Small interfering RNAs in psiSTRIKE vector	The vector-derived small interfering RNAs targeted to the NS1 genes effectively inhibited CPV-2 replication, suppressing specific mRNAs.	F81	[37]
CPV-2	*Bacillus* sp. P34 peptide	No antiviral activity was detected.	CRFK	[68]
CPV-2	Animal feed additives (Curcumin, bisdemethoxycurcumin, demethoxycurcumin, linoleic acid, tannic acid, α-tocopherol, extracted turmeric, yerba mate, and sesame cake)	NS1 endonuclease activities.	Not applicable - FRET substrate	[69]
PPV	2-amino-4,4a-dihydro-4a-7-dimethyl-3Hphenoxazine-3-one and 3-amino-1,4a-dihydro-4a-8-dimethyl-2H-phenoxazine-2-one	The antiviral mechanisms are still unknown.	ESK	[77]
PPV	High molecular weight hyaluronic acid	Probably involved general/non-specific host cell-virus interaction at membrane level, such as virus entry or release.	PK15	[78]

The role of computational tools

Current antiviral drug discovery for parvovirus heavily relies on computational methods, particularly *in silico* approaches employing artificial intelligence (AI). The notable increase in the use of AI is accompanied by the growing availability of structural, chemical, and relevant biological data to a growing number of therapeutic targets [79]. AI demonstrates effective utility across various stages of drug discovery, including design, synthesis, screening, polypharmacology, and repurposing [80].

Methods employing structure- and ligand-based approaches for virtual screening of compounds in virtual chemical spaces have been recognized for their advantages, including improved profile analysis, rapid elimination of lead-free compounds, and identification of drug candidates at reduced costs [80]. Among these methods, molecular docking stands out as a crucial tool in structural molecular biology and computational drug design (Fig. 2). Despite its widespread use, the effectiveness and reliability of molecular docking have been subjects of examination. Computational determination of the binding affinity between a protein structure and a ligand, a central aspect of docking, depends on exploring all conceivable binding positions in the binding pocket of the target protein. The optimal binding geometry is evaluated using predefined scoring functions [81]. Although molecular docking holds promise for virtual screening of extensive compound libraries and for facilitating lead optimization, its limitations and potential sources of bias must be critically evaluated. The reliance on scoring functions raises questions about the accuracy and predictive power of results, necessitating a careful assessment of their role in the drug discovery process [82].

While many molecular docking tools offer high flexibility to ligands, they often impose fixed or limited flexibility to the protein, especially for residues in or near the active site. This limitation stems from the computational complexity inherent in granting full molecular flexibility to proteins in terms of both space and time. Despite the technique's widespread applicability, it fails to capture the true flexibility of proteins and ligand molecules in solution, where conformational changes play a pivotal role. Recognizing this limitation, ongoing efforts aim to incorporate an increasing number of parameters to enhance the accuracy of predictions [81]. The integration of this method with other computational drug design techniques, such as pharmacophore modeling and molecular dynamics, can achieve a more realistic representation and improve the prediction of optimal protein-drug complexes [83].

Automation of certain tasks in drug development, manufacturing, and supply chain, clinical trials, and distribution will occur over time, necessitating the training of AI using extensive datasets. However, it is crucial to acknowledge that human intervention remains indispensable for the successful implementation, development, and operation of AI platforms [80]. While navigating this evolving landscape, critical examination is paramount to address the inherent limitations and complexities associated with integrating AI into the various facets of the anti-parvovirus drug development process.

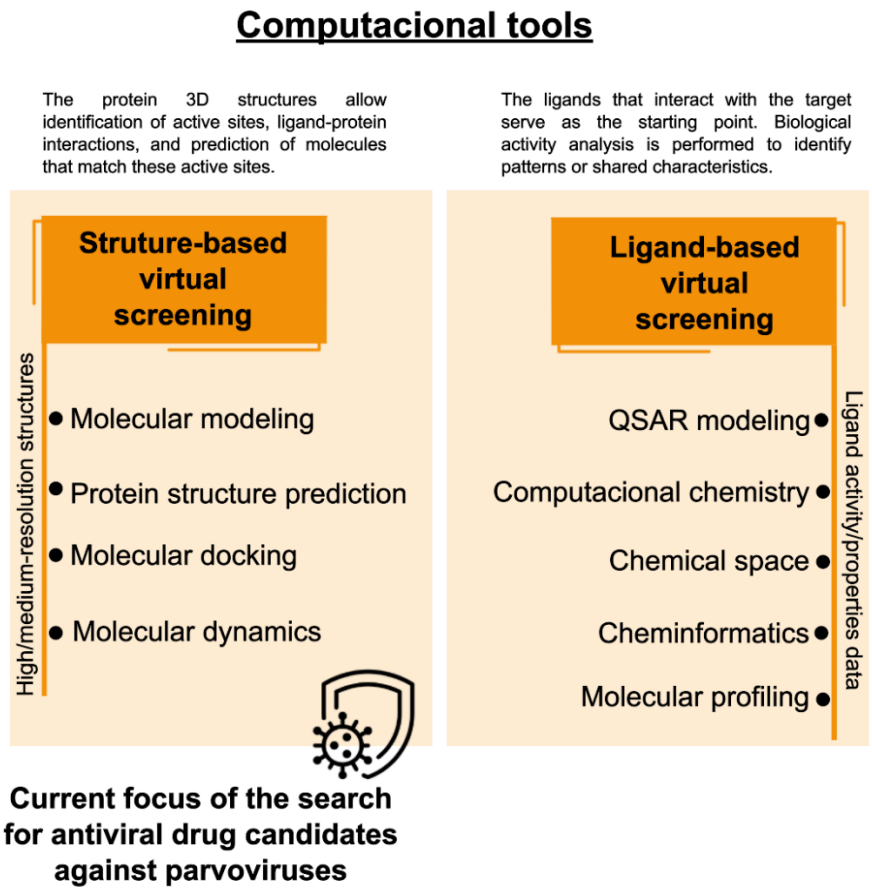

Fig. 2 Categories of two different computational tools used for drug discovery, highlighting the most commonly employed approaches in the search for drugs against parvoviruses

Conclusion

The urgent need for effective antiviral therapies against parvoviruses in both human and veterinary medicine underscores the significance of continuous research in this domain. Given the diverse and complex nature of these viruses, a thorough understanding of

their replication mechanisms and interactions with host cells is imperative to identify potential targets for drug development.

While several antiviral agents targeting significant parvoviruses have been documented, a considerable proportion remains in the early stages of *in vitro* testing. Recent advancements in *in silico* methodologies, such as molecular docking and AI-driven drug discovery, offer promising pathways to accelerate the discovery of potential antiviral candidates. The virtual screening of extensive compound libraries and the prediction of binding affinities between viral proteins and potential drugs empower researchers to hasten the lead optimization and drug design processes.

Effective collaboration between experimental and computational researchers is pivotal in the development of antiviral drugs. While AI can automate specific aspects of drug discovery, human intervention and expertise remain essential to interpret and validate results, guiding the development and implementation of AI platforms.

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Declarations

Conflict of interest The authors declare no conflict of interest

Ethical approval Not applicable

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4.2 Capítulo II

O manuscrito deverá ser submetido na revista "Brazilian Journal of Microbiology" no estilo "short communication".

Assessment of single mutations in the VP2 capsid protein of canine parvovirus

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Abstract

Canine parvovirus (CPV-2) is a highly contagious virus that primarily causes gastroenteritis in carnivores, especially in dogs and cats. This virus has undergone antigenic evolution, resulting in the emergence of CPV-2a, CPV-2b, and CPV-2c variants. A key factor influencing these variants is residue 426 in the VP2 capsid protein. In this study, we constructed CPV-2 mutants with common VP2 mutations, including F267Y, S297A, V300G, D305Y, Y324I, N426D, N426E, and T440A, to assess their impact on viral fitness. Additionally, we used the N426E mutant for a viral neutralization assay and compared it with a standard strain to evaluate how this mutation affects the virus's antigenic properties. Our findings showed that the mutants exhibited varied replication patterns compared to the parental strains, with some mutations, such as N426D, N426E, and T440A, enhancing viral fitness. Polyclonal antibodies generated protective titers against both the standard CPV-2 and the CPV-2 N426E mutant, indicating potential cross-protection. Further investigation into additional mutants and combined mutations is crucial for refining intervention strategies and deepening our understanding of viral dynamics.

Keywords CPV-2; VP2 protein; Antigenic evolution

Introduction

Canine parvovirus (CPV-2), a member of the *Parvoviridae* family, is a pathogen that causes gastroenteritis and myocardial disease in carnivores, especially in dogs and cats (Parrish and Carmichael, 1986). CPV-2 is a nonenveloped DNA virus with its genome divided into two coding frames: the 3′ end coding for nonstructural proteins (NS1 and NS2), and the 5′ end coding for viral capsid proteins (VP1 and VP2) (Hao et al., 2022).

CPV-2 emerged as a new pathogen in dogs during the late 1970s, likely originating as a variant of a related virus from another host, possibly feline panleukopenia virus (FPV) (Truyen, 1999). Shortly after its emergence, two antigenic variants appeared distinguishable by specific monoclonal antibodies. Designated as CPV-2a and CPV-2b, they differed from the original CPV-2 strain by five or six amino

acid residues of the VP2 capsid protein. In 2000, a new antigenic variant called CPV-2c was identified in Italy and rapidly spread throughout the world (Decaro and Buonavoglia, 2012).

The main determinant of CPV-2 variants are mutations at residue 426 (Silva et al., 2022), with potential amino acid changes such as Asn, Asp and Glu, each linked to distinct antigenic variants (Allison et al., 2016). This critical residue is located in the VP2 region between residues 267 and 498, encompassing the GH loop located between the βG and βH chains. Due to its exposure on the capsid surface, this region is notably prone to mutations (Decaro and Buonavoglia, 2012). Furthermore, positions 87, 139, 267, 297, 300, 324, 347, 440, and 555 of the viral capsid protein VP2 contribute to the classification of CPV-2 into variants and subtypes (Leal et al., 2024). With its antigenicity constantly drifting, an increasing number of further mutations of VP2 have been described and various viral mutants have been identified (Zhou et al., 2017).

In the present study, we constructed and evaluated a series of CPV-2 mutants, each harboring a common mutation of the VP2 protein, to assess their impact on viral fitness and antigenic properties.

Materials and methods

Construction of mutant viruses

An infectious plasmid clone of the virulent CPV-2 447 strain, inserted in plasmid M13, served as the parental strain for generating virus mutants (Shackelton et al., 2005). Site-specific substitutions of the VP2 capsid protein were introduced using the site-directed mutagenesis kit (Invitrogen, United States of America), employing specific overlapping primers designed to introduce point mutations (as detailed in Fig. 1a). These amino acids were chosen for evaluation due to their extensive occurrence in CPV-2 strains isolated worldwide over the past 10 years (2013-2023) (Fig. 1b). In addition, another infectious plasmid clone of the ancient CPV-2 strain 265 was included in the study for comparison (GenBank accession number M38245.1) (Parrish, 1991).

Competent *Escherichia coli* TOP10 cells were transformed with the plasmids. Subsequently, nucleic acid concentration was assessed using a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, USA) and Qubit Fluorometer, according to the manufacturers' guidelines.

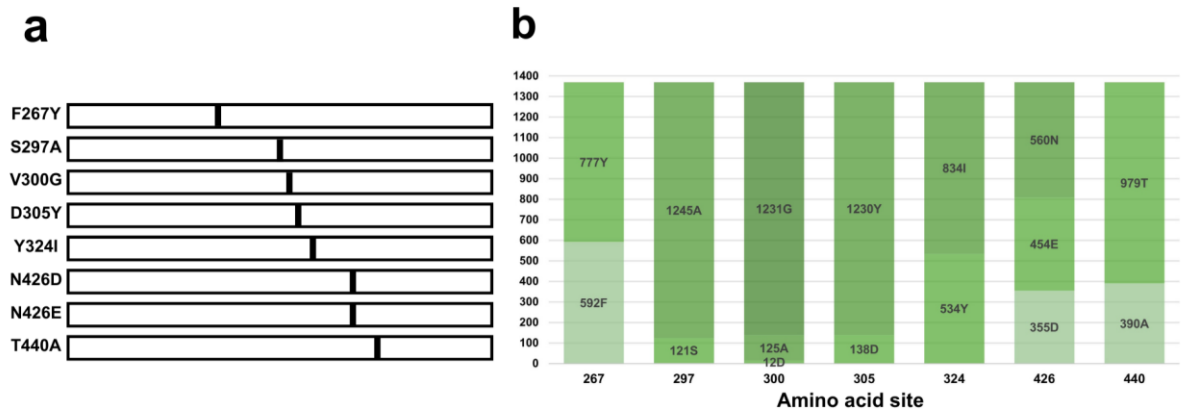

Fig. 1 (a) Mutant strains constructed from the parental strain 447. The numbers and black quadrants indicate the position of the modified amino acid. (b) Analysis of the amino acid profile present in important locations of the VP2 protein in the last 10 years around the world, using 1369 sequences deposited in GenBank

Transfection and propagation

For transfection of the plasmids, feline kidney cells (CRFK) were seeded at a density of $1x10^4$ cells/mL 24 hours prior in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FCS), penicillin (100 U/mL) and streptomycin (100 mg/mL). Transfection with the plasmids (each separately, in triplicate) was performed using the LipofectamineTM 3000 reagent (Invitrogen, United States of America). A negative and a positive virus control (1:10) were included in the experiments. The cells were then incubated at 37°C in a humidified environment with 5% CO_2. To confirm successful transfection, after 48 hours, the cells were fixed with acetone/methanol (1:1, v/v), blocked with FCS (3%) and incubated with polyclonal antibodies against CPV-2. Positive cells were visualized through immunofluorescence using fluorescein-conjugated anti-canine VP2 antibody.

For viral propagation, CRFK cells were seeded 24 hours prior at a density of $3×10^3$ cells/cm². The cells were washed once with phosphate-buffered saline. Then, each mutant and parental strain (10 µL) were added in triplicate and allowed to adsorb to the cells for 1 h at 37°C. After adsorption, fresh DMEM was inserted again and the cells were further incubated at 37°C. Upon reaching confluency, supernatants were collected. In the third passage, cells were seeded 24 hours before ($7x10^4$ cells/cm²) and

35 µL of each mutant and parental strains was added, this time in 12-well plates, employing the same infection protocol. Once the culture was confluent, the entire volume from passage 3 was harvested and stored for subsequent experiments. DNA extraction from supernatants was performed using the DNeasy Blood and Tissue Qiagen kit (Thermo Fisher Scientific, USA). Immunofluorescence and qPCR were conducted for virus quantification and visualization of infected cells in each passage (Streck et al., 2013).

