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Estudos sobre vacinologia e evolução do vírus da cinomose canina

Autora: Renata da Fontoura Budaszewski
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ESTUDOS SOBRE VACINOLOGIA E EVOLUÇÃO DO VÍRUS DA CINOMOSE CANINA

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DEDICATÓRIA

Dedico esta tese ao meu avô Ney, pela eterna inspiração.

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EPÍGRAFE

“O progresso é feito por tentativa e erro; os erros são geralmente centenas de vezes mais numerosos que os sucessos; no entanto, normalmente são deixados inenarrados”

William Ramsay

Resumo

O vírus da cinomose canina (CDV) é um importante patógeno de cães domésticos e carnívoros selvagens. A infecção pelo CDV é relevante a nível mundial e está associada com alta morbidade e mortalidade. Em diversos países a cinomose é considerada controlada pelo uso de vacinas, no entanto, no Brasil ainda é endêmica, principalmente devido ao grande número de animais não domiciliados. Além disso, surtos em cães e várias espécies de animais silvestres ocorrem com frequência, dizimando populações ameaçadas. As vacinas vivas atenuadas são seguras para cães, mas seu uso não é aconselhado em espécies altamente suscetíveis à infecção pelo CDV. Também os relatos de surtos de cinomose em cães supostamente vacinados levantam a hipótese de que as vacinas disponíveis no mercado podem não ser eficientes frente a algumas cepas de campo. Com o objetivo de gerar dados acerca dos mecanismos de evolução do CDV e desenvolver e testar a eficácia de uma vacina bivalente inativada contra o vírus da raiva (RABV) e CDV a presente tese será apresentada na forma de dois artigos científicos. Ainda, um artigo de revisão sobre os modelos animais utilizados para obtenção de informações sobre o vírus do sarampo utilizando a infecção de CDV em furões e cães foi publicada e será apresentada na presente tese. No primeiro artigo, foi analisada a ocorrência de recombinação homóloga em genomas de CDV e detectou-se oito possíveis vírus recombinantes, incluindo um evento de recombinação entre uma cepa de campo e uma cepa vacinal atenuada, sugerindo que o uso de vacinação com vírus vivo atenuado pode influenciar a evolução do CDV. No segundo trabalho, uma vacina recombinante bivalente inativada baseada em RABV expressando as glicoproteínas do envelope do CDV, hemaglutinina e proteína de fusão, mostrou-se eficiente na proteção contra infecção por CDV em furões quando utilizado um protocolo *prime/boost*. Finalmente, foi publicada uma revisão de literatura sobre os modelos animais utilizados para obtenção de informações sobre a patogênese do vírus do sarampo utilizando a infecção com o vírus da cinomose.

Palavras-chave: vírus da cinomose canina, recombinação homóloga, vacina, vírus da raiva, modelos animais

ABSTRACT

Canine distemper virus (CDV) is an important pathogen of domestic dogs and wild carnivores. CDV infection is globally relevant and it is associated with high morbidity and mortality. In several countries, distemper is considered controlled by vaccination, however, in Brazil it is still endemic, mainly due to the large number of non-domiciliated animals. In addition, outbreaks in dogs and various species of wild animals occur frequently, decimating threatened populations. Live attenuated vaccines are safe for dogs, but their use is not advised in species that are highly susceptible to CDV infection. Also, reports of canine distemper in supposedly vaccinated dogs raise the hypothesis that commercially available vaccines may not be effective against some wild type strains. In order to investigate the mechanisms of CDV evolution and to develop and assess the efficacy of an inactivated bivalent vaccine against rabies virus and CDV, this thesis will be presented in the form of two scientific papers. Furthermore, a review article on the animal models used to gain information on measles virus using CDV infection in ferrets and dogs has been published and will be presented in this thesis. In the first paper, the occurrence of homologous recombination in CDV genomes was analyzed and eight possible recombinant viruses were detected, including a recombination event between a wild type strain and an attenuated vaccine strain, suggesting that the use vaccines based on attenuated live virus may influence CDV evolution. In the second study, an inactivated bivalent recombinant vaccine based on RABV expressing CDV envelope glycoproteins, hemagglutinin and fusion protein, proved to be effective in protecting against CDV infection in ferrets when using a prime/boost protocol. Finally, a literature review was published on the animal models used to obtain information on the pathogenesis of measles virus using infection with canine distemper virus.

Keywords: canine distemper virus, homologous recombination, vaccine, rabies virus, animal models

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

C	Proteína C
CDV	Vírus da cinomose canina
CeMV	Morbilivírus dos cetáceos
DNA	Ácido desoxirribonucleico
F	Gene/proteína de fusão
FeMV	Morbilivírus dos felinos
Fsp	Peptídeo sinal da proteína de fusão
H	Gene/proteína da hemaglutinina
ISCOM	Complexos imunoestimulantes
kDa	Quilodaltons
L	Gene/proteína da grande polimerase
M	Gene/proteína da matriz
MeV	Vírus do sarampo
MLV	Vírus vivo modificado
N	Gene/proteína do nucleocapsídeo
nm	Nanômetro
nt	Nucleotídeo
OIE	Organização Mundial de Saúde Animal
ORF	Fase de leitura aberta
P	Gene/proteína da fosfoproteína
PDV	Vírus da cinomose dos focídeos
PRRV	Vírus da peste dos pequenos ruminantes
PVRL4	Receptor relacionado a poliovírus 4
RABV	Vírus da raiva

rCDV	Vacina recombinante baseada em vetor canaripox
RdRp	RNA-polimerase dependente de RNA
RNA	Ácido ribonucleico
RNP	Complexo ribonucleoproteína
RPV	Vírus da peste bovina
SLAM	Molécula sinalizadora da ativação de linfócitos
SNC	Sistema nervosa central
UTR	Região não traduzidas
V	Gene/proteína V

SUMÁRIO

1. INTRODUÇÃO

O vírus da cinomose canina (CDV) é um membro do gênero *Morbillivirus* da família *Paramyxoviridae*. O RNA genômico codifica seis proteínas estruturais: proteína da fusão (F), proteína da hemaglutinina (H), proteína da matriz (M), fosfoproteína (P), proteína da grande polimerase (L) e proteína do nucleocapsídeo (N) (RIMA, 1983; APPEL, 1987).

O CDV causa uma doença epizoótica em carnívoros. Historicamente é relacionado à infecção de membros da família *Canidae*, no entanto, nos últimos 30 anos, vários relatos sugerem se tratar de um vírus bastante promíscuo, saltando a barreira entre espécies de hospedeiros inúmeras vezes (ROELKE-PARKER et al., 1996; WOOLHOUSE; HAYDON; ANTIA, 2005; SUN et al., 2010).

O gene H tem a maior variação genética e é, portanto, um alvo adequado para investigar o polimorfismo do CDV e para estudos de epidemiologia molecular (HAAS et al., 1997; HASHIMOTO; UNE; MOCHIZUKI, 2001; VON MESSLING et al., 2001). Com base na variabilidade do gene H, cepas de CDV segregam em pelo menos nove grandes linhagens genéticas geograficamente relacionadas: *America-1* (incluindo vacinas atenuadas), *America-2*, *Asia-1*, *Asia-2*, *Europe Wildlife*, *Arctic*, *South Africa*, *Europe/South America-1* e *South America-2* (HAAS et al., 1997; MOCHIZUKI et al., 1999; MARTELLA et al., 2006; CALDERÓN et al., 2007; PANZERA et al., 2012; BUDASZEWSKI et al., 2014). Vários estudos analisam as diferenças entre as cepas de campo dos diferentes genótipos de CDV. No entanto, pouco se sabe sobre o efeito do uso de cepas vacinais atenuadas na evolução das cepas de campo.

Em países desenvolvidos, o uso extensivo de vacinas vivas atenuadas reduziu drasticamente a incidência da cinomose em cães, no entanto, a gama de hospedeiros suscetíveis continua a expandir. Apesar de vacinas de vírus atenuado serem eficazes e seguras para o uso em cães, o uso em espécies altamente suscetíveis ao CDV, como pandas e furões, não é recomendado por terem virulência suficiente para causar doença severa e morte nessas espécies (BARRETT et al., 1999). Ainda, uma vacina recombinante baseada no vetor canaripox, que expressa as proteínas H e F do CDV, é segura para todas as espécies de hospedeiros mas induz uma proteção menos duradoura em cães (STEPHENSEN et al., 1997), exigindo a re-imunização frequente, o que torna o seu uso inviável para animais silvestres.

A fim de gerar informações acerca da imunidade humoral anti-CDV e de mecanismos pelos quais o uso de vacinação baseada em cepas atenuadas pode influenciar a sua evolução, a presente tese será apresentada na forma de dois artigos científicos. O primeiro objetivo do presente trabalho foi o desenvolvimento e avaliação de uma vacina inativada bivalente baseada no vírus da raiva (RABV) expressando as glicoproteínas H/F de CDV para a proteção de cães e animais selvagens de forma segura e eficiente e avaliação da contribuição de anticorpos anti-H e anti-F para a proteção contra o CDV. O segundo objetivo da presente tese foi a realização de uma extensa análise filogenética e de recombinação utilizando todos os genomas completos de CDV disponíveis para detecção de possíveis eventos de recombinação homóloga. Ainda, um artigo de revisão de literatura sobre o uso da infecção por CDV em modelos animais para estudo da patogênese de morbilivírus foi incluída neste trabalho.

2. REVISÃO BIBLIOGRÁFICA

2.1. Taxonomia e morfologia

Os morbilivírus são membros da ordem *Mononegavirales* pertencentes à família *Paramyxoviridae* dentro da subfamília *Paramyxovirinae*. O gênero *Morbillivirus* inclui atualmente seis espécies, sendo os quais o vírus do sarampo (MeV), vírus da cinomose canina (CDV), o recentemente descoberto morbilivírus dos felinos (FeMV), morbilivírus dos cetáceos (CeMV), vírus da cinomose dos focídeos (PDV), vírus da peste dos pequenos ruminantes (PPRV) e vírus da peste bovina (RPV), o qual foi declarado oficialmente erradicado pela Organização Mundial de Saúde Animal (OIE) em 2011. Filogeneticamente, CDV e PDV são os mais estreitamente relacionados (**Figura 1**).

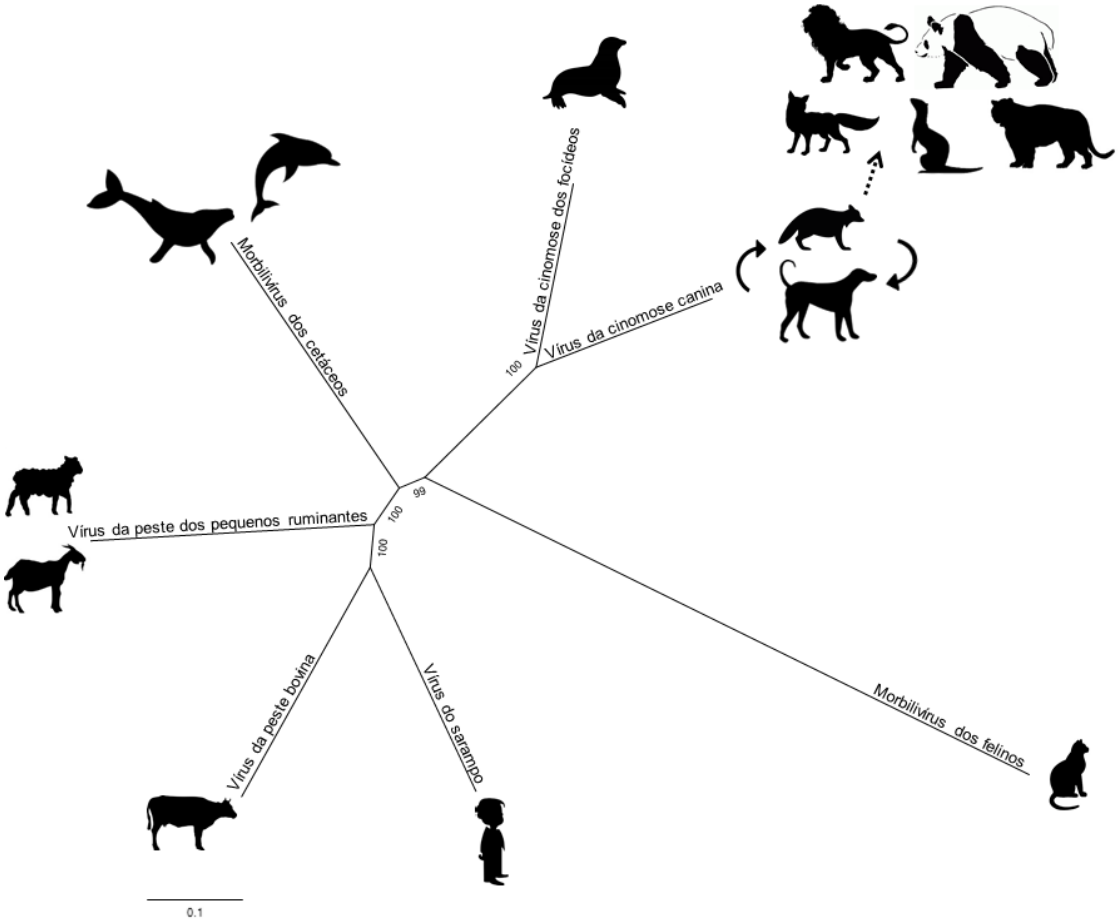

Figura 1. Árvore filogenética das diferentes espécies virais do gênero *Morbillivirus* com seus respectivos hospedeiros. Fonte: adaptado de Budaszewski & von Messling, 2016.

Técnicas moleculares são utilizadas para estudos de epidemiologia e dinâmica da circulação das diferentes cepas de CDV. Por ser a proteína mais variável, a análise filogenética da hemaglutinina é a base para classificação do vírus em diferentes genótipos. Os genótipos, no geral, correspondem à distribuição geográfica do vírus e não diferenciam espécies de hospedeiros. Nove genótipos principais são comumente descritos, apesar de discrepâncias entre diferentes autores: *America-1*, o qual inclui as cepas vacinais; *America-2*, inclui os isolados da América do Norte; *Europe*, o qual inclui isolados principalmente obtidos de cães da Europa, que também observou-se circulando na América do Sul (PANZERA et al., 2012), podendo então ser denominado *Europe/South America-1*; *Europe Wildlife*, inclui principalmente isolados obtidos de animais selvagens na Europa; *Arctic*, inclui isolados obtidos do Ártico, no entanto, também inclui isolados europeus; *Asia-1* e *Asia-2*, incluem isolados obtidos de diversos países asiáticos, divididos em dois grupos distintos; *South Africa*, inclui isolados obtidos na África do Sul; e *South America-2*, que inclui isolados de diferentes países da América do Sul (**Figura 2**) (MOCHIZUKI et al., 1999; CALDERÓN et al., 2007; KAPIL et al., 2008; GAMIZ et al., 2011; NEGRÃO et al., 2013; BUDASZEWSKI et al, 2014; ESPINAL et al., 2014, FISCHER et al., 2016).

Outras linhagens já foram descritas, havendo relatos de um novo genótipo circulando na América do Norte (RILEY; WILKES, 2015), um terceiro na América do Sul, chamado *South America-3* (ESPINAL et al., 2014; SARUTE et al., 2014), dois novos genótipos na Ásia, denominados *Asia-3* (ZHAO et al., 2010) e *Asia-4* (RADTANAKATIKANON et al., 2013; BI et al., 2015), e o genótipo *Africa-2* (KE et al., 2015). Além destes, frequentemente são descritas sequências relacionadas à cepa vacinal Rockborn, formando um *cluster* denominado *Rockborn-like* (MARTELLA et al., 2011; BUDASZEWSKI et al., 2014; KE et al., 2015).

A análise filogenética de outros fragmentos do genoma também é comumente realizada, incluindo a análise da região Fsp (peptídeo sinal da proteína F) (SARUTE et al., 2013; SARUTE et al., 2014), da proteína F (RADTANAKATIKANON et al., 2013; ROMANUTTI et al, 2016), proteína N (YOSHIDA et al., 1999; TAN et al., 2011) e proteína P (RADTANAKATIKANON et al., 2013).

2.2. Genoma, transcrição e replicação

Os vírions são pleomórficos, com diâmetro de cerca de 150 nm e consistem de um envelope lipídico que circunda o nucleocapsídeo. O envelope é derivado da membrana plasmática da célula hospedeira e contém as glicoproteínas transmembranares que formam projeções do tipo espícula (**Figura 3**). A proteína da matriz é não glicosilada e intimamente associada à membrana na sua superfície interna, interagindo com a proteína do nucleocapsídeo e com as caudas citoplasmáticas das glicoproteínas de superfície. As proteínas do nucleocapsídeo estão firmemente associadas ao RNA viral, protegendo-o.

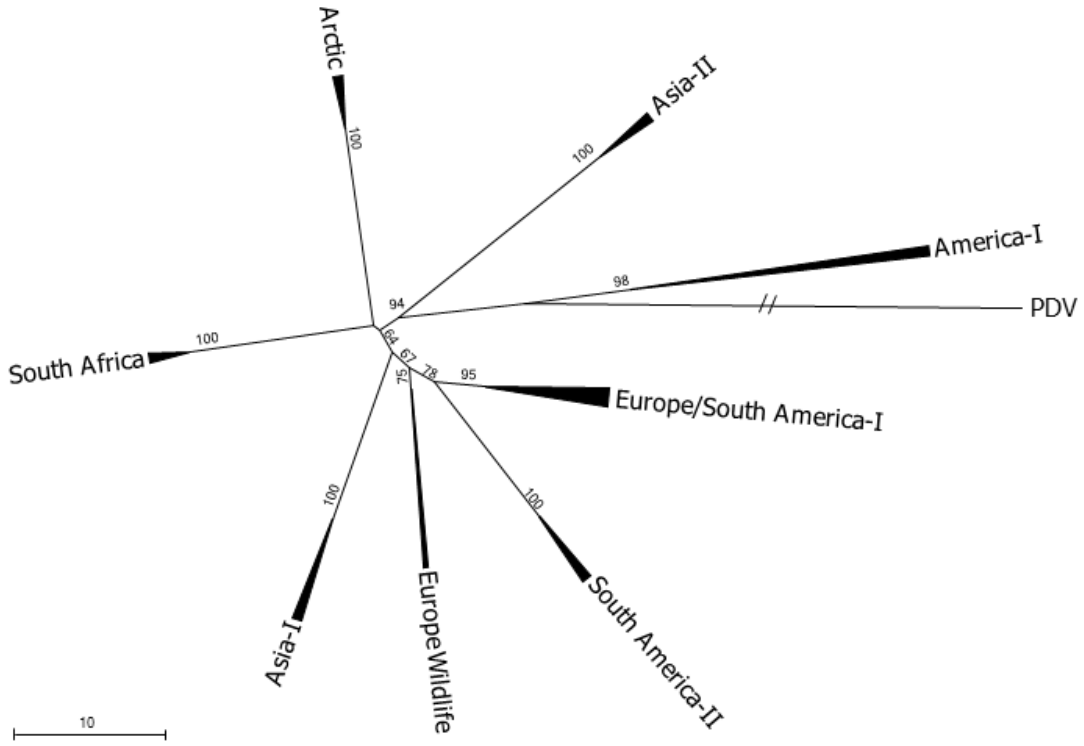

Figura 2. Árvore filogenética ilustrando os genótipos de CDV mais comumente descritos na literatura. Análise realizada baseada em sequências parciais de nucleotídeos do gene da hemaglutinina através do software MEGA6 utilizando o método Neighbor-Joining e modelo de substituição Tamura-3. Valores de *bootstrap* (1000 réplicas) >60 (60%) estão indicados nos nodos internos. Fonte: Budaszewski, R. F.

O genoma é constituído de RNA de fita simples com sentido negativo, contendo 15.690 nucleotídeos (nt). Cada proteína do nucleocapsídeo abrange um fragmento de seis nt do genoma, razão pela qual todos os morbilivírus seguem à "regra de seis", ou seja, o comprimento completo do genoma é necessariamente múltiplo de seis. O genoma é flanqueado por uma sequência líder na extremidade 5’ e uma sequência *trailer* (reboque)

na extremidade 3’, essenciais para a replicação viral. O genoma contém seis unidades de transcrição dispostas linearmente e são separadas por regiões não traduzidas (UTRs), que contêm sinais essenciais de transcrição e tradução. As unidades de transcrição codificam pelo menos oito proteínas: nucleocapsídeo, fosfoproteína, de matriz, de fusão, hemaglutinina e grande polimerase. Fases de leitura aberta (ORFs) sobrepostas originam as proteínas adicionais V e C.

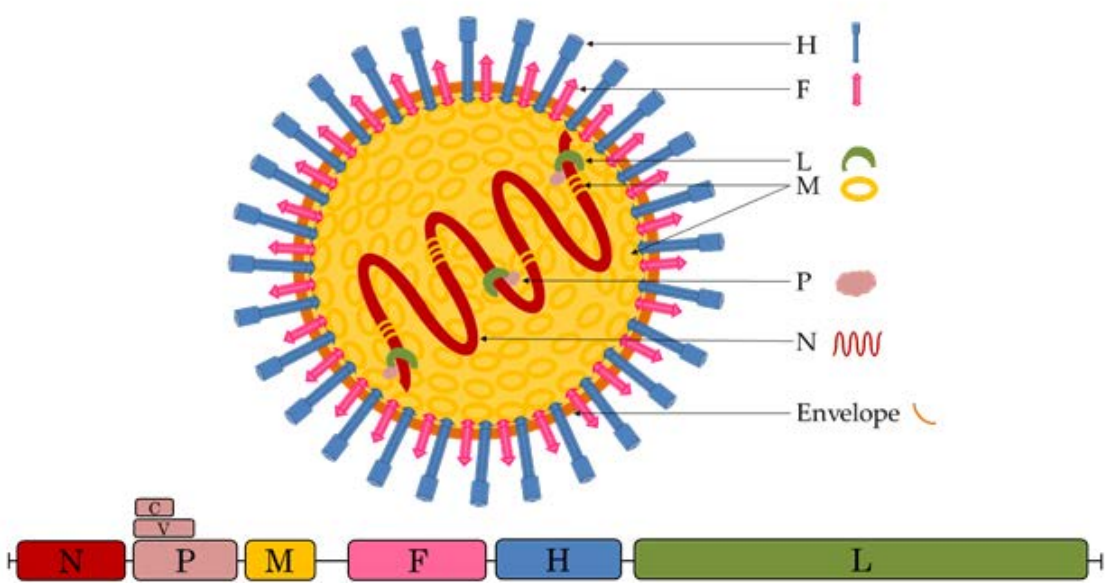

Figura 3. Diagrama da estrutura e genoma do CDV. O vírus é envelopado e contém duas glicoproteínas de membrana, H e F. A proteína da matriz interage com suas caudas citoplasmáticas e com a proteína do nucleocapsídeo, a qual reveste o genoma. As proteínas P e L estão intimamente associadas ao N, formando o complexo RNP. Fonte: Budaszewski, R. F.

2.3. Proteínas virais

2.3.1. Proteínas do complexo RNP (ribonucleoproteína)

2.3.1.1. Nucleocapsídeo

O gene N situa-se na extremidade 3' do genoma. A sua ORF codifica uma proteína de 523 aminoácidos com peso molecular de cerca de 58 kDa (RIMA, 1983). Uma vez que o genoma é transcrito de modo sequencial, a proteína N é a mais abundantemente produzida e está firmemente associada com o ácido nucleico. Ela serve como molde para a síntese de RNA encapsidando o genoma em um nucleocapsídeo RNase-resistente. A proteína do nucleocapsídeo associa-se à polimerase (P-L) durante a transcrição e

replicação formando o complexo RNP, e com a proteína H durante a maturação do vírion (LISTON et al., 2014).

2.3.1.2. Fosfoproteína

O gene P codifica três proteínas: P, um cofator essencial da polimerase, e C e V, as quais são produzidas por tradução alternativa de ORFs sobrepostas (BELLINI et al., 1985). A proteína V possui um papel importante na inibição imunológica agindo como antagonista de interferon e inibidor de resposta de citocinas, enquanto a proteína C atua como um fator de infectividade (VON MESSLING; SVITEK; CATTANEO, 2006).

2.3.1.3. Polimerase

A polimerase (L) é responsável pelas funções catalíticas de RNA-polimerase dependente de RNA (RdRp). É essencialmente conservada, no entanto, ao analisar alinhamentos de proteínas L de diferentes morbilivírus, percebe-se a presença de duas regiões altamente variáveis, denominadas dobradiças (*hinges* - *hinge* 1 e *hinge* 2) que podem formar limites de domínios (MCILHATTON; CURRAN; RIMA, 1997; DUPREX; COLLINS; RIMA, 2002). Estas regiões variáveis encontram-se entre três domínios conservados, designados como I, II e III (MCILHATTON; CURRAN; RIMA, 1997). Acredita-se que o complexo RdRp, o qual inclui a ribonucleoproteína e a fosfoproteína, executa todas as atividades requeridas para o *capping* e metilação do RNA.

2.3.2. Proteínas do envelope

2.3.2.1. Hemaglutinina

A proteína H é uma glicoproteína de membrana integral de tipo II e é responsável pela ligação ao receptor da célula hospedeira, ativando assim a proteína de fusão. Ela é formada por uma cabeça globular C-terminal que se estende a partir do núcleo viral em uma haste longa transmembranar com uma curta cauda citoplasmática N-terminal. A maior parte dos resíduos de aminoácidos situados na região de ligação ao receptor SLAM (*signaling lymphocytic activation molecule* ou molécula sinalizadora da ativação de linfócitos) são carregados negativamente formando uma porção ácida. Por outro lado, os resíduos do receptor SLAM que interagem com a proteína H estão carregados positivamente formando uma porção básica. Estas áreas da proteína H e do SLAM são

altamente conservadas em morbilivírus e seus hospedeiros, respectivamente (HASHIGUCHI et al., 2007).

Anticorpos neutralizantes produzidos contra a hemaglutinina são importantes para a imunidade protetora, incluindo os resíduos do sítio de ligação ao SLAM (COLF; JUO; GARCIA, 2007; HASHIGUCHI et al., 2007). Estudos utilizando vírus recombinantes indicam que a proteína H, e não a proteína F, é a principal determinante da eficiência de fusão e, *in vitro*, determina a citopatogenicidade (VON MESSLING et al., 2001). *In vitro* a fusogenicidade é observada como formação de sincício (**Figura 4**) (VON MESSLING et al., 2001). A proficiência de formação de sincícios por certas cepas de CDV e seu nível de atenuação podem ser correlacionadas: quanto mais atenuada a cepa, maior a sua fusogenicidade (COSBY et al., 1981). Ela determina o efeito citopático e tropismo por certos tecidos (STERN et al., 1995; VON MESSLING et al., 2001).

Entre as proteínas codificadas pelo CDV, a proteína H é a mais variável (assim como quando comparada aos outros morbilívírus) a qual parece ser a causa da grande gama de hospedeiros.

2.3.2.2. Proteína de fusão

O gene F codifica para a menor glicoproteína dos morbilivírus a qual é sintetizada como um precursor inativo, F0. Ela precisa sofrer um processo de clivagem proteolítica pela protease furina intracelular para ser convertida em sua forma biologicamente ativa. Os glicopolipeptídeos resultantes F1 e F2 são mantidos intimamente associados por pontes dissulfeto (SCHEID; CHOPPIN, 1977). As proteínas de fusão são sintetizadas como monômeros, mas durante o processo de fusão são dispostas como trímeros, formando poros de homotrímeros inseridos na membrana da célula alvo (HERNANDEZ et al., 1996). Após a trimerização, a clivagem ocorre e a proteína torna-se ativada, um passo crítico para a infectividade. Assim, proteínas de fusão ativadas são compostas por uma subunidade ancorada à membrana (F1) e uma subunidade distal à membrana (F2). A ativação ocorre em pH neutro e inicia-se com a ligação da proteína H ao receptor da célula. A proteína de ligação, em seguida, interage com F, e esta interação conduz ao seu rearranjo e formação de uma estrutura estável após inserção à membrana celular (BAKER et al., 1999).

A UTR entre as proteínas M e F tem um comprimento aumentado, e enquanto no vírus do sarampo ela é em sua totalidade uma região não traduzida, em outros morbilivírus

vários códons de iniciação são encontrados, razão pelo qual essa longa região é chamada Fsp (*F protein signal peptide* – peptídeo sinal da proteína F). Estudos utilizando vírus recombinantes com deleção dessa região ou com substituição por outras UTRs de CDV demonstraram que a sua deleção não causa alterações *in vitro* e *in vivo*¸ enquanto sua substituição alterou a virulência, resultando em animais infectados que se recuperaram ou sofreram infecção do SNC (ANDERSON; VON MESSLING, 2008).

2.3.2.3. Proteína da matriz

A proteína M é a proteína mais abundante no vírion e interage com o envelope lipídico, as caudas citoplasmáticas das glicoproteínas H e F, e a RNP. Assim, a proteína M atua como uma ligação estrutural entre eles, com um papel fundamental na montagem e brotamento das partículas virais.

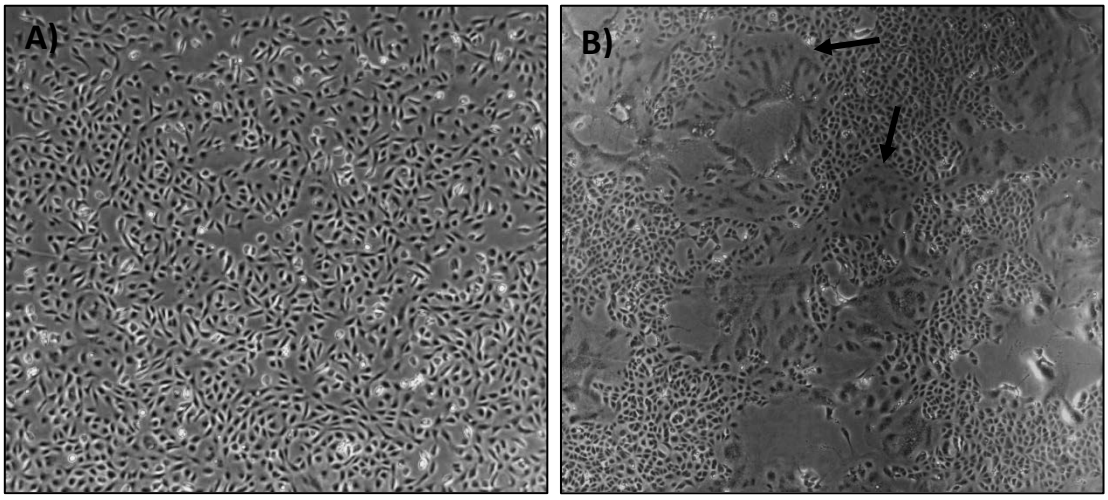

Figura 4. Observação de sincícios em células VerodogSLAMtag. Em A) células com 80% de confluência e aparência normal. Em B) células infectadas com CDV apresentando a formação de sincícios (setas). Aumento = 40X. Fonte: Budaszewski, R. F.

2.4. Mecanismos de variabilidade genética

A principal causa da variabilidade genética dos morbilívirus, assim como de outros vírus de RNA não-segmentados, são mutações pontuais causadas por erros intrínsecos da polimerase. Os erros da polimerase geram uma grande quantidade de variantes genéticas as quais sofrem pressão de seleção, sendo ou não mantidos no genoma. Foi demonstrado que uma forte pressão de seleção nos aminoácidos 530 e 549 da hemaglutinina, resíduos que fazem parte da região responsável pela ligação ao receptor celular, está associada com múltiplos eventos de *spillover* - quando ganha capacidade de infectar novos hospedeiros - para novas espécies. A grande maioria das

substituições de aminoácidos detectadas nestas regiões está associada à vírus isolados de hospedeiros silvestres (MCCARTHY; SHAW; GOODMAN, 2007).

Eventos de recombinação homóloga também são importantes mecanismos de evolução viral, gerando variantes virais sob as quais a pressão de seleção atua. Ela ocorre quando pelo menos duas variantes virais co-infectam uma célula do hospedeiro e trocam segmentos genômicos durante a replicação. A recombinação é homóloga quando ocorre nos mesmos pontos (chamados também de *breakpoints*) em ambos os genomas parentais (LAI et al., 1992).

Dois mecanismos de recombinação são aceitos para vírus de RNA, um replicativo e um não-replicativo. O modelo replicativo envolve a transferência do complexo de replicação de uma fita de RNA para outra, enquanto o modelo não replicativo exige uma quebra na fita de RNA que facilite a formação de um genoma híbrido. O modelo mais aceito para vírus de RNA é o chamado “escolha de cópia” (do inglês *copy choice*) (SIMON-LORIERE; HOLMES, 2011), em que a enzima responsável pela replicação troca de uma fita de RNA (doadora) para outra (receptora) durante a síntese, enquanto permanece ligada à cadeia nascente, assim gerando uma molécula de RNA com dois ancestrais. O nível de identidade entre a fita doadora e a receptora, a carga viral, a cinética de transcrição e estruturas secundárias são fatores que influenciam essa troca (BAIRD et al., 2006).

Apesar de recorrente em vírus RNA de sentido positivo (LAI, 1992; WOROBEY; HOLMES, 1999), tais eventos são menos comuns em vírus RNA de sentido negativo (CHARE et al., 2003; HAN; WOROBEY, 2011) e considerados especialmente raros em paramixovírus devido à organização do seu genoma. Eventos de recombinação detectados em paramixovírus geram controvérsia pela dificuldade de elucidar seu mecanismo, no entanto, relatos em diferentes espécies virais sugerem que sua ocorrência não é incomum.

Possíveis eventos de recombinação já foram descritos para algumas espécies de morbilivírus, incluindo CDV (MCCARTHY, SHAW, GOODMAN, 2007; HAN et al., 2008a), MeV (SCHIERUP et al., 2005) e FeMV (PARK et al., 2014). Considerando outros paramixovírus, pode-se citar o vírus da doença de Newcastle com diversos relatos (HAN et al., 2008b; QIN et al., 2008; ZHANG et al., 2010; YIN et al., 2011; CHONG et al., 2011; SATHARASINGHE et al., 2016).

Em relação ao CDV, os principais sinais de recombinação envolvem as glicoproteínas H e F, o que significa um grande ganho evolutivo ao obter maior variação nas proteínas responsáveis pelo tropismo celular as quais estão sujeitas à ação de anticorpos neutralizantes. O envolvimento destas proteínas também deve ser considerado ao realizar análise filogenética com fins de genotipagem, pois tais eventos provocam incongruências na filogenia que podem causar classificações errôneas (ARENAS; POSADA, 2010). Em casos de sinais de recombinação positivos, é sempre importante considerar o contexto epidemiológico e a localização dos *breakpoints*, para identificar falsos-positivo artificialmente gerados em laboratório (AFONSO et al., 2008; HAN; WOROBEY, 2011; SONG et al., 2011).

2.5. Hospedeiros

Embora a maioria dos morbilivírus possua uma gama limitada de hospedeiros, o CDV pode ser considerado promíscuo, infectando muitos carnívoros e com *spillover* esporádico para novas espécies. É endêmico em cães domésticos e guaxinins (MACHIDA et al., 1993), e pode causar doença severa e eliminar populações de animais selvagens. Há relatos de mortalidade em massa em furões-de-patas-negras (THORNE; WILLIAMS, 1988; WILLIAMS et al., 1988), raposas (BALLARD et al., 2001; TIMM et al., 2009), cães selvagens (ALEXANDER; APPEL, 1994), martas (ZHAO et al., 2010), coiotes (DAVIDSON et al., 1992), hienas (ALEXANDER et al., 1995) entre outros.

É de grande importância para algumas espécies de animais selvagens cativos em zoológicos e santuários, os quais são altamente sensíveis à infecção e para os quais não há uma vacina segura disponível no momento. Surtos de CDV em espécies ameaçadas como pandas vermelhos (BUSH et al., 1976) e pandas gigantes (GUO et al., 2013) são de grande preocupação. A cinomose é também um risco para grandes felinos, com surtos notificados causando mortalidade de leões, leopardos, tigres e onças em zoológicos americanos (APPEL et al., 1994) e no Parque Nacional do Serengeti, Tanzânia (ROELKE-PARKER et al., 1996). É interessante observar que os gatos domésticos podem soroconverter (APPEL et al., 1974), mas não há relato de doença relacionada à infecção natural. Recentemente, o CDV foi capaz de infectar também primatas não humanos, com vários surtos sendo relatados (SUN et al., 2010; QIU et al., 2011), principalmente na Ásia. As taxas de morbidade e mortalidade também variam muito,

sendo os furões domésticos ainda mais suscetíveis ao CDV que os cães, para os quais a letalidade de cepas de campo pode atingir 100% (VON MESSLING et al., 2003).

2.6. Patogenia, receptores e tropismo tecidual

Até o momento, duas proteínas celulares foram identificadas como receptores de CDV: SLAM (também conhecido como CD150) (TATSUO et al., 2000) e, recentemente, nectina-4 (também conhecido como PVRL4 - *poliovirus receptor-related 4* ou receptor relacionado a poliovirus 4) (MÜHLEBACH et al., 2011; NOYCE et al., 2011). As mesmas moléculas servem como receptores para outros morbilivírus em seus respectivos hospedeiros, o que poderia explicar por que espécies de vírus diferentes apresentam tropismo tecidual equivalentes.

A infecção celular é iniciada pela ligação da glicoproteína H aos seus receptores celulares. O CDV utiliza a glicoproteína de membrana SLAM como o principal receptor de entrada (**Figura 5a**) (TATSUO et al., 2000). Esta é expressa seletivamente nas células do sistema imune (timócitos, linfócitos T e B ativados, células dendríticas maduras, macrófagos e plaquetas) e possui dois domínios extracelulares da superfamília de imunoglobulinas, V e C2. A proteína H interage com seu domínio variável e também interage com baixa afinidade com outras moléculas SLAM agindo como um auto-ligante, o que facilita a propagação do vírus. Sua distribuição e função podem explicar o tropismo e a natureza imunossupressora desses vírus (MAVADDAT et al., 2000). Vale ressaltar que alguns morbilivírus são capazes de usar SLAM de espécies não hospedeiras como receptores, embora com eficiência reduzida (TATSUO et al., 2000).

Um segundo receptor celular, nectina-4, desempenha um papel importante na disseminação agindo como receptor de saída, facilitando a amplificação e subsequente liberação do vírus pelas secreções das vias aéreas (**Figura 5b**) (MÜHLEBACH et al., 2011; NOYCE et al., 2011), uma vez que as gotículas respiratórias de hospedeiros infectados funcionam como veículos de transmissão para disseminação de vírus infeccioso às células epiteliais do trato respiratório de hospedeiros susceptíveis. A nectina-4 é uma proteína de adesão que contém domínios semelhantes a imunoglobulinas, assim como SLAM. A nectina-4 também já foi detectada em cérebros de cães, e o CDV

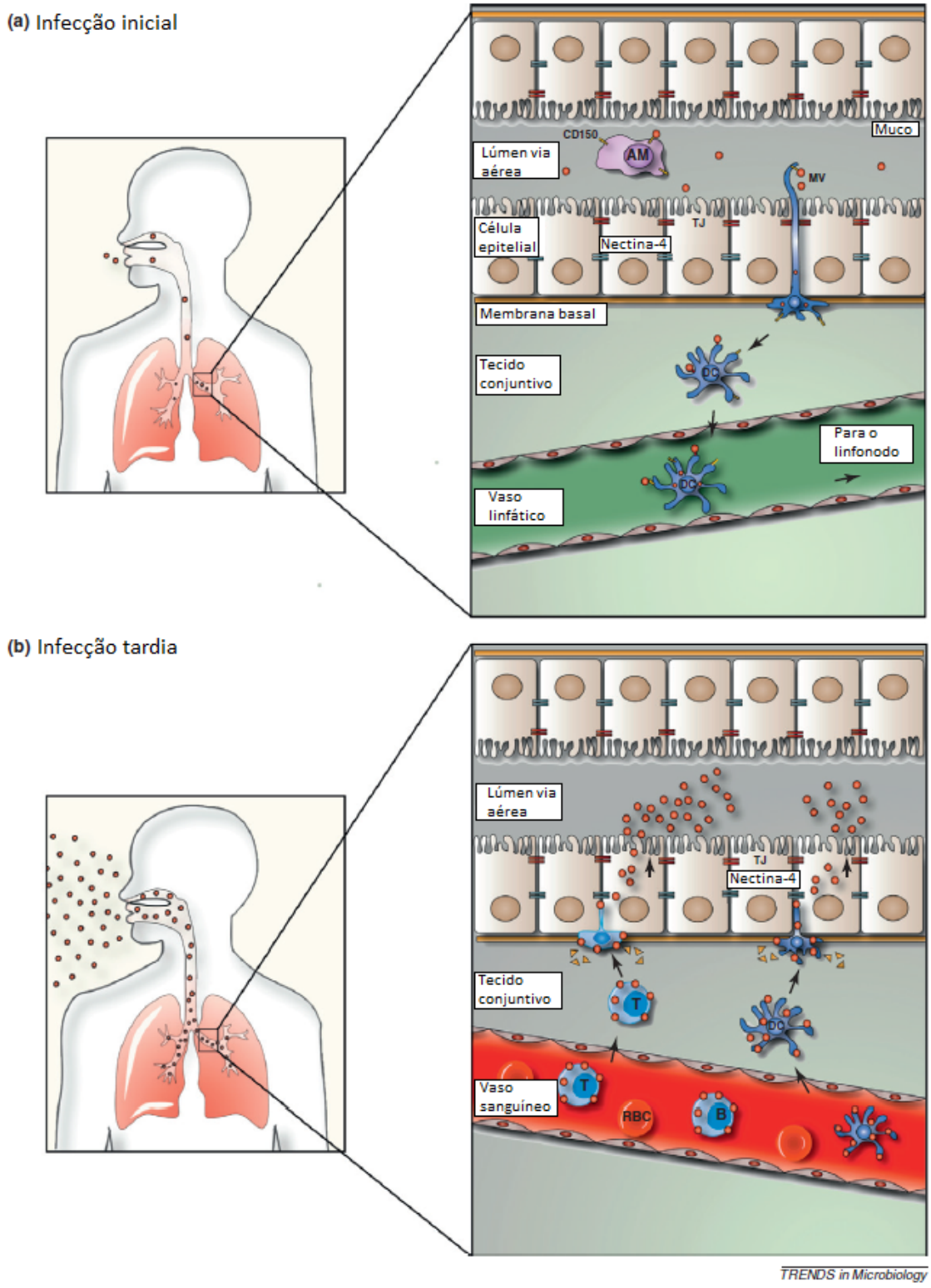

Figura 5. Receptores de entrada e saída de morbilivírus. A) No momento inicial da infecção, o vírus infecta o hospedeiro utilizando o receptor CD150 expresso em macrófagos alveolares (AMs) ou células dendríticas (DCs) presentes no trato respiratório, e é em seguida transportado pelas células imunes infectadas através dos vasos linfáticos aos linfonodos locais, onde infectam linfócitos B e T, replicam e causam a primeira viremia. B) Em um momento tardio da infecção, linfócitos e células dendríticas migram aos pulmões, induzem a degradação da membrana basal e circulam entre as células epiteliais, as quais infectam ao entrar em contato com a nectina-4 expressas nas junções aderentes (TJ). Há replicação viral e disseminação no lúmen das vias aéreas

através da superfície apical, e o vírus é exalado pelo hospedeiro. MV = vírus do sarampo; RBC = células sanguíneas vermelhas. Fonte: adaptado de Noyce & Richardson (2012).

é preferencialmente detectado em neurônios que expressam este receptor, no entanto, o seu papel na infecção do SNC ainda não está esclarecido (PRATAKPIRIYA et al., 2012).

O vírus infecta o hospedeiro através do trato respiratório, pela inalação de aerossóis, e a replicação primária ocorre no tecido linfóide do trato respiratório superior [células que possuem a glicoproteína SLAM (SLAM+)] (DE SWART et al., 2007; LEMON et al., 2011). A progressão da infecção depende da resposta imune do animal. Anticorpos IgM são produzidos nas primeiras duas semanas após infecção (VANDEVELDE; ZURBRIGGEN, 2005) e a magnitude da resposta humoral pode ser correlacionada com a progressão da doença. A imunidade protetora humoral é baseada, primeiramente, na produção de anticorpos anti-N, e em seguida pela produção de anticorpos anti-H e anti-F (MIELE; KRAKOWKA, 1983; RIMA et al., 1991). Quando o animal não gera uma resposta imune humoral adequada, este desenvolve uma infecção aguda com sinais clínicos frequentemente letais, enquanto animais que produzem anticorpos em um título detectável entre 1-2 semanas, conseguem eliminar o vírus ou desenvolvem uma infecção persistente. Uma infecção letal experimental em furões, os quais não são capazes de controlar a infecção por cepas de campo de CDV, demonstrou o comprometimento do sistema imune. Primeiramente, linfócitos T e B circulantes são infectados, seguidos de linfócitos residentes em tecidos linfóides secundários. Após, timócitos primários são infectados, e em uma semana o sistema imune dos animais é completamente comprometido, levando à imunossupressão, e assim o vírus replica intensamente e se distribui por todo o organismo (VON MESSLING; MILOSEVIC; CATTANEO, 2004).

Observa-se um primeiro pico febril 3-6 dias após a infecção, o qual indica a distribuição do vírus a todos os tecidos linfáticos com a primeira viremia (IWATSUKI et al., 1995; ZHU et al., 1997). A segunda viremia ocorre vários dias depois, quando se observam sinais clínicos devido à infecção de células epiteliais como início de erupção cutânea, seguido de secreção nasal, conjuntivite e anorexia (BLIXENKRONE-MØLLER, 1989; VON MESSLING et al., 2003). Devido à imunossupressão e a linfopenia, comumente infecções secundárias resultam em sinais gastrointestinais e respiratórios. Cães e furões infectados, dependendo da cepa e duração da doença, podem desenvolver encefalomielite aguda (BONAMI; RUDD; VON MESSLING, 2007). Além disso,

hiperqueratose do plano nasal e coxins são frequentemente observados. Na fase final da infecção, o vírus utiliza a nectina-4 como receptor de saída para atravessar as barreiras epiteliais e garantir a transmissão para hospedeiros suscetíveis (MÜHLEBACH et al., 2011).

2.7. Controle

A única forma eficiente de controle da cinomose é através da vacinação sistemática. Apesar do sucesso atingido ao erradicar o vírus da peste bovina e do presente esforço para erradicação do vírus do sarampo, o mesmo não é realístico para o vírus da cinomose, devido ao grande número de hospedeiros suscetíveis.

Um título de soroneutralização ≥100 é considerado padrão para imunidade protetora contra o CDV em cães (BUDD, 1981; MONTALI et al., 1983). Apesar da soroconversão ser o ideal, alguns relatos demonstraram que títulos ≤100 podem ser protetores contra infecção (MONTALI et al., 1983; WIMSATT et al., 2003). Além disso, o tempo para soroconversão varia entre as diferentes espécies de hospedeiros - de 48 horas pós-vacinação em furões até 41 dias em martas-pescadoras (*Martes pennanti*) (PEPER et al., 2016).

Animais vacinados com vacina de vírus vivo modificado/atenuado (MLV - *modified live vaccine*) desenvolvem forte resposta humoral e celular de longa duração, semelhante à infecção natural, e estão protegidos contra o desafio. Animais vacinados com vacina inativada ou com vírus do sarampo não são capazes de desenvolver imunidade celular detectável e produzem resposta humoral parcial (APPEL et al., 1984). Em um estudo utilizando vacinas de subunidades, cães vacinados com CDV em ISCOMs (*immune-stimulating complexes* – complexos imunoestimulantes), que continham as proteínas H e F, desenvolveram imunidade protetora contra infecção. Já cães vacinados com o vírus do sarampo em ISCOMs contendo sua proteína F purificada ou vírus completo purificado, desenvolveram somente imunidade parcial contra infecção por CDV (DE VRIES et al., 1988).

A grande maioria das vacinas comerciais é baseada em cepas MLV. A cepa Onderstepoort, isolada na década de 50, é a mais comumente utilizada nas formulações. Outras cepas comumente utilizadas são a Snyder Hill e Lederle (MARTELLA; BUONAVOGLIA, 2008). A cepa Rockborn ainda é encontrada em algumas formulações no mercado, apesar de relatos de reter patogenicidade (MARTELLA et al., 2011). Em

relação à duração da imunidade vacinal, estudos indicam que cães vacinados com MLV estão protegidos por pelo menos 3-4 anos, sendo que 100% dos animais sobrevivem ao desafio e 90% não desenvolvem sinais clínicos de cinomose (ABDELMAGID et al., 2004; GORE et al., 2004).

Além das vacinas MLV, há também disponível no mercado uma vacina recombinante baseada em vetor canaripox (rCDV) que expressa as proteínas H e F da cepa Onderstepoort (PARDO; BAUMAN; MACKOWIAK, 1997). A vacina é eficiente e segura, pois o vírus não replica eficientemente em linfócitos e no SNC de mamíferos e não há risco de reversão à virulência. No entanto, um estudo de vacinação em tigres comparou a imunidade humoral induzida por uma vacina MLV e a vacina rCDV, e observou que os animais vacinados com a MLV desenvolveram uma resposta imune humoral mais robusta e duradoura (SADLER et al., 2016). Em alguns países, esta vacina também é recomendada para uso em furões domésticos; no entanto, por ser uma vacina polivalente, alguns veterinários não a recomendam para esses animais.

Preferencialmente, furões e animais selvagens não devem ser vacinados com vacinas MLV, pois estas mesmo atenuadas podem permanecer patogênicas para as espécies altamente suscetíveis à infecção, com risco de causar doença induzida pela vacinação (DURCHFELD et al., 1990). Novas tecnologias estão sendo desenvolvidas para atender a essa necessidade, principalmente vacinas baseadas em vírus recombinantes e vacinas de DNA.

Além do vetor canaripox já descrito, outros vírus têm sido utilizados para geração de recombinantes, incluindo adenovírus (FISCHER et al., 2002), vírus da raiva (WANG et al., 2012; WANG et al., 2014) e vírus vaccínia (WILD et al., 1993; STEPHENSEN et al., 1997; WELTER et al., 1999; WELTER et al., 2000). As vacinas de subunidades têm se mostrado seguras e eficientes, sendo baseadas em plasmídeos de DNA codificando as proteínas N, H ou F de CDV (SIXT et al., 1998; CHERPILLOD et al., 2000; DAHL et al., 2004; NIELSEN et al., 2009; NIELSEN et al., 2012). Estudos relatam indução de anticorpos neutralizantes após duas doses e proteção contra desafio em filhotes de marta (JENSEN et al., 2009). Além disso, uma vacina recombinante baseada em adenovírus, quando administrada por via subcutânea (FISCHER et al., 2002), e uma vacina de DNA (JENSEN et al., 2015) demonstraram ser eficientes em induzir proteção em filhotes na presença de imunidade passiva.

Uma desvantagem das vacinas de DNA, ao comparar com alternativas mais potentes, é gerar um nível limitado de proteção. Esta limitação pode ser eficientemente superada com diferentes estratégias, como co-expressão de citocinas, direcionamento a células específicas do sistema imune, administração por diferentes métodos e adição de adjuvantes, no entanto, poucas estratégias têm sido estudadas para vacinas de CDV. Até o momento, alguns adjuvantes foram utilizados como lipídios catiônicos (FISCHER et al., 2003) e ISCOMs (DE VRIES et al., 1988).

Estudo realizado por Norrby e colaboradores (1986) comparou a resposta imune desenvolvida em cães imunizados com as proteínas H e F purificadas, separadamente. Após desafio com uma cepa virulenta, observou-se que animais de ambos os grupos desenvolveram forte resposta imune humoral e celular, no entanto, somente animais imunizados com a proteína H desenvolveram sinais clínicos, sugerindo que a proteína F é capaz de bloquear a infecção e, quando ocorre replicação, pode prevenir o aparecimento de sinais clínicos.

Apesar de diversos relatos de doença em cães vacinados terem sido publicados, ainda não está esclarecido se as falhas vacinais são decorrentes de baixa reatividade cruzada entre as cepas atenuadas e as cepas de campo circulantes, ou devido a falhas em protocolos vacinais e/ou armazenamento dos produtos. Na maioria dos casos de cães filhotes recém vacinados que desenvolvem a doença, esta é causada por infecção por cepa de campo antes do desenvolvimento da imunidade ativa. A interferência dos anticorpos maternais através da imunidade passiva é uma das maiores dificuldades para vacinação de filhotes, podendo impedir a imunização ativa (MARTELLA; ELLIA; BUONAVOGLIA, 2008).

Considerando que a titulação de anticorpos maternais em cada filhote é impraticável, o protocolo vacinal recomendado para vacinas MLV é vacinação a cada 3-4 semanas entre as primeiras 8 a 16 semanas de idade. Assim, assegura-se que o filhote receba pelo menos uma dose vacinal em uma idade em que a imunidade materna é insuficiente para impedir a imunização ativa. A primovacinação com qualquer agente infeccioso antes das 6 semanas de idade, mesmo na ausência de imunidade passiva, não é recomendada devido à imaturidade imunológica (SCHULTZ; CONKLIN, 1998; WELBORN et al., 2011).

3. ARTIGOS CIENTÍFICOS

Os materiais e métodos e os respectivos resultados que compõem esta tese serão apresentados a seguir na forma de artigos científicos.

3.1. Artigo 1 – Influence of vaccine strains on the evolution of canine distemper virus

O presente projeto foi finalizado e um artigo científico foi publicado na revista *Infection, Genetics and Evolution*. Este trabalho será apresentado no formato de publicação. O material suplementar correspondente pode ser encontrado na sessão “Anexos”.

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Research paper

Influence of vaccine strains on the evolution of canine distemper virus

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ABSTRACT

Canine distemper virus (CDV) is a major dog pathogen belonging to the genus *Morbillivirus* of the family *Paramyxoviridae*. CDV causes disease and high mortality in dogs and wild carnivores. Although homologous recombination has been demonstrated in many members of *Paramyxoviridae*, these events have rarely been reported for CDV. To detect potential recombination events, the complete CDV genomes available in GenBank up to June 2015 were screened using distinct algorithms to detect genetic conversions and incongruent phylogenies. Eight putative recombinant viruses derived from different CDV genotypes and different hosts were detected. The breakpoints of the recombinant strains were primarily located on fusion and hemagglutinin glycoproteins. These results suggest that homologous recombination is a frequent phenomenon in morbillivirus populations under natural replication, and CDV vaccine strains might play an important role in shaping the evolution of this virus.

1. Introduction

Canine distemper virus (CDV) affects a great number of animal species, primarily terrestrial carnivores. In dogs, CDV causes a systemic disease with respiratory, nervous and digestive signs. CDV is a small, enveloped virus belonging to the genus *Morbillivirus* within the family *Paramyxoviridae* of the Mononegavirales order, possessing a non-segmented single-stranded negative RNA genome (15,690 kb long) encoding six structural proteins: hemagglutinin (H), fusion (F), envelope-associated matrix (M), phospho- (P), large polymerase (L) and nucleocapsid (N) (Lamb and Parks, 2013). Among these, the F and H glycoproteins are protective antigens that induce humoral antibodies (Hirama et al., 2003; Wild and Buckland, 1997).

Based on the H gene, which has the highest genetic variability, CDV strains segregate into at least ten major geographically related genetic lineages: America-I (including the vaccine strains), America-II, Asia-I, Asia-II, Europe Wildlife, Arctic, South Africa, South America-I/Europe, South America-II and Rockborn-like (Budaszewski et al., 2014; Calderón et al., 2007; Martella et al., 2006; Panzera et al., 2012; Woma et al., 2010). Moreover, in Mexico and Asia, some CDV strains substantially diverge and might represent novel geographically related groups (Gámiz et al., 2011; Radtanakatikanon et al., 2013).

The introduction and extensive use of live-attenuated CDV vaccines in the 1950s has drastically reduced the incidence of canine distemper in dogs. However, CDV infections and diseases in immunized dogs have been observed on several occasions (Martella et al., 2011). Wild-type CDV strains are genetically divergent from the vaccine strains, and a number of studies have described antigenic differences between these variants, especially considering the influence of N-linked glycans at the H protein (Sawatsky and von Messling, 2010). However, the influence of vaccine immunity on CDV evolution remains unknown.

Copy choice recombination plays an important role in the evolution of positive sense RNA viruses (Lai, 1992; Nagy and Simon, 1997; Weber et al., 2015), whereas negative sense RNA viruses were characterized by low rates of recombination (Chare et al., 2003; Schierup et al., 2005). For the *Paramyxoviridae* family, recombination events have previously been documented in some species, primarily in measles virus (Schierup et al., 2005), respiratory syncytial virus (Spann et al., 2003) and Newcastle disease virus (Chare et al., 2003; Miller et al., 2009), but there are limited studies concerning CDV (Han et al., 2008; Ke et al., 2015; McCarthy et al., 2007). Thus, the aim of the present study was to perform extensive phylogenetic and recombination analyses using the available complete CDV genomes of different host species to detect potential recombination events that might shape viral genetic diversity.

2. Material and methods

2.1. Datasets

All CDV complete genome sequences deposited in GenBank (up to June 2015) were retrieved from the National Center for Biotechnology

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1567-1348/

Table 1
GenBank accession numbers of the complete CDV genome sequences used in the recombination analysis.