Stock viruses were titrated by an immunofluorescence 50% tissue culture infective dose ($TCID_{50}$) assay in CRFK cells (Allison et al., 2016).

Infectivity assay

CRFK cells were seeded at a density of 1×10^3 cells/cm² in a 96-well plate and infected at a multiplicity of infection (MOI) of 0.1 $TCID_{50}$ (Allison et al., 2016), in triplicate. Supernatants were harvested at 48- and 72-hours post-infection (hpi), and plates were stained using the immunofluorescence assay described earlier. Infected cells in all wells were counted. In addition, the genetic material from the supernatants was extracted and qPCR was performed as described previously. The results of the mutants were compared with those of parental strains and positive virus control.

Generation of polyclonal antibodies

Thirty rats were immunized with six commercial vaccines to generate polyclonal antibodies against CPV-2. They were divided into groups of five, with an extra negative control group. Each received three booster injections at 15-day intervals, while the negative control group received saline. Blood samples were taken before each booster and the final procedure was done 15 days after the last boost. Serum samples were heat-inactivated at 56°C for 30 minutes and stored at -20°C. This study was approved by the Ethical Committee on the Use of Animals of UCS (number 06/2022).

Virus neutralization assay

All serum samples from rats underwent serial dilution in 5-log steps, with 0.1 mL of each dilution combined with an equal volume of 200 $TCID_{50}$ of standard CPV-2 strain

or CPV-2 N426E mutant. After 2 h incubation at 37°C, 100 μL of the serum-virus mixture was added to CRFK cells seeded 24 hours prior to the experiment at a density of 1×10^4 cells/mL in 96-well plates, in duplicate. The cells were grown in DMEM supplemented with penicillin and streptomycin, and the plates were incubated for 5 days at 37°C in a humid environment containing 5% CO_2. After this incubation period, the cells were fixed and the immunofluorescence assay was performed.

Statistical analyzes

The data were subjected to statistical analysis using SPSS software (IBM Company, USA). ANOVA was initially used, followed by Tukey's post hoc test to compare Ct (cycle thresholds) values and positive cells between different treatments. For VN analyzes the Wilcoxon test was used.

Results and discussion

CPV-2 has been continuously transmitted for more than 40 years since its emergence, undergoing continuous genetic variations (Hao et al., 2022). Understanding the molecular biology of CPV-2, particularly the significance of point mutations in the VP2 protein, is crucial for disease management and prevention. In this study, we constructed a range of CPV-2 VP2 mutants to evaluate their cellular fitness.

The different mutations were prepared in the genetic background of one representative carnivore parvovirus. The mutated plasmids (designated as F267Y, S297A, V300G, D305Y, Y324I, N426D, N426E and T440A) and parental strains were successfully transfected, leading to the recovery of infectious virus with adequate titers for subsequent experiments. When assessing viral fitness using qPCR results, differences in genome replication were evident among most mutants compared to the parental strain 447 at 48 and 72 hpi, as illustrated in Fig. 2 and 3. While the majority of mutants exhibited a similar fitness pattern to that of the parental strain 265, only mutant CPV-2 Y324I showed comparable results to parental strain 447 after 72 hpi (1.2× less copies than 447) (Fig. 3). Field strains carrying the 324I mutation emerged in 2006 and have become increasingly common (Zhou et al., 2017). This site undergoes positive selection and the Y324I substitution appears to enhance binding with the TfR type I (Alexis et al., 2021).

The comparison between the results of the viral infectivity assay via qPCR and immunofluorescence revealed differences (Fig. 4). For example, mutant CPV-2 D305Y could only be detected at 48 hpi in qPCR, whereas the same mutant exhibited positive cells at both 48 and 72 hours hpi, yielding comparable results to those of the parental strains. Similar patterns were noted with other mutants, particularly with mutant T440A. High Ct values in the supernatant, juxtaposed with the notable presence of fluorescent positive cells, underline the predominance of the mutant within the cell monolayer. In the mutants studied, he hypothesized that they may display unique replication patterns, resulting in a less efficient release of viral particles into the medium while actively replicating within host cells. On the other hand, the absence of this feature in the positive virus control suggests that the live virus has an inherent ability to infect, replicate and spread in host cells, leading to a higher concentration of viral particles in the supernatant.

The sites 297, 300, 305 and 324 are situated within regions positioned at the three-fold site of the capsid subunit, which is considered responsible for species specificity (Lee et al., 2019). The profiles 297A, 300G, 305Y and 324I are highly prevalent and widely distributed globally (de Oliveira Santana et al., 2022). Site 267 is located at the base of the three-fold spike and does not reside on the VP2 surface; however, the substitution 267Y can induce structural changes that might alter surface conformation (de Oliveira Santana et al., 2022). Regarding fitness, when considering positive cells, mutants CPV-2 F267Y and S297A at 48 and 72 hpi, as well as Y324I at 72 hpi, exhibited higher fitness compared to the parental strains 265 and 447.

The sites 426 and 440 are situated within loop 4 of VP2, a region known for its high immunogenicity within the capsid (Alexis et al., 2021). Site 426 has been identified as the major mutation site for CPV-2 evolution (Zhou et al., 2017). In this study, the parental strains displayed an asparagine at residue 426, indicating the CPV-2a variant, while the mutants CPV-2 N426D and CPV-2 N426E represented the CPV-2b and CPV-2c variants, respectively. Interestingly, mutant CPV-2 N426D exhibited higher fitness than both parental strains at 72 hpi when positive cells were taken into account, whereas mutant N426E displayed higher fitness compared to both parental strains at both 48 and 72 hpi. Remarkably, the fitness of mutant N426E at 72 hpi was comparable to that of the positive virus control. Following this trend, mutant CPV-2 T440A also demonstrated enhanced fitness compared to the parental strains. The substitutions N426E and T440A entail electrostatic or polarity modifications, indicative

of a non-conservative substitution (de Oliveira Santana et al., 2022). T440A promotes immune evasion against the host's immune response via antigenic drift and may contribute to the ineffectiveness of traditional vaccines (Alexis et al., 2021). In this investigation, substitutions at both sites seem to confer some advantage.

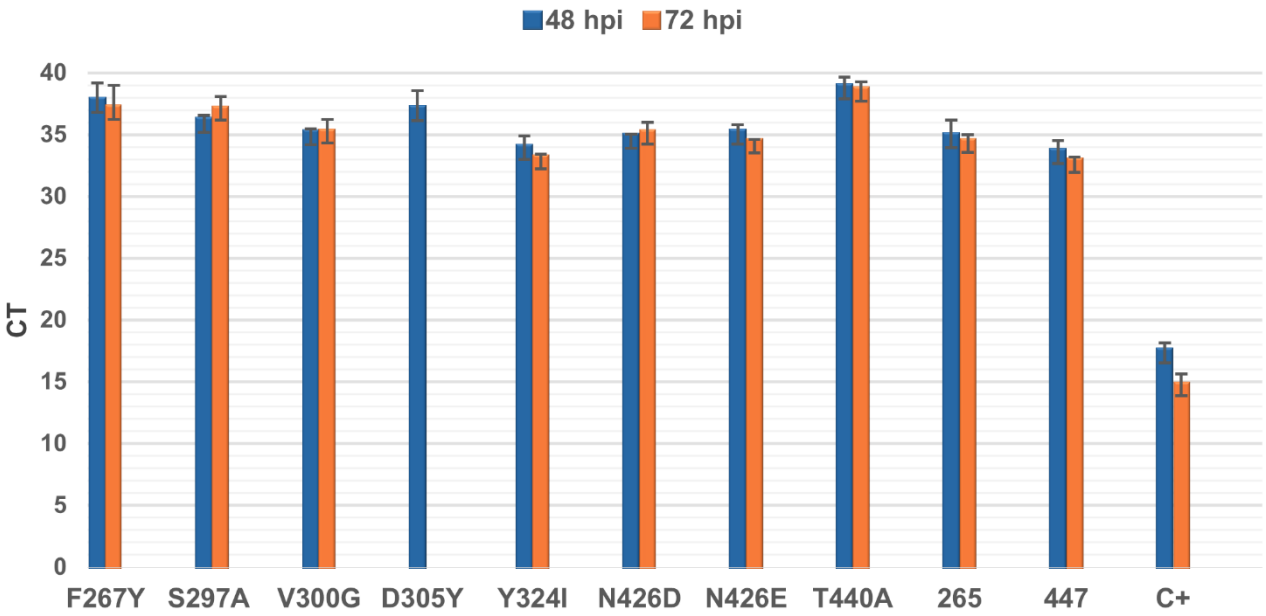

Fig. 2 Replication of the CPV-2 mutants, parental strains and positive virus control as Ct values measured by qPCR at 48 and 72 hpi from the supernatant. Lines represent the mean ± standard deviation

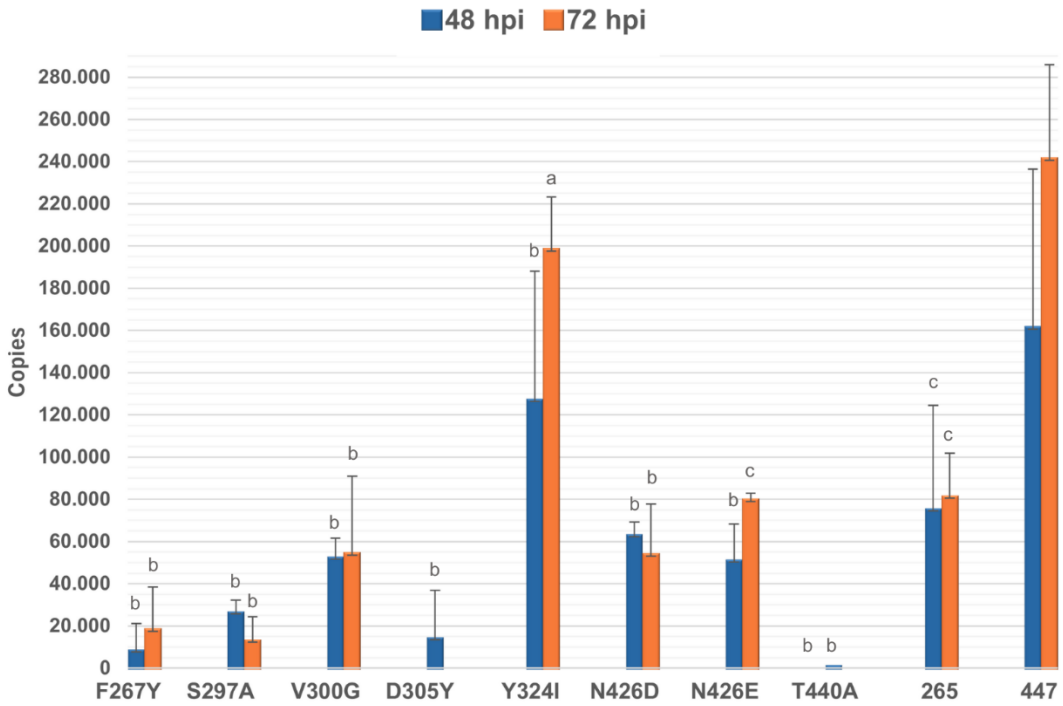

Fig. 3 Replication of the CPV-2 mutants, parental strains and positive virus control expressed as viral genome copy equivalents measured by qPCR at 48 and 72 hpi from the supernatant. Lines expressed the mean ± standard deviation. Means were compared using ANOVA followed by Tukey's post hoc test ($p < 0.05$)

[a] Statistical difference of 265 at 48 hpi and 72 hpi

[b] Statistical difference of 447 at 48 hpi and 72 hpi

[c] Statistical difference of 447 at 72 hpi

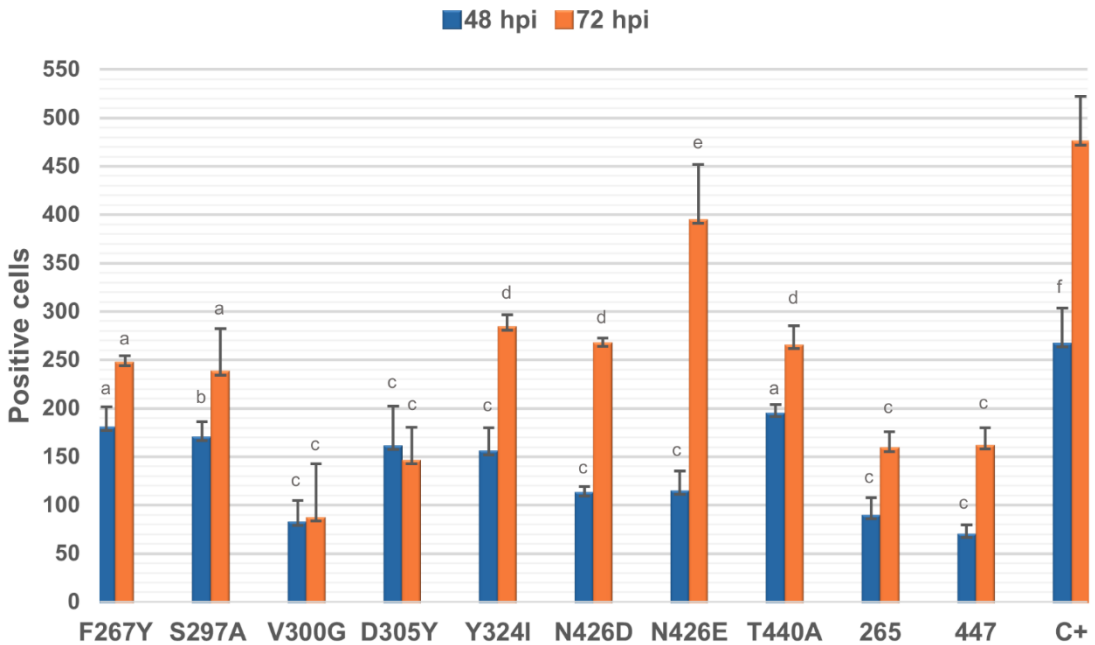

Fig. 4 Replication of CPV-2 mutants, parental strains and positive virus control, represented by the number of positive cells obtained through immunofluorescence at 48 and 72 hpi. Lines expressed the mean ± standard deviation. Means were compared using ANOVA followed by Tukey's post hoc test ($p < 0.05$)

[a] Statistical difference compared to the parental strains 265 and 447 at 48 hpi, as well as to the positive virus control at 72 hpi

[b] Statistical difference compared to the parental strain 447 at 48 hpi and the positive virus control at 72 hpi

[c] Statistical difference compared to the positive virus control at both 48 and 72 hpi

[d] Statistical difference compared to the parental strains 265 and 447 at both 48 and 72 hpi, as well as to the positive virus control, at 72 hpi

[e] Statistical difference compared to the parental strains 265 and 447 at both 48 and 72 hpi, as well as to the positive virus control, at 48 hpi

[f] Statistical difference compared to the positive virus control at 72 hpi

The VN titre is the reciprocal of the highest serum dilution that completely neutralizes the virus (Cavalli et al., 2008). VN assays were conducted to assess the neutralization potential of polyclonal antibodies against both a standard strain of CPV-2 and the mutant CPV-2 N426E. This was done with the objective of identifying any potential immune evasion, given that most vaccines are designed based on the classical CPV-2 or its variant CPV-2b (Silva et al., 2022). For this, serum samples from rats immunized with six distinct CPV-2 vaccines were utilized. The VN results demonstrated that all vaccines could elicit protective titers (≥ 1:80, equivalent to ≥6.32 log2) (Vasu et al., 2019) (Table 1). Although individual variations were evident, the vaccines neutralized both the standard CPV-2 strain and a CPV-2 N426E mutant strain, without any notable decrease in the neutralizing activity of the polyclonal antibodies. Considering that the CPV-2 N426E mutant is representative of the CPV-2c variant, our results suggest cross-protection. This is in line with the findings of Larson and Schultz (2008) and Wilson et al. (2014). Conversely, Cavalli et al. (2008) noted a substantial reduction in VN titers against CPV-2a, CPV-2b and CPV-2c in animals vaccinated with CPV-2 when compared to the homologous virus. Similarly, Kang et al. (2008) observed limited cross-reactivity between CPV-2 and its variants, not only through VN but also by HI. These discrepancies emphasize the need for careful consideration.