Accession no.	Strain	Host[a]	Country[a]	Genotype[b]	Year[a]	Reference
AY443350.1	00-2601	Raccoon	USA	America-II	2000	Lednicky et al., 2004
AY649446.1	01-2689	Raccoon	USA	America-II	2001	Lednicky et al., 2004
EU716337.1	164071	Dog	USA	America-II	2004	Unpublished
KJ123771.1	171391-513	Dog	USA	America-II	2004	Unpublished
AF164967.1	A75/17 (Cornell)	Dog	USA	America-II	–	Unpublished
AY386316.1	5804	Dog	Germany	South America-I/Europe	–	von Messling et al., 2003
KJ466106.1	CDV SY	Raccoon	China	Asia-I	2012	Cheng et al., 2015
KC427278.1	Hebei	Mink	China	Asia-I	2008	Unpublished
HM852904.1	MKY-KM08	Rhesus monkey	China	Asia-I	2008	Qiu et al., 2011
AB687720.2	CYN07-dV	Cynomolgus monkey	Japan	Asia-I	2008	Sakai et al., 2013
AB687721.2	CYN07-hV	Cynomolgus monkey	Japan	Asia-I	2008	Sakai et al., 2013
JN896331.1	PS	Dog	China	Asia-I	2010	Yi et al., 2013
JX681125.1	HLJ1-06	Fox	China	Asia-I	2006	Jiang et al., 2013
AB474397.1	007Lm	Dog	Japan	Asia-II	–	Unpublished
AB476401.1	011C	Dog	Japan	Asia-II	–	Sultan et al., 2009
AB476402.1	50Con	Dog	Japan	Asia-II	–	Sultan et al., 2009
AB475099.1	55L	Dog	Japan	Asia-II	–	Sultan et al., 2009
AB475097.1	M25CR	Dog	Japan	Asia-II	–	Sultan et al., 2009
KF914669.1	CDV2784/2013	Dog	Italy	Artic	2013	Marcacci et al., 2014
AY445077.2	98-2645	Raccoon	USA	America-I	1998	Lednicky et al., 2004
AY542312.2	98-2646	Raccoon	USA	America-I	1998	Lednicky et al., 2004
AY466011.2	98-2654	Raccoon	USA	America-I	1998	Lednicky et al., 2004
AF305419.1	Onderstepoort	Dog (attenuated)	–	America-I	–	Gassen et al., 2000
EU726268.1	CDV3	Mink (attenuated)	China	America-I	–	Unpublished
HM046486.1	Phoca/Caspian/2007	Seal	Kazakhstan	America-I	2007	Unpublished
HM063009.1	Shuskiy	Mink	Kazakhstan	America-I	1989	Unpublished
JN896987.1	Snyder_Hill	Dog (attenuated)	USA	America-I	–	Ludlow et al., 2012

[a] Data retrieved from GenBank or corresponding reference, when available.
[b] Genotype according to the phylogenetic analysis of the complete H gene.

Information (NCBI; http://www.ncbi.nlm.nih.gov/nucleotide) using 'canine distemper virus complete genome' as key words. Fifty-seven complete CDV genomic sequences were retrieved, edited using BioEdit version 7.2.5 and aligned with ClustalW using MEGA 6 software (Tamura et al., 2013). To avoid evolutionary discrepancies, identical sequences and attenuated isolates other than the vaccine strains were removed from the dataset, resulting in twenty-seven complete genomic sequences (Table 1).

Table 2
Putative recombination events detected using the software RDP4 in canine distemper virus.

Recombinant		00-2601 (AY443350.1)	00-2601 (AY443350.1)	01-2689 (AY649446.1)	01-2689 (AY649446.1)	171391-513 (KJ123771.1)	171391-513 (KJ123771.1)
Major parent		01-2689 (AY649446.1) (99%)	01-2689 (AY649446.1) (99.8%)	5804 (AY386316.1) (96.4%)	Unknown strain (00-2601[a])	5804 (AY386316.1) (96%)	164071 (EU716337.1) (99.3%)
Minor parent		Unknown strain (164071[a])	Unknown strain (171391-513[a])	00-2601 (AY443350.1) (100%)	164071 (EU716337.1) (99.6%)	00-2601 (AY443350.1) (99.7%)	Unknown strain (01-2689[a])
P-values determined by							
RDP4	RDP	9.743×10^{-21}	2.773×10^{-10}	5.921×10^{-07}	3.601×10^{-06}	5.921×10^{-07}	1.317×10^{-07}
	GENECONV	1.562×10^{-19}	5.923×10^{-12}	3.561×10^{-07}	4.543×10^{-05}	3.561×10^{-07}	ND
	BootScan	2.692×10^{-20}	1.136×10^{-12}	7.452×10^{-10}	ND	7.452×10^{-10}	2.990×10^{-06}
	MaxChi	2.287×10^{-10}	1.138×10^{-05}	1.212×10^{-06}	ND	1.212×10^{-06}	ND
	Chimaera	2.204×10^{-09}	4.069×10^{-05}	1.722×10^{-05}	ND	1.722×10^{-05}	ND
	Siscan	3.844×10^{-40}	1.200×10^{-28}	8.551×10^{-14}	3.636×10^{-05}	8.551×10^{-14}	1.975×10^{-06}
	3Seq	5.148×10^{-18}	ND	ND	4.679×10^{-03}	ND	2.963×10^{-03}
Fragment							
SH test	A	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	B	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	C	0.0665	0.0000	0.0831	0.0701	0.0598	0.0000
	Concatened tree	0.8415	0.7934	0.8139	0.8477	0.8022	0.7738
ELW test	A	0.0067	0.0000	0.0000	0.0000	0.0000	0.0000
	B	0.0000	0.0277	0.0000	0.0000	0.0000	0.0000
	C	0.7598	0.0000	0.9662	0.9746	0.9668	0.9538
	Concatened tree	0.2335	0.9723	0.0338	0.0254	0.0332	0.0462
Beginning breakpoint (position in alignment)		4936 (99% CI: 4897–4966)	9095 (99% CI: 8090–10642)	Undetermined (2525; 99% CI: 2485–2686)	Undetermined (5741; 99% CI: 2470–5821)	Undetermined (2475; 99% CI: 2485–2686)	Undetermined (5800; 99% CI: 2470–5821)
Ending breakpoint (position in alignment)		6956 (99% CI: 6888–7010)	2223 (99% CI: 1787–2234)	Undetermined (4529; 99% CI: 7033–7095)	7065 (99% CI: 6979–7151)	Undetermined (4529; 99% CI: 7033–7095)	7073 (99% CI: 6979–7151)
Recombination rate		0.033003	0.018414	0.003564	0.026	0.007321	0.013130

ND: Recombination not detected with this algorithm.
[a]Strain considered as parent to perform bootscan analysis.

2.2. Recombination analysis

To detect recombination events over the entire CDV genome, we used the statistical methods included in the RDP4 software package with default settings: RDP (Martin and Rybicki, 2000), GENECONV (Padidam et al., 1999), BootScan (Martin et al., 2005), MaxChi (Smith, 1992), Chimaera (Posada and Crandall, 2001), SiScan (Gibbs et al., 2000) and 3Seq (Boni et al., 2007). As different algorithms might not be completely consistent, any breakpoint supported by four or more methods with P values $P \leq 0.01$ was set as a positive recombination signature. The beginning and ending breakpoints of the potential recombinant sequences were also defined using RDP4 software. Using the putative recombinant sequence as a query, similarity plot and bootscanning analyses were also performed using the SimPlot program (version 3.5.1) (Lole et al., 1999) to confirm or infer the parental strains. When one of the putative parents could not be defined using RDP4 software, a potential parent was selected through similarity based on phylogenetic analysis. The Edmonston measles virus strain (K01711.1) was used as an outgroup for the bootscanning analysis.

The recombination rate for each putative recombination event detected was estimated by LAMARC package v. 2.1.10 (Kuhner, 2006). The maximum-likelihood estimation and the Watterson's (1975) summary-statistic estimator were computed as implemented in the software. Run conditions were 10 initial chains of 10,000 steps each and 2 final chains of 200,000 steps each, discarding the first 1000 steps of each chain and then sampling every 20th step.

2.3. Phylogenetic analysis

Phylogenetic analysis was performed to further evaluate putative recombinant strains and visualize potential relationships between these sequences and the other sequences available. The beginning and ending breakpoints of the potential recombinant strains were used to define the cutoff and segregate the genomes in three segments to perform independent analyses. MEGA6 software was used to obtain phylogenetic inferences, performed after inferring the best-fit DNA model for each alignment, through maximum likelihood model and the Tamura 3-parameter distance correction in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 5). The robustness of the hypothesis was examined using 1000 non-parametric bootstrap analyses. The Shimodaira-Hasegawa (SH) (Shimodaira and Hasegawa, 1999) and expected likelihood weight (ELW) (Strimmer and Rambaut, 2002) tests were used to verify if the topologies were significantly different between the three fragment trees and the concatenated tree, which was set as the reference or best topology model. The analyses were performed with phangorn package (Schliep, 2011) within R (R Core Team, 2015) and Tree-Puzzle v.5.3 (Schmidt et al., 2002), respectively, and it was required that at least two segments showed a statistically significant difference in tree topologies (P values $P \leq 0.01$) to be considered incongruent with the dataset under analysis.

Additionally, a phylogenetic analysis of the 27 complete H gene sequences included in the dataset for recombination analyses was performed with the addition of 69 sequences retrieved from GenBank for genotype determination, including sequences from different geographic areas. MEGA6 was used for phylogeny inference according to the maximum likelihood algorithm. The rate of variation among sites was modeled with a gamma distribution (shape parameter = 5). Statistical support was provided using 1000 non-parametric bootstrap analyses.

All the sequence alignments used to perform the analysis for RDP4 and construct the phylogenetic trees are available in Figshare (http://figshare.com/), with the DOI http://dx.doi.org/10.6084/m9.figshare.1409646.

98-2645 (AY445077.2)	98-2645 (AY445077.2)	98-2646 (AY542312.2)	98-2646 (AY542312.2)	98-2654 (AY466011.2)	98-2654 (AY466011.2)	007Lm (AB474397.1)	HLJ1-06 (JX681125.1)
Phoca/Caspian/2007 (HM046486.1) (93.8%)	01-2689 (AY649446.1) (98%)	Phoca/Caspian/2007 (HM046486.1) (93.8%)	01-2689 (AY649446.1) (98%)	Phoca/Caspian/2007 (HM046486.1) (93.8%)	01-2689 (AY649446.1) (98.1%)	011C (AB476401.1) (99.5%)	CDV SY (KJ466106.1) (98.8%)
5804 (AY386316.1) (98.4%)	Snyder Hill (98.9%)	5804 (AY386316.1) (97.5%)	Snyder Hill (98.8%)	5804 (AY386316.1) (98.4%)	Snyder Hill (98.8%)	01-2689 (AY649446.1) (100%)	Phoca/Caspian/2007 (HM046486.1) (99.9%)
2.369×10^{-49}	9.728×10^{-07}	2.369×10^{-49}	9.728×10^{-07}	2.369×10^{-49}	6.722×10^{-29}	3.548×10^{-14}	9.728×10^{-07}
4.727×10^{-47}	6.345×10^{-08}	4.727×10^{-47}	6.345×10^{-08}	4.727×10^{-47}	3.393×10^{-27}	1.033×10^{-12}	6.345×10^{-08}
3.276×10^{-50}	ND	3.276×10^{-50}	ND	3.276×10^{-50}	4.712×10^{-25}	3.161×10^{-14}	ND
1.149×10^{-20}	4.136×10^{-05}	1.149×10^{-20}	4.136×10^{-05}	1.149×10^{-20}	4.134×10^{-09}	1.143×10^{-05}	4.136×10^{-05}
4.465×10^{-07}	9.613×10^{-04}	4.465×10^{-07}	9.613×10^{-04}	4.465×10^{-07}	4.054×10^{-09}	9.131×10^{-06}	9.613×10^{-04}
9.134×10^{-29}	1.827×10^{-15}	9.134×10^{-29}	1.827×10^{-15}	9.134×10^{-29}	1.265×10^{-08}	4.483×10^{-05}	1.827×10^{-15}
7.911×10^{-36}	ND	7.911×10^{-36}	ND	7.911×10^{-36}	ND	ND	ND
0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.3877	0.0000
0.0000	0.0003	0.0000	0.0164	0.0000	0.0000	0.0000	0.0000
0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
0.7840	0.7421	0.7821	0.7397	0.7789	0.7821	0.8129	0.6018
0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.9577	0.0000
0.0000	0.9675	0.0000	0.9686	0.0000	0.0000	0.0000	0.9593
0.0021	0.0000	0.0011	0.0000	0.0011	0.9475	0.0000	0.0000
0.9979	0.0325	0.9989	0.0314	0.9989	0.0525	0.0423	0.0407
15574 (99% CI: 15147–15175)	7126 (99% CI: 7045–7142)	15154 (99% CI: 15147–15175)	7126 (99% CI: 7045–7142)	15574 (99% CI: 15147–15175)	7096 (99% CI: 7045–7142)	7049 (99% CI: 6977–7098)	12922 (99% CI: 12873–12949)
1602 (99% CI: Undetermined)	9026 (99% CI: 8995–9034)	1602 (99% CI: Undetermined)	9026 (99% CI: 8995–9034)	1602 (99% CI: Undetermined)	9026 (99% CI: 8995–9034)	7546 (99% CI: 7534–7609)	14260 (99% CI: 14214–14292)
0.006662	1.78e-06	0.012242	0.002555	0.0066	2.32e-06	0.0066	4.37e-06

3. Results

Based on the 27 complete CDV genomes, we observed at least eight potential recombinant strains, involving nine unique putative recombination events [00-2601 (AY443350.1), 01-2689 (AY649446.1), 171391-513 (KJ123771.1), 98-2645 (AY445077.2), 98-2646 (AY542312.2), 98-2654 (AY466011.2), 007Lm (AB474397.1) and HLJ1-06 (JX681125.1)] (Figure S1), with *P* values lower than 0.01, detected using at least four of the seven algorithms. The recombination rates were estimated for each event and the rate estimates varied between 0.026 to 4.37e-06 as shown in Table 2. The potential major and minor parents defined through RDP4 of the putative recombinants and the beginning and ending breakpoints are described in Table 2.

Further evidence for recombination in CDV was demonstrated through the occurrence of incongruent phylogenetic trees. Forty-six different estimations from different genomic regions between potential recombinant strains and their major and minor parents were constructed, visually analyzed and the incongruences found were further statistically calculated with the SH and ELW tests, which showed significant results for at least two fragments of each dataset (Table 2).

Additionally, a phylogenetic tree of 96 complete sequences of the H gene was constructed to define the genotypes of each putative recombinant. The genotypes are listed in Table 1.

For the strains 98–2645, 98–2646 and 98–2654, classified as genotype America-I based on the complete H gene phylogeny, the same recombination events were detected, with minor differences in beginning and ending breakpoints (Table 2). For the first event, strain 01-2689 was considered closer to the major parent and strain Snyder Hill (America-I) was considered the minor parent. The putative recombination region was the H gene. In the tree of this region (7126–9026 nt), the strains clustered with vaccine strains of genotype America-I, close to the Snyder Hill strain, a putative minor parent (Fig. 1, Figures S2 H and K). For the other trees, the strains formed a separate group clustered between wild type and vaccine strains. For the second event, strain Phoca/Caspian/2007 was considered the major parent and strain 5804 was defined as the minor parent. The putative recombination region comprised a large fragment of the N gene. Phylogenetic analysis showed that in the tree of region 1–1602 nt, the putative recombinants clustered with strain 5804, as on the second tree, and formed a group clustered with strains of America-I genotype, including its putative major parent. In the third tree, these recombinants formed a cluster clearly separate from both putative parents (Figures S2 G, I and J). Similarity plots and bootscanning analyses were performed with each putative recombinant strain as query, and the results indicated that putative recombination events between strains 01-2689, Snyder Hill, 5804 and Phoca/Caspian/2007 occurred (Figures S3 G-K and S4 G-K).

Strain 007Lm (Asia-II) potentially had one recombination event. Strain 11C (Asia-I) was considered the major parent, and strain 01-2689 was considered the minor parent. The putative recombination region comprised the 5 region of the H gene. The putative recombinant strain was used as the query sequence on similarity plots and bootscanning analyses, indicating putative recombination events between strains 01-2689, Snyder Hill, 5804 and Phoca/Caspian/2007. The phylogenetic analysis showed that the fragment corresponding to the putative recombinant region clustered with strain 01-2689, closer to other strains of genotype America-II. For the other two fragments, the putative recombinant clustered with strains of genotype Asia-II, including strain 011C (Fig. 2).

The putative recombinant strain HLJ1-06 (Asia-I) had CDV SY strain (Asia-I) as the major parent and Phoca/Caspian/2007 (America-I) as the minor parent. The putative recombinant region was located within the L gene. The putative recombinant was used as a query for the similarity plot and bootscanning analyses, indicating putative recombination events between strains CDV SY and Phoca/Caspian/2007 (Figure S3 L and S4 L). Phylogenetic trees of three fragments were constructed (Figure S2 L). The first tree, including positions 1–12,921 nt, showed that this strain clustered with vaccine strains of genotype America-I, which includes the putative parent strain Phoca/Caspian/2007. For the second tree, this strain also clustered with genotype America-I strains, and for the third tree, this strain clustered with genotype Asia-I strains, including its putative minor parent.

Strain 00-2601 (genotype America-II) was considered a putative recombinant with two potential events. For the first recombination event, strain 01-2689 (America-II) was considered a potential major parent, but the minor parent could not be identified within the dataset (this strain was therefore defined as unknown). The seven algorithms detected recombination signals involving these strains. The putative region of recombination was located in the F gene. The phylogenetic analysis revealed two trees with putative recombinants clustering with strain 01-2689 (Figure S2 A). In the other tree, the putative recombinant strain clustered with strains of genotype America-I, primarily including vaccine-attenuated strains. For the second event, strain 01-2689 was also considered to be the potential major parent and the minor parent could not be identified. For this event, six of the seven algorithms supported the recombination signals. The putative recombinant region comprised a large portion of the P gene and complete M, F and H genes (Figure S2 B). Similarity plot and bootscanning analyses were performed with strain 00-2601 as a query and also indicated a putative recombination event between strains 01-2689, 164071 and 171391-513 (Figures S3 A–B and S4 A–B). The phylogenetic tree of this event revealed a putative recombinant clustering with strain 01-2689 on two trees, and on the third tree, this recombinant was clearly separated from not only the America-II genotype but also the America-I genotype. It was not possible to infer a minor parent for this event.

Strain 01-2689 potentially descends from at least three putative parents, as a mosaic of strains 5804 (South America-I/Europe), 00-2601, 164,071 (America-II) and a potential fourth parent that could not be identified in the dataset. For the first event, strain 5804 was considered the major parent and strain 00-2601 was considered the minor parent. The putative region of recombination could not be determined because the RDP4 software could not identify beginning and ending breakpoints. For the phylogenetic analysis, positions within regions 2525–2686 and 4529–7095 were used, with a confidence interval of 99%. The first tree showed that the putative recombinant clustered with strains of America-II genotype and was related to strain 5804 of genotype South America-I/Europe. On the other two trees, this recombinant clustered with strain 00-2601 and was distant from strain 5804, which clustered with Asia-I strains (Figure S2 C). For the second event, strain 164071 was considered the minor parent, as the putative region of recombination within the F protein. The major parent and the beginning breakpoint could not be identified. For the phylogenetic analysis, the sequences within positions 5741–7065 were used as breaking points, with a confidence interval of 99%. The tree of the recombinant region revealed clustering with strain 164071, a putative minor parent. In the other two trees, this strain was closer to strain 00-2601 (Figure S2 D). All of the strains in this cluster belonged to the America-II genotype. Similarity plot and bootscanning analyses were performed with the putative recombinant as a query, and the results indicated that putative recombination events between strains 00-2601, 164071 and 5804 (Figures S3 C–D and S4 C–D).

The same events might have shaped strain 171391-513, of the America-II genotype, although the software determined slightly different breakpoints for this strain (Figures S2 E–F), and strain 164071 was considered the major parent for the second event (Table 2). The phylogenetic analysis of the second event showed that for the first and third fragments analyzed (1–5799 nt and 7073–15,690 nt, respectively), 171391-513 clustered with strain 164071 and other isolates of genotype America-II. For the putative recombinant region, strain 171391-513 clustered with isolate 01-2689, also of genotype America-II. Similarity plot and bootscanning analyses were performed with the putative recombinant as a query and indicated

putative recombination events between strains 00-2601, 164071 and 5804 (Figures S3 E–F and S4 E–F).

Figures for all of the putative recombinant events and graphics can be accessed as Supplementary material.

4. Discussion

It has been suggested that the evolution dynamics of non-segmented RNA viruses are based on a lack of RNA-dependent RNA polymerase proofreading or recombination events (Worobey and Holmes, 1999). Although mutation is the ultimate source of genetic variation, recombination acts on mutation to shape the genetic structure of populations, but for non-segmented negative-sense RNA viruses, including CDV, this phenomenon is considered rare (Han and Worobey, 2011). However, in recent years, studies have reported recombination throughout the entire genome of paramyxoviruses (Han et al., 2008; Lai, 1992; Song et al., 2011). These natural events have been previously described in different members of *Paramyxoviridae*, such as *Avulavirus* (McCarthy et al., 2007; Miller et al., 2009), *Rubulavirus* (Lai, 1992), *Pneumovirus* (Spann et al., 2003) and in some species of the *Morbillivirus* genus, such as measles virus (McCarthy et al., 2007; Schierup et al., 2005) and CDV (Han et al., 2008; McCarthy et al., 2007).

Studies of CDV typically analyze gene sequences, showing that the recombination signals are stronger for the F gene (McCarthy et al., 2007), with evidence of recombination also on the H gene (Han et al., 2008; Ke et al., 2015). Since recombination hot spots can hardly be detected through the analysis of partial genomes, multiple-sequence analyses of complete viral genome sequences are needed to identify potential recombination breakpoints. Recently, recombination events between sequences of the H gene of wildtype and vaccine strains were identified (Ke et al., 2015). However, not all of these sequences are available at complete genomes, so those putative recombinants could not be included in our analysis.

In the present study, recombination was examined using different but complementary approaches. Strong evidence for the occurrence of homologous recombination between CDV strains of different genotypes and different hosts was supported by at least four of the seven algorithms used to detect genetic conversion through similarity plots, bootscanning and phylogenetic inferences. Sets of phylogenetic trees for different genomic regions, considered a gold-standard bioinformatics approach to demonstrate the presence of recombination (Boni et al., 2010), were used to detect incongruences. Notably, the minor and major parent sequences were defined through RDP4 based on pairwise comparisons between the query (putative recombinant) and the other sequences in the dataset, suggesting that the putative recombinant and the potential parental sequences are descendants of a common ancestor, likely better represented as a common node in the phylogeny.

When phylogenetic trees at each breakpoint were constructed, clear evidence of recombination was observed in eight of the 27 sequences. Since visual analysis could be misleading, the statistical significance of the incongruences was calculated using the SH and ELW tests, as suggested by a recent review (Pérez-Losada et al., 2015). If there is no recombination, we expect that a single topology applies to all segments in the same way as in the concatenated phylogeny, defined as the best model. Four ML trees (three segments corresponding to the breakpoints

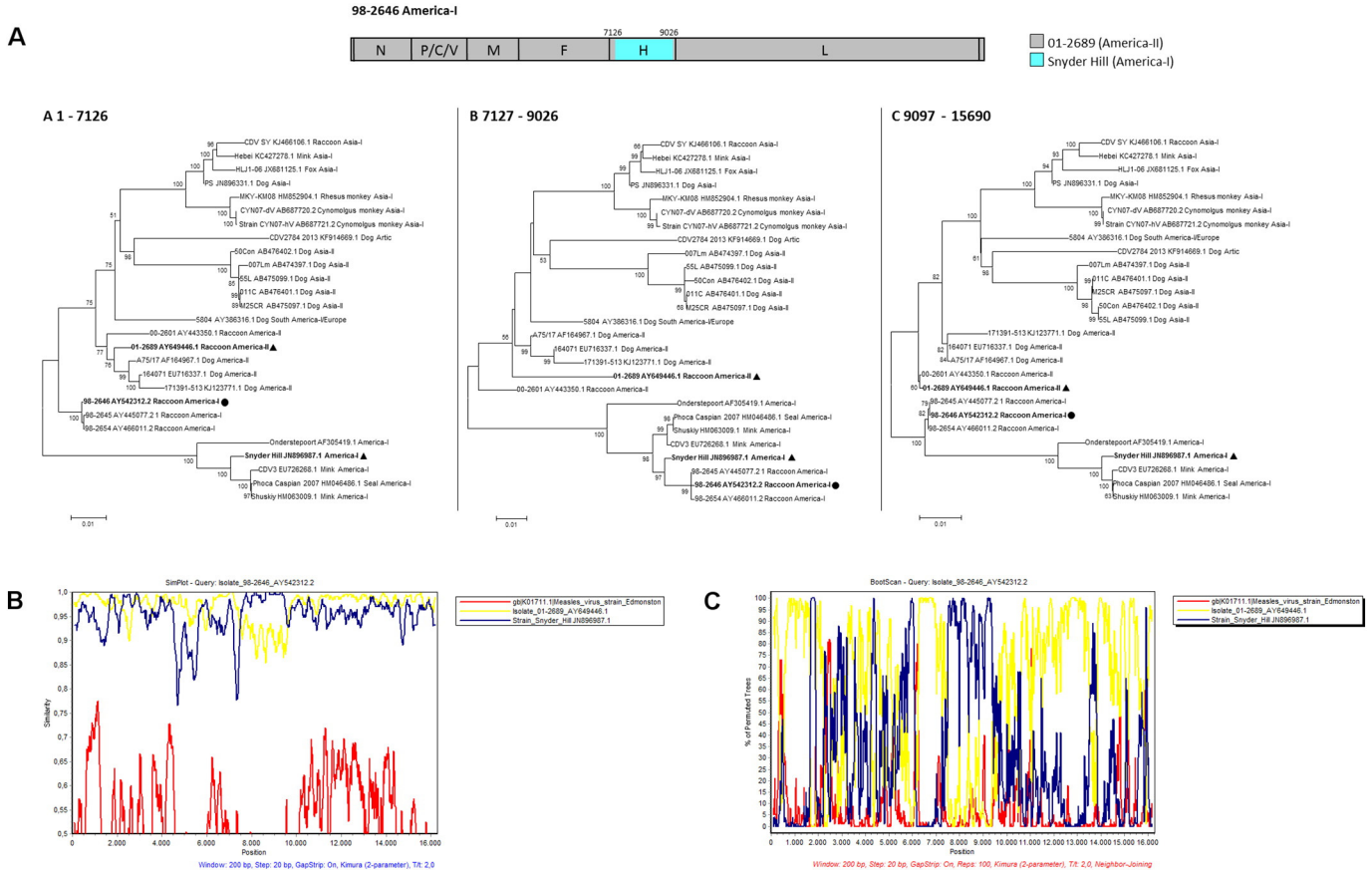

Fig. 1. Schematic representation of first recombination event of putative recombinant strain 98-2646. The genome organization of the potential recombinant virus with the breakpoints and segments of the genome derived for major and minor parents are represented. The relationship between the putative recombinant (●) and putative parents (▲) in the different segments of the genome was shown in the neighbor-joining trees, where bootstrap values ≥50% are represented (A). The results from similarity plot analysis of the putative recombinant strain as a query are shown. The y-axis shows the percentage of identity within a 200-bp-wide sliding window, with a 20-bp step size between plots (B). The results of the bootscanning analysis. The y-axis provides the percentage of permutated trees using a 200-bp-wide sliding window, with a 20-bp step size between plots (C).

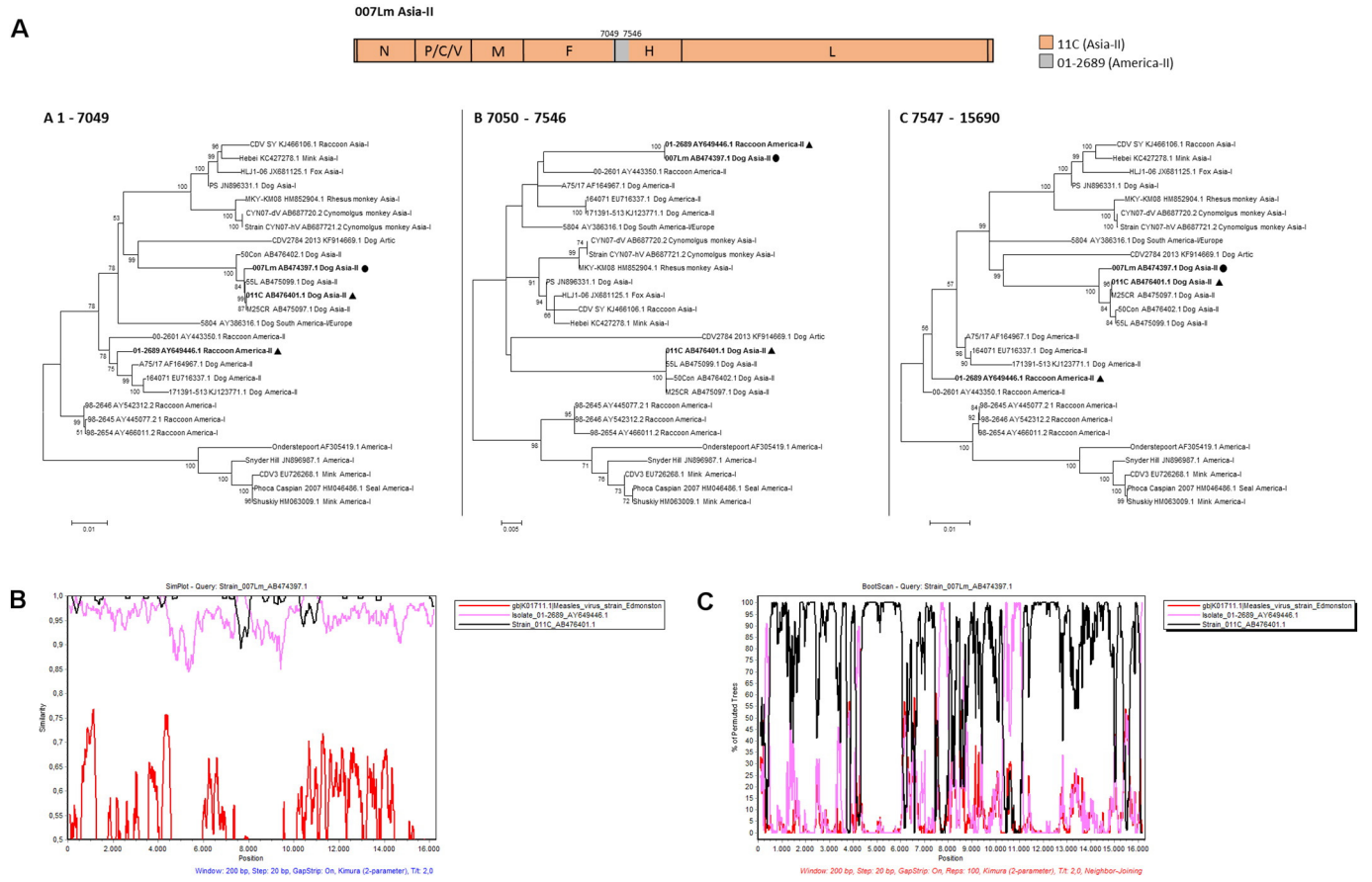

Fig. 2. Schematic representation of putative recombinant strain 007Lm. The genome organization of the potential recombinant virus with the breakpoints and the segments of the genome derived for major and minor parents are represented. The relationship between the putative recombinant (●) and putative parents (▲) in the different segments of the genome is shown in the neighbor-joining trees, where bootstrap values ≥50% are represented (A). The results of the SimPlot analysis of the putative recombinant strain as a query are shown. The y-axis shows the percentage of identity within a 200-bp-wide sliding window, with a 20-bp step size between plots (B). The results of the bootscanning analysis. The y-axis shows the percentage of permutated trees using a 200-bp-wide sliding window, with a 20-bp step size between plots (C).

and concatenation of all genes) were constructed. A statistically significant rejection indicates that the particular tree does not explain the data as well as the best tree for that dataset. This method confirmed all the recombination events with at least two incongruent trees in each putative recombinant, using both methods. These events were detected throughout the genome but were more frequently in F and H genes, consistent with the results of previous studies (Han et al., 2008; McCarthy et al., 2007).

Six of the eight putative recombinants were involved in multi-recombination events. Interestingly, the recombinants were primarily isolated from free-ranging raccoons of USA, and the recombination events were concentrated in viruses of genotypes America-I (genotype of viruses widely used as vaccines) and America-II (field strains isolated from dogs and wild carnivores). Notably, the Snyder Hill vaccine strain was considered the putative parent of a recombination event detected in three isolates (98-2645, 98-2646 and 98-2654) harvested from USA free-ranging raccoons in 1998 (Lednicky et al., 2004). These raccoons lived on zoo grounds or preserved forest areas around zoos. The authors observed that these isolates were less virulent than other raccoon strains (00-2601 and 01-2689) isolated in the same study group after a high mortality distemper outbreak that occurred in 2000–2001. Strains 00–2601 and 01–2689 were more virulent than the previous strains, inducing an increase in mortality, with different histological presentation, and segregated with America-II wild type strains on phylogenetic analysis, suggesting that these genetic differences induced the increased virulence. The results of the present study were not only consistent with this hypothesis but also suggested that this genetic difference reflected a natural recombination event.

Putative recombinant strains 98-2645, -2646 and -2654 possessed the H gene as the recombinant region inherited from the vaccine strain Snyder Hill. Because the hemagglutinin glycoprotein is responsible for virus attachment to the SLAM (CD150) cell receptor, it is likely that the recombinant strain would become less cytophatic. However, these recombinant strains remained pathogenic, inducing symptoms such as pneumonia and encephalitis in these raccoons. Nevertheless, several reports have shown that cell-attenuated CDV vaccines still cause disease in wild carnivores (Deem et al., 2000). The long-term use of attenuated vaccines in dogs might affect the evolution of the virus in the field. Whether the recombination event occurred before or after the massive use of vaccination is an interesting question that should be further addressed.

In addition, the results of the present analysis identified two putative recombinant strains (007Lm and HLJ1-06) belonging to Asiatic genotypes (Asia-I and Asia-II), each with unique recombination events. Strain 007Lm (Asia-II) is a virulent strain isolated from a vaccinated Labrador retriever in Japan, which causes severe hemorrhagic diarrhea with oculonasal discharge and epileptic seizure (Lan et al., 2005). The results suggested that this virus emerged from a recombination event between strains 11C (Asia-II), also isolated from a Japanese dog, and 01-2689 (America-II), isolated from an American raccoon, as the recombinant region of the H gene (nt 7049–7546) with 100% identity to the 01-2689 strain. This potential event emphasizes the need for considering recombination events when performing genotyping analyses as the H gene is frequently used for this purpose. Thus, CDV genotyping should always be performed with complete gene analyses as a partial sequence could lead to misidentification.

Strain 5804 was the only complete CDV genome sequence of the genotype South America-I/Europe available in GenBank at the time of the present analysis, and interestingly, this sequence might be involved in at least five of the eight putative recombinants, indicating that 5804 might be an ancestral strain. Historical records suggested that distemper was imported from Peru into Spain during the 17th century, and this virus subsequently spread throughout Europe (Blancou, 2004), while a recent publication tracked the emergence of CDV to North America around the 1880s. It was suggested that an ancestral virus diversified in two clades, one that spread world-wide and originated eight of current genotypes and the other gave rise to the America-I lineage, which comprehends most vaccine and vaccine-related strains (Panzera et al., 2015). The high genetic identity between the South American and European strains is also an indicative of a common ancestor, which could explain the involvement of strain 5804 as a putative parent for most recombinants.

Isolate 171391-513 was recently reported as a putative recombinant (Ke et al., 2015) of strain 164071 with an unknown strain when analyzing datasets of the H sequence. Interestingly, in our study, we detected the same recombination signal but with different break-points, indicating a recombination within the F protein. Moreover, our complete genome analysis could identify a second event of recombination within the P and M proteins, with strains 5804 and 00-2601 as putative parents, albite with a lower recombination rate.

Recombination events should be further investigated in the future. More importantly, whole genome sequencing following co-infection experiments with wild type and vaccine strains would be useful to experimentally determine whether recombination indeed occurs in CDV and how frequent recombination might be. Also, the effect of possible recombination events between wildtype and vaccine strains should be considered when designing vaccines based on the evolution of the virus (i.e., vaccines based on centralized genes) (Arenas and Posada, 2010).

In conclusion, these results provide strong evidence that recombination occurs between circulating CDV in natural populations, but recombination might also occur between vaccine and field strains of the virus, suggesting that CDV vaccination might play an important role in shaping virus evolution.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2016.04.014.

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3.2. Artigo 2 - Inactivated recombinant rabies viruses expressing the canine distemper virus glycoproteins induce protective immunity against both pathogens

O presente projeto foi finalizado e um artigo foi aceito para publicação na revista *Journal of Virology*. Este trabalho será apresentado no formato de manuscrito aceito.

Inactivated Recombinant Rabies Viruses Displaying the Canine Distemper Virus Glycoproteins Induce Protective Immunity Against Both Pathogens

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Running title: Efficacy of bivalent RABV-CDV vaccines

ABSTRACT

The development of multivalent vaccines is an attractive methodology for the simultaneous prevention of several infectious diseases in vulnerable populations. Canine distemper (CDV) and rabies (RABV) viruses both cause lethal disease in wild and domestic carnivores. While RABV vaccines are inactivated, the live-attenuated CDV vaccines retain residual virulence for highly susceptible wild life species. In the current study, we have developed recombinant bivalent vaccine candidates based on recombinant vaccine strain rabies virus particles, which concurrently display the protective CDV and RABV glycoprotein antigens. The recombinant viruses replicated to near wild type titers and the heterologous glycoproteins were efficiently expressed and incorporated in the viral particles. Immunization of ferrets with beta-propiolactone inactivated recombinant virus particles elicited protective RABV antibody titers, and animals immunized with a combination of CDV attachment and fusion protein-expressing recombinant viruses were protected from lethal CDV challenge. However, animals that were only immunized with a RABV expressing the attachment protein of the CDV vaccine strain Onderstepoort succumbed to the infection with a more recent wild type strain, indicating that immune responses to the more conserved fusion protein contribute to protection against heterologous CDV strains.

IMPORTANCE

Rabies and canine distemper virus (CDV) cause high mortality and death in many carnivores. While rabies vaccines are inactivated and thus have an excellent safety profile and high stability, live-attenuated CDV vaccines can retain residual virulence in highly susceptible species. Here we generated recombinant inactivated rabies viruses that carry one of the CDV glycoproteins on their surface. Ferrets immunized twice with a mix of recombinant rabies viruses carrying the CDV fusion and attachment glycoproteins resulted in protection from lethal CDV challenge, whereas all animals receiving recombinant rabies viruses carrying only the CDV attachment protein following the same immunization scheme died. Irrespective of the CDV antigens used, all animals developed protective titers against rabies virus, illustrating that a bivalent rabies-based vaccine against CDV induces protective immune responses against both pathogens.

INTRODUCTION

Canine distemper virus (CDV) is a member of the morbillivirus genus that infects a broad range of terrestrial and aquatic carnivores. In addition to dogs, raccoons, and foxes, which are the primary reservoir species for the virus, infections have been reported in wild and domestic ferrets, mink, and different species of large cats, bears, and seals (1). Disease severity varies in the different species but mortality rates above 90% are common in wild carnivores, and CDV outbreaks can push endangered species to the brink of extinction and cause great losses among susceptible animals during outbreaks in zoos or wild life refuges (2-6).

As with its close relatives, measles virus (MeV) and pest-de-petits-ruminants virus (PPRV), highly efficient live-attenuated vaccines have been developed that prevent CDV infection and disease. Most commercially available vaccines are based on the Onderstepoort strain, which was developed in the 1950s by repeated passage in embryonated chicken eggs and different mammalian cell lines (7, 8). However, since these live-attenuated vaccines retain their ability to replicate in the vaccinated animal, they can cause severe distemper disease in highly susceptible species (9). Aside from a vectored vaccine based on a CDV attachment (H) and fusion (F) protein-expressing recombinant canarypox virus (10-12) that has been licensed for use in ferrets, there are currently no prophylactic options for valuable zoo animals and wild life.

The main antigenic determinants of CDV are H protein, which binds the cellular receptors signaling lymphocyte activation molecule (SLAM) (13) on lymphocytes and nectin-4 (14, 15) on epithelial cells, and the F protein, which fuses the host and viral membranes to initiate infection (16). Antibodies against either protein can have neutralizing activity, but the majority of neutralizing antibodies are directed against the H protein (17). While the F protein is highly conserved among different CDV strains and even between morbilliviruses, the H protein has

undergone considerable genetic change over the last decades (18). Even though all strains belong to the same serotype, different phylogenetic lineages have been identified. Strains isolated in the same geographic location generally fall into the same lineage, and then cluster by isolation year within those lineages (19). Increasing sequence information from previously underrepresented regions in Africa, South America, and Asia, further supports this model (20-22). The increasing sequence variation between the original vaccine strains and currently circulating isolates is frequently discussed as a possible cause for reduced vaccine efficacy, but there is currently no experimental evidence to support these speculations.

To investigate the contribution of H and F protein-specific antibodies to protection and to explore a new avenue for the development of an inactivated CDV vaccine, we generated recombinant rabies viruses (RABV) carrying vaccine and wild type CDV glycoproteins. RABV is a rhabdovirus in the genus *Lyssavirus* that causes fatal neurological disease if not treated in a timely manner, but disease and death can be prevented with an effective inactivated vaccine. The RABV SAD B19 vaccine strain (23) used in this study has been further attenuated by mutation of arginine to glycine at position 333 of the G glycoprotein (24, 25). Since the production and β-propiolactone inactivation process of RABV vaccines is well established (26), they represent an excellent platform for development of inactivated bivalent vaccines that also protect against other pathogens (23, 27). Towards this, we assessed the safety and efficacy of the different RABV-CDV vaccine candidates in ferrets.

RESULTS

Canine distemper virus glycoproteins are expressed and incorporated into recombinant rabies viruses. To compare the immune responses elicited against glycoproteins of

97 the CDV vaccine strain OS and the more recent wild type strain 5804P, we generated
98 recombinant RABV based on the SAD-B19 vaccine strain of RABV (BNSP-333) (24)
99 expressing either the F or H glycoproteins of these two CDV strains (Fig. 1). Transgene
100 expression silencing had been observed in vesicular stomatitis virus (VSV) expressing the MeV
101 F protein (28, 29), therefore to ensure transgene expression in our system we first analyzed cell
102 lysates from Vero cells infected at an MOI of 10 for 48 h. All of the recombinant viruses
103 expressed the respective CDV proteins with equivalent expression levels for proteins of OS and
104 5804P origin (Fig. 2A), with the OS H protein migrating at the expected lower molecular weight
105 due to the lack of several N-glycosylation sites (Fig. 2A, anti-Hcyt immunoblot) (30). As in
106 lysates from CDV-infected cells (30), uncleaved F0 precursors and H proteins at different
107 glycosylation stages were also seen (Fig. 2A, anti-Fcyt and anti-Hcyt). In samples from H
108 protein-expressing RABVs, lower RABV matrix (M) protein levels were observed, likely
109 reflecting the slower replication kinetics of these viruses (Fig. 3A).
110 We next sought to determine to what extent the CDV antigens were incorporated into the
111 recombinant RABV particles. Analysis of sucrose-purified particles by SDS-PAGE revealed that
112 proportionately more 5804P than OS H protein was incorporated (Fig. 2B, anti-Hcyt). In
113 contrast, total F protein expression levels (Fig. 2A, anti-Fcyt) and their corresponding
114 incorporation into viral particles (Fig. 2A and B, anti-Fcyt) was similar for both strains. As
115 expected, only fully glycosylated CDV H proteins and mature F1 proteins were incorporated into
116 recombinant RABV particles (compare Fig. 2B, anti-Hcyt and anti-Fcyt). SYPRO ruby analysis
117 of sucrose-purified particles illustrated a comparable overall purity of the vaccine preparations,
118 and confirmed CDV protein incorporation into RABV particles, with the OS H protein migrating
119 close to the RABV glycoprotein (G) and the CDV F_1 proteins migrating in the same region as the

120 RABV phosphoprotein (P) (Fig. 2C). Taken together, the origin of the CDV F proteins has little
121 effect on its expression and incorporation by RABV while the 5804P H protein is incorporated
122 more effectively than the OS H protein.

123 **CDV transgene expression slightly reduces rRABV replication.** We next determined
124 the growth kinetics of the respective recombinant viruses in Vero cells. When inoculated at a low
125 MOI of 0.01, we observed that all of the recombinant viruses reached similar peak titers by 96 h
126 post-infection, even though replication of both H- and the F5804P-expressing viruses was
127 delayed at the early time points (Fig. 3A). This slight delay is likely due to the position of the
128 additional transcription unit immediately downstream of the nucleoprotein gene, which ensures
129 the high expression levels needed for an inactivated bivalent vaccine but may also increase the
130 fitness cost associated with the expression of the inserted gene (31, 32). At a MOI of 10, all
131 viruses followed similar growth kinetics and reached peak titers within the same range as the
132 BNSP-333 control virus, with the H-expressing viruses reaching slightly lower titers (Fig. 3B).
133 These data indicate that the expression of the additional CDV glycoproteins slightly reduces
134 overall RABV growth, but that the titers of all of the viruses were adequate to produce sufficient
135 material for vaccination studies in ferrets.

136 **Immunization with both CDV glycoproteins protects against lethal CDV challenge.**
137 There is increasing genetic divergence between the OS and circulating H gene sequences (33).
138 To compare the protection from lethal challenge with the more recent wild type strain 5804P
139 conferred by the OS H protein alone or in combination with the OS F with the matched 5804P
140 glycoproteins, ferrets were immunized once or twice in a 4-week interval with a combination of
141 BNSP-333-Hos and –Fos, or twice with BNSP-333-Hos or a combination of BNSP-333-H5804P
142 and –F5804P. Animals immunized twice with BNSP-333 alone were included as negative

143 control. Five weeks after the last immunization, all animals were challenged with a lethal dose of
144 the CDV wild type strain 5804PeH (Fig. 4A). While all animals immunized with BNSP-333-Hos
145 alone succumbed to the disease within two weeks, similar to the BNSP-333 vaccinated control
146 animals (Fig. 4B), groups that received two injections of BNSP-333 vectors expressing CDV F
147 and H proteins developed at most a mild and transient rash and were protected from death (Fig.
148 4B). A single immunization with Fos- and Hos-expressing BNSP-333 virions reduced mortality
149 by 75% (Fig. 4B), with three out of four animals surviving, and two animals developing
150 moderate to severe signs of disease including rash and conjunctivitis.

151 Animals that succumbed to the disease were unable to control virus replication in PBMCs
152 (Fig. 4C), and experienced dramatic leukopenia (Fig. 4D). While the control animals also
153 gradually lost weight, animals immunized with BNSP-333-Hos maintained a stable body weight
154 throughout the infection (Fig. 4B). Among the survivors, the group immunized twice with 5804P
155 glycoproteins experienced the shortest-lived cell-associated viremia (Fig. 4C), which was also
156 significantly ($p<0.005$) than in all other groups, and the least drop in leukocyte counts (Fig. 4D).
157 Even though two immunizations with the OS glycoproteins were sufficient to confer complete
158 protection, the antibody response was less efficient than that elicited by the 5804P glycoproteins
159 in controlling cell-associated viremia and leukopenia (Fig. 4C and D), and this effect was even
160 more pronounced in the group that received only one immunization. This indicates that the
161 immune response against the CDV F protein contributes importantly to the protection conferred
162 by immunization with inactivated CDV glycoprotein-expressing RABV.

163 **Two immunizations are required to induce detectable anti-CDV antibody responses.**
164 To gain insights in the antibody levels induced by the different immunization schemes, total and
165 neutralizing anti-CDV antibody kinetics against OS and 5804P were evaluated. At the time of

166 the second immunization, five weeks before challenge, only titers close to the detection limits
167 could be detected in individual animals distributed across all groups immunized with CDV
168 glycoprotein-expressing BNSP-333 (Fig. 5A-D, week -5). Neither at this time point nor at the
169 time of challenge, the differences in antibody titers reached statistical significance. At the time of
170 challenge, only the group immunized twice with the 5804P glycoprotein-containing vaccines had
171 also mounted a total IgG response against the challenge virus 5804P (Fig. 5A). Even though
172 these antibodies had no neutralizing capacity (Fig. 5B), all animals were able to rapidly clear
173 viremia (Fig. 4C) and showed little to no clinical signs (Fig. 4B). In addition to these animals, all
174 groups that received two immunizations developed robust total IgG and low level neutralizing
175 antibodies against the CDV OS vaccine strain (Fig. 5C and D, week 0). However, only the group
176 immunized with the combination of OS H and F proteins was sufficiently protected to clear the
177 virus and survive the challenge (Fig. 4 B and C), while the animals that had only received the OS
178 H protein-containing vaccine all succumbed to the disease (Fig. 4B). Together with the
179 observation that 75% of the animals immunized only once with the combined 5804P H and F
180 protein-containing vaccine survived despite lower total and neutralizing antibody levels (Fig. 4B,
181 and 5C and D), further supports a role for the contribution of F protein-mediated immunity to
182 protection. After the challenge, total IgG antibodies increased at least 100-fold ($p<0.01$)
183 compared to the respective pre-challenge values in all surviving animals, and robust neutralizing
184 antibody responses against both strains were detected (Fig. 5A-D, week 4), suggesting that the
185 preexisting immunity supported a rapid recall response even against mismatched strains in these
186 animals.

187 **All vaccines induce a robust and sustained protective response against RABV.** An
188 important added advantage of a bivalent inactivated RABV-CDV vaccine lies in the

simultaneous protection from two important virus infections. To predict the protection against RABV conferred by the different vaccine candidates, antibody titers were quantified using the WHO international reference. We found that a single immunization with any of the vaccine candidates was sufficient to induce antibody responses above the protective threshold in the majority of the animals (Fig. 6, week -5), and that all generated protective titers after the second vaccination (Fig. 6, week 0). After CDV challenge, a slight but not statistically significant ($p>0.1$) decline in anti-RABV antibodies was observed in survivors (Fig. 6, week 4). Nonetheless, even the reduced titers were still within the protective range for RABV immunity.

DISCUSSION

Rabies and distemper are among the most devastating viral infections in carnivores. For both viruses, efficient vaccines are commercially available. However, live-attenuated CDV vaccines have limited thermo-stability and may retain residual virulence in highly susceptible wild life species (34, 35). In contrast, inactivated RABV vaccines are stable for extended periods of time and have an excellent safety profile (36, 37). To explore the potential of CDV glycoprotein-expressing RABV as a bivalent inactivated vaccine, we generated recombinant BSNP-333 RABVs carrying CDV H or F glycoproteins of either the vaccine strain OS or the wild type strain 5804P. All the recombinant viruses replicated at near wild type efficiency allowing the production at quantities needed for immunization studies and the respective CDV glycoprotein was incorporated at levels similar to the rabies proteins. All immunized ferrets developed anti-RABV antibodies above the protective cut off, but only groups immunized with a combination of CDV F and H protein-containing inactivated RABVs were also protected from lethal CDV challenge.

212 **The RABV-based vector is a promising platform for Morbillivirus vaccines.**
213 Development of inactivated Morbillivirus vaccines was abandoned after enhanced disease upon
214 exposure to the virus was seen in children immunized with formalin-inactivated MeV vaccines
215 (38). For CDV, the residual virulence of live-attenuated vaccines for highly susceptible species
216 has led to continued interest in this approach. The potential of various recombinant vaccine
217 platforms expressing CDV glycoproteins has been explored (39, 40), and a CDV H and F
218 protein-expressing canary pox vector is licensed in several countries for use in dogs and ferrets
219 (11). While all of these approaches undergo at least a single round of infection in the vaccinee,
220 the RABV vaccine platform is inactivated and as such no longer considered a GMO. In addition,
221 licensed inactivated RABV vaccines have been stability-optimized for tropical climates, thereby
222 addressing a major drawback of all live-attenuated morbillivirus vaccines, which are notoriously
223 temperature sensitive (35). We found that all vaccine candidates induced protective antibody
224 responses against RABV after two immunizations, similar to results obtained with this vector
225 platform in combination with other heterologous antigens in various species (31, 41). When
226 vectors expressing the CDV F and H proteins were combined, this immunization schedule also
227 conferred complete protection from lethal CDV disease, indicating that RABV might thus not
228 only be an attractive bivalent vaccine platform in combination with CDV but also with other
229 morbilliviruses including MeV.
230 **Immune responses against the Morbillivirus F protein contribute to protection from**
231 **heterologous strains.** Morbillivirus infections including live-attenuated vaccines confer a robust
232 and likely life-long protection. Neutralizing antibody titers above 120 are considered protective,
233 even in the absence of T cell-mediated immunity, and conversely, a Morbillivirus infection-
234 induced T cell response alone is sufficient to protect from reinfection (42-44). Previous studies

have demonstrated that the H protein is by far the most important target for neutralizing antibodies (17, 45, 46), so many vaccine development approaches have chosen to focus on this protein. However, the increasing diversity of CDV H proteins among circulating strains from different geographic regions as well as compared to the vaccine strain H protein suggest variation in antigenic epitopes (33, 47). Consistent with these speculations, we observed that immunization with the vaccine strain H protein alone induced an antibody response against the vaccine but not the heterologous wild type strain, and did not confer protection in a subsequent lethal wild type challenge. In contrast, immunization with the vaccine H and F proteins was protective, even though pre-challenge antibodies profiles were similar in both groups. These results demonstrate the critical role of immune responses directed against the F protein for control of CDV and likely other Morbillivirus infections (17). As the F proteins are highly conserved (48), immune responses against this protein may also contribute to the continued efficacy of live-attenuated Morbillivirus vaccines based on strains isolated many decades ago.

Potential for protection of humans and wildlife from zoonotic infections. In contrast to many other vector platforms, the immune response against the RABV vector constitutes an added value, as it confers protection from rabies (49). Wild carnivores are considered an important RABV reservoir, which, often via infection of domestic dogs, are the root cause for human rabies in developing countries (50, 51). Conversely, CDV outbreaks in wild life species are thought to originate from contact with infected domestic at the interface between inhabited and uninhabited regions (2, 52). Vaccination of domestic dog populations in rural areas is thus a promising strategy to reduce or even prevent human rabies cases (53-55). Use of an inactivated bivalent RABV/CDV vaccine in these campaigns could at the same time reduce the risk of CDV

257 introduction in at risk wildlife species and thus improve public health while contributing to
258 conservation efforts.
259
260 **MATERIALS AND METHODS**
261 **Cells and viruses.** Vero cells were maintained in Dulbecco's modified Eagle medium
262 (DMEM; Mediatech, Inc., Manassas, VA) supplemented with 1% penicillin-streptomycin
263 (Mediatech) and 5% fetal calf serum. VerodogSLAMtag cells (1) were maintained in Dulbecco's
264 modified Eagle's medium (DMEM: Invitrogen) supplemented with 5% fetal calf serum
265 (Invitrogen) and 1% L-glutamin. BHK 21 cells were grown in MEM-Earle, supplemented with 2
266 mM L-glutamine, non-essential amino acids and 10% FCS (Lonza, Belgium). The CDV wild-
267 type strain 5804P (56), its enhanced green fluorescent protein (eGFP)-expressing derivative
268 5804PeH (57), and the eGFP-expressing vaccine strain Onderstepoort (OSeH) (58) were grown
269 in VerodogSLAMtag cells. CDV F and H proteins were detected using the rabbit anti-Fcyt or
270 anti-Hcyt antibodies described previously (59), and peptide-specific antibodies described
271 elsewhere (26) were used to detect RABV M and actin.
272 **Plasmid construction.** Full-length genomic cDNA constructs were based on the
273 pcBNSP-333 backbone described previously (24, 60). The open reading frames from CDV F and
274 H genes were cloned from the following plasmids, pCG-Fos, pCG-F5804P, pCG-Hos, and pCG-
275 H5804P (59) while inserting a 5' BsiWI and 3' NheI restriction site (Fig. 1).
276 **Virus purification and inactivation.** Recombinant RABVs were recovered on Vero
277 cells as described previously (61). Briefly, per 6-well plate, a DNA mixture consisting of 5 µg
278 full-length cDNA, 2.5 µg pT7T-N, 1.25 µg pT7T-P, 1.25 µg pT7T-L, 1 µg pT7T-G and 1.5 µg
279 pCAGGS-T7 were mixed with X-tremeGENE 9 (Roche, Indianapolis, IN) at a ratio of 1:2 and

incubated for 15 min at room temperature before being added to Vero cells plated the day before at $5x10^5$ cells/well (62, 63). The cells were then incubated in a humidified incubator supplemented with 5% CO_2 at 34ºC for 3 h, heat-shocked at 42ºC for 3 h and returned to 34ºC for 4 days. At such time, the cells were expanded into 60mm dishes and incubated for an additional 2 days. Afterwards, 1 ml of supernatant was harvested from each of the 60 mm dishes and overlaid on fresh Vero cells and incubated at 34ºC for 2 days before being screened for virus recovery.

Virus stocks were prepared by infecting Vero cells at a multiplicity of infection (MOI) of 0.01 in OptiPro medium (Invitrogen, Carlsband, CA) supplemented with 1% penicillin-streptomycin and 4 mM L-glutamine (Thermo-HyClone, Rockford, IL). Supernatant harvests were collected at days 3, 6, 9 and 12 post-infection. Supernatants were first concentrated using a Research II*i* tangential flow filtration system (Spectrum Labs, Rancho Dominguez, CA) using a 300 kDa mPES column, then purified by ultracentrifugation at 25,000 rpm for 2 hours at 4ºC through a 20% sucrose cushion in an SW32-Ti rotor (Beckman Coulter, Inc., Brea, CA). Pelleted viruses were resuspended in D-PBS (Mediatech) overnight at 4ºC. Viruses were inactivated by incubation with β-propiolactone (Sigma) as described previously (26).

SDS-PAGE analysis. Western blot analysis was performed on Vero cells infected at a MOI of 10 with the viruses indicated for 48 h before being washed twice with PBS and lysed with RIPA buffer (50 mM Tris, pH 8, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100) supplemented with HALT protease inhibitors (Thermo). Total cellular protein (5 µg) or lysate from sucrose-purified particles (1 µg) was separated by 10% SDS-PAGE before transfer to polyvinylidine fluoride membranes using Towbin transfer buffer, then subjected to enhanced chemiluminescence detection using the antibodies indicated in the figure legend. SYPRO protein

analysis was conducted by fractioning 4 µg of sucrose-purified particles by 10% SDS-PAGE before staining with SYPRO ruby protein stain according to the manufacturer's directions (Lonza, Allendale, NJ).