Table 1 Neutralization activity of polyclonal serum samples from rats immunized with six different vaccines against 200 $TCID_{50}$ of the reference strain CPV-2 and mutant CPV-2 N426E

Vaccine	Standard CPV-2 Log_2 mean ± SD	CPV-2 N426E Log_2 mean ± SD
1	8.92 ± 0.5	9.52 ± 0.4
2	8.92 ± 1.1	8.32 ± 1
3	8.52 ± 1.6	8.32 ± 0.7
4	9.92 ± 0.5	9.12 ± 0.4
5	7.57 ± 1.0	7.32 ± 0.8
6	8.12 ± 1.0	7.92 ± 1.1

Although our findings provide valuable information, it is important to recognize that analysis of individual mutations in VP2 may not comprehensively capture the complex interactions between the various sites of this protein. During our experiments, we observed varying replication patterns among mutants, highlighting the diversity of responses that mutations can trigger. Notably, some mutations, such as N426D, N426E and T440A, appeared to improve viral fitness. However, we emphasize the importance of carrying out additional investigations to evaluate the impact of combined mutations in this protein and in other regions of the viral genome. Such studies would significantly improve our understanding of viral dynamics and could inform future intervention strategies. Furthermore, other mutants should be tested against monoclonal and polyclonal antibodies to draw more accurate conclusions about the impact of these mutations on the antigenicity of the virus.

Authors contributions TSL and BPG performed experiments. TSL, AAEW, UT and AFS wrote the manuscript. All authors critically revised the manuscript and approved the final version.

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Data availability Data available on request from the authors.

Declarations

Conflict of interest None to declare.

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4.3 Capítulo III

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In silico designing of multi-epitope vaccine against canine parvovirus using reverse vaccinology

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Abstract

Canine parvovirus (CPV-2) is a highly contagious virus affecting dogs worldwide, posing a significant threat. The VP2 protein stands out as the predominant and highly immunogenic structural component of CPV-2. Soon after its emergence, CPV-2 was replaced by variants known as CPV-2a, 2b and 2c, marked by changes in amino acid residue 426 of VP2. Additional amino acid alterations have been identified within VP2, with certain modifications serving as signatures of emerging variants. In Brazil, CPV-2 outbreaks persist with diverse VP2 profiles. Vaccination is the main preventive measure against the virus. However, the emergence of substitutions presents challenges to conventional vaccine methods. Commercial vaccines are formulated with strains that usually do not match those currently circulating in the field. To address this, the study aimed to investigate CPV-2 variants in Brazil, predict epitopes, and design an *in silico* vaccine tailored to local variants employing reverse vaccinology. The methodology involved data collection, genetic sequence analysis, and amino acid comparison between field strains and vaccines, followed by the prediction of B and T cell epitope regions. The predicted epitopes were evaluated for antigenicity, allergenicity and toxicity. The final vaccine construct consisted of selected epitopes linked to an adjuvant and optimized for expression in *Escherichia coli*. Structural predictions confirmed the stability and antigenicity of the vaccine, while molecular docking demonstrated interaction with the canine toll-like receptor 4. Molecular dynamics simulations indicated a stable complex formation. *In silico* immune simulations demonstrated a progressive immune response post-vaccination, including increased antibody production and T-helper cell activity. The multi-epitope vaccine design targeted prevalent CPV-2 variants in Brazil and potentially other regions globally. However, experimental validation is essential to confirm our *in silico* findings.

Keywords Canine parvovirus . Epitope . Immunoinformatics . Brazil . Vaccine

Introduction

Canine parvovirus type 2 (CPV-2) is one of the most common causes of morbidity and mortality in dogs worldwide [1]. CPV-2 is a non-enveloped and single-stranded DNA

virus that belongs to the genus *Protoparvovirus*, family *Parvoviridae*, that infects rapidly dividing cells of the gastrointestinal tract, bone marrow, lymphoid tissue and cardiac myocytes [2, 3]. The CPV-2 genome contains two open reading frames (ORFs). One of them encodes the non-structural proteins NS1 and NS2 and the other encodes the structural proteins VP1 and VP2 [4]. VP2 is the major capsid protein and also the major antigenic protein, determining viral tissue tropism and host range [5].

Soon after its emergence in the 1970s, the original CPV-2 was quickly and totally replaced by different CPV-2 variants named based on differences observed at VP2 amino acid residue 426 such as CPV-2a (Asn), CPV-2b (Asp) and CPV-2c (Glu) [6]. In addition to the mutation in residue 426, other amino acid changes have been detected in VP2, including L87M, T101I, V139I, F267Y, S297A, A300G, Y324I, A347T, T440A and I555V, with some of these alterations serving as signatures of the new variants [7]. In Brazil, canine parvovirus outbreaks are still very common and there is a wide variety of VP2 profiles in circulation [7, 8].

Vaccination is the main way to prevent CPV-2. Most veterinary vaccines are developed using standard methods, such as inactivating the pathogen using chemical or physical methods and then injecting the killed organism directly into animals. Alternatively, molecular tools are used to selectively modify a pathogen to limit its virulence, resulting in an attenuated version that can be used as a vaccine [9]. In the context of CPV-2, most existing vaccines are made up of modified live viruses derived from both the original CPV-2 strain and its variant CPV-2b [10, 11]. However, these traditional vaccine approaches may not effectively adapt to the emergence of mutations. The presence of new mutations in specific amino acids of VP2, located in regions of high antigenicity and in the receptor-binding area, has been described as potentiating the immune escape of CPV-2 [12].

Genome-based vaccinology, known as reverse vaccinology, has made a significant impact on the development of multi-epitope vaccines [13]. The continuous advancements in sequencing technologies have made it possible to efficiently acquire pathogen genome sequences at low cost and in a short period, which also reduces the time required for vaccine design [14]. Moreover, bioinformatics tools enable the identification of conserved sequences that can serve as promising vaccine candidates, especially against microorganisms that undergo continuous genetic alterations [15].

The objective of this study was to investigate amino acid substitutions and selection pressures within VP2 of CPV-2 strains isolated in Brazil. Additionally, we

aimed to predict potential epitopes using computer-based immunoinformatics methods. These analyses were conducted with the ultimate goal of developing an *in silico* vaccine candidate against CPV-2, specifically targeting the variants circulating within Brazilian territory.

Materials and methods

In this study, all computational tasks were executed using both online and offline tools concurrently from July 2023 to December 2023. The schematic representation of the methodological steps is provided in Fig. 1.

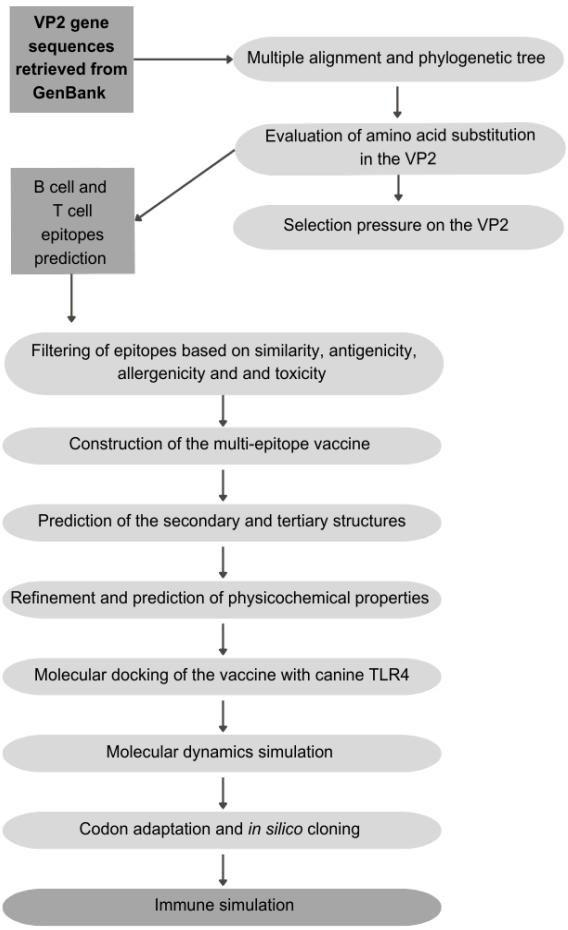

Fig. 1 This schematic representation presents the step-by-step process for designing a multi-epitope vaccine against CPV-2 employing immunoinformatics tools. The process began with protein sequence retrieval and progressed through amino acid evaluation of VP2, selection pressure analysis, epitope prediction, secondary and tertiary vaccine structure prediction, refinement, molecular docking and simulation. It ends with codon adaptation, *in silico* cloning and immunological simulation

GenBank data collection

Whole-genome sequences (WGS) (n = 29) and complete nucleotide sequences of the VP2 gene (n = 91) of CPV-2 strains collected from 1980 to 2023 in dogs in Brazil were retrieved from GenBank (ncbi.nlm.nih.gov/genbank/). In addition, VP2 gene sequences from commercial vaccines (n=8) available in Brazil were included in the analysis dataset (Table S1, supplementary material).

Multiple sequence alignment and phylogenetic tree construct

Multiple sequence alignment of WGS and VP2 gene were performed by muscle in MEGA-X software v10.0.05. Then, a maximum-likelihood (ML) tree was constructed using MEGA-X according to the best-fitting model. Jones–Taylor–Thornton (JTT) + Gamma model and 1000 bootstrap replicates were used. The phylogenetic tree was visualized and colored with Figtree v.1.4.3.

Amino acid evaluation of VP2 protein

The sequences were aligned in MEGA-X. After that, amino acids were evaluated into conserved and variable sites along the VP2 protein, compared with an ancestral strain CPV-N (M19296.1) and vaccine strains. Substitutions were considered significant if they were present in more than 40% of the sequences. Then, a consensus sequence was generated contemplating the relevant substitutions found.

Selection pressure on the VP2

Positive selection sites on the VP2 were determined using the following methods on the Datamonkey webserver (http://www.datamonkey.org): Single-Likelihood Ancestor Counting (SLAC), Fast Unconstrained Bayesian Approximation (FUBAR) and Mixed Effects Model of Evolution (MEME). A site was considered as a positive selection position only if it was identified by at least two algorithms, and with $p < 0.1$ in SLAC, $p < 0.05$ in MEME and posterior probability > 0.9 in FUBAR were considered significant [16].

Analysis of selection pressure on VP2 protein was performed using MEGAX software, estimating synonymous (dS) and nonsynonymous (dN) substitution rates for

the aligned gene. The dN/dS ratio < 1 represents negative selection and the dN/dS ratio > 1 represents positive selection.

Linear B-cell epitope prediction

B-cells are fundamental in the adaptive immune system, given their ability to identify and provide lasting protection against infectious pathogens or cancer cells [17]. The BepiPred-2.0 web server (https://services.healthtech.dtu.dk/service.php?BepiPred-2.0) was utilized for predicting B-cell epitopes derived from the VP2 consensus sequence. This server is based on a random forest algorithm trained on epitopes annotated from antibody-antigen protein structures [17]. The residues with scores above the threshold (default value is 0.5) are predicted to be part of an epitope.

Prediction of T-cell epitopes

Major histocompatibility complex (MHC) molecules are expressed on the cell surface, where they present peptides to T-cells, playing a crucial role in shaping the immune responses mediated by these cells [18]. NetMHCpan-4.1 (https://services.healthtech.dtu.dk/service.php?NetMHCpan-4.1) is a tool for predicting peptide binding to MHC molecules with known sequences utilizing artificial neural networks. The canine MHC molecules DLA-8803401, DLA-8850101 and DLA-8850801 were selected to predict the epitopes for the VP2 consensus sequence. All parameters were set to default in the webserver, and only strongly binding peptides were considered (percent rank below 0.5%).

Antigenicity, allergenicity and toxicity prediction

Antigenicity, allergenicity and toxicity were evaluated for the predicted epitopes of VP2 consensus sequence. Antigenicity was evaluated using VaxiJen v2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html), setting a threshold of 0.4 (default) and the target organism selected was the virus. AllerTOP v2.0 (https://www.ddg-pharmfac.net/AllerTOP/) was used to determine allergenicity, while ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/design.php) was applied to assess the toxicity of these regions. In ToxinPred tool, the chosen parameters were: prediction

method SVM (Swiss-Prot) + Motif based; E-value cut-off for motif-based method of 10 and physicochemical properties to be displayed was “all”.

Multi-epitope vaccine construct

The most antigenic, non-allergenic and non-toxic epitopes were selected to design the vaccine construct. The construct was attached with a 50S ribosomal protein L7/L12 (NCBI accession no. P9WHE and UniProt ID P0A7K2) as an adjuvant to the N-terminal through EAAAK linker to improve the antigenicity [19]. Furthermore, the epitopes were joined with each other through GPGPG linker.