Virus growth analysis. Vero cell monolayers in 6-well plates were infected at a MOI of either 10 (single-step) or 0.01 (multi-step) for 2 h in serum-free DMEM. The inoculum was then removed and the monolayers were washed three times with PBS to remove non-adsorbed virus and the media was replaced with 3 ml complete media. Samples of 100 µl were harvested from cell supernatants at the times indicated and the virus titer was quantified in duplicate on Vero cells.

Vaccination protocol and wild-type virus challenge. Unvaccinated male and female ferrets (*Mustela putorius furo*) 16 weeks or older were divided into five groups of four animals and immunized using the following strategies: immunization with a single dose of combined RABV containing Fos and Hos, or two doses of RABV-Hos, combined RABV-Fos and -Hos or RABV-F5804P and -H5804P. One group of animals was immunized twice with only the BNSP-333 vector as a negative control (Table 1). Blood samples were collected before the first and second immunizations, the challenge and weekly thereafter. Two months after the initial immunization, animals were challenged by intranasal inoculation of 2×10^5 50% tissue culture infectious doses ($TCID_{50}$) 5804PeH which expresses the enhanced green fluorescent protein from an addition gene inserted between the viral H and L genes (57). Body temperature and clinical signs were recorded daily, and blood samples were collected twice weekly for the first two weeks and weekly thereafter. Clinical signs were graded as described previously (56), and all animals reaching experimental end points were euthanized. For the total white blood cell count, 10 µl of heparinized blood was added to 990 µl of a 3% solution of acetic acid, and

nucleated cells were counted. Cell-associated viremia was quantified by limited dilution of white blood cells isolated from the same blood sample after hypotonic erythrocyte lysis, and expressed as $TCID_{50}$ per 10^6 PBMCs (64).

CDV-specific antibody responses. Total anti-CDV IgG antibodies were quantified by immunoperoxidase monolayer assay (IPMA) as previously described (65). Briefly, VerodogSLAMtag cells were seeded in 96-well plates and simultaneously infected with either 5804PeH or OSeH at a MOI of 0.01 and incubated at 37°C for 2 days until easily detectable syncytia appeared. The plates were then washed once with phosphate-buffered saline, dried, and fixed at 65°C for 8 h. Antibody titers were quantified by adding serial twofold dilutions to the plates, starting at a dilution of 1:10 or 1:100 in PBS, followed by incubation with a peroxidase-labeled anti-ferret IgG secondary antibody (Bethyl Laboratories, Montgomery, TX). Virus-specific staining was visualized with 3-amino-9-ethylcarbazole solution (Sigma, Taufkirchen, Germany), and titers were expressed as reciprocals of the highest antibody dilution at which viral antigen was detected by light microscopy.

Neutralizing antibodies were quantified by virus neutralization assay. Briefly, serial dilutions of serum samples starting at a dilution of 1:10 were incubated with 10^2 $TCID_{50}$ of 5804PeH or OSeH and incubated for 20 min, before 3×10^4 VerodogSLAMtag cells were added to each well. The neutralizing titer was expressed as the reciprocal of the dilution at which no cytopathic effect was observed after 3 days.

Anti-RABV antibody quantification. RABV neutralizing antibodies were quantified using a modified rapid fluorescent focus inhibition test as previously described (66). Briefly, four serial two-fold dilutions of test sera and the standardized rabies anti-immunoglobulin (RAI, NIBSC, Hertfordshire, UK) adjusted to 2 IU/ml were prepared in 96-well plates. 50 µl of each

dilution were pipetted into the wells and mixed with 50 µl of 100 $TCID_{50}$ of rabies challenge virus standard CVS-27 (ATCC VR-321, Behringwerke, Marburg, Germany). Virus back titrations as well as untreated cells were included in each test as controls. The mixtures were incubated at 37°C for 1 h followed by addition of 100 µl of a BHK 21 cell suspension (2.5×10^5 cells/ml) to each well. After incubation at 37°C for 48 h, the cells were washed with PBS, fixed with 80% acetone and stained by incubation with fluorescein-conjugated anti-rabies antibody (IBL International, Hamburg, Germany) for 30 min at 37°C. Each well was analyzed for presence of fluorescent cells, and the titers were calculated using the CombiStats software by comparison to the RAI immunoglobulin titers. The percent infected cells per well was determined, and international units of antibody were calculated based on the results obtained with RAI as the WHO standard, by which the dilution of the WHO standard that results in a 50% tissue culture inhibitory dose for the challenge virus is equivalent to 2 IU of neutralizing antibody.

Statistical analyses. One-way ANOVA with multiple group comparisons and Tukey post-test was used to determine the statistical significance of differences in post-challenge cell-associated viremia, total IgG and neutralizing antibody responses against the OS and 5804P CDV strains, and the neutralizing antibody response against RABV.

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FIGURE LEGENDS

Figure 1: Diagrams of the CDV glycoprotein-expressing recombinant RABVs generated for this study. The glycoprotein genes from vaccine strain CDV Onderstepoort (Fos and Hos) or pathogenic CDV 5804P (F5804P and H5804P) were cloned into the BNSP-333 vector, which contains the R333G mutation that eliminates neurotropism. White rectangles represent the RABV genes encoding the nucleoprotein (N), phosphoprotein (P), matrix (M) protein, glycoprotein (G), and polymerase (L) protein. The position of the respective CDV glycoprotein gene is indicated by a grey rectangle, and the restriction sites used for cloning as well as the position of the attenuating mutation at position 333 in the G protein are shown.

Figure 2: Western blot and particle composition analysis of recombinant RABV-CDV viruses. (**A**) Vero cell lysates or (**B**) sucrose purified virus particles were fractioned on 10% SDS-PAGE and analyzed with antibodies that detected the cytoplasmic tail of CDV-F (anti-Fcyt), CDV-H (anti-Hcyt), RABV-M (anti-RABV M) or β-actin (anti-actin). (**C**) SYPRO ruby stained SDS-PAGE of 4 µg sucrose purified particles. Stars indicate the putative CDV protein bands.

Figure 3: Growth analysis of recombinant RABV-CDV viruses. Vero cells were infected at an MOI of 0.01 (**A**) or 10 (**B**) and samples were collected at the time points indicated. Titers are expressed as focus-forming units (FFU/ml). The horizontal dashed line represents the detection limit of the assay.

Figure 4: Vaccination strategy and lethal challenge experiment. (**A**) Experimental timeline. Five weeks after the last immunization, four groups vaccinated with combinations are the RABV

vaccine vectors containing the CDV glycoproteins, as well as a control group vaccinated with only the RABV vaccine, were challenged intranasally with 2×10^5 $TCID_{50}$ of the CDV wild type strain 5804PeH. (**B**) Virulence index. Each box represents one animal, and black represents the highest (2), gray an intermediate (1), and white the lowest (0) score, as detailed in Materials and Methods. (**C**) Course of cell-associated viremia expressed as $\log_{10}$ 50% tissue culture infectious doses ($TCID_{50}$) per 10^6 PBMCs. The star indicates a statistically significant difference of $p<0.005$. (**D**) Total leukocyte counts from infected animals expressed as 10^3 leukocytes per mm^3. Days post-infection are indicated on the *x* axes of the graphs, symbols represent group means, error bars indicate the standard deviation.

Figure 5: Anti-CDV antibody responses. (**A** and **B**) Total IgG (**A**) and neutralizing antibody titers (**B**) against the 5804P wild-type strain. (**C** and **D**) Total IgG (**C**) and neutralizing antibody titers (**D**) against the OS vaccine strain. Total IgG was measured by IPMA and neutralizing antibody titers against the respective virus were quantified as outlined in materials and methods. Each symbol represents the mean of each group, and error bars indicate the standard deviation. The time of challenge with the CDV wild type strain 5804PeH is indicated by a dotted line.

Figure 6: Neutralizing antibody titers against RABV. Total IgG antibodies were quantified by IPMA and neutralizing antibodies were determined by virus neutralization assay against both wild-type and vaccine CDV strains. Titers are expressed as reciprocals of the highest antibody dilution at which viral antigen or syncytium formation was observed. Neutralizing antibody titers against RABV were quantified by a modified rapid fluorescent foci inhibition test as described in materials and methods. Each symbol represents the mean of each group, and error bars indicate

659 the standard deviation. The dashed horizontal line at 0.5 IU/ml indicates the minimum
660 neutralizing antibody level required to ensure protection against RABV infection, and the dashed
661 vertical line marks the time of challenge with the CDV wild type strain 5804PeH.
662

Figure 1

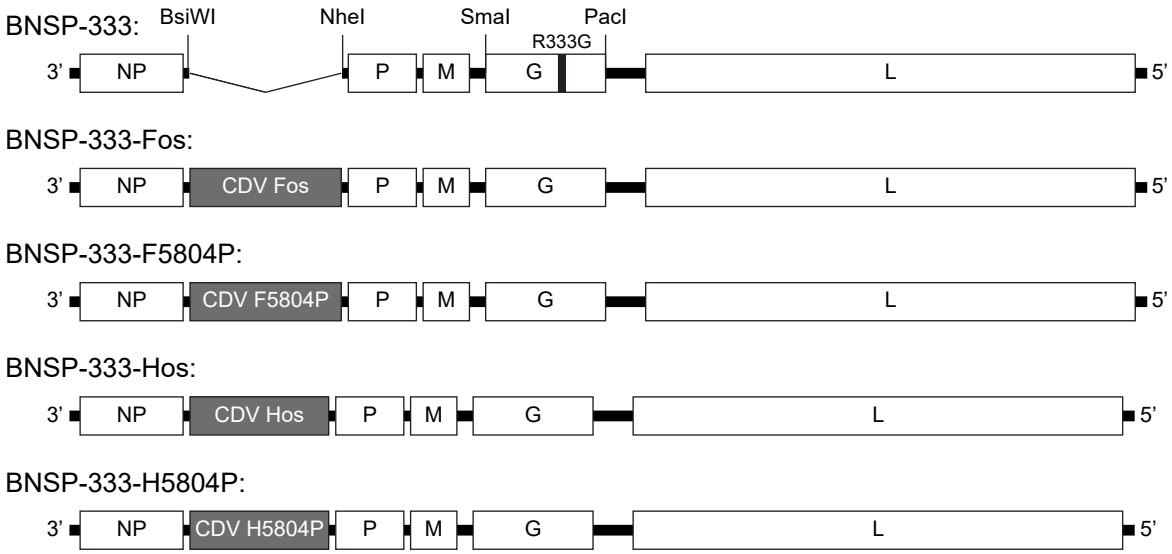
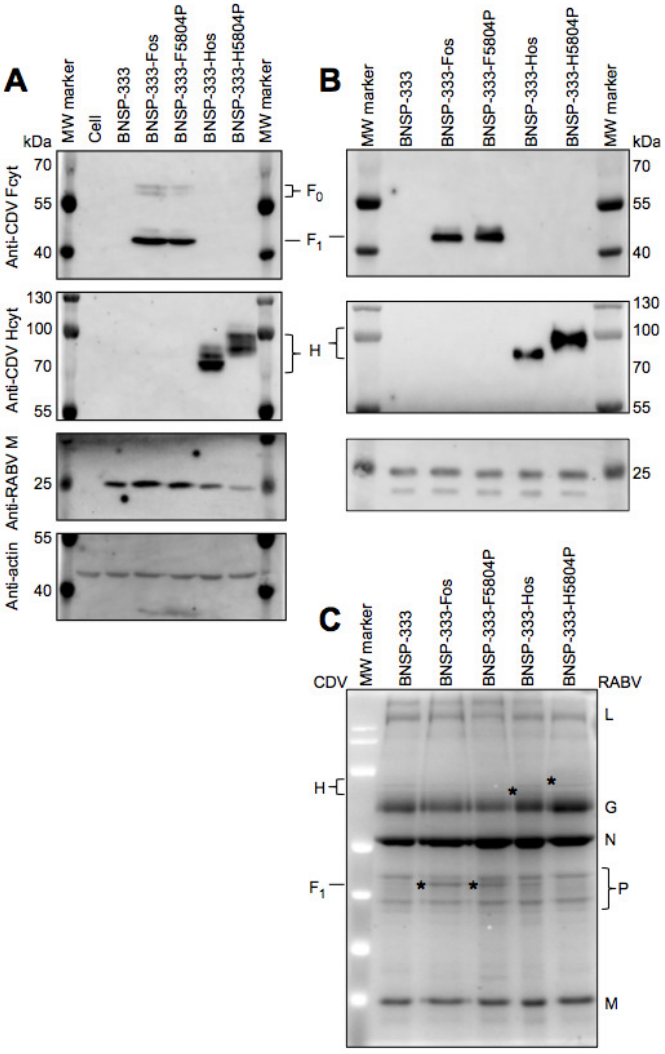


Figure 2



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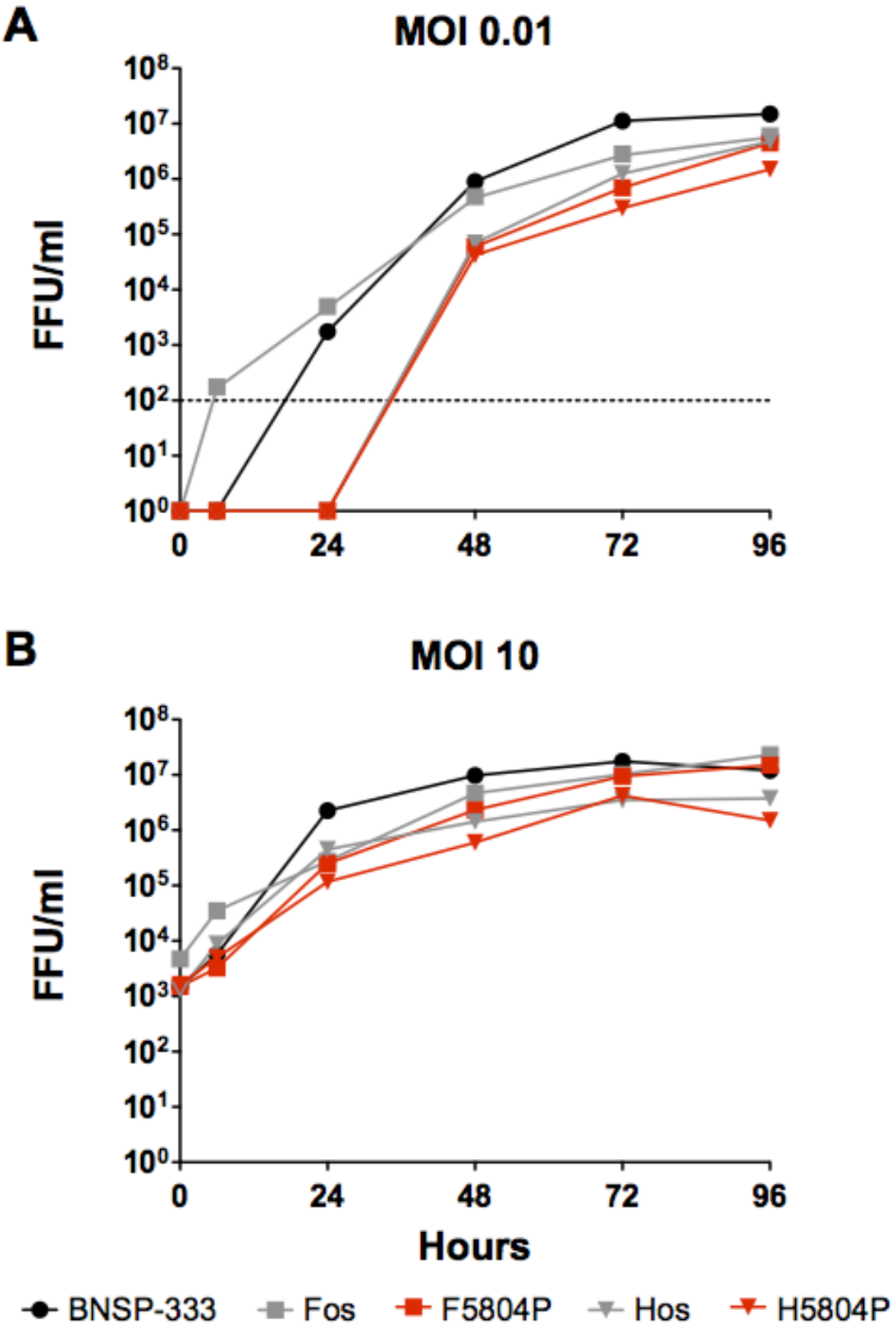


Figure 4

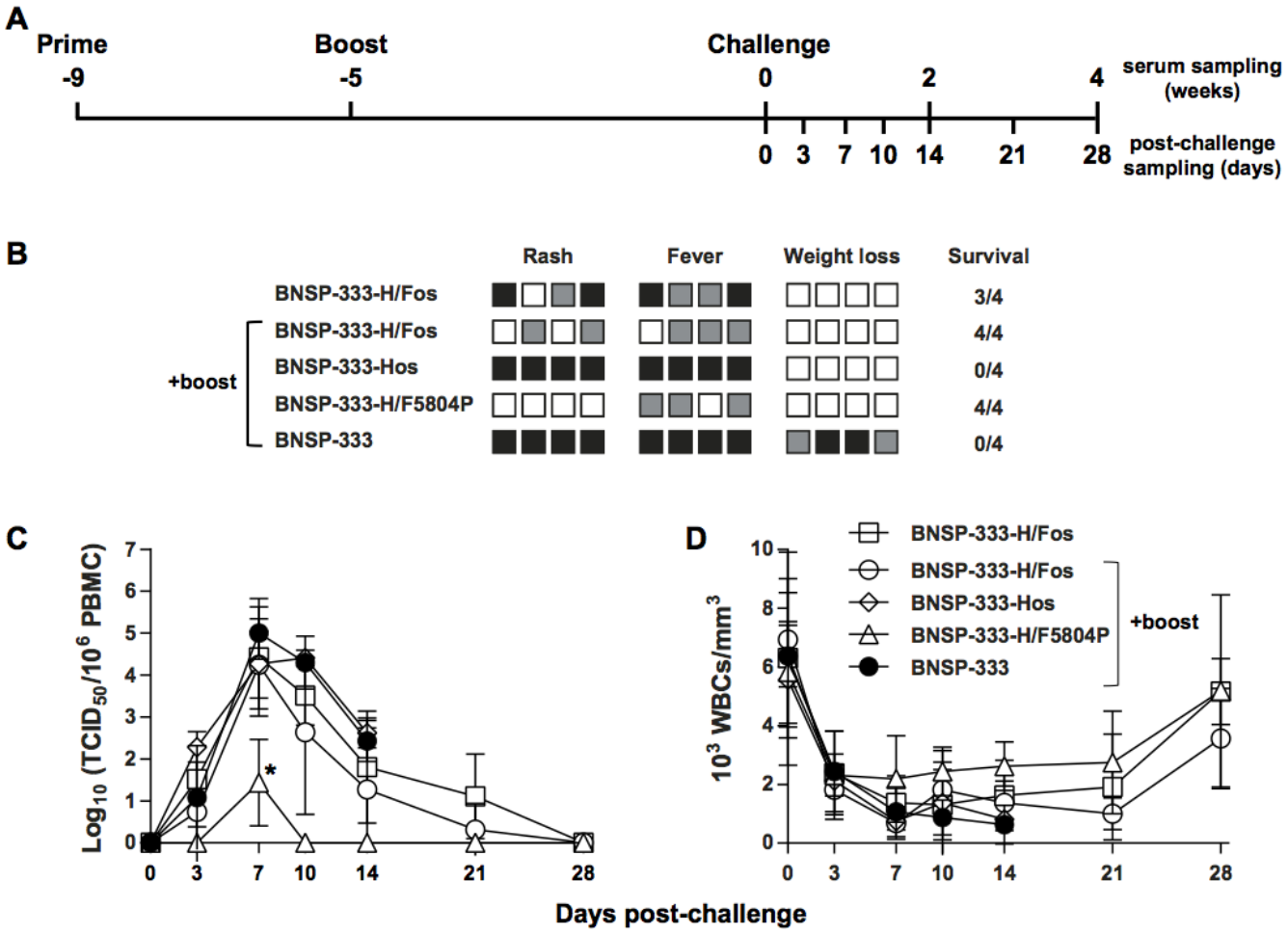


Figure 5

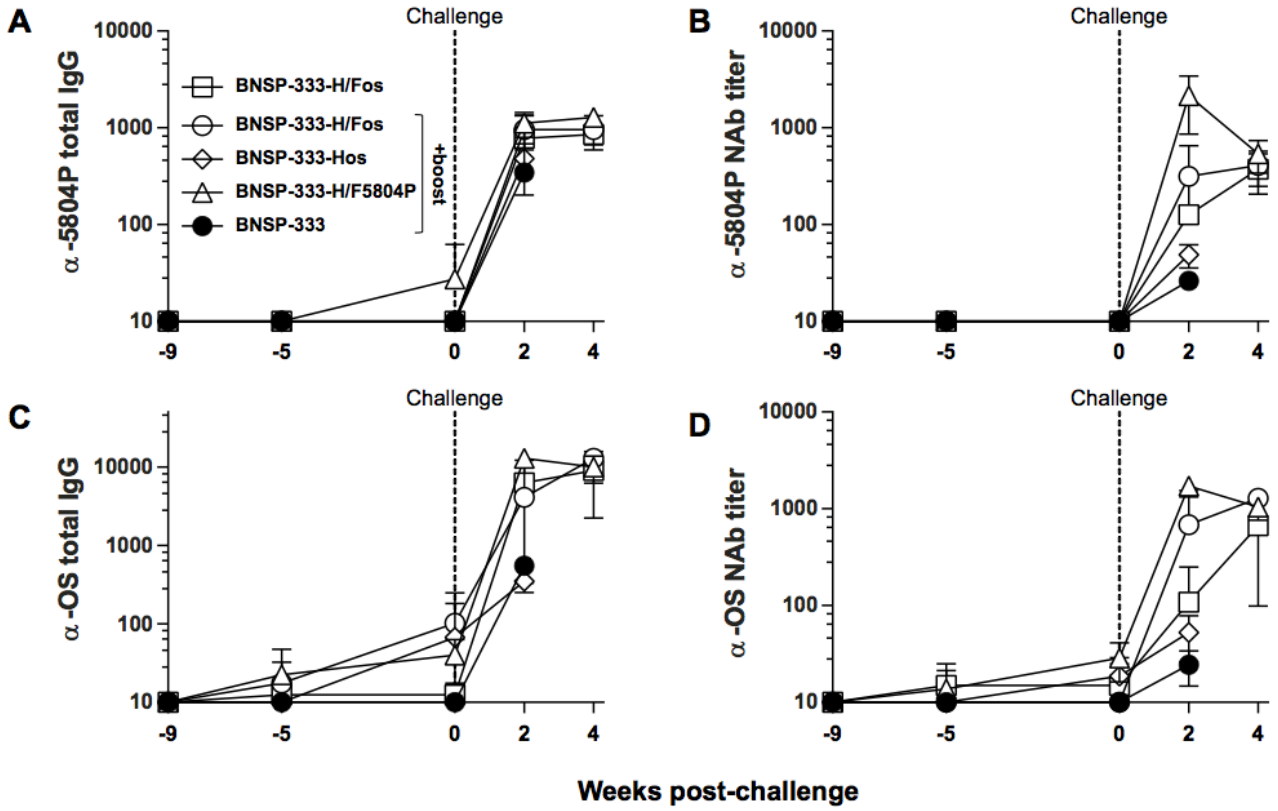
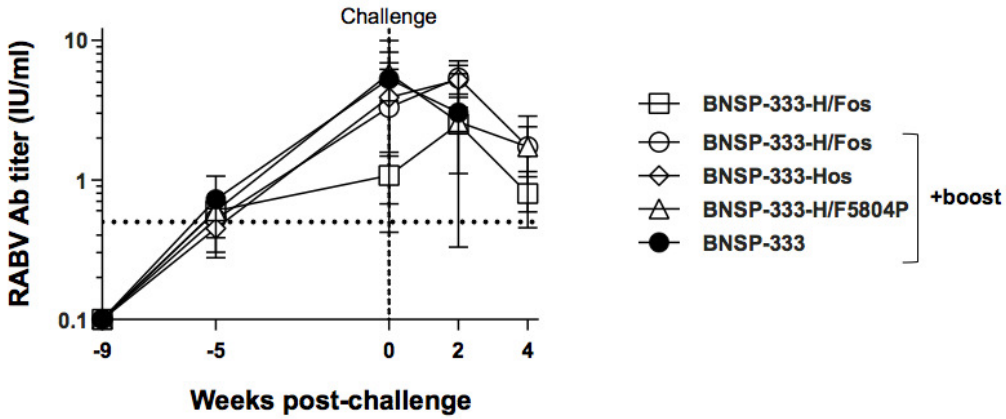


Figure 6



3.3. Artigo 3 - Morbillivirus experimental animal models: measles virus pathogenesis insights from canine distemper virus

Artigo de revisão publicado na revista *Viruses*. O presente artigo será apresentado a seguir no formato de publicação.

viruses

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Review

Morbillivirus Experimental Animal Models: Measles Virus Pathogenesis Insights from Canine Distemper Virus

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Abstract: Morbilliviruses share considerable structural and functional similarities. Even though disease severity varies among the respective host species, the underlying pathogenesis and the clinical signs are comparable. Thus, insights gained with one morbillivirus often apply to the other members of the genus. Since the *Canine distemper virus* (CDV) causes severe and often lethal disease in dogs and ferrets, it is an attractive model to characterize morbillivirus pathogenesis mechanisms and to evaluate the efficacy of new prophylactic and therapeutic approaches. This review compares the cellular tropism, pathogenesis, mechanisms of persistence and immunosuppression of the *Measles virus* (MeV) and CDV. It then summarizes the contributions made by studies on the CDV in dogs and ferrets to our understanding of MeV pathogenesis and to vaccine and drugs development.

Keywords: morbillivirus genus; canine distemper virus; animal models; pathogenesis

1. Introduction

Morbilliviruses are enveloped, negative-stranded RNA viruses which cause a moderate to severe respiratory and gastrointestinal disease and long-lasting immunosuppression in their respective hosts. Despite the availability of a safe and cost-effective vaccine, the *Measles virus* (MeV) remains one of the leading causes of death among young children in developing countries [1]. MeV infections are also associated with neurological complications that may occur during the acute disease phase, but also many years later, due to long-term persistence in the central nervous system (CNS).

Since humans are the only reservoir for MeV, it is a promising target for global eradication, and a world-wide eradication campaign is ongoing [1]. The successful eradication of the closely related *Rinderpest virus* (RPV) in 2011 demonstrates the general feasibility of this approach [2]. However, decreasing adherence to vaccination in industrialized countries has resulted in a re-emergence of the disease. The increase in outbreaks in North America and Europe in recent years has led to these regions missing eradication targets, endangering the success of the overall campaign [3]. To develop new prophylactic and therapeutic strategies that support these efforts, a better understanding of MeV pathogenesis and immune interference is required.

While the narrow host range, which includes only humans and non-human primates, makes MeV eradication possible, it also limits the characterization of its pathogenesis. A surrogate model based on the study of the *Canine distemper virus* (CDV)—a closely related morbillivirus that infects a broad range of carnivores including ferrets or dogs—represents an attractive alternative. CDV causes a similar overall pathogenesis in its different hosts, but the disease severity varies from moderate in

dogs, to completely lethal in highly susceptible species, such as ferrets and many wild carnivores [4,5]. The clinical signs include fever, often a characteristic rash, diarrhea, nasal discharge, conjunctivitis, and generalized immunosuppression, thereby reproducing the disease spectrum seen in MeV patients (Figure 1). Furthermore, acute and delayed neurologic complications are frequently observed [6]. This review will provide an update on the contribution of these models to our understanding of the routes of infection, receptors, tissue tropism, mechanisms of immunosuppression, and viral persistence in the CNS, and also an update on the progress that has been made in drug and vaccine development.

Figure 1. Morbilliviruses (*Measles virus* (MeV) in blue, *Canine distemper virus* (CDV) in red) display similar tropism and tissue distribution in their respective hosts.

2. Morbillivirus Taxonomy and Life Cycle

Morbilliviruses belong to the *Paramyxoviridae* family as a member of the *Mononegavirales* order. In addition to MeV, CDV, and RPV, the *Morbillivirus* genus currently includes four species, namely the *Cetacean morbillivirus* (CeMV), the *Phocine distemper virus* (PDV), the *Peste-des-petits-ruminant virus* (PPRV) and the recently added *Feline morbillivirus* (FeMV). Phylogenetically, the CDV and PDV are the most closely related (Figure 2), suggesting that the PDV emerged from the CDV several thousands of years ago by contact with terrestrial carnivores [7]. The CeMV forms a separate branch that includes isolates from dolphins and porpoises, while the MeV is most closely related to the ruminant morbilliviruses: RPV and PPRV. The RPV has been suggested as the progenitor virus of the *Morbillivirus* genus [8].

All morbilliviruses share the same particle structure and genome organization. Viral particles are pleomorphic, with a diameter of approximately 150 nm. They consist of a lipid envelope composed of the fusion (F) and attachment (H) transmembrane glycoproteins and are lined by the matrix (M) protein. The viral genomic RNA is encapsidated by the nucleoprotein (N), and forms, together with the phosphoprotein (P) and the polymerase (L) protein, the ribonucleoprotein-complex (RNP), which is surrounded by the viral envelope during the budding process at the plasma membrane [9]. The morbillivirus genome size lies at around 16,000 nt, and the genome contains six transcription units arranged linearly in the order 3'leader-N-P-M-F-H-L-5'trailer, which are separated by intergenic regions. The transcription units give rise to at least eight proteins, since the *P* gene also encodes the C and V proteins by use of an alternate open reading frame and RNA editing, respectively [10].