Prediction of the secondary and tertiary structures of the final vaccine construct

The secondary structure of the final vaccine formulation was predicted as helix, b-sheet and loop using the PSIPRED 4.0 webserver (http://bioinf.cs.ucl.ac.uk/psipred/).

The tertiary structure was predicted in I-Tasser webserver (https://zhanggroup.org/I-TASSER/about.html). This webserver first generates fragments of three-dimensional (3D) atomic models from the primary sequence by multiple threading alignments. Then, the fragments are reassembled into full-length models by replica-exchange Monte Carlo simulations [19]. The structure was refined using GalaxyRefine (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE) and YASARA programs. The evaluation of the stereochemical quality of a protein structure was done in PROCHECK webserver (https://saves.mbi.ucla.edu/).

Physicochemical properties and solubility prediction

The physicochemical properties of the construct were predicted in ProtParam (https://web.expasy.org/protparam/). The parameters evaluated were amino acid composition, theoretical isoelectric point (pI), instability index, *in vitro* and *in vivo* half-life, aliphatic index, molecular weight and grand average of hydropathicity. Protein-sol server (https://protein-sol.manchester.ac.uk) was used to evaluate the solubility of the vaccine construct.

Molecular docking of designed vaccine constructs with toll-like receptor

Toll-like receptors (TLRs) are immune receptors whose activation leads to intracellular signaling pathway, being the first molecules to come in contact with pathogens. The adjuvant chosen for the construction of the vaccine has the ability to activate TLR4 [19]. Therefore, molecular docking of the multi-epitope vaccine construct with canine TLR4 (NP_001002950.3) was performed using the HDOCK webserver (http://hdock.phys.hust.edu.cn/) in order to evaluate the interaction between ligand and receptor and consequently the activation of an immune response. Before docking, the tertiary structure of canine TLR4 was predicted in I-Tasser (using 3FXI_A as a template), followed by refinement and evaluation of stereochemical quality as described above.

Molecular dynamics simulation of vaccine-TLR4 complex

Molecular dynamics (MD) simulation was conducted by utilizing the Linux-based GROMACS 2023.4 package to assess the conformational stability of the vaccine-receptor docked complex [20]. The simulation was executed with the CHARMM27 force field. The complex was solved by using SPC water model in a cubic box. A total of 65698 water molecules and 23 NA molecules were added to neutralize the system before performing the equilibration step for 100 ps. Finally, the molecular dynamics simulation was carried out for 30 ns. RMSD (Root Mean Square Deviation of the atomic position) for backbone, RMSF (Root Mean Square Fluctuations) of residues and Rg (Radius Gyration) of the vaccine-receptor complex were analyzed.

Codon adaptation and *in silico* cloning of designed vaccine construct

Codon optimization of the designed multi-epitope vaccine construct was performed in the Java Codon Adaptation Tool (JCat) server (http://www.prodoric.de/JCat) for gene expression in the *Escherichia coli* (strain K12) host. This process is important to increase the protein expression in a prokaryotic host. The parameters avoiding rho-independent transcription termination, prokaryotic ribosome binding site and restriction enzyme cleavage sites were selected in the additional options section.

Then, the optimized nucleotide sequence of the vaccine construct was cloned into the *E. coli* pET-28a+ vector by SnapGene Tool and *Hind*III and *Bam*HI restriction sites were inserted at the N- and C-terminals of the sequence, respectively. In addition, a 6xHis tag was inserted to facilitate subsequent purification and identification steps.

In silico immune simulation

To evaluate the probable immune response profile of the constructed vaccine, *in silico* immune simulations were done in C-ImmSim server (http://150.146.2.1/C-IMMSIM/index.php), which predict the mammalian immune system. The vaccine construction sequence was uploaded to the server. The other simulation parameters were kept as default and three injections (1000 antigens per dose) at the time steps of 1, 84 (equivalent to 4 weeks) and 168 (equivalent to 8 weeks) were used (each time-step corresponds to an 8-hour interval in real-life, with time-step 1 representing the injection occurring at time = 0) [21].

Results

Phylogenetic analysis and mapping of VP2 amino acid substitutions

In order to gain a deeper insight into the genetic relationships among canine parvovirus isolates in Brazil, we conducted a comparative analysis of 120 CPV-2 VP2 sequences and 8 vaccine strains. The analysis revealed four main clades (Fig. 2). The first clade comprised seventeen strains, including all vaccine strains, older strains (1980-1986) and a strain collected in 2020. This cluster predominantly consisted of CPV-2a strains, with two representatives of CPV-2b. The second clade was the largest, with fifty-four sequences, almost entirely composed of the CPV-2a variant, with only two isolates classified as CPV-2c. Collection dates varied widely from 1990 to 2020. The third clade included twenty-nine more recent strains (2013-2022) and representatives of the three variants, with a predominance of CPV-2b. The fourth clade contained twenty-eight sequences collected from 2010 to 2020 and was primarily composed of CPV-2c variants, with two isolates classified as CPV-2a.

It is noteworthy that, despite the co-circulation of the three variants of CPV-2 in Brazilian territory, the most prevalent is CPV-2a (68/120), followed by CPV-2c (31/120) and CPV-2b (21/120).

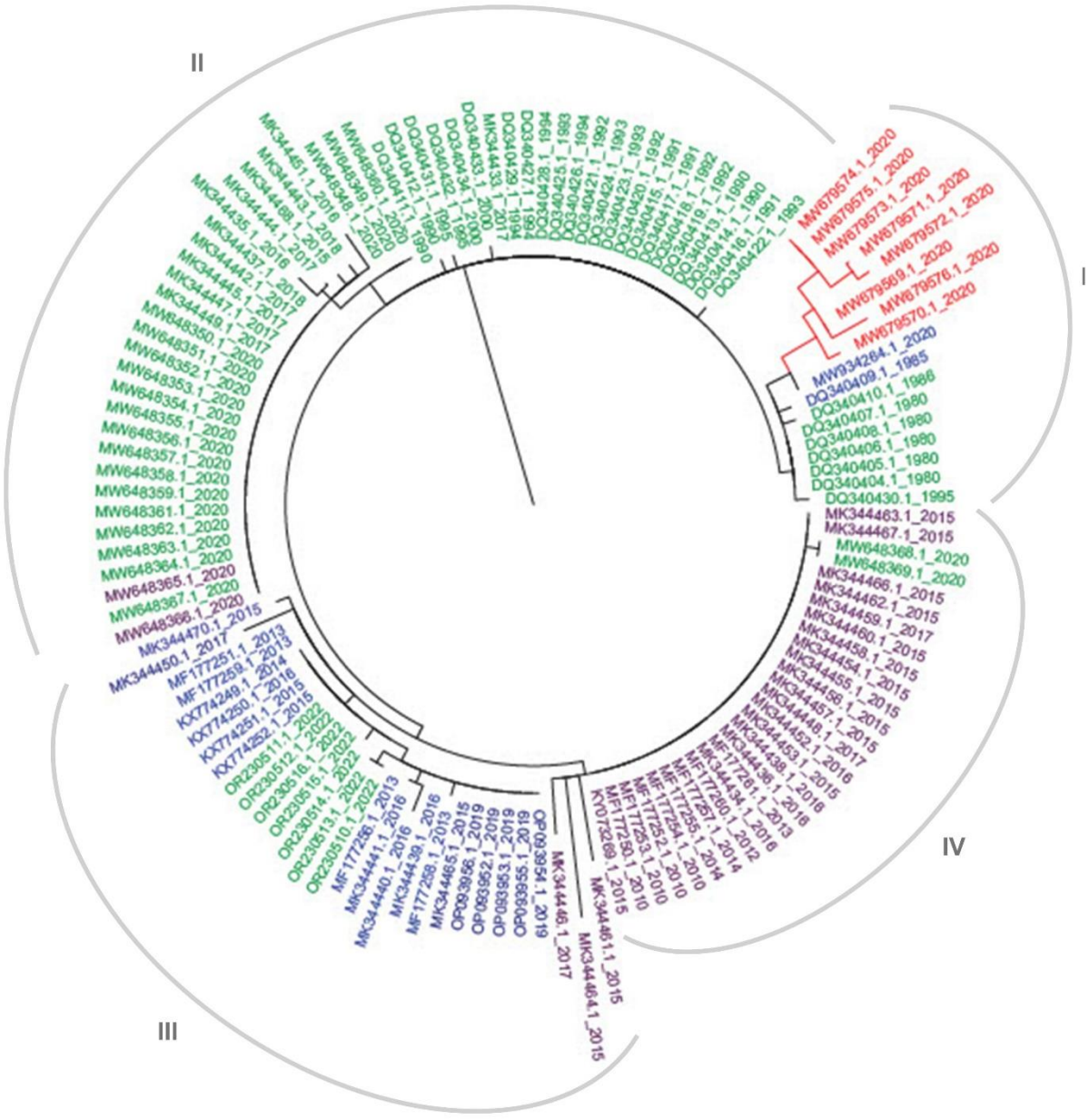

Fig. 2 Phylogenetic tree using the maximum likelihood (ML) method. The evolutionary distances were computed using the Jones-Taylor-Thornton (JTT) matrix-based substitution model. Phylogenetic inferences were carried out using complete nucleotide sequences of the VP2 gene of CPV-2 isolated in Brazil from 1980 to 2023. Each sequence was identified according to GenBank accession number and the isolation year. Accession numbers in red are vaccine strains; in green, blue and purple are the viral variants CVP-2a, CVP-2b and CVP-2c, respectively

The amino acid substitutions of the consensus sequence of the VP2 protein were compared with the ancestral strain CPV-N and the vaccine strains (Table 1). As

expected, characteristic amino acid changes associated with CPV-2 antigenic variants were identified, with substitutions such as M87L, I101T, A300G and D305Y present in practically all sequences.

At sites 297, significant substitutions were detected. The predominant change observed at site 297 was S297A (81,7%), with occasional occurrences of S297N (11,7%). Although most sequences at site 440 matched the vaccine strains (76,7%), T440A substitution was obtained in 23,3% of the sequences. Additionally, the Y367D substitution, observed in all field strains, was also detected in the vaccine strains. Notably, residue 426 serves as a distinguishing factor among CPV-2 antigenic variants, so alterations at this site were not considered mutations. All vaccines exhibited an N residue at this site.

Table 1 Amino acid changes in the consensus sequence compared to CPV-N (M19296.1) and vaccine strains

Identification	Amino acid residue								
	87	101	297	300	305	324	367	426	440
CPV-N	M	I	S	A	D	Y	Y	N	T
VP2 consensus sequence	L (100%)	T (100%)	A (81,7%) N (11,7%) S (6,6%)	A (0,8%) G (99,2%)	D (0,8%) Y (99,2%)	Y (56,7%) I (25%) L (18,3%)	D (100%)	N (51,7%) E (25,8%) D (22,5%)	T (76,7%) A (23,3%)
Vaccine strains	M (100%)	I (100%)	S (100%)	A (75%) D (25%)	D (100%)	Y (100%)	D (100%)	N (100%)	T (100%)

Selection pressure on the VP2

Two sites (297 and 324) in VP2 were confirmed by FUBAR and MEME methods to be under positive selection (Table 2). The overall mean differences of dN-dS were −3.65 for VP2 gene, indicating that the protein was under negative selection.

Table 2 Selection pressure on VP2 in the sequence by different methods in canine parvovirus 2 sequences from Brazil

Site	Selection type	Method	dN-dS	Prob[α<β]	Method	β+	dN-dS	*p*-value
297	Positive	FUBAR*	9.789	0.917	MEME**	242.05	0.00	0.00
324	Positive		26.739	0.951		5820.87	0.00	0.00

*Posterior probability ≥ 0.90
**p-value < 0.05

Prediction of B-cell and T-cell epitopes

For the generated VP2 consensus sequence, Bepipred identified seventeen B-cell epitopes of different lengths above the threshold score (Table 3). Predicted T-cell epitopes are described in Table 4. The DLA-8803401 allele revealed 14 epitope regions, while DLA-8850101 exhibited 13 regions and DLA-8850801 displayed the fewest, with only 7.

Table 3 Prediction of linear B-cell epitopes for the VP2 consensus sequence using the BepiPred 2.0 server

Epitope sequence	Start	End	Length
GAVQPDGGQPAVRNERATGSGNGSGGGGGGSGGVGISTGTFNNQTEFKFLEN	4	56	53
NLDKTAVNGNMALDD	86	100	15
ELHL	135	138	4
ESATQPPTKVYN	155	166	12
ETL	193	195	3
WKP	200	202	3
QWDRTLIPSHTGTSGTPTNIYHGTDP	213	238	26
FDCKPCRL	268	275	8
NSLPQAEGGTNFGYIGVQQDKRRGVTQMGNTN	292	323	32
MRPAEVGYSAPYYSFEASTQGPFKTPIAAGRGGAQTDENQAADGDPR	331	377	47
QHGQKTTTTGETPERFTYIAHQDTGRYPEGDWIQNINFNLPVTNDNVLLPTDPIGGKTG INYTNIFN	383	449	67
NNVPPV	458	463	6
DTDLKPR	475	481	7
TNEYDPDASANMSR	507	520	14
ASHTWNPIQQMSINVDNQFNY	541	561	21
GG	567	568	2
EKSQLAP	574	580	7

Table 4 T-cell epitopes indicated by NetMHCpan 4.1 using canine alleles for the consensus sequence

DLA allele	Start	End	Score
DLA- 8803401	163	173	0.413
	201	210	0.495
	501	510	0.129
	43	51	0.245
	157	165	0.103
	189	197	0.036
	283	291	0.388
	286	294	0.480
	330	338	0.042
	449	457	0.286
	456	464	0.047
	543	551	0.108
	570	578	0.487
	576	584	0.306
DLA- 8850101	163	173	0.162
	245	254	0.361
	448	457	0.431
	128	136	0.156
	137	145	0.394
	169	177	0.359
	244	252	0.493
	286	294	0.161
	414	422	0.383
	449	457	0.111
	543	551	0.367
	570	578	0.474
	571	579	0.365
DLA-8850801	501	510	0.121
	248	256	0.310
	286	294	0.398
	449	457	0.382
	456	454	0.248
	543	551	0.297
	571	579	0.360

Multi-epitope vaccine construct and structural analysis

Five epitope regions were carefully chosen to compose the final vaccine candidate. These selections were based on specific criteria: they had to be recognized as epitopes and/or located within the variable regions of the viral capsid. Additionally, antigenicity, allergenicity and toxicity were evaluated, ensuring that only regions exhibiting high antigenicity, non-allergenicity and non-toxicity were incorporated into the vaccine construction (Table 5).

While most field strains did not show substitutions at positions 324 and 440, and the amino acids at these positions matched those of the vaccines (324Y and 440T), we chose to introduce substitutions. Specifically, we replaced 324Y with Y324I and 440T with T440A. This decision was influenced by the positive selection pressure at site 324, with the Y324I substitution already prevalent in most strains isolated in Brazil over the past five years (22 out of 37 isolates, 59.5%). This was also observed at site 440, where the T440A substitution was predominantly detected in strains isolated in the country during the same period (19 out of 37 isolates, 51,4%).

The epitopes were linked by GPGPG linkers whereas the adjuvant was added to the N-terminal of the vaccine construct using an EAAAK linker (Fig. 3). The final designed vaccine construct comprised 308 amino acid residues, with an antigenicity score reported as 0.5459.

The secondary structure prediction of the multi-epitope vaccine revealed a composition of 26% α-helix, 14% β-sheet and 60% coils (Fig. 4). The selected tertiary model for the vaccine and canine TLR4 exhibited the highest C-scores (-3.65 and -0.37, respectively) (Fig. 5 and 6). After refinement steps, the PROCHECK server yielded G-scores of 0.11 and -0.03 for the vaccine and canine TLR4, respectively, indicating favorable backbone and side chain conformations. Ramachandran plot analysis for the vaccine indicated 83.9% of residues in the favorable region, with 13.7% in the allowed region and 2.4% in the outlier region. The overall quality factor assessed by the ERRAT server for the vaccine was 91.95%. For canine TLR4, the Ramachandran plot revealed 75.2% of residues in the favorable region, 21.8% in the allowed region and 2% in the outlier region. The overall quality factor determined by ERRAT was 84.41%.

Table 5 Selected regions for the final multi-epitope vaccine construct: Antigenicity, allergenicity, and

toxicity evaluation

Region	Amino acids composition	Antigenicity*	Allergenicity	Toxicity
84-100	VNNLDKTAVNGNMALDD	antigenic (0.47)	non-allergenic	non-toxic
213 -238	QWDRTLIPSHTGTSGTPTNIYHGTDP	antigenic (0.50)	non-allergenic	non-toxic
292-326	NSLPQAEGGTNFGYIGVQQDKRRGVTQ MGNTNIIT	antigenic (0.73)	non-allergenic	non-toxic
383-447	QHGQKTTTTGETPERFTYIAHQDTGRYP EGDWIQNINFNLPVTNDNVLLPTDPIGGK AGINYTNI	antigenic (0.52)	non-allergenic	non-toxic
502-520	VAPNLTNEYDPDASANMSR	antigenic (0.71)	non-allergenic	non-toxic

*Threshold > 0.4

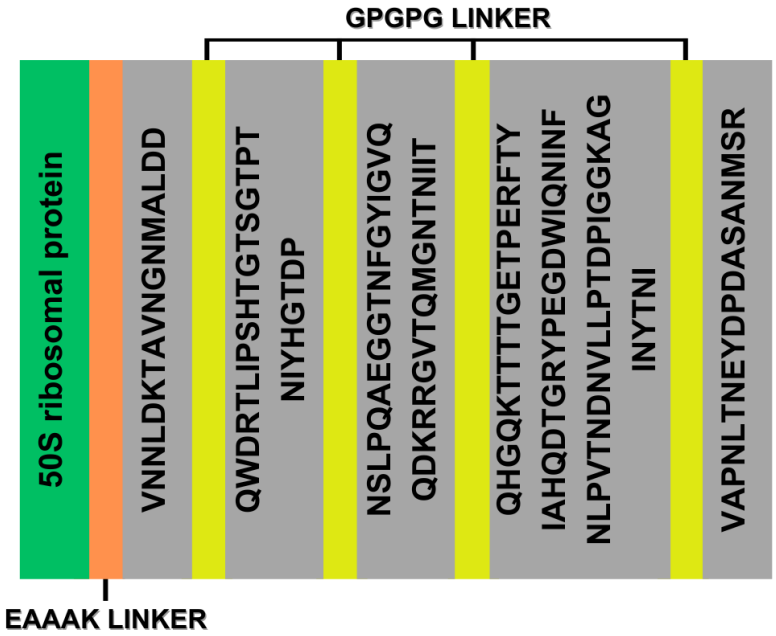

Fig. 3 Schematic representation of the final multi-epitope vaccine construct. The construct sequence consists of an adjuvant followed by epitopes, with the adjuvant linked to the epitopes via an EAAAK linker. The epitopes are connected by a GPGPG linker

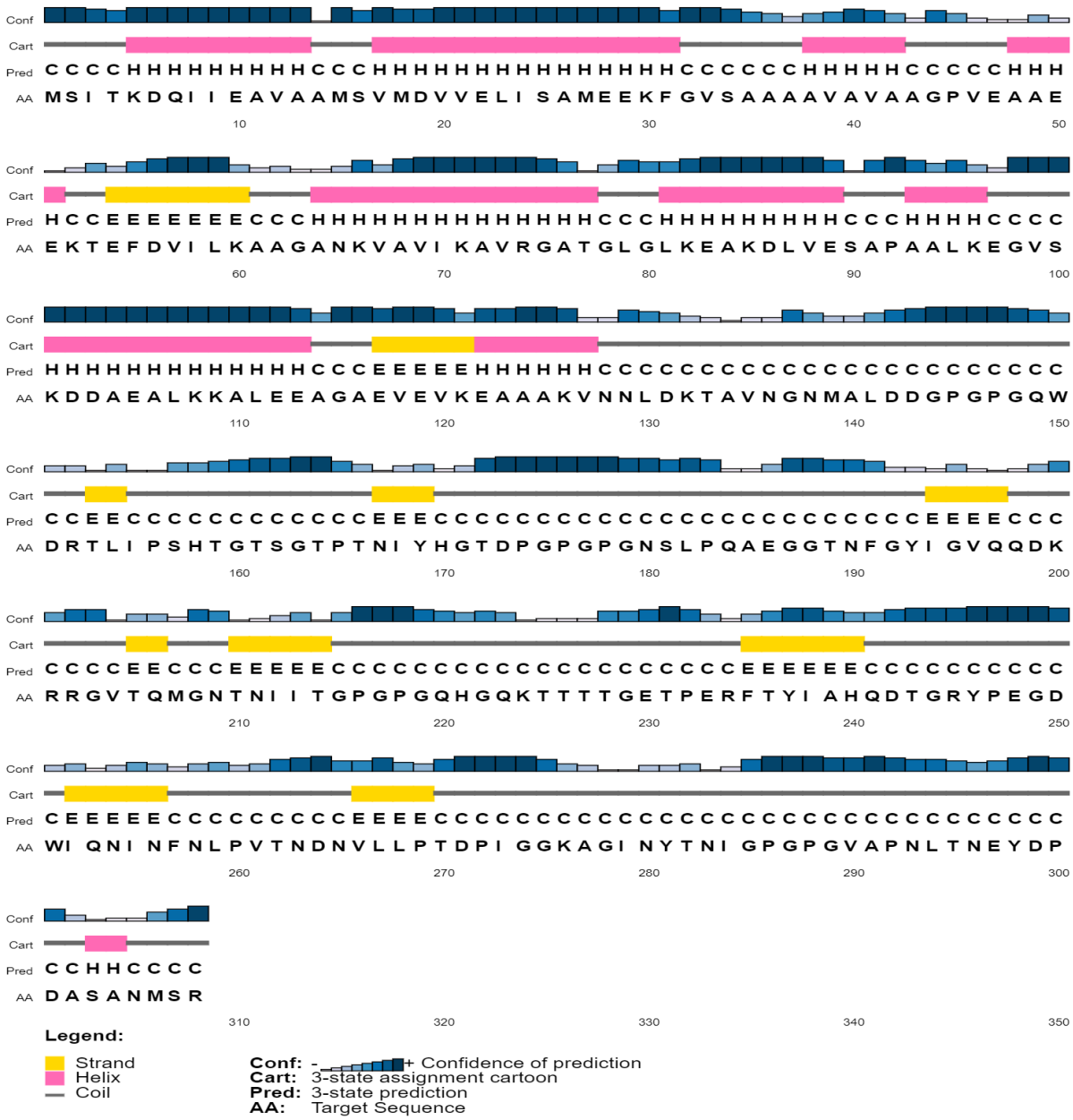

Fig. 4 Prediction of secondary structure of the vaccine construction by PSIPRED. The predicted secondary structure is estimated to contain 26% α-helix, 14% β-strand, and 60% coils

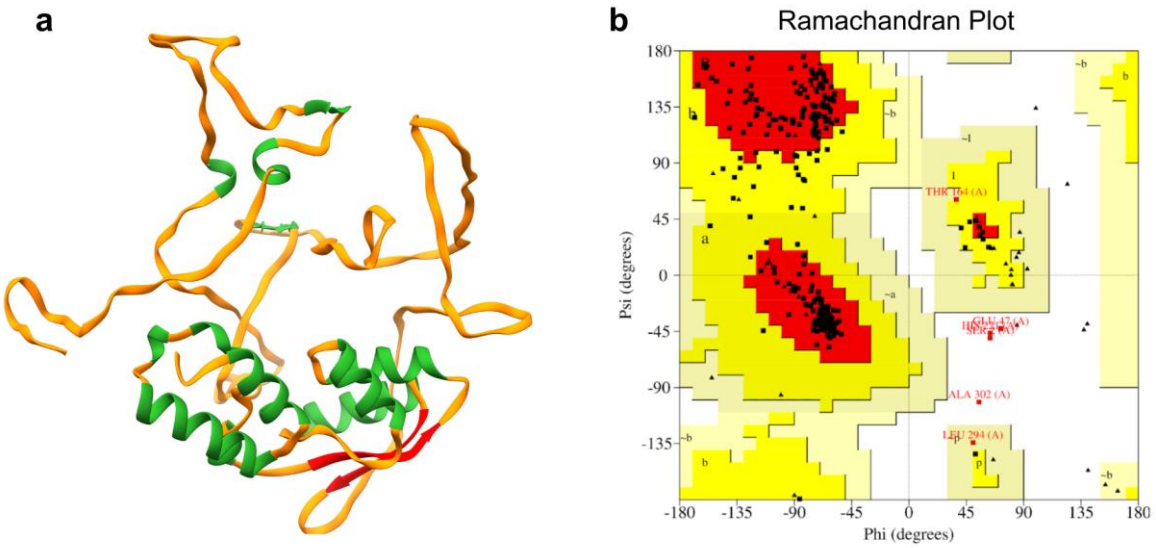

Fig. 5 (**a**) Refined tertiary structure of the vaccine construction. The secondary structural elements in the model are α-helix, β-strand, and coils colored in green, red and yellow, respectively. (**b)** Ramachandran plot of the refined structure of the vaccine construct

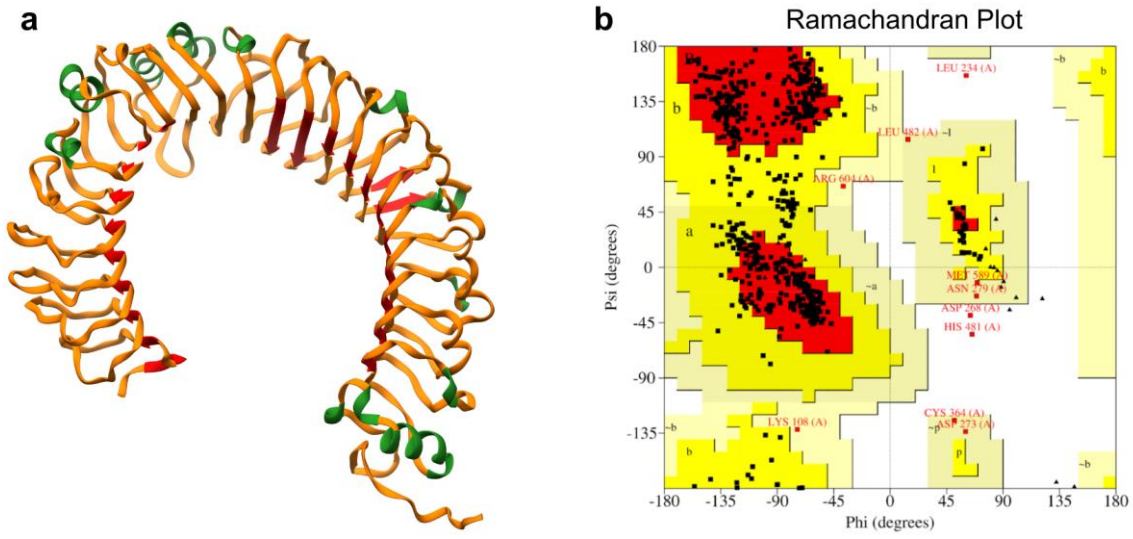

Fig. 6 (**a**) Refined tertiary structure of the canine TLR4 based on the template with PDB ID 3FXI. The α-helix, β-strand, and coils are colored in green, red and yellow, respectively. (**b**) Ramachandran plot of the refined structure of the canine TLR4

Physicochemical properties and solubility prediction

The molecular weight of the final vaccine construct is 31.87 kDa. The predicted theoretical pI is 4.70. The vaccine consists of 40 negatively charged residues and 25 positively charged residues. Half-life was estimated to be 30h mammalian reticulocytes *in vitro*, > 20h yeast *in vivo* and > 10 h *E. coli in vivo*. The formula is $C_{1384}H_{2209}N_{387}O_{461}S_7$. The instability index was computed to be 26.59 and classifies the protein as stable. The aliphatic index was estimated to be 76.10, indicating thermostability. Furthermore, the last property is GRAVY which was predicted to be -0.365. Negative GRAVY values show that the protein is hydrophilic and presents strong interactions with water molecules [22]. The final vaccine was evaluated as a soluble protein with a solubility score of 0.892.

Codon optimization and *in silico* cloning

The final designed vaccine construct was used to generate *in silico* cloning model for *E. coli* (K12) expression. Following codon optimization, the codon adaptation index (CAI) value of the vaccine was 1.0 and the GC content was 53.03%. To enable integration into the *E. coli* pET28a+ expression vector, two restriction sites (*Hind*III and *Bam*HI) were inserted at the N- and C-terminals of the sequence, respectively, forming a clone with a total length of 6274 bp. In addition, a 6xHis tag was inserted at both ends (Fig. 7).