There is considerable homology on the amino acid and even nucleotide level, which results in high levels of structural and functional conservation among morbilliviruses. On the one hand, this allows the application of structural data available for MeV proteins to those of other morbilliviruses [11,12], and on the other hand, it enables the use of chimeric viruses or in vitro trans-complementation for the characterization of functional domains [13,14].

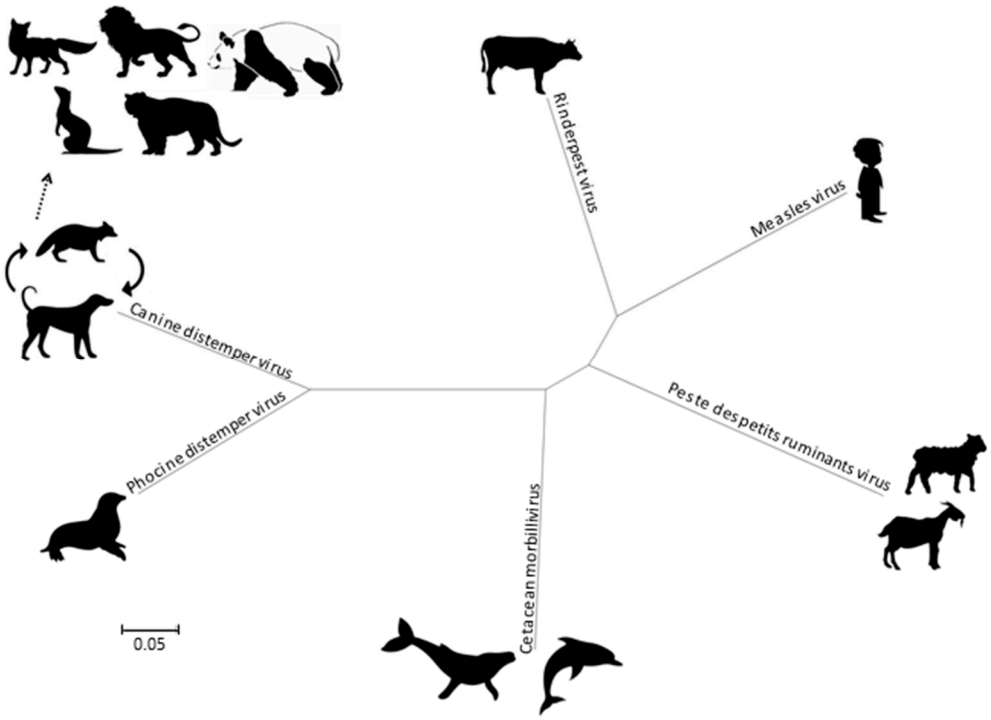

Figure 2. Phylogenetic tree, based on complete genomes of morbilliviruses. Molecular Evolutionary Genetics Analysis 6 (MEGA6) was used for phylogeny inference according to the maximum likelihood algorithm.

3. Pathogenesis

All morbilliviruses are transmitted by aerosol. Taking advantage of eGFP-expressing viruses, resident immune cells in the respiratory tract were identified as initial target cells [15], and the subsequent spread to local immune organs and systemic dissemination coincides with a first fever peak observed three to six days after infection [16]. This stage of infection is critically dependent on the interaction of the virus with the immune cell receptor CD150 or signaling lymphocyte activation molecule (SLAMF1, [17]). CD150 has two extracellular immunoglobulin-superfamily domains, V and C2, and the cytoplasmic tail carries several tyrosine phosphorylation motifs involved in intracellular signaling [18–20]. Structure-function studies revealed that the H protein interacts with its variable domain (Figure 3), and that the residues involved in this interaction are located in close structural proximity in the MeV and CDV H proteins [11,21]. Mutation of these residues resulted in viruses that no longer infected immune cells but retained wild type entry and replication efficiency in epithelial cells. In ferrets, these SLAMblind viruses were unable to establish a systemic infection or cause clinical signs of disease [22], but elicited a protective immune response, suggesting that limited replication, possibly in epithelial cells close to the inoculation site, had occurred. A similar phenotype was observed in primates infected with SLAMblind MeV [23], further validating these findings.

The efficiency of immune recognition by the respective host determines the extent of virus amplification in immune cells and thereby the level of spread to epithelial cells. In ferrets, which are unable to control wild type viruses, infection levels exceeding 80% of B or T cells can be observed in certain immune tissues [24], whereas levels below 10% are usually found in MeV-infected macaques [25]. Consequently, there is a massive infection of epithelial tissues in wild type virus-infected ferrets, leading to severe respiratory and gastro-intestinal signs of disease and death within two to five weeks [16,26], whereas most primates develop only localized infection of epithelial cells and mild clinical signs [25]. Upon the identification of nectin-4—an adhesion protein that also contains immunoglobulin-like domains (Figure 3)—as a morbillivirus epithelial cell receptor [27–30],

nectin-4blind viruses were generated that retained their ability to infect immune cells and resulted in wild type peripheral blood mononuclear cell (PBMC) infection levels and immunosuppression in ferrets. However, no rash or other clinical signs were observed, and no virus was shed [31]. The corresponding MeV in primates also remained immunosuppressive and was no longer shed [32], again illustrating the common role of receptor interactions in both viruses. Furthermore, studies with these selective receptor-blind viruses revealed that immunosuppression is caused by immune cell infection while clinical signs, and likely also transmission, result from epithelial cell infection. This link between specific target cell populations and defined clinical aspects of the disease provides starting points for therapeutic and outbreak control strategies.

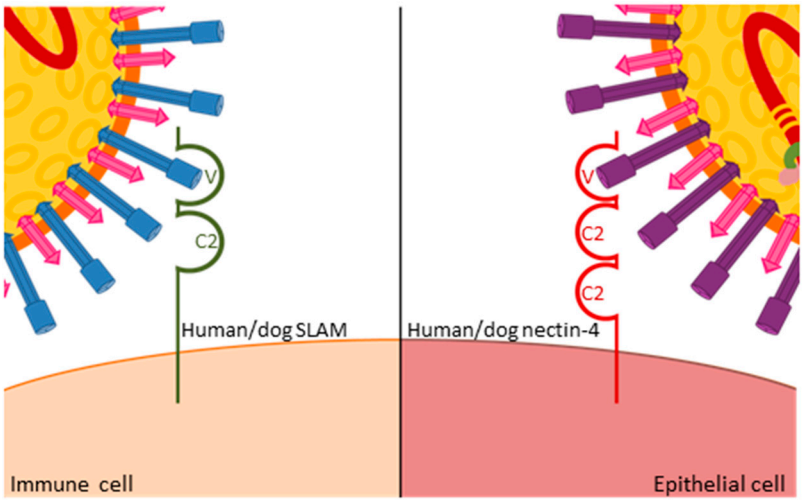

Figure 3. Similar interactions of MeV and CDV H proteins with the variable domain of human and dog CD150 receptors on immune cells, and human and dog nectin-4 receptors on epithelial cells, respectively.

4. Immunosuppression

The severe transient immunosuppression, which occurs at variable levels and can last from weeks to months after the resolution of the disease, is one of the hallmarks of morbillivirus infections. While a profound leukopenia during the acute infection phase is typical for many viral infections, morbilliviruses additionally induce an anergy-like state in immune cells that prevents their ex vivo activation by non-specific stimuli and leads to the loss of delayed-type hypersensitivity responses [33]. It is this long-term immunosuppression that leaves patients with an increased susceptibility to secondary infections such as pneumonia and gastroenteritis, which significantly contribute to MeV-associated morbidity and mortality.

In CDV-infected dogs and ferrets and MeV-infected macaques, leukopenia is first observed two to four days after infection, when the replication in immune cells is just beginning; it peaks at the onset of clinical signs, and gradually resolves with the immune response-mediated control and elimination of the virus (Figure 4) [34–36]. It coincides with the depletion of immune cells from lymphatic tissues and an increase of cells in an early apoptotic state [24]. While leukocyte numbers normalize quickly after resolution of the disease, the anergic state persists over a prolonged period of time, which indicates that it is not only caused by a direct mechanism of immune cell destruction, but that indirect mechanisms are involved [37]. Studies in patients revealed a Th2-biased response, which, together with increased production of the immunosuppressive cytokine interleukin (IL)-10, may be involved in these alterations [38,39], but this phenomenon remains to be investigated in more detail in CDV or MeV animal models.

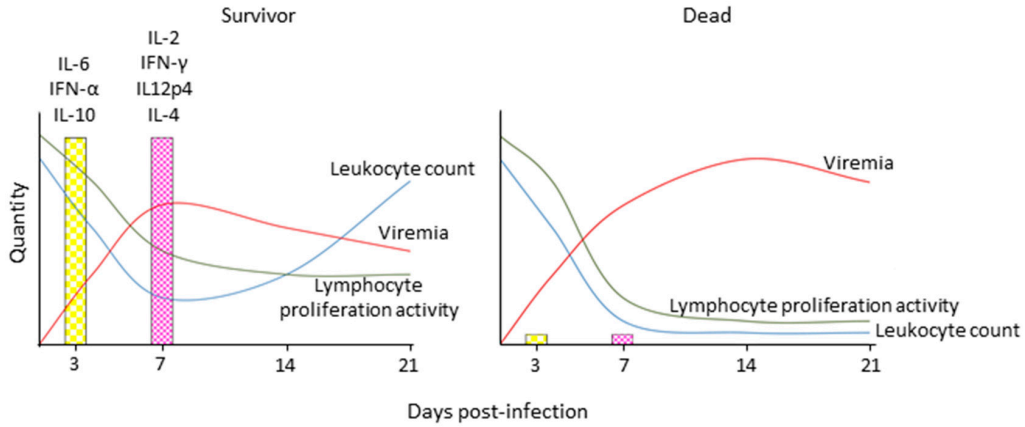

Figure 4. Schematic depiction of immune response profiles associated with different morbillivirus disease outcomes. Survivors display robust innate immune activation and experience transient immune suppression as indicated by a transient drop in leukocytes and lymphocyte proliferation activity upon non-specific stimulation, robust induction of innate and then adaptive cytokine responses, and control of cell associated viremia, while animals that succumb to the disease experience severe leukopenia and complete loss of lymphocyte proliferation activity, and are unable to activate innate immune responses and control the virus. IL: interleukin; IFN: interferon.

In contrast, the viral interference with innate immune activation has been extensively characterized. A comparison of cytokine profiles revealed a complete lack of innate immune activation in animals that succumbed to infection, whereas those that survived displayed a broad upregulation of cytokines associated with innate and later adaptive immune responses [40]. Consistent with many other members of the *Paramyxoviridae* family, the morbillivirus V protein inhibits innate immune signaling pathways on several levels [41,42]. Deletion of the V protein results in attenuation of lethal CDV wild type strains in ferrets, and loss of inhibition of lymphocyte proliferation, but the leukopenia is still observed [22]. A more detailed investigation of the contribution of different interferon (IFN) signaling pathways revealed that interference with signal transducer and activator of transcription 2 (STAT2) and melanoma differentiation-associated gene 5 (mda5) signaling was essential for effective immunosuppression and a lethal disease phenotype, while restored STAT1 signaling alone did not attenuate the virus sufficiently to lead to survival [43]. However, a STAT1blind MeV was attenuated in macaques [44], demonstrating a role of STAT1 signaling in Morbillivirus pathogenesis. Similarly, a C protein-deleted CDV retained full lethality in ferrets [22], while the corresponding MeV was clearly attenuated in macaques [45]. Both cases illustrate that, because of their high sensitivity to CDV, ferrets may thus not be ideally suited to characterize minor attenuation factors.

5. Neurologic Complications

MeV infection can lead to several rare but potentially lethal neurologic complications: acute disseminated encephalomyelitis (ADEM), also known as post-infectious encephalomyelitis (PIE); measles inclusion body encephalitis (MIBE); and subacute sclerosing panencephalitis (SSPE) [46]. In contrast, neuroinvasion is frequently observed during the acute phase of CDV infections, and dogs that survived a natural infection occasionally develop old dog encephalitis (ODE) that shares similarities with SSPE [47]. While around 30% of dogs infected with CDV will develop neurologic complications [48], certain strains cause close to 100% neuroinvasion in ferrets [26,49], making them an attractive model to characterize the underlying mechanisms.

ADEM, as a consequence of MeV infection, occurs in 1 in 1000 cases, and up to 25% of affected patients die, with around 33% of survivors experiencing chronic sequelae [46]. Only few infected cells are usually detected in the CNS, and an abnormal immune response to myelin basic protein is considered the primary pathomechanism [50]. In CDV-infected dogs, first infected cells are found at

the interface between endothelial or epithelial cells and CNS cells around two to three weeks after infection, at the same time as epithelial cell infection is seen in other tissues [51]. If the virus is not cleared, focal infection of neurons and glial cells in the grey and white matter ensues four to five weeks after infection, with a concomitant onset of demyelination in these areas [52]. There is increasing evidence that this demyelination primarily results from an antiviral response in the CNS [53–55], even though virus-induced death of infected cells may also be a contributing factor [56]. While the presence of a virus in the CNS seen in CDV-infected animals clearly differs from the findings in ADEM patients, where little to no CNS infection is seen, the characterization of the immune mediated demyelination in this model may still provide valuable insights into the mechanisms involved in ADEM.

MIBE typically affects immunocompromised patients and is most often reported in HIV positive children, leukemia patients, and transplant patients undergoing immunosuppressive therapy [57,58]. The onset is usually within weeks or months after the acute infection, and the disease is characterized by behavioral changes and seizures [46]. Since ferrets are unable to mount an effective immune response to CDV infection, the neuroinvasion seen in that model likely reproduces key aspects of MIBE pathogenesis, especially cases with an onset during the acute phase. Time course studies in ferrets infected with neurovirulent strains revealed the importance of anterograde neuroinvasion via the olfactory bulb in addition to the previously described hematogenous spread, illustrating a direct entry route from the olfactory mucosa in the upper respiratory tract into the CNS [26,49,59]. The observation that the extent of immunosuppression determines not only disease duration but also the neurovirulence of the respective virus, further substantiates the link between the efficiency of the host immune response in controlling the infection and the incidence of neuroinvasion [60].

SSPE primarily affects patients infected in their first year of life and is exceedingly rare, with an incidence rate of around one in 10,000 cases [61,62]. It progresses slowly with demyelination of multiple areas of the brain. Initial symptoms include subtle behavioral changes, followed by myoclonic seizures leading invariably to death [46]. The mechanisms underlying this CNS persistence are poorly understood, but SSPE virus particle assembly is severely impaired due to hypermutations in the *M* and *F* gene regions [63,64]. While the pathogenesis of old dog encephalitis is similar, it is hitherto unknown if the virus accumulates similar hypermutations. Since only a small subset of animals that survive a CDV infection develop this complication, a canine model has not been pursued so far. Shortly after SSPE viruses were first isolated, their neurovirulence in ferrets was compared with wild type isolates, revealing that certain SSPE strains led to encephalitis in all intracerebrally inoculated animals, with one of the strains even causing CNS persistence of several months [65]. Further investigations demonstrated that the incidence of persistent infections could be increased if the animals received an MeV vaccine prior to inoculation, and that the histological changes and the antibody responses reproduced those seen in SSPE patients [66]. However, this model has not been further developed or used in recent years. Instead, an in vitro culture system of different CNS cell types is being increasingly used to study the mechanisms involved in morbillivirus CNS persistence and to explore therapeutic strategies [67–69].

6. Vaccine and Drug Development

The live-attenuated MeV vaccines licensed in the 1960s represent one of the most successful public health interventions and remain efficacious today. However, the exceptionally high MeV basic reproductive number (R_0), close to 20, requires at least a 95% coverage in a population to prevent transmission [70]. Even though exact values have not been determined, the similarities among morbilliviruses make it highly likely that their R_0 are also similar. CDV vaccines were first marketed around the same time as MeV vaccines and are now an integral part of the standard puppy vaccination schedule [71]. In countries with widespread vaccination, CDV cases in dogs have become rare, but a CDV eradication program is currently not feasible due to its diverse wildlife reservoir.

Morbillivirus vaccines have so far retained their efficacy, since the antigenic stability of these viruses is remarkably high compared to other RNA viruses, likely due to constraints on their

glycoproteins [72]. Nonetheless, the development of a new generation of vaccines is actively ongoing to explore the potential of morbilliviruses as vaccine platforms, and, in the case of CDV, to increase safety for highly sensitive species. Morbilliviruses stably incorporate additional genes in their genome, and immunization with such a bi- or multivalent virus elicits humoral and cellular immune responses against the added proteins [73]. Strong immunogenicity of the first such MeV vaccine carrying a chikungunya virus antigen has been seen in a clinical phase I trial [74], and the efficacy of a leishmania antigen-expressing CDV has been demonstrated in dogs [75], illustrating the potential of this platform.

Even though the live-attenuated CDV vaccines are fully apathogenic in dogs, they can cause severe clinical distemper in highly sensitive species such as black-footed ferrets or other wild carnivores [76,77], highlighting the limitations of arbitrary attenuation. For those species, different vaccine approaches have been evaluated, including DNA vaccines [78], non-replication competent and replication-competent vector vaccines [79,80], as well as rationally attenuated vaccines [81,82]. A CDV F and H protein-expressing canarypox-based vaccine has been licensed for several years now, and has been successfully used in various wildlife species [83,84]. In addition, these studies have significantly contributed to our understanding of morbillivirus correlates of protection and the role of maternal immunity. The efficacy of DNA and vectored vaccines that express the CDV H protein alone revealed that immune responses against this protein are sufficient to protect against morbillivirus infections [85–87]. Many of these vectors also displayed superior immunogenicity in the presence of maternal antibodies compared to live-attenuated vaccines [88,89], providing strategies that may also become relevant for MeV eradication.

The resurgence of MeV in many industrialized countries has rekindled the interest in developing therapeutic strategies against these viruses, and the similar life cycles and availability of a sensitive small animal model make CDV an attractive surrogate system to assess the safety and efficacy of promising candidates. Furthermore, therapeutic interventions to control CDV infection may also be considered for valuable animals. Among the most promising candidates is an orally bioavailable small molecule inhibitor of the MeV polymerase that was able to rescue ferrets from lethal CDV challenge when given shortly after infection [90]. Strategies targeting viral entry or fusion, which also use small molecule inhibitors [91,92], as well as the first generation of host-targeting molecules [93], are reaching the state of preclinical testing, which will likely also involve one of the CDV surrogate models.

A meta-analysis of several studies has recently drawn attention to the potential of post-exposure passive immune transfer to reduce MeV-associated deaths [94], and a canine hyperimmune serum was marketed in Europe for CDV therapy. Finally, monoclonal antibody therapy might also yield promising results against morbilliviruses. Earlier proof-of-concept studies in mice demonstrate the efficacy of monoclonal antibodies directed against CDV glycoproteins [95], and a development of similar MeV-specific antibodies might become attractive as eradication progresses.

7. Conclusions/Perspectives

The study of the CDV in its natural hosts is a powerful complement to the investigation of MeV in non-human primates or rodent models and has significantly contributed to our understanding of morbillivirus pathogenesis. The high sensitivity of ferrets to the CDV infection enables the identification of virulence and attenuation determinants, as well as the safety and efficacy assessment of new therapeutics and vaccines. The naturally occurring progressive CNS infection in dogs surviving the acute CDV infection provides unique insights into the pathogenesis of this devastating complication and may yield novel therapeutic strategies. Since dogs, as companion animals, develop many of the same cancers and degenerative disorders as seen in people, they also represent attractive translational models for morbillivirus-based gene therapy approaches. The comparative analysis in different virus-host models will be essential to answer still-open questions about antigenetic stability, immunosuppression and persistence and will continue to improve our understanding of these important viruses.

Conflicts of Interest: The authors declare no conflict of interest.

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4. CONCLUSÕES

Como conclusões dos trabalhos que compõem a presente tese, pode-se citar:

- Oito possíveis eventos de recombinação homóloga foram detectados em sequências completas de CDV, incluindo eventos entre cepas de campo e vacinais, e os *breakpoints* foram mais comumente encontrados nos genes F e H.
- Eventos de recombinação homóloga em morbilivírus, especificamente no CDV, aparentam ser mais frequentes do que anteriormente relatado na literatura.
- Eventos de recombinação entre cepas de campo e vacinais podem ser importantes mecanismos na evolução do CDV.
- A proteína H isoladamente não é suficiente para induzir proteção, mesmo com *boost*, sugerindo que a proteína F tem um papel crucial na indução de proteção.
- Ambas glicoproteínas do CDV, H e F, independentemente se originada de cepa de campo ou de cepa atenuada, são necessárias para desenvolver uma proteção completa contra desafio por CDV, assim como um protocolo de *prime/boost* também é necessário.
- A vacina bivalente contra raiva e CDV pode ser uma alternativa eficiente e segura para vacinação de animais altamente suscetíveis à infecção por CDV.

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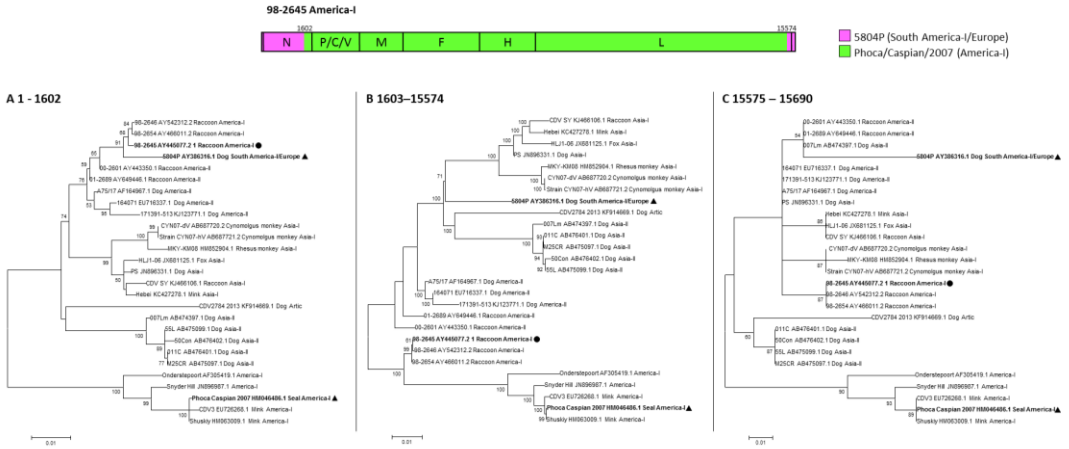
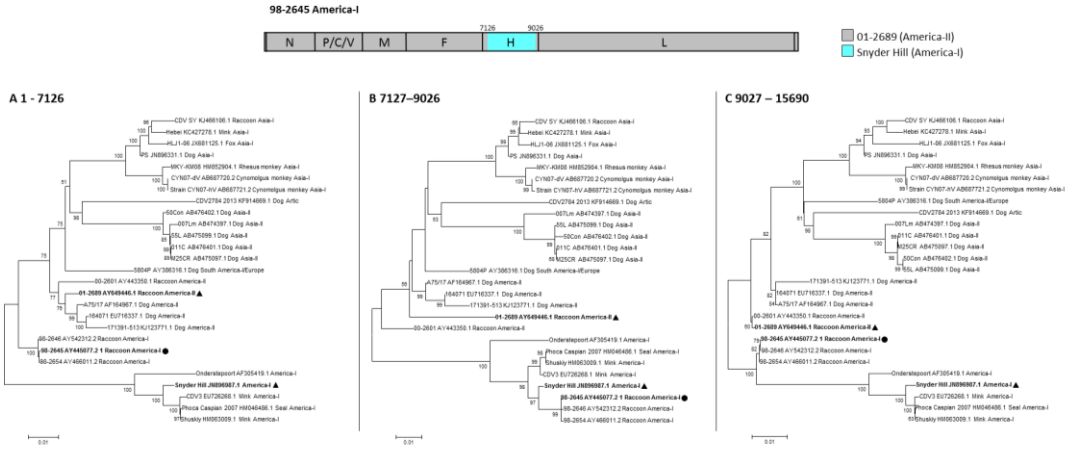
Anexo – Material suplementar referente ao Artigo 1

Supplemental material

Schematic representation of the putative recombinant strains. The genome organization of the potential recombinant virus with the breakpoints and segments of the genome derived for major and minor parents are schematically represented. The relationship between the putative recombinant (●) and putative parents (▲) in different segments of the genome was shown in the neighbor-joining trees, where bootstrap values ≥50% are represented.

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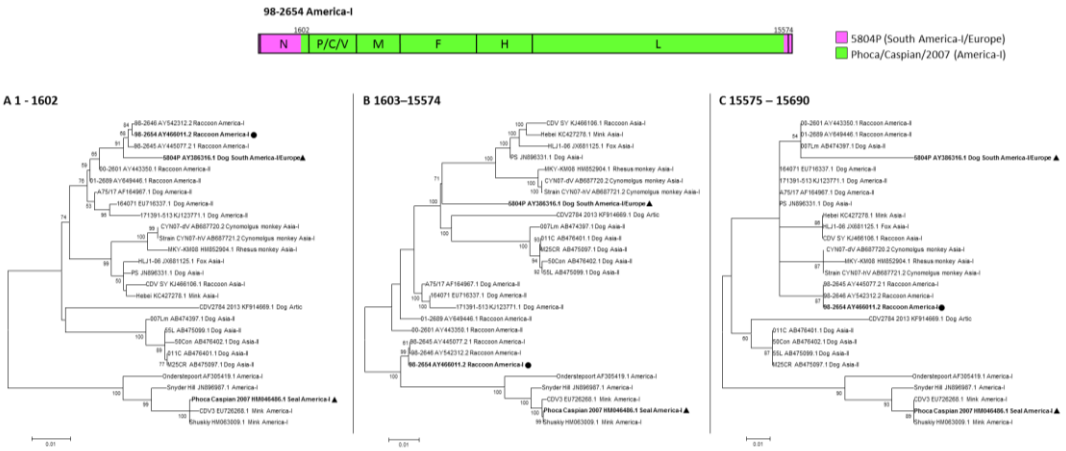
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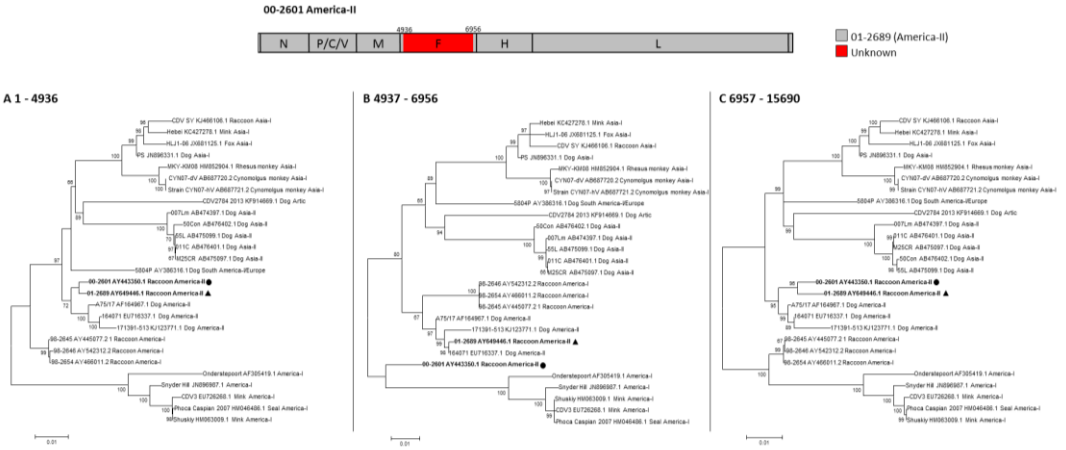
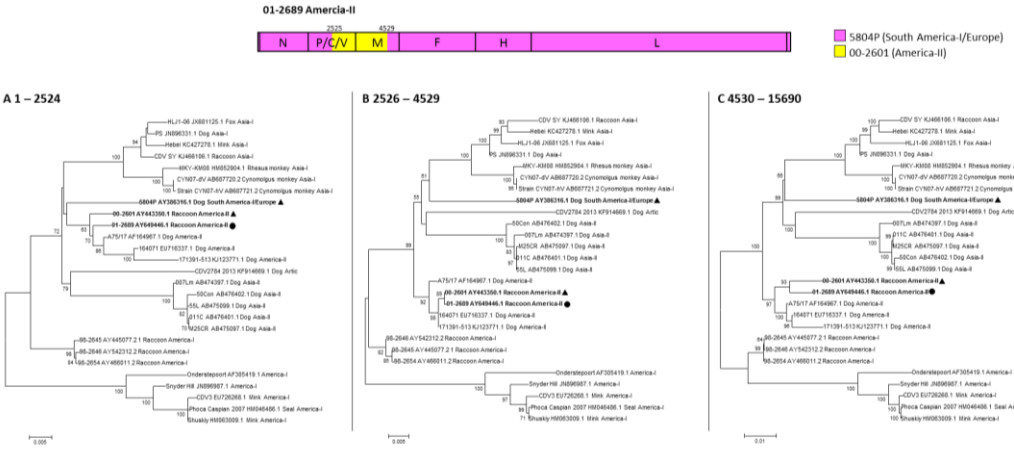
C 15575 – 15690

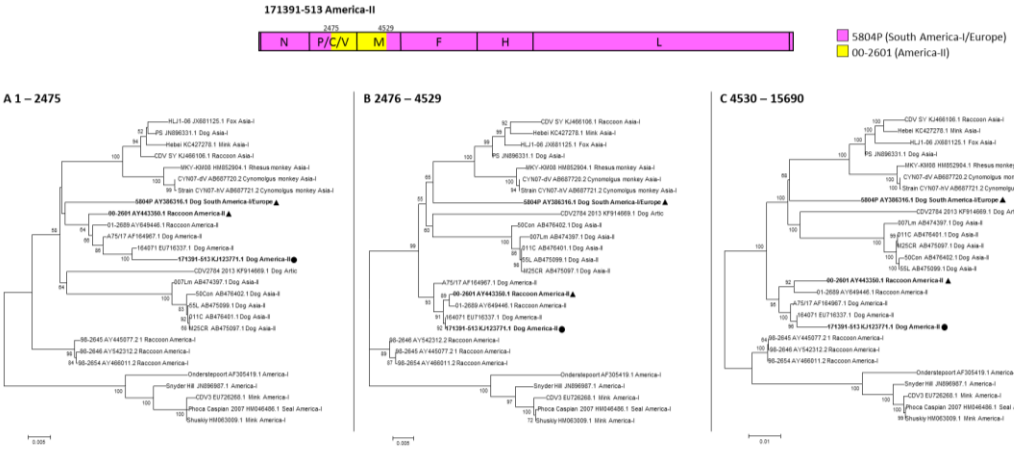
10


00-2601 America-II
4936
6956
N
P/C/V
M
F
H
L
01-2689 (America-II)
Unknown
A 1 - 4936
B 4937 - 6956
C 6957 - 15690

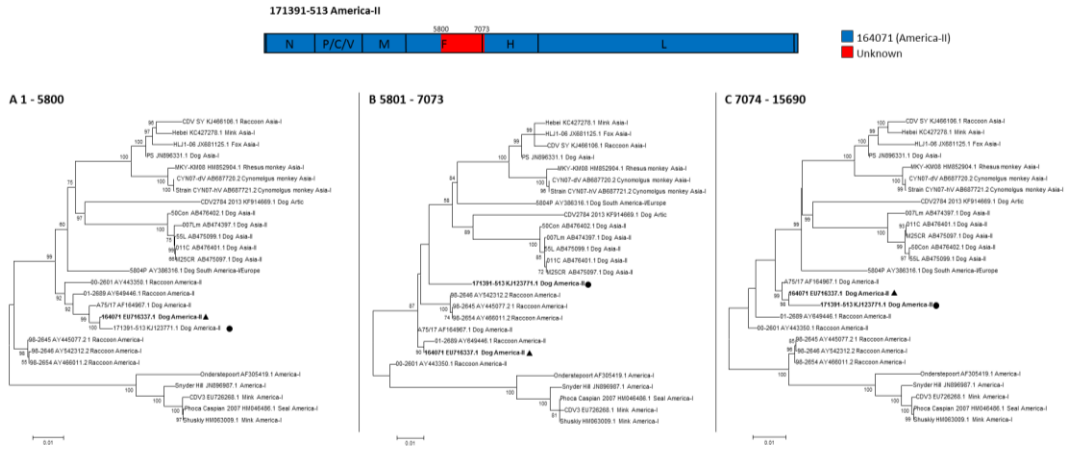
11


01-2689 Amercia-II
2525
4529
N
P/C/V
M
F
H
L
5804P (South America-I/Europe)
00-2601 (America-II)
A 1 – 2524
B 2526 – 4529
C 4530 – 15690

12


171391-513 America-II
2475
4529
N
P/C/V
M
F
H
L
5804P (South America-I/Europe)
00-2601 (America-II)
A 1 – 2475
B 2476 – 4529
C 4530 – 15690

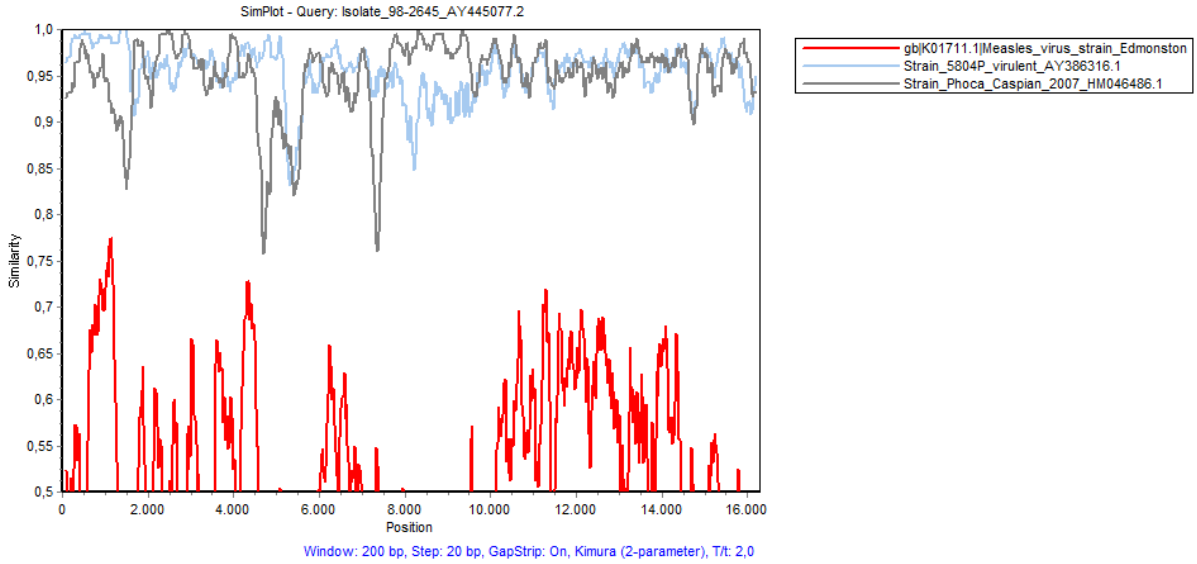
13

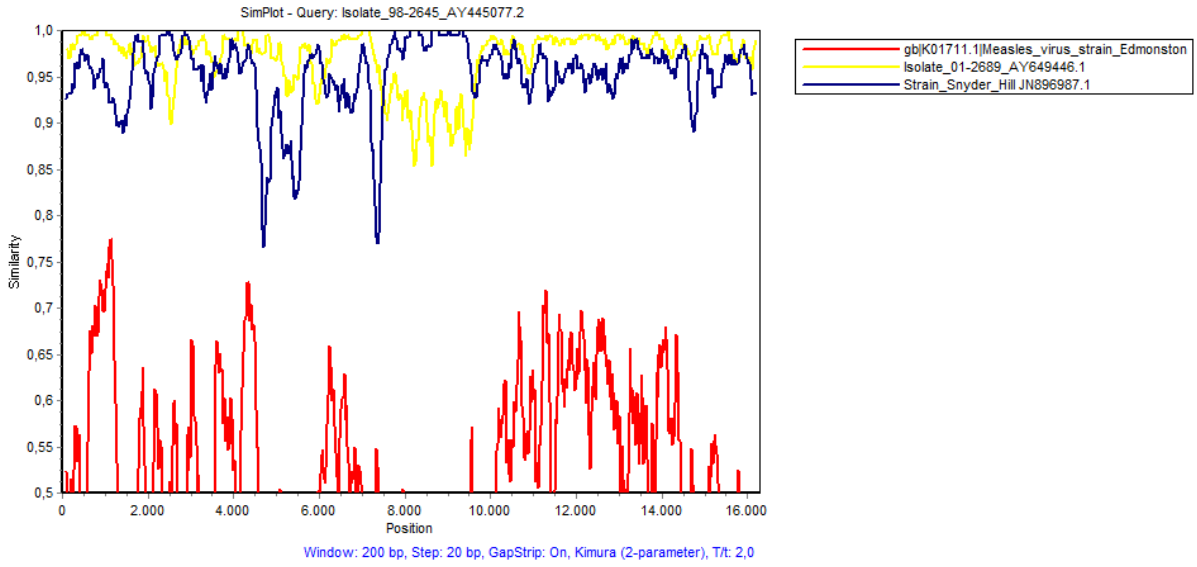

171391-513 America-II
5800
7073
N
P/C/V
M
F
H
L
164071 (America-II)
Unknown
A 1 - 5800
B 5801 - 7073
C 7074 - 15690

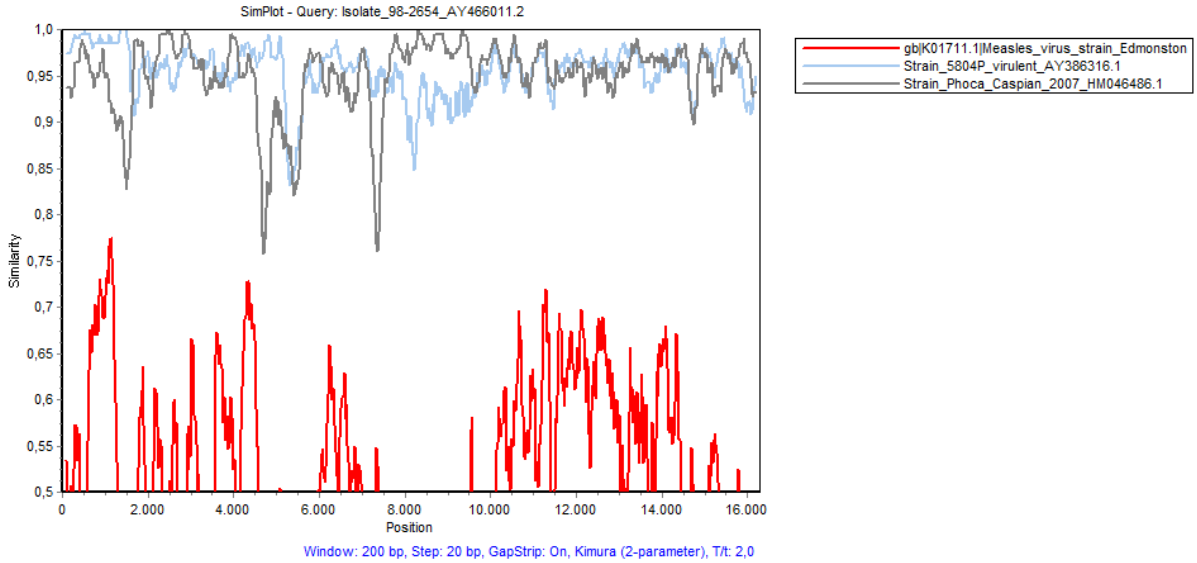
14

15

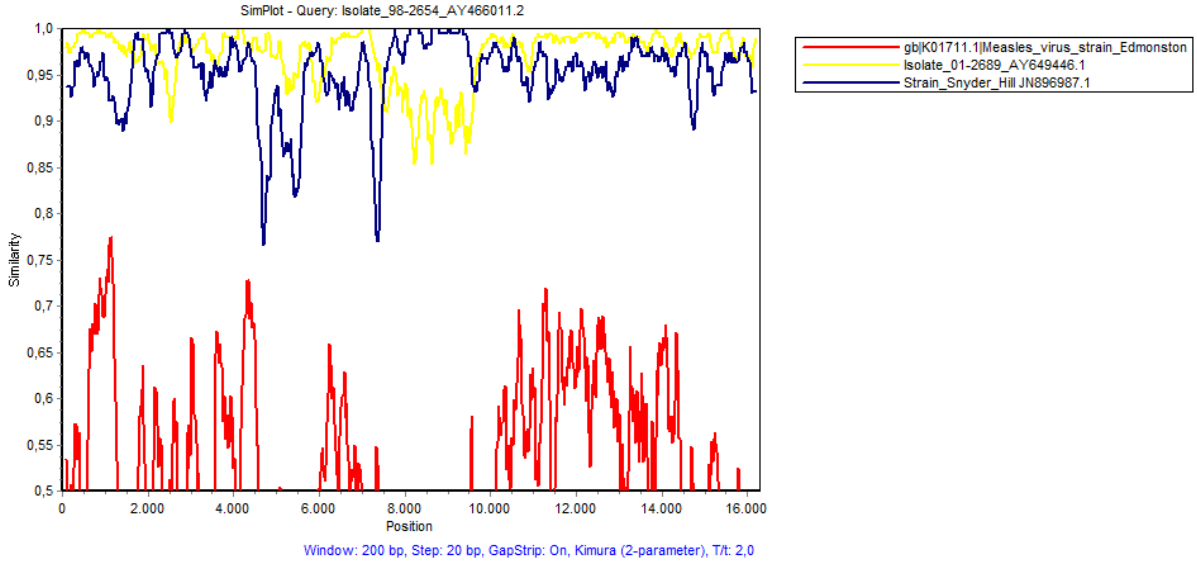
The results of the SimPlot analysis using putative recombinant strains as query are shown below. The y-axis shows the percentage of identity within a 200-bp-wide sliding window, with a 20-bp step size between plots.



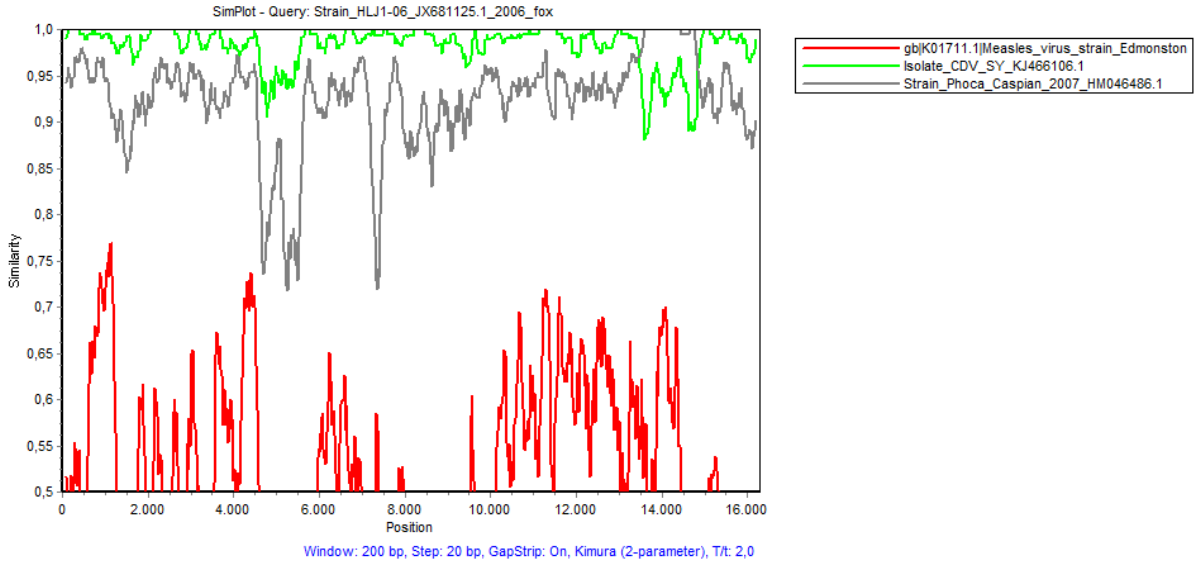



SimPlot - Query: Isolate_98-2654_AY466011.2
gb|K01711.1|Measles_virus_strain_Edmonston
Strain_5804P_virulent_AY386316.1
Strain_Phoca_Caspian_2007_HM046486.1
Similarity
1,0
0,95
0,9
0,85
0,8
0,75
0,7
0,65
0,6
0,55
0,5
0
2.000
4.000
6.000
8.000
10.000
12.000
14.000
16.000
Position
Window: 200 bp, Step: 20 bp, GapStrip: On, Kimura (2-parameter), T/t: 2,0

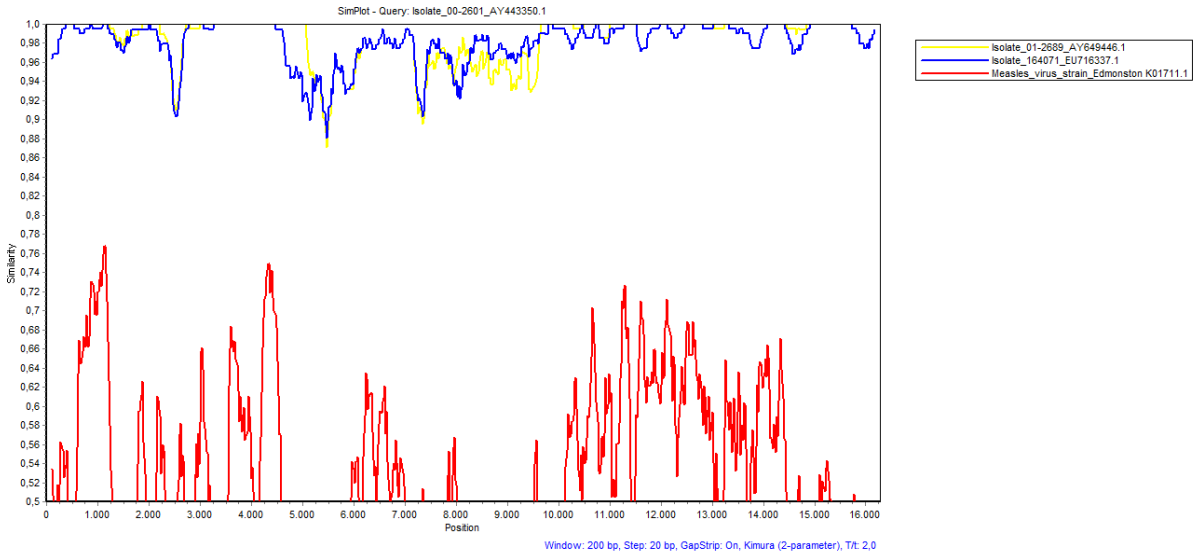
22


SimPlot - Query: Isolate_98-2654_AY466011.2
gb|K01711.1|Measles_virus_strain_Edmonston
Isolate_01-2689_AY649446.1
Strain_Snyder_Hill JN896987.1
Similarity
1,0
0,95
0,9
0,85
0,8
0,75
0,7
0,65
0,6
0,55
0,5
0
2.000
4.000
6.000
8.000
10.000
12.000
14.000
16.000
Position
Window: 200 bp, Step: 20 bp, GapStrip: On, Kimura (2-parameter), T/t: 2,0

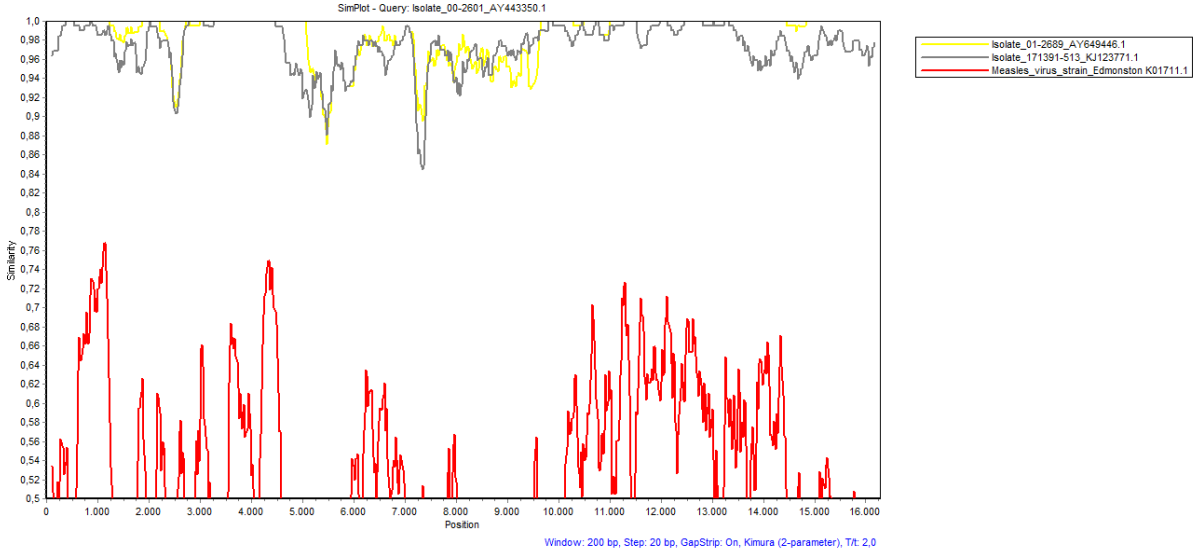
23


SimPlot - Query: Strain_HLJ1-06_JX681125.1_2006_fox
gb|K01711.1|Measles_virus_strain_Edmonston
Isolate_CDV_SY_KJ466106.1
Strain_Phoca_Caspian_2007_HM046486.1
Similarity
Position
Window: 200 bp, Step: 20 bp, GapStrip: On, Kimura (2-parameter), T/t: 2,0

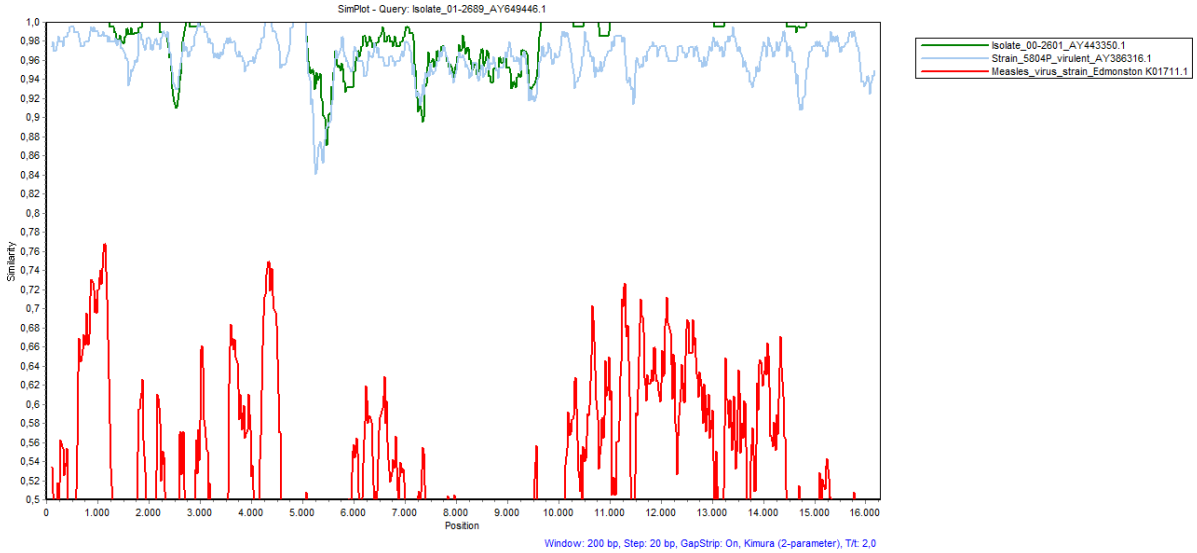
24


SimPlot - Query: Isolate_00-2601_AY443350.1
Isolate_01-2689_AY649446.1
Isolate_164071_EU716337.1
Measles_virus_strain_Edmonston K01711.1
Similarity
Position
Window: 200 bp, Step: 20 bp, GapStrip: On, Kimura (2-parameter), T/t: 2,0

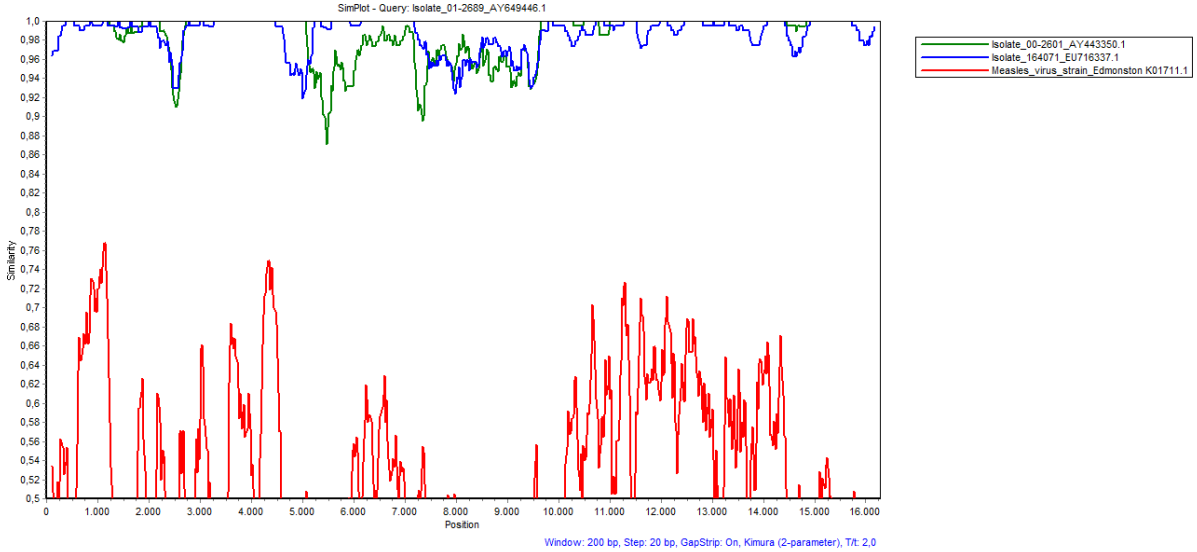
25


SimPlot - Query: Isolate_00-2601_AY443350.1
Isolate_01-2689_AY649446.1
Isolate_171391-513_KJ123771.1
Measles_virus_strain_Edmonston K01711.1
Similarity
Position
Window: 200 bp, Step: 20 bp, GapStrip: On, Kimura (2-parameter), T/t: 2,0

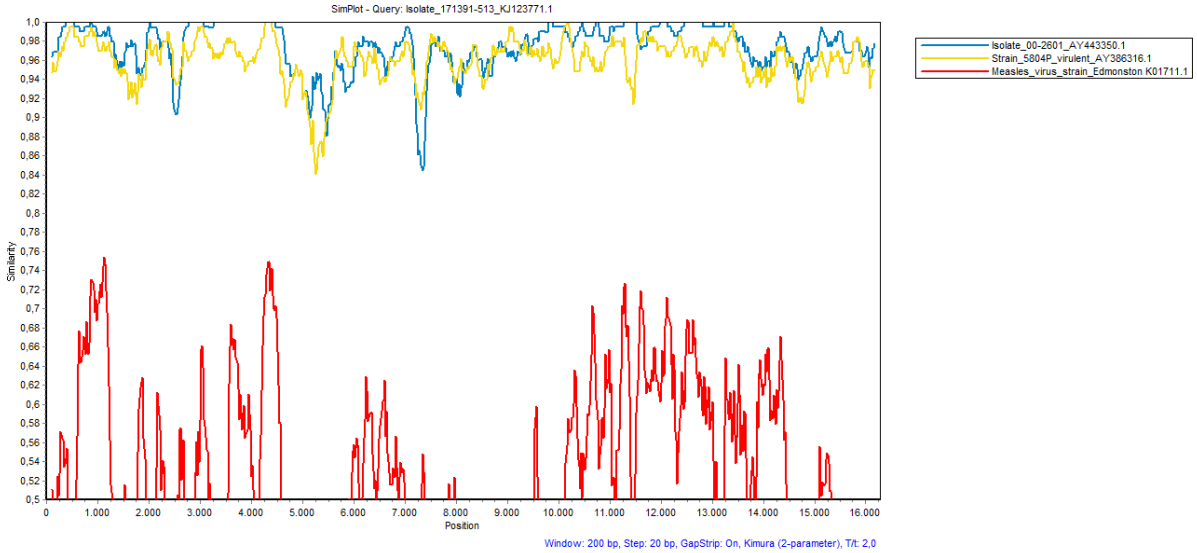
26


SimPlot - Query: Isolate_01-2689_AY649446.1
Isolate_00-2601_AY443350.1
Strain_5804P_virulent_AY386316.1
Measles_virus_strain_Edmonston K01711.1
Similarity
Position
Window: 200 bp, Step: 20 bp, GapStrip: On, Kimura (2-parameter), T/t: 2,0