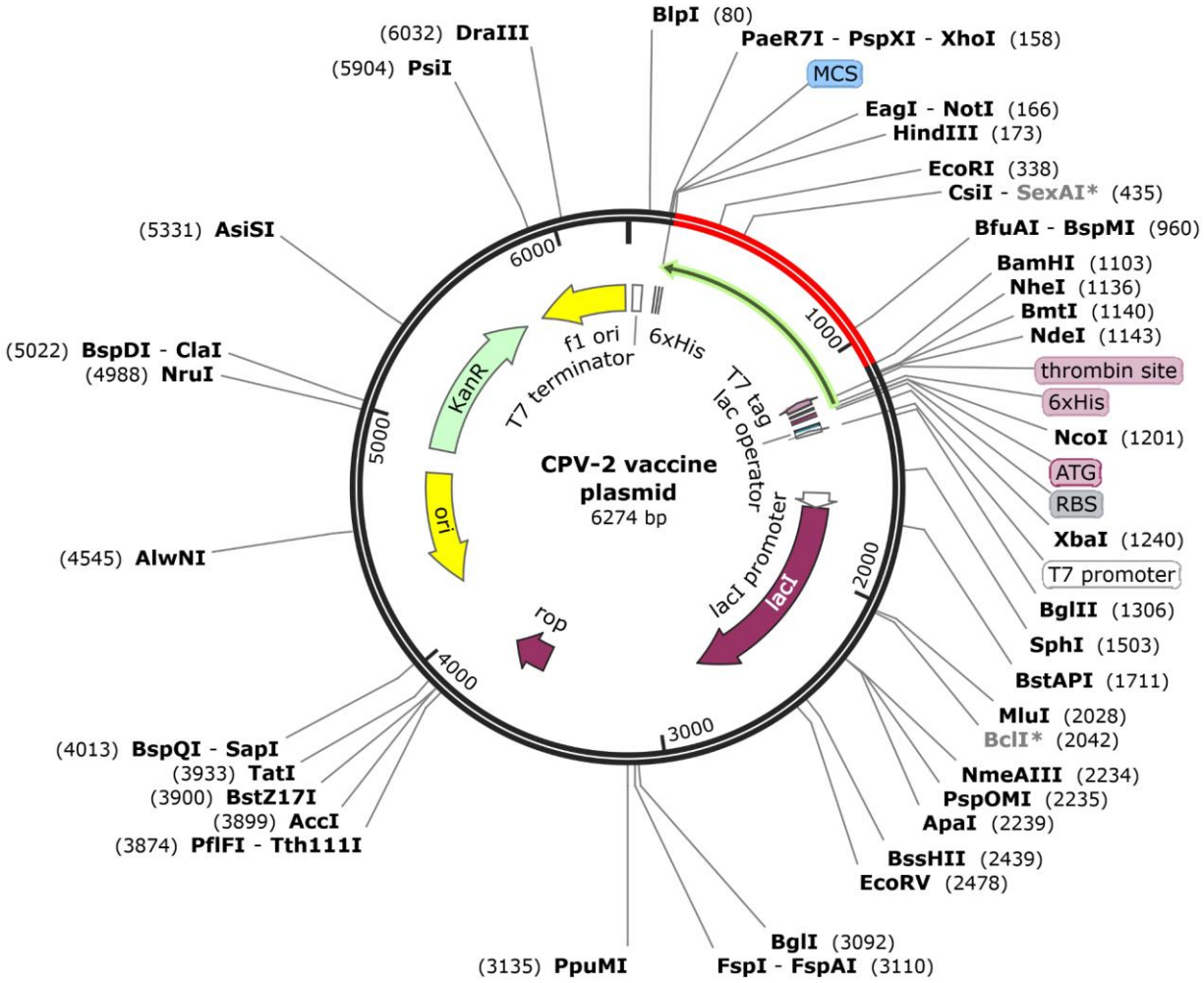

Fig. 7 *In silico* restriction cloning of the vaccine constructed into the pET28a+ vector. The vaccine coding region is colored in red and is a 924 bp gene sequence generated by the Java Codon Adaptation Tool server. This sequence was inserted between the *Hind*III and *Bam*HI restriction sites, forming a clone with a total length of 6274 bp

Molecular docking and molecular dynamics simulations of vaccine-TLR4 complex

The utilization of the HDOCK docking server enabled the molecular docking process for the vaccine designed with canine TLR4, yielding a set of 10 generated models. The selected docked complex was model 1, with the minimum docking energy and therefore the most probable binding position (-283.01). Notably, this selected model also displayed the highest confidence score of 0.9346. A confidence score above 0.7 is indicative that two molecules are likely to bind (Fig. 8).

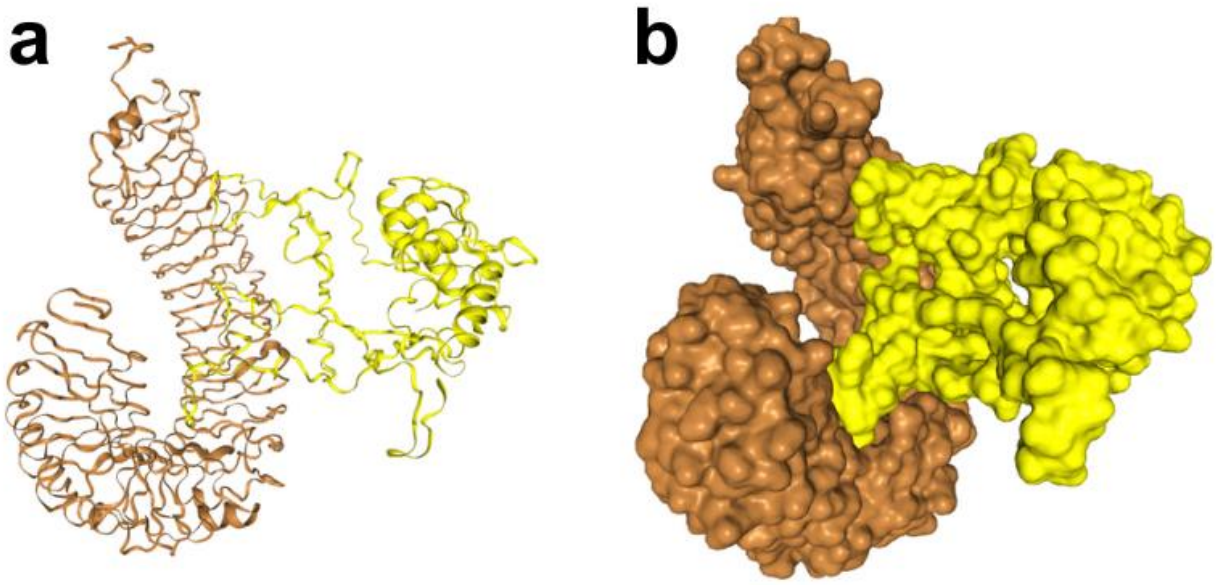

Fig. 8 The docked complex of the vaccine model and the canine TLR4 immune receptor. The vaccine protein is in yellow and the canine TLR4 receptor is in brown. It is possible to see the complex in (**a**) cartoon and (**b**) surface

To validate the effective interaction between canine TLR4 and the multi-epitope vaccine, a MD simulation was performed (Fig. 9). The radius of gyration (Rg) serves as a metric to determine the degree of compactness. The Rg derived from the docked complex remained mostly compact (around 35 to 36 Å) along the MD simulation period of 30 ns with a drop in Rg observed between 10 and 18 ns (Fig. 9a) which may indicate increased compactness. This is corroborated by a rise in intermolecular hydrogen bond (H-bond) between the side chains of vaccine protein and TLR4 peaking at around 18 ns (Fig. 9b), suggesting the role of H-bonds in increasing the compactness of the complex. Additionally, root mean square deviation (RMSD) of the vaccine-TLR4 complex fitting to its backbone showed deviations initially only to stabilize later at around 18 ns onward (Fig. 9c). The RMSD values ranged from 2 Å to 6 Å along the 30 ns time interval. The trend of convergence in the RMSD plot indicates the stability of the ligand-receptor complex in a dynamic state. Root means square fluctuation (RMSF) helps to explain

regional flexibility of the complex throughout the simulation to provide further understanding into the degree to which ligand binding influences the complex's flexibility. The RMSF plot showed low residual fluctuations for both the vaccine (Fig. 9d) and TLR4 (Fig. 9e), except at the non-interacting terminal ends of canine TLR4 and the C-terminal residues of the vaccine construct. This could be attributed to higher solvent exposure of the terminal residues compared to the inner regions and/or lower resolution of the secondary structure (e.g., lack of α-helix and β-strands).

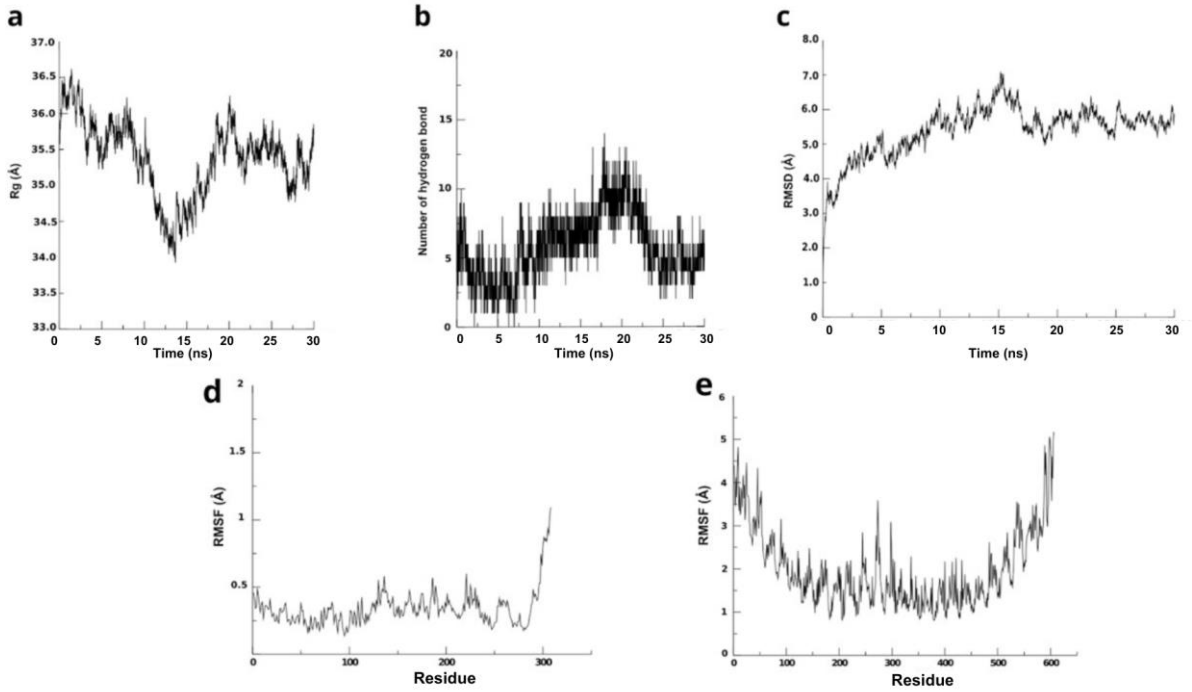

Fig. 9 Molecular dynamics simulation of the vaccine-canine TLR4 complex, presenting: (**a**) profile of the radius of gyration, (**b**) hydrogen bond, (**c**) RMSD profile, (**d**) RMSF for vaccine construction and (**e**) RMSF for canine TLR4 receptor

In silico immune simulation

The C-ImmSim server was employed to carry out an immune simulation, which demonstrated an immunological reaction after to hypothetically administering three doses of the vaccine construct (Fig. 10). After the first dose, the presence of a very low level of antibodies is observed. But following the second and third dose, the level of IgM+IgG increased, peaking after the third dose (Fig. 10a). It was also possible to observe after subsequent doses a reduction in the concentration of antigen, combined with a gradual increase in the production of memory B-cells with persistence (Fig. 10a-b). The IFN-γ level exhibited increments after each dose. By the end of the fourth week,

the IFN-γ level had retreated to its basel state, but the booster dose triggered IFN-γ expression again. IL-2, another cytokine, was also produced and its expression peaked after the second dose (Fig. 10c). Furthermore, an expansion of memory T helper cells was observed, reaching a peak after the administration of the second dose and remaining at levels greater than half throughout the simulated period (Fig. 10d).

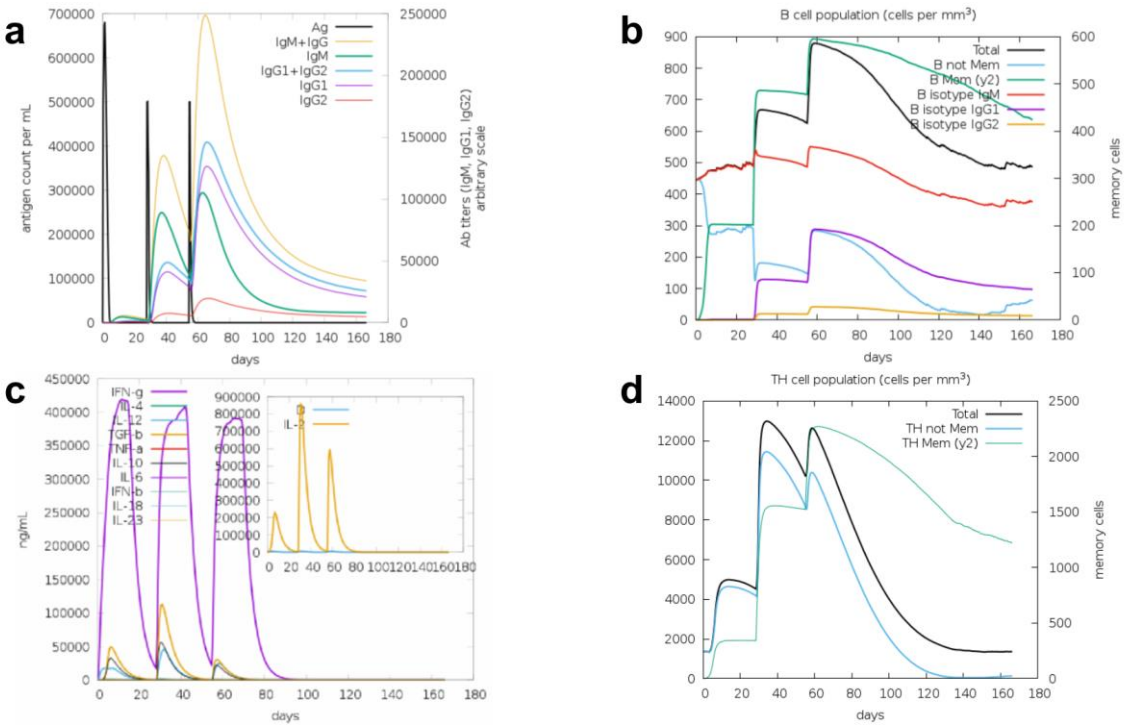

Fig. 10 *In silico* immune simulation with the multi-epitope vaccine construct. (**a**) Antigen, immunoglobulins and immunocomplexes after antigen injections. (**b**) B-cell populations after antigen injections. (**c**) Concentration of cytokines and interleukins in response to antigen injections. (**d**) Production of T-helper cells after antigen injections

Discussion

Over the past two decades, the co-circulation of various variants and subtypes of CPV-2 has been documented within the dog population in Brazil, including those that have been vaccinated [7, 8, 23, 24]. This diversity of CPV-2 lineages, as confirmed in this study, can be attributed to inadequate viral control measures and the significant movement of humans and animals within the country and across its borders [23].