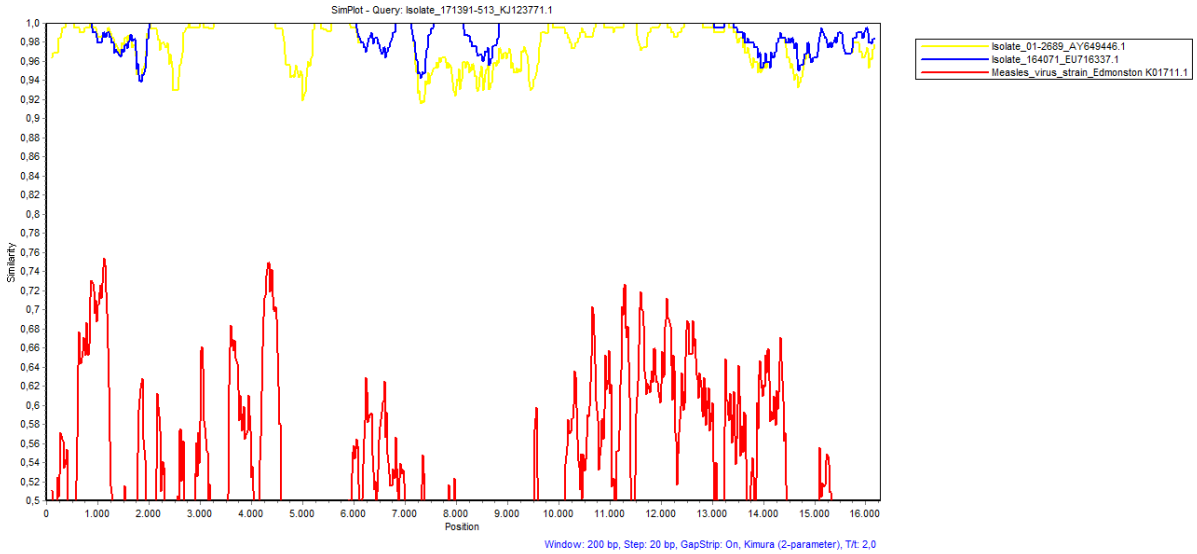
27


SimPlot - Query: Isolate_01-2689_AY649446.1
Isolate_00-2601_AY443350.1
Isolate_164071_EU716337.1
Measles_virus_strain_Edmonston K01711.1
Similarity
Position
Window: 200 bp, Step: 20 bp, GapStrip: On, Kimura (2-parameter), T/t: 2,0

28


SimPlot - Query: Isolate_171391-513_KJ123771.1
Isolate_00-2601_AY443350.1
Strain_5804P_virulent_AY386316.1
Measles_virus_strain_Edmonston K01711.1
Similarity
Position
Window: 200 bp, Step: 20 bp, GapStrip: On, Kimura (2-parameter), T/t: 2,0

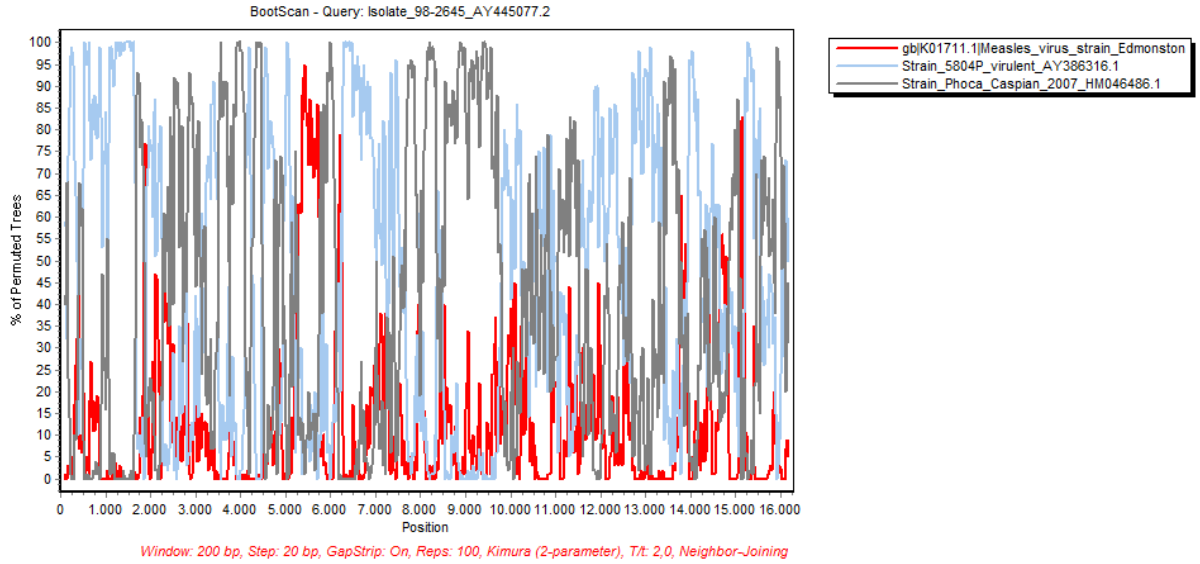
29

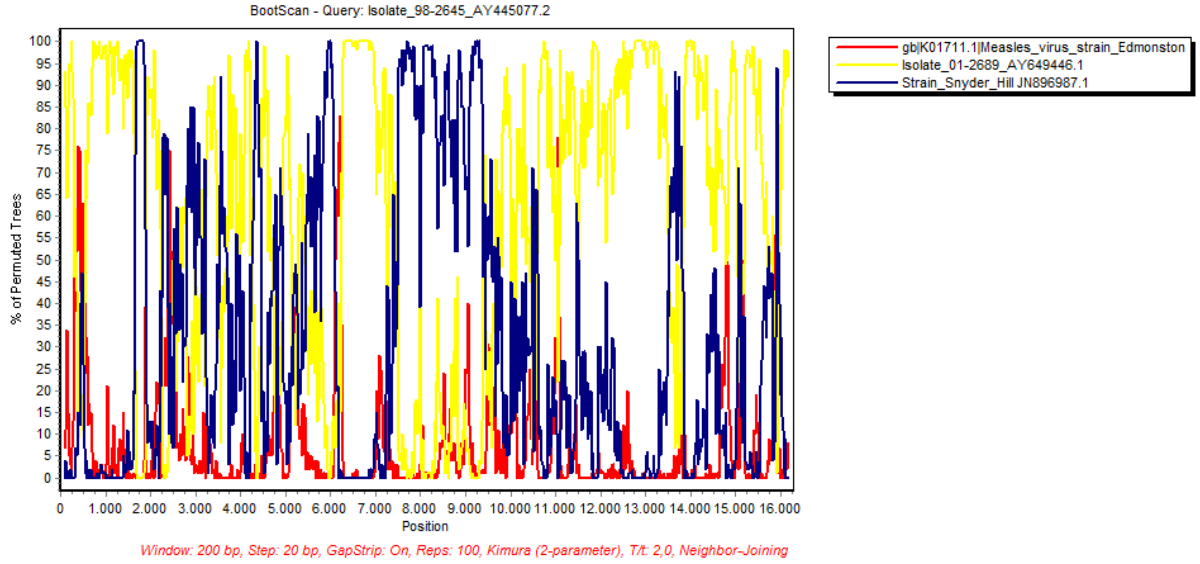

SimPlot - Query: Isolate_171391-513_KJ123771.1
Isolate_01-2689_AY649446.1
Isolate_164071_EU716337.1
Measles_virus_strain_Edmonston K01711.1
Similarity
Position
Window: 200 bp, Step: 20 bp, GapStrip: On, Kimura (2-parameter), T/t: 2,0

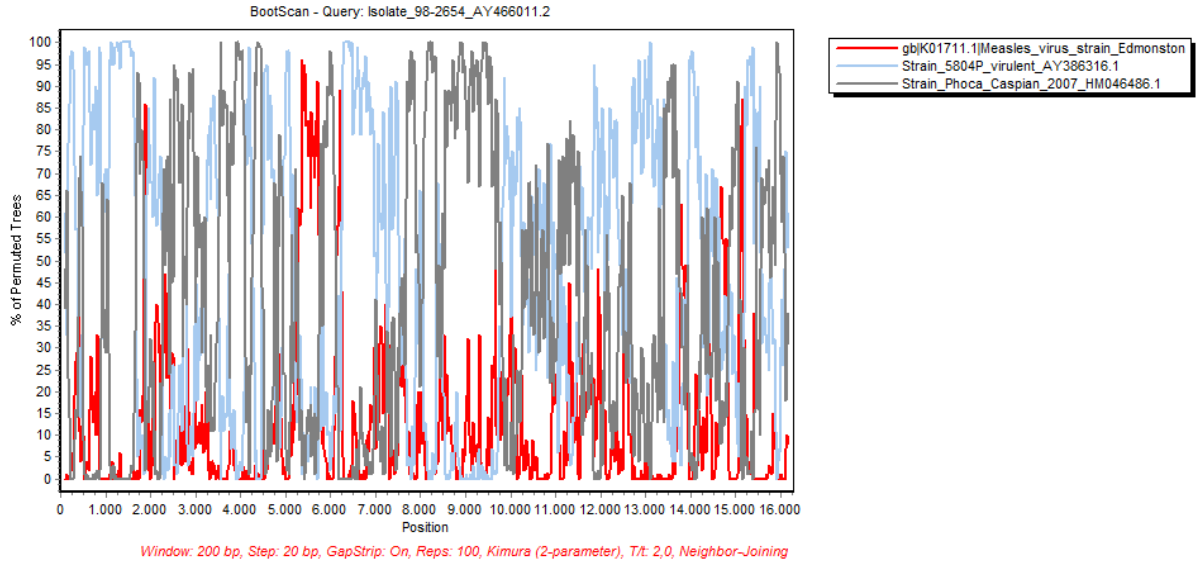
30

31

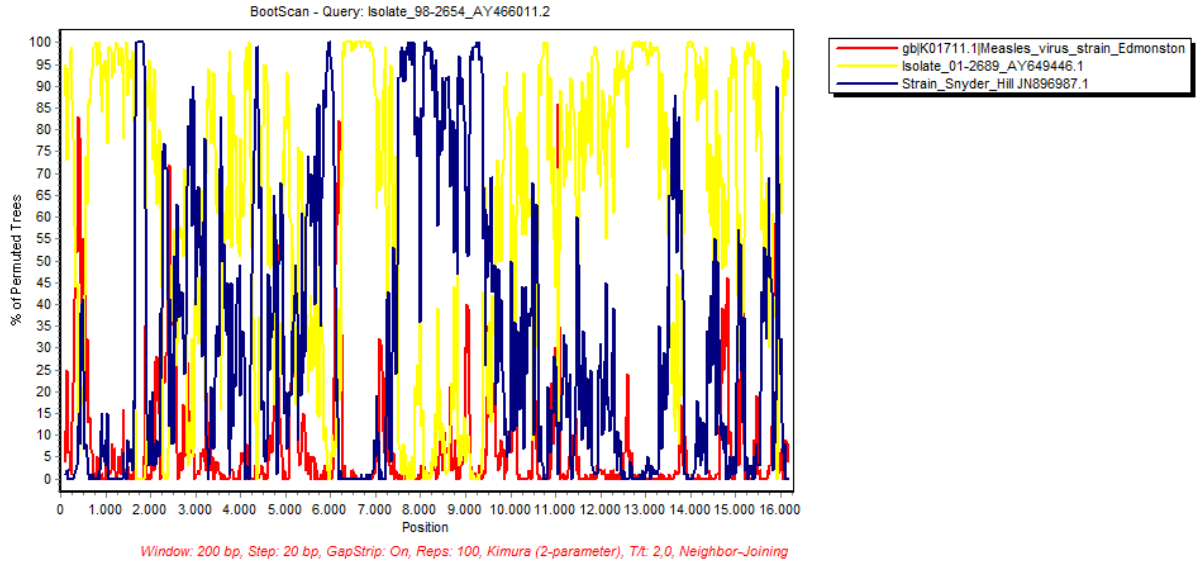
The results of Bootscanning using putative recombinant strains as a query are shown below. The y-axis shows the percentage of permutated trees using a 200-bp-wide sliding window, with a 20-bp step size between plots.



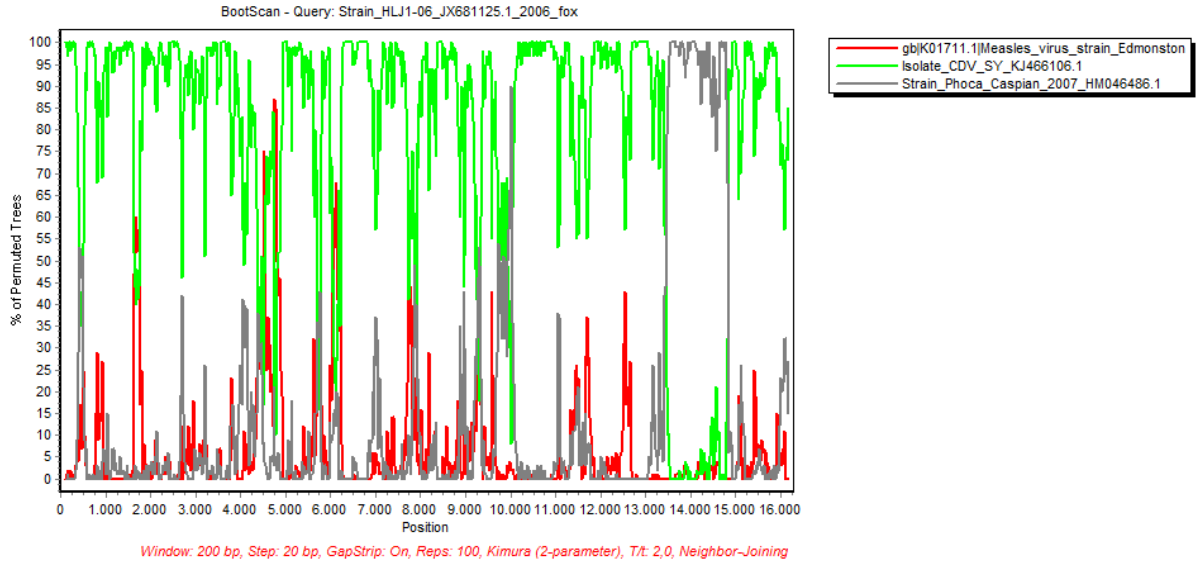



BootScan - Query: Isolate_98-2654_AY466011.2
% of Permuted Trees
Position
gb|K01711.1|Measles_virus_strain_Edmonston
Strain_5804P_virulent_AY386316.1
Strain_Phoca_Caspian_2007_HM046486.1
Window: 200 bp, Step: 20 bp, GapStrip: On, Reps: 100, Kimura (2-parameter), T/t: 2,0, Neighbor-Joining

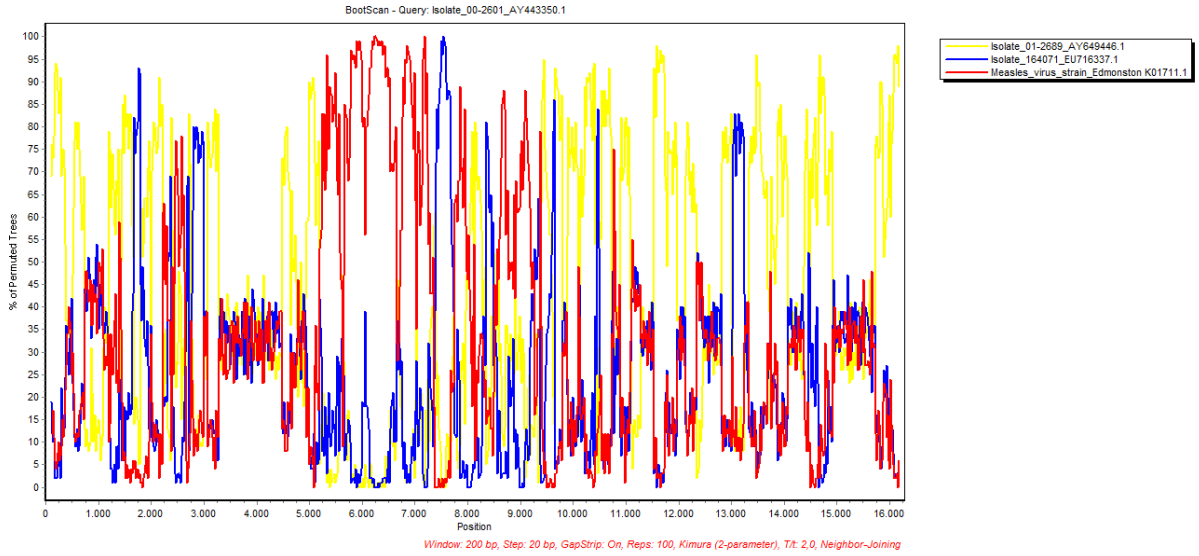
37


BootScan - Query: Isolate_98-2654_AY466011.2
% of Permuted Trees
Position
gb|K01711.1|Measles_virus_strain_Edmonston
Isolate_01-2689_AY649446.1
Strain_Snyder_Hill JN896987.1
Window: 200 bp, Step: 20 bp, GapStrip: On, Reps: 100, Kimura (2-parameter), T/t: 2,0, Neighbor-Joining

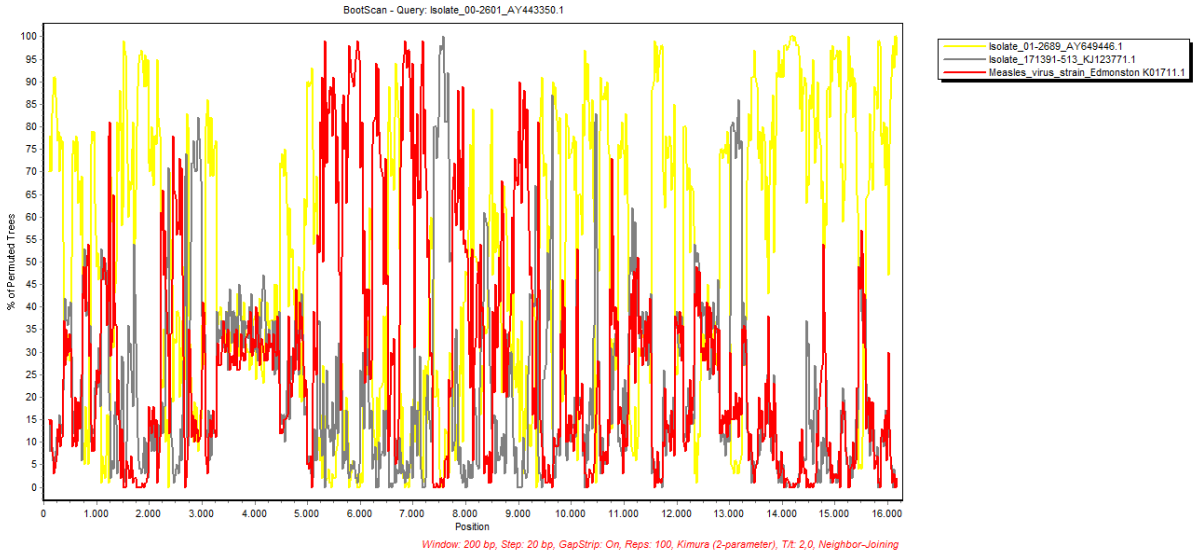
38


BootScan - Query: Strain_HLJ1-06_JX681125.1_2006_fox
% of Permuted Trees
Position
gb|K01711.1|Measles_virus_strain_Edmonston
Isolate_CDV_SY_KJ466106.1
Strain_Phoca_Caspian_2007_HM046486.1
Window: 200 bp, Step: 20 bp, GapStrip: On, Reps: 100, Kimura (2-parameter), T/t: 2,0, Neighbor-Joining

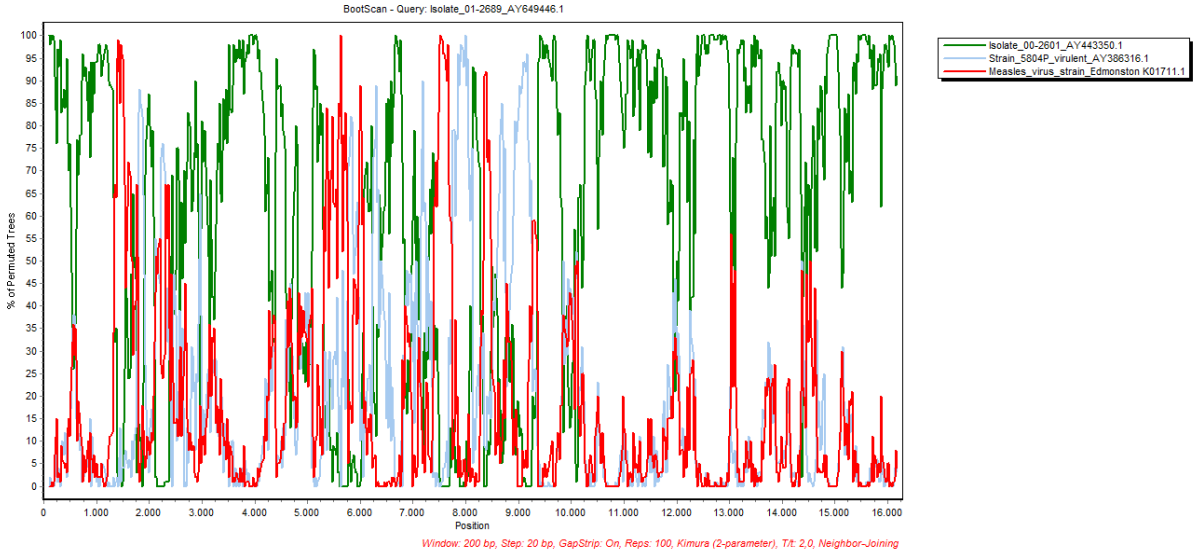
39


BootScan - Query: Isolate_00-2601_AY443350.1
% of Permuted Trees
Position
Isolate_01-2689_AY649446.1
Isolate_164071_EU716337.1
Measles_virus_strain_Edmonston K01711.1
Window: 200 bp, Step: 20 bp, GapStrip: On, Reps: 100, Kimura (2-parameter), T/t: 2,0, Neighbor-Joining

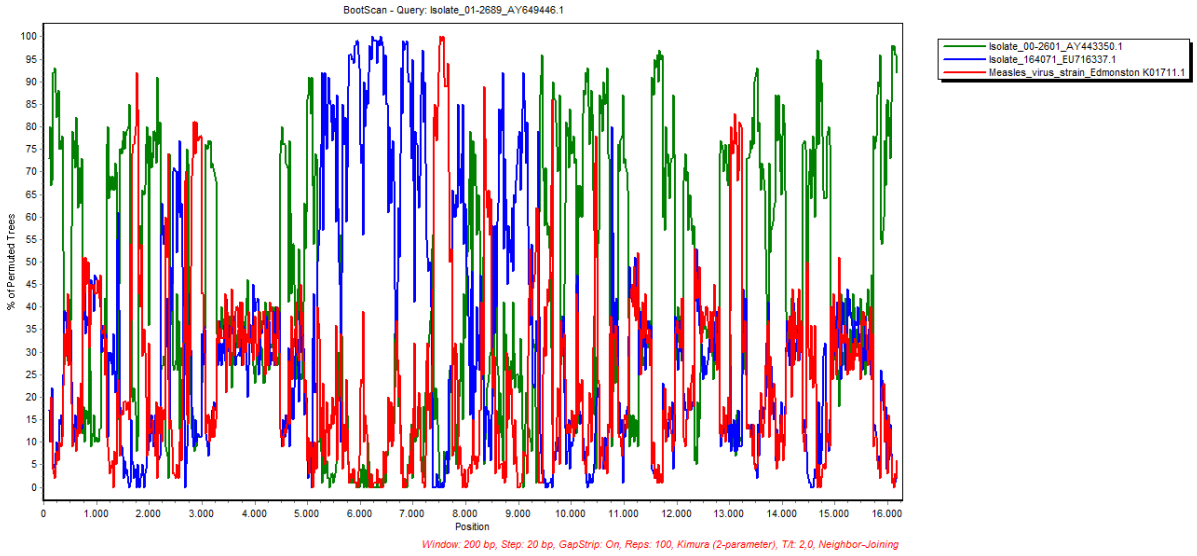
40


BootScan - Query: Isolate_00-2601_AY443350.1
% of Permuted Trees
Position
Isolate_01-2689_AY649446.1
Isolate_171391-513_KJ123771.1
Measles_virus_strain_Edmonston K01711.1
Window: 200 bp, Step: 20 bp, GapStrip: On, Reps: 100, Kimura (2-parameter), T/t: 2,0, Neighbor-Joining

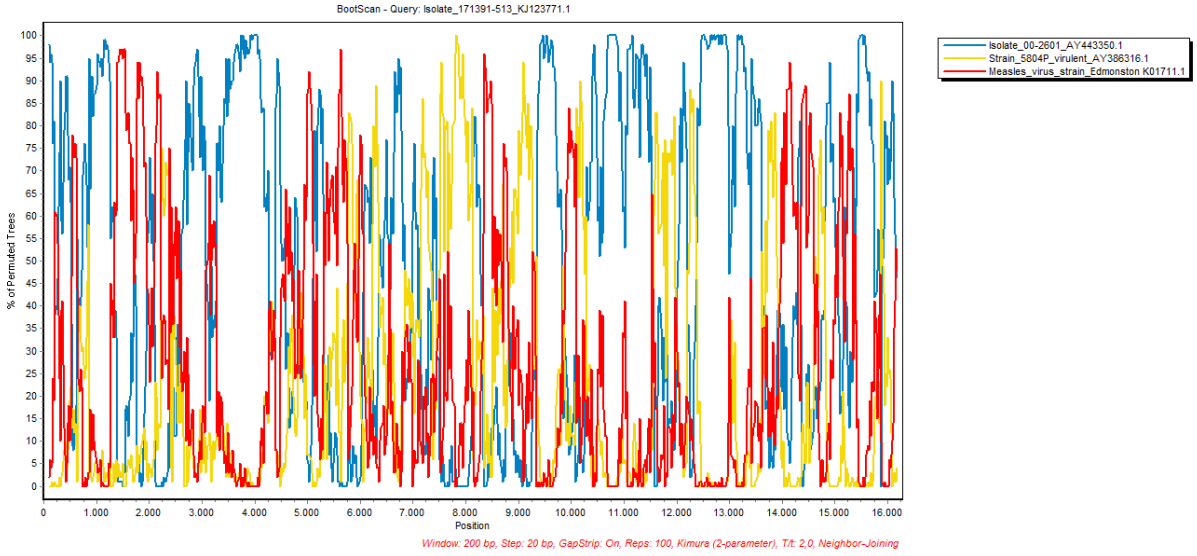
41


BootScan - Query: Isolate_01-2689_AY649446.1
% of Permuted Trees
Position
Isolate_00-2601_AY443350.1
Strain_5804P_virulent_AY386316.1
Measles_virus_strain_Edmonston K01711.1
Window: 200 bp, Step: 20 bp, GapStrip: On, Reps: 100, Kimura (2-parameter), T/t: 2,0, Neighbor-Joining

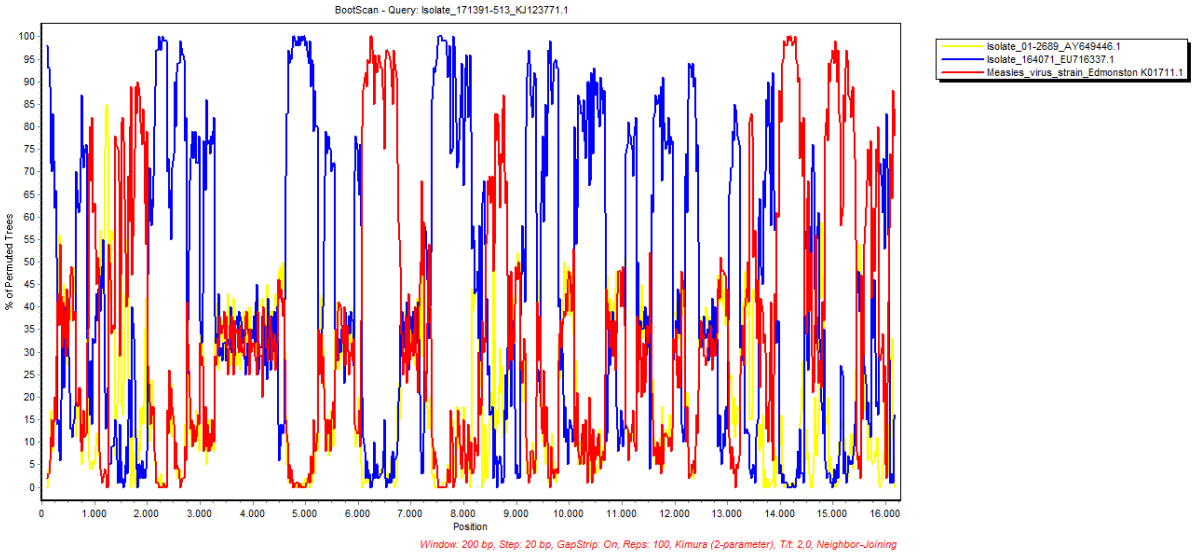
42


BootScan - Query: Isolate_01-2689_AY649446.1
% of Permuted Trees
Position
Isolate_00-2601_AY443350.1
Isolate_164071_EU716337.1
Measles_virus_strain_Edmonston K01711.1
Window: 200 bp, Step: 20 bp, GapStrip: On, Reps: 100, Kimura (2-parameter), T/t: 2,0, Neighbor-Joining

43


BootScan - Query: Isolate_171391-513_KJ123771.1
% of Permuted Trees
Position
Isolate_00-2601_AY443350.1
Strain_5804P_virulent_AY386316.1
Measles_virus_strain_Edmonston K01711.1
Window: 200 bp, Step: 20 bp, GapStrip: On, Reps: 100, Kimura (2-parameter), T/t: 2,0, Neighbor-Joining

44


BootScan - Query: Isolate_171391-513_KJ123771.1
% of Permuted Trees
Position
Isolate_01-2689_AY649446.1
Isolate_164071_EU716337.1
Measles_virus_strain_Edmonston K01711.1
Window: 200 bp, Step: 20 bp, GapStrip: On, Reps: 100, Kimura (2-parameter), T/t: 2,0, Neighbor-Joining

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