The resulting phylogenetic tree from our study revealed distinct clades, illustrating the coexistence of CPV-2 variants in Brazil, with CPV-2a being the most

predominant, followed by CPV-2c and CPV-2b being less frequently observed. Interestingly, all vaccine sequences clustered together, primarily comprising older CPV-2a strains. This clustering emphasizes the importance of updating vaccines to contain the most recent antigenic types for better control, as changes in the capsid protein over the last 50 years have led to the occurrence of distinct capsid profiles, which should be considered in the development of future vaccines against the virus [25].

The amino acid evaluation performed in this study revealed several sites with non-synonymous substitutions, as well as selection pressure results similar to those previously described [12]. Characteristic amino acid alterations linked to CPV-2 antigenic variants, such as M87L, I101T, A300G and D305Y, were consistently observed across all sequences [26]. Despite the well-documented nature of these substitutions, our comparison with commercially available vaccines in Brazil revealed notable amino acid mismatches between vaccine strains and field isolates, as previously described [25]. Other significant substitutions were detected at sites 297, 324 and 440. Of particular concern is site 297, which exhibits strong positive selection, indicating a notable impact on both host adaptation and viral immune evasion [27]. This site is prominently located on the surface of VP2, specifically on the triple spike (loop 3), where it plays a crucial role [12]. Alongside amino acids 93 and 323, it contributes to recognizing the canine transferrin receptor-1 (TfR-1) [28]. Moreover, substitutions at the S297A site have been instrumental in driving the emergence of new CPV-2a and CPV-2b variants, often coinciding with substitutions at other sites such as 267, 324 and 440 [12]. Another site identified under positive selection was 324 [7]. Although the majority of field strains in Brazil matched with the vaccine strains (56.7%), the Y324I substitution has become increasingly common in the country in recent years, prompting us to introduce this substitution in the vaccine construct. This substitution appears to increase binding affinity with TfR-1 and has been associated with reduced efficacy of traditional vaccines [12]. The substitution T440A also was introduced into the vaccine, since that amino acid residue is situated in loop 4 of the VP2 protein, which comprises highly exposed sites on the VP2 surface and corresponds to one of the most immunogenic regions of the capsid [5, 12]. This substitution has been identified globally in CPV-2a, CPV-2b and CPV-2c variants and has been gaining importance in Brazil in recent years [12].

Vaccination is an effective intervention against CPV-2 spread. However, the emergence of new substitutions in specific amino acids of VP2, particularly within

highly antigenic regions, facilitates immune evasion [12]. Moreover, conventional vaccine development typically demands substantial financial investments and lengthy development periods [29].

In silico methods offer a streamlined approach to screening optimal vaccine candidates [30]. Particularly, peptide vaccines designed to prompt immune responses derive considerable advantage from computational prediction of candidate epitopes, ensuring both cost-effectiveness and safety [29]. Structural proteins interact with cell receptors on viral particles, making them great targets in epitope-based peptide vaccine development [19]. In our investigation, various prediction tools were employed to explore potential B and T-cell epitopes within the VP2 consensus sequence, taking into account the notable substitutions identified in Brazilian strains. Our selection criteria enabled the identification of a final set of epitopes with high affinity for B-cells and potential recognition by T-cells. This aspect is crucial in vaccine development, given the importance of neutralizing antibodies against the pathogen and the supportive role of helper T-cells in sustained antibody production, as well as in maintaining the activation of cytotoxic T lymphocytes responses [21]. Foroutan et al. [31] employed the same B and T-cells prediction tools in their designed vaccine candidate against *Toxoplasma gondii*. Furthermore, they conducted laboratory validation of their vaccine, demonstrating robust humoral and cellular responses in mice.

Compared to live vaccines, peptide vaccines tend to have lower immunogenicity [32]. To resolve this limitation, adjuvants are included with the aim of reinforcing the immunogenicity of peptide vaccines, consequently promoting an increase in antibody production [33]. In this study, the selected adjuvant, a TLR4 agonist, is known for its immunostimulatory properties [19]. The adjuvant and epitope regions were connected using linkers, which have been shown to facilitate the effective separation of individual epitopes *in vivo* [29]. Subsequently, the multi-epitope vaccine was subjected to meticulous evaluation, including antigenicity, allergenicity and toxicity predictions, resulting in an antigenic, non-allergenic and non-toxic vaccine construct. Further analysis of the physicochemical properties revealed a molecular weight of 31.87 kDa. Proteins with a molecular weight below 110 kDa are favored in vaccine development due to their easier and faster expression, as well as rapid purification [34]. Additionally, the predicted isoelectric point of the subunit was 4.70, indicating its weakly acidic nature. Stability assessment yielded an instability index of 26.59, suggesting protein stability, as values below 40 are typically indicative of stability [33]. The aliphatic index

falls within the range of 66.5 to 84.33, indicating thermal stability [35]. The vaccine showed hydrophilic nature with a GRAVY value of -0.365, indicating strong interactions with water molecules [22]. Moreover, it displayed favorable solubility, with a value of 0.627, confirming its suitability as a good vaccine candidate [29]. These parameters are crucial for guiding the expression processes. Finally, our vaccine candidate demonstrated a half-life exceeding 20 hours in yeast and over 10 hours in *E. coli*, suggesting prolonged exposure to the immune system [29]. The physicochemical properties predicted in our study were comparable to those predicted by Foroutan et al. [31], who conducted laboratory validation of their vaccine, demonstrating good results *in vivo*. Interestingly, our vaccine candidate exhibited a superior instability index and aliphatic index compared to the values reported by these authors.

The secondary structure analysis of the vaccine construct revealed a predominant coil formation (60%), potentially enhancing protein flexibility and antibody-binding efficacy [29]. Additionally, the vaccine comprised 26% α-helix and 14% β-strand structures. Following the prediction of the 3D model, refinement techniques were applied to optimize both global and local structures [32]. The refined tertiary structure exhibited high quality, as depicted by the Ramachandran plot, with 83.9% of residues falling within the favored region, 13.7% within the allowed region, and 2.4% considered outliers. Finally, the overall quality factor assessed by the ERRAT server reached 91.95% indicating an acceptable standard for further docking studies [35].

Molecular docking was conducted using the HDOCK server, which provides a wide range of tools for robust and efficient protein–protein docking, including homology search, template-based modeling and structure prediction [36]. Model 1 was identified as the best complex based on binding energy (-283.01) and confidence score (0.9346). To assess the stability of the canine TLR4-vaccine construct complex, a 30 ns molecular dynamics simulation was performed using the GROMACS 2023.4 software package. The simulation results indicated a stable interaction between the vaccine construct and the canine TLR4 receptor [21]. Analysis of the RMSF plot indicated localized fluctuations in terminal residues which are non-interacting, whereas interacting residual regions have low fluctuations along the simulation period [37]. Additionally, the radius of gyration plot demonstrated the compactness of the vaccine construct-TLR4 complex throughout the 30 ns simulation [19].

Three injections of 1000 antigen molecules without LPS were simulated with a 4-week interval and the immune response was monitored for 160 days. Following the first dose, a minimal level of IgM was observed. However, subsequent doses increased both IgM and IgG levels. Similarly, the levels of memory B-cells increased after each dose, while memory T-cell levels increased following the second dose but remained stable after the third dose. These results suggest that the vaccine construct primarily stimulates the production of IgG, IgM and stimulate B-cells and T-cells memory cells, with effects presumed to persist over several months based on simulation findings [19, 37].

Subsequent phases of the reverse vaccinology approach should involve assessing the recombinant immunogenic protein expressed in the *E. coli* system (strain K12), as proposed here, followed by *in vitro* testing and evaluation in challenge models. The *in silico* designed vaccine demonstrated significant immunogenic potential, indicating its suitability for *in vitro* experimental evaluation in the upcoming phase of the study.

Conclusion

In conclusion, our study provides information on the evolutionary dynamics of CPV-2 in Brazil, highlighting potential limitations in the efficacy of currently available vaccines against local strains. Additionally, we developed a multi-epitope-based vaccine against CPV-2 using immunoinformatics approaches. Through this method, we chose five epitopes within the capsid protein VP2 (84-100, 213-238, 292-326, 383-447, 502-520) and constructed a vaccine candidate using computational tools. These methods significantly reduce the time-consuming process of trial and error peptide screening for vaccine candidate identification, offering a cost-effective and easily conductible method prior to wet-lab trials. The immunogenic, physicochemical and structural properties of our vaccine candidate show promise for yielding positive outcomes in both *in vitro* and *in vivo* assays in the near future, thus opening new avenues in veterinary research.

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Declarations

Ethics approval and consent to participate Not needed.

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5 DISCUSSÃO GERAL

A família *Parvoviridae* compreende uma ampla e altamente diversificada variedade de vírus com genoma de DNA linear de fita simples (Gallinella, 2019). Dentro da subfamília *Parvovirinae*, encontram-se os parvovírus que infectam vertebrados, responsáveis por uma ampla variedade de doenças em humanos e animais. O parvovírus mais significativo para os seres humanos é o B19V, especialmente preocupante para crianças e indivíduos com sistema imunológico comprometido. Na medicina veterinária, o CPV-2 e o PPV se destacam, causando enterite hemorrágica e distúrbios reprodutivos, respectivamente.

A parvovirose canina é uma doença altamente contagiosa causada pelo CPV-2, afetando cães em todo mundo (Tuteja et al., 2022). Esta enfermidade é de particular importância devido à rápida disseminação em populações caninas densamente concentradas, como em abrigos e canis, e também pode afetar animais de estimação em ambientes domésticos, representando um risco significativo para a saúde pública veterinária. Caracteriza-se por uma gastroenterite aguda grave, que pode levar a complicações severas e, em casos extremos, resultar em óbito (Silva et al., 2022).

O tratamento para a parvovirose canina é de suporte e sintomático. As principais abordagens terapêuticas incluem fluidoterapia para combater a desidratação, administração de antibióticos para prevenir infecções secundárias, medicação antiemética para controlar vômitos e suporte nutricional para manter a nutrição adequada (Mylonakis et al., 2016). Diversas alternativas terapêuticas foram exploradas para avaliar o potencial contra o CPV-2 e essas investigações foram compiladas em uma revisão detalhada apresentada no Artigo I.

Apesar de não haver, até o momento, um tratamento específico disponível, essa revisão oferece uma visão abrangente das pesquisas em andamento na área. Essas investigações representam um passo indispensável na busca por futuros antivirais, destacando a importância contínua da pesquisa para enfrentar essa lacuna clínica. No entanto, uma proporção significativa desses estudos permanece em estágios iniciais de testes *in vitro*. Os avanços recentes em metodologias computacionais, como o acoplamento molecular e a descoberta de medicamentos baseada em inteligência artificial, oferecem caminhos promissores para acelerar a identificação de potenciais candidatos antivirais.

Para que alguns candidatos sejam liberados para tratamento *in vivo*, um processo criterioso de validação em modelo animal e, posteriormente, em ensaios clínicos seria essencial. Além disso, o financiamento da indústria é crucial, considerando que esses processos geralmente são onerosos. Também seria fundamental desenvolver processos de fabricação em larga escala e garantir a comercialização desses candidatos.

O Artigo II descreve a construção de uma série de mutantes, cada um abrigando uma mutação comum da proteína VP2, a fim de avaliar seu impacto na capacidade replicativa do vírus. Essa compreensão é de grande importância, considerando a história evolutiva deste vírus. O CPV-2 tem sido transmitido continuamente há mais de 40 anos (Hao et al., 2022). O primeiro caso foi relatado em 1979 e a partir de então o vírus sofreu diversas modificações genéticas na proteína do capsídeo VP2, dando origem às suas três variantes (CPV-2a, CPV-2b e CPV-2c) (Tuteja et al., 2022). Essas variantes substituíram completamente a cepa original do vírus. Mutações no resíduo 426 da VP2 são os principais determinantes das variantes do CPV-2. Outras mudanças de aminoácidos em posições específicas na proteína também classificam o CPV-2 em variantes e subtipos (Leal et al., 2024). Com mudanças antigênicas em curso, novas mutações na VP2 estão constantemente surgindo, principalmente na região entre os resíduos 267 e 498. Esta região é particularmente suscetível a mutações devido à sua exposição na superfície do capsídeo (Decaro e Buonavoglia, 2012).

Durante os experimentos de clonagem, as principais dificuldades enfrentadas foram relacionadas à padronização da transfecção dos plasmídeos com Lipofectamina. A transfecção de células aderentes usando esse agente requer uma otimização cuidadosa da quantidade de plasmídeo, quantidade do reagente e número de células, e essa otimização é específica para cada linhagem celular (Ferraris e Burg, 2007). O sucesso da transfecção foi alcançado após várias repetições, durante as quais variáveis individuais e combinadas foram ajustadas até que resultados esperados fossem obtidos.

Os resultados encontrados para a capacidade replicativa dos mutantes no Artigo II diferiram quando comparados por qPCR e imunofluorescência. Essa discrepância pode ser atribuída ao fato de os mutantes apresentarem padrões de replicação únicos, resultando em uma liberação menos eficiente de partículas virais no meio enquanto se replicam ativamente nas células hospedeiras. Notavelmente, os mutantes portadores das mutações N426D, N426E e T440A exibiram uma melhor capacidade replicativa quando comparados às cepas parentais.

Para complementar as análises, o mutante CPV-2 N426E foi ainda empregado no ensaio de neutralização viral, visando investigar a capacidade dos soros policlonais de ratos imunizados com vacinas baseadas no CPV-2 de neutralizar o mutante. Os resultados obtidos foram comparados com os de uma cepa padrão de CPV-2 e sugeriram uma possível proteção cruzada. Considerando que o mutante CPV-2 N426E é representativo da variante CPV-2c, esse achado está alinhado com as conclusões de Larson e Schultz (2008) e Wilson et al. (2014), indicando que as vacinas disponíveis podem conferir uma proteção cruzada contra cepas variantes. Em contraste, Cavalli et al. (2008) e Kang et al. (2008) observaram que animais vacinados com CPV-2 apresentaram títulos significativamente mais baixos de anticorpos neutralizantes contra CPV-2a, CPV-2b e CPV-2c em comparação com o vírus homólogo. Para uma compreensão mais aprofundada, é essencial conduzir investigações adicionais com outros mutantes e explorar mutações combinadas nesta proteína. É igualmente importante testar essas mutações utilizando tanto anticorpos monoclonais quanto policlonais. Esses estudos ampliariam nossa compreensão da dinâmica viral e orientariam futuras estratégias de intervenção.

Embora o CPV-2 original tenha sido completamente substituído pelas variantes antigênicas alguns anos após o seu aparecimento, o CPV-2 original ainda é utilizado na maioria das vacinas comerciais (Cavalli et al., 2008). Dessa forma, existe a preocupação de que as vacinas utilizadas atualmente para prevenir a infecção possam não proteger eficazmente os cães contra as atuais variantes antigênicas de CPV-2. A avaliação da variabilidade de aminoácidos nas cepas empregadas nesses imunógenos é crucial para a formulação de estratégias imunológicas eficazes contra o vírus.

Conforme demonstrado no Artigo III, nenhuma das vacinas contra CPV-2 comercializadas no Brasil compartilhava semelhança completa à proteína antigênica VP2. Em vez disso, estas apresentavam notável variabilidade de aminoácidos quando comparadas às cepas de campo do CPV-2 isoladas no país. Embora os resultados do Artigo II apontem para uma possível proteção cruzada entre as variantes, é crucial ponderar sobre o risco potencial das variações nas sequências de aminoácidos da vacina em relação às propriedades antigênicas. Isso pode possibilitar na introdução de novos vírus, perfis distintos de pressão seletiva e recombinação genética entre cepas vacinais e selvagens (Silva et al., 2022). Assim, a vacinologia reversa pode fornecer uma abordagem valiosa para o desenvolvimento de novas vacinas contra vírus, acelerando a criação de imunizantes que conferem proteção precisa contra as cepas prevalentes no

campo (Monterrubio-López e Delgadillo-Gutiérrez, 2021). Consequentemente, como resultado de uma série de análises utilizando ferramentas de bioinformática, foi proposta uma vacina multi-epítopo contra o CPV-2 no Artigo III. A escolha de aminoácidos que comporiam a vacina foi proposta para condizer com as variantes do CPV-2 que circulam no território nacional.

Cinco regiões de epítopos dentro da proteína VP2 foram escolhidas para compor a vacina (84-100, 213-238, 292-326, 383-447, 502-520). As propriedades imunogênicas, físico-químicas e estruturais da vacina proposta demonstram potencial para produzir resultados positivos em ensaios *in vitro* e *in vivo* em um futuro próximo. É importante destacar que, por serem consideradas alternativas seguras (Bayani et al., 2023), essa vacina peptídica pode ser viável para uso em animais imunocomprometidos e cadelas prenhas, em substituição às vacinas de vírus vivo modificado.

Em uma investigação anterior, Paul et al. (2023) desenvolveram uma vacina multi-epítopo *in silico* contra o CPV-2, incorporando regiões conservadas da VP2 em sua formulação. A vacina resultante mostrou capacidade de induzir uma resposta imune eficaz nas simulações realizadas. Essas descobertas destacam o forte potencial da proteína VP2, especialmente de suas regiões de epítopos, para o desenvolvimento de vacinas de subunidades. Dado que as mutações identificadas no presente estudo extrapolam as fronteiras do Brasil, o uso desse imunógeno pode ser apropriado para outras regiões geográficas. Além disso, é importante ressaltar que as substituições de aminoácidos na composição da vacina podem ser facilmente implementadas quando necessário, produzindo assim uma proteção mais robusta contra cepas virais circulantes (Maleki et al., 2021).

6 CONCLUSÃO

- A revisão das alternativas terapêuticas promissoras para importantes membros da subfamília *Parvovirinae*, incluindo B19V, CPV-2 e PPV, revelou uma variedade de estratégias em desenvolvimento, ressaltando a necessidade de avanços contínuos para melhorar o prognóstico dos indivíduos infectados.
- A avaliação das mutações pontuais na proteína VP2 do CPV-2 demonstrou comportamentos diversos quando testadas *in vitro*. Mutações como N426D/E e T440A mostraram um aumento na capacidade replicativa do vírus, proporcionando uma melhor compreensão da relação entre a variabilidade genética do vírus e sua antigenicidade.
- As análises das sequências de CPV-2 isoladas no Brasil revelaram a co-circulação de três variantes e um perfil variado da proteína VP2 ao longo do tempo. As sequências analisadas mostraram diferenças significativas em comparação com as sequencias presentes nas vacinas disponíveis no país, indicando a necessidade de atualização das vacinas.
- A sequência da vacina candidata, desenvolvida para incluir as alterações de aminoácidos mais comuns nas cepas brasileiras, apresentou alta antigenicidade e manteve-se não tóxica e não alergênica nos testes realizados.
- A vacina candidata demonstrou capacidade de se ligar eficazmente ao receptor TLR4, e o complexo formado manteve estabilidade, sugerindo um bom potencial para elicitar uma resposta imunológica.
- As simulações imunológicas *in silico* indicaram uma resposta imune robusta após a vacinação, com aumento significativo na produção de anticorpos e na atividade das células T auxiliares, reforçando a possibilidade de sucesso da vacina em estudos *in vivo*.

7 PERSPECTIVAS

A continuidade deste trabalho é importante para confirmar os resultados obtidos até agora. As perspectivas futuras incluem:

- Realizar os testes de neutralização viral utilizando todos os mutantes gerados no Artigo II.

- Realização dos testes *in vitro* da vacina projetada no Artigo III.
- Avaliação da eficácia da vacina projetada no Artigo III em um modelo animal apropriado.
- Avaliação da eficácia da vacina projetada no Artigo III em cães.

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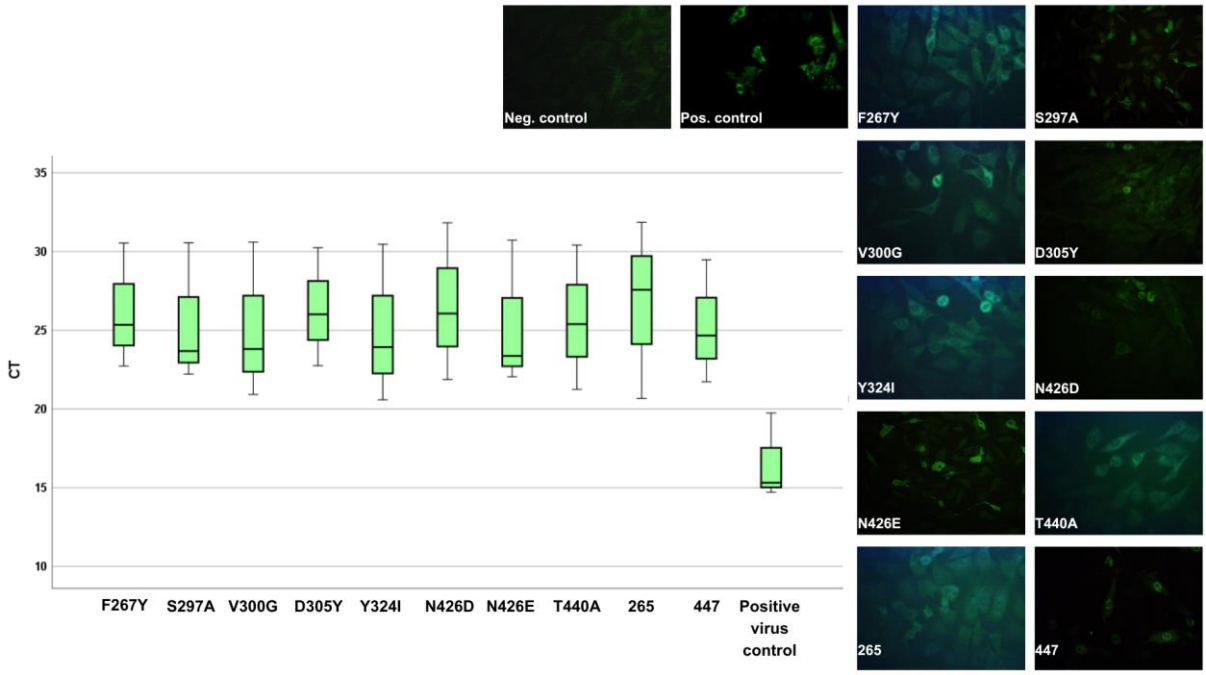
ANEXOS



Anexo I – Gráfico referente ao Artigo II mostrando os valores de Ct dos mutantes, cepas parentais e controle positivo do vírus da primeira à terceira passagens. Nas imagens adjacentes são apresentados os resultados da imunofluorescência da terceira passagem de mutantes, cepas parentais, controle positivo do vírus e controle negativo.

Durante o período do doutorado também foram publicados outros trabalhos relacionados ao projeto de mestrado desenvolvido pela aluna. Estes trabalhos estão apresentados aqui.

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Original Article
Livestock Diseases

Species identification and antimicrobial susceptibility profile of bacteria associated with cow mastitis in southern Brazil[1]

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ABSTRACT.- Lopes T.S., Fussieger C., Rizzo F.A., Silveira S., Lunge V.R. & Streck A.F. 2022. **Species identification and antimicrobial susceptibily profile of bacteria associated with cow mastitis in southern Brazil.** *Pesquisa Veterinária Brasileira 42:e06958, 2022.* Laboratório de Diagnóstico em Medicina Veterinária, Universidade de Caxias do Sul, Rua Francisco Getúlio Vargas 1130, Campus-Sede, Caxias do Sul, RS 95070-560, Brazil. E-mail: afstreck@ucs.br

Bovine mastitis is the most common disease in dairy cattle and responsible for economic losses in the milk industry. The present study aimed to identify the main species and to evaluate the antimicrobial susceptibility of bacterial isolates from cow herds with mastitis in dairy farms from southern Brazil. A total of 107 milk samples were collected from different cow herds in one important dairy producing region in southern Brazil, including farms located in ten cities from the Northeast region in the Rio Grande do Sul state. Bacterial strains were isolated and submitted to presumptive identification by classical bacteriological methods. Bacterial species were also identified by MALDI-TOF MS and antimicrobial susceptibility testing was performed with 12 antimicrobials commonly used in dairy farms. Fifty-one bacterial strains were isolated and the presumptive identification demonstrated the occurrence of *Staphylococcus* spp. (82.3%), *Bacillus* spp. (3.9%), *Klebsiella* spp. (3.9%), *Streptococcus* spp. (3.9%), *Corynebacterium* sp. (2%), *Enterococcus* sp. (2%) and *Serratia* sp. (2%). Forty-one isolates were successfully identified in the MALDI-TOF analysis, including 35 isolates from eleven different bacterial species. Importantly, there were eight different *Staphylococcus* species, with a high frequency of *Staphylococcus chromogenes* (48.6%) and *Staphylococcus aureus* (20%). Overall, bacterial isolates demonstrated resistance to penicillin (46.3%), tetracycline (39%), amoxicillin (36.6%), ampicillin (34.1%) and sulfamethoxazole/trimethoprim (31.7%). Enrofloxacin was the unique antimicrobial that all isolates were susceptible. In addition, there were six multidrug resistant isolates (five *S. chromogenes* and one *S. aureus*). This study highlights that bacterial pathogens with resistance to several antimicrobials were identified in cows from dairy farms in a very important milk producing region located in southern Brazil. Microbial identification of the bovine mastitis pathogens and determination of the antimicrobial profile is necessary for the rational use of the medicines.

INDEX TERMS: Species identification, antimicrobial resistance, mastitis, milk, *Staphylococcus*, MALDI-TOF MS, public health, cattle, Brazil.

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VETERINARY MICROBIOLOGY - RESEARCH PAPER

Antimicrobial activity of essential oils against *Staphylococcus aureus* and *Staphylococcus chromogenes* isolated from bovine mastitis

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Abstract

Staphylococcus aureus and *Staphylococcus chromogenes* are pathogens frequently detected in bovine mastitis. Treatment and prevention of this disease have been usually carried on with antimicrobials. However, the emergence of bacterial isolates with antimicrobial resistance has aroused interest in new therapeutic alternatives. Plant essential oils (EOs) have been largely studied as antibacterial treatments. In the present study, EOs from five plants were evaluated for their antibacterial activities against *S. aureus* and *S. chromogenes*. Bacterial isolates were obtained in a previous study of clinical cases of bovine mastitis. EOs from lemongrass, eucalyptus, lavender, peppermint, and thyme were obtained by hydrodistillation and their chemical compositions were evaluated by gas chromatography (GC). Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were evaluated for all EOs. The results demonstrated that citral (40.9%), myrcene (24.7%), and geraniol (1.9%) were detected in lemongrass EO; 1,8-cineole (76.9%), α-pinene (8.2%), and ledene (5.1%) in eucalyptus EO; 1,8-cineole (45.2%), camphor (18.2%), and fenchone (14.6%) in lavender EO; L-menthol (38.5%), menthofuran (16.3%), and citronellal (10.6%) in peppermint EO; and thymol (44.2%), p-cymene (24.6%) and 1,8-cineole (9.9%) in thyme EO. More effective antibacterial activities were observed only with the use of lemongrass (MIC and MBC ranging from 0.39 to 3.12 mg/mL and 0.39 to 6.35 mg/mL, respectively) and thyme (MIC and MBC ranging from 0.39 to 1.56 mg/mL and 0.39 to 3.12 mg/mL, respectively). Peppermint, lavender and eucalyptus EOs did not show bactericidal activities. In conclusion, lemongrass and thyme EOs are promising antibacterial alternatives against *Staphylococcus* species associated with bovine mastitis.

Keywords Antimicrobial resistance · Alternative treatments · Food security · Mastitis pathogens