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VARIABILIDADE GENÉTICA DO VÍRUS DA LEUCEMIA VIRAL FELINA (FELV) E AVALIAÇÃO DE FATORES VIRAIS E DO HOSPEDEIRO NA INFECÇÃO PELOS VÍRUS DA IMUNODEFICIÊNCIA FELINA (FIV) E FELV.

LUCÍA CANO-ORTIZ

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Orientadora: Ana Cláudia Franco
Coorientador: Dennis Maletich Junqueira

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“Only art and science make us suspect the existence of life to a higher level, and maybe also instill hope thereof”.

Ludwig van Beethoven

Agradecimentos, agradecimientos, thanks…

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RESUMO

Os retrovírus são patógenos mundialmente distribuídos com grande importância na medicina veterinária e humana. Em felinos domésticos existem dois retrovírus que causam doenças: o vírus de leucemia felina (FeLV) e o vírus da imunodeficiência felina (FIV). Tanto o FIV quanto o FeLV estão associado a síndromes imunossupressoras, apresentação de doenças oportunistas e desenvolvimento de tumores. O FeLV é uma das principais causas de doença e morte em gatos, sendo assim mais patogênico do que o FIV. Muitos aspectos importantes da patogênese do FeLV ainda não foram elucidados, mas sabe-se que o curso da doença desenvolvida varia entre indivíduos. Esta variação é determinada por fatores tanto virais quanto do próprio hospedeiro. Sobre o vírus, informações sobre variantes específicas são muitas vezes suficientes para prever o resultado da doença. Além disso, no caso dos retrovírus, a proteína do envelope é um fator chave que define o subtipo ou subgrupo viral. A glicoproteína do envelope possui duas subunidades: superfície (SU) e transmembrana (TM). A glicoproteína de superfície determina o tropismo celular e é estruturalmente conformada por regiões conservadas e variáveis que são importantes na interação vírus-células.

Para estabelecer uma infecção eficiente, os retrovírus precisam neutralizar a atividade de diversas proteínas celulares, conhecidas como fatores de restrição, através de proteínas virais que interferem ou suprimem a dita atividade. Além disso, os fatores de restrição determinam em parte o repertório de hospedeiros e assim limitam a transmissão entre espécies. Os fatores de restrição são menos eficientes contra os vírus do hospedeiro natural.

A tese apresenta dois estudos que visam contribuir para a compreensão do FeLV. O primeiro trabalho é um estudo *in sílico* de bioinformática e filogenética do gene de superfície do envelope (*SU*) onde propõe-se um método altamente suportado para a classificação genética do FeLV. O segundo trabalho é um trabalho *in vitro* sobre o efeito do fator de restrição SERINC5 humano e felino (huSER5 e feSER5) sobre a infecciosidade do vírus da imunodeficiência humana – 1 (HIV-1), FIV e FeLV. Além disso, foi avaliada a capacidade de contra atacar o efeito inibitório das SER5s pelas proteínas virais Nef do HIV-1, glycoGag e Env do FeLV-A e do FeLV-B.

Palavras-chave: vírus da leucemia felina, filogenética, variante viral, SERINC5.

ABSTRACT

Retroviruses are globally distributed pathogens with great importance in veterinary and human medicine. In domestic cats there are two retroviruses that cause disease: feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV). Both FIV and FeLV are associated with immunosuppressive syndromes, opportunistic diseases and tumors development. FeLV is a major cause of disease and death in cats and thus is more pathogenic than FIV. Many important aspects of the pathogenesis of FeLV remain to be unveiled, but it is known that the course of the disease varies between individuals. This variation is determined by both viral and host factors. Viral aspects, such as information on specific variants is often sufficient to predict the outcome of the disease. Furthermore, in retroviruses the envelope protein is a key factor defining the viral subtype or subgroup. The envelope glycoprotein possesses two subunits: surface (SU) and transmembrane (TM). The surface glycoprotein determines cell tropism and is structurally conformed by conserved and variable regions that are important in the virus-cell interaction.

To establish an efficient infection, retroviruses need to neutralize the activity of several cellular proteins known as restriction factors. Viral proteins are capable of interfere with or suppress these host mechanisms. In addition, restriction factors partly determine the host repertoire and thus limit transmission between species. However, restriction factors are less efficient against natural host viruses.

This thesis presents two studies that aim at contributing to the understanding of FeLV. The first work is a bioinformatics and phylogenetic study in silico of the envelope surface gene (SU). This paper proposes a highly supported method for genetic classification of FeLV. The second work is an in vitro study on the effect of the restriction factors human and the feline SERINC5 (huSER5 and feSER5) on the human immunodeficiency virus-1 (HIV-1), FIV and FeLV infectivity. In addition, the ability to counteract the inhibitory effect of SER5s by the viral proteins Nef from HIV-1, glycoGag and Env from FeLV-A and FeLV-B was studied.

Keywords: *feline leukemia virus, phylogenetics, viral variant, SERINC5*

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LISTA DE ABREVIATURAS

A3	APOBEC 3
APOBEC	*Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like*
CA	Capsídeo
CD	*Cluster of differentiation*
DU	dUTPase
EIAV	Vírus da anemia infecciosa equina
enFeLV	FeLV endógeno
env	Envelope
FAIDS	Síndrome da imunodeficiência adquirida felina
FeLV	Vírus da leucemia felina
FIV	Vírus da imunodeficiência felina
gag	*Group-specific antigen*
HIV	Vírus da imunodeficiência humana
HTS	*High-throughput sequencing*
IN	Integrase
LTR	*Long Terminal Repeats*
MA	Matriz
MLV	Vírus da leucemia murina
mRNA	RNA mensageiro
NC	Nucleocapsídeo
PBS	*Primer Binding Site*
PCR	*Polymerase chain reaction*
pol	*Polymerase*
PPT	*Polypurine tract*
PR	*Protease*
Psi ψ	*Packaging Signal*
PSIV	*Prosimian immunodeficiency virus*
qPCR	*Quantitative polymerase chain reaction*
R	Região repetida
RBS	*Receptor Binding Site*
RELIK	*Rabbit endogenous lentivirus type K*
RH	Ribonuclease H
RNA	*Ribonucleic acid*
RT	Transcriptase reversa
RT-qPCR	*Reverse transcription quantitative polymerase chain reaction*
SERINC	*Serine incorporator*
SIV	Vírus da imunodeficiência dos símios
ssRNA	*Single strand* RNA
SU	*Surface*
TM	*Transmembrane*
tRNA	RNA transportador

U3	Região única 3'
U5	Região única 5'

SUMÁRIO

1. INTRODUÇÃO

Os retrovírus compõem uma família de vírus RNA de grande importância na medicina veterinária e humana. Graças à descoberta do vírus da imunodeficiência humana (HIV), diversas áreas de pesquisa como a imunologia, oncologia, biologia molecular, terapia gênica, entre outras, foram desenvolvidas. Em felinos domésticos, dois retrovírus desencadeiam doenças: o vírus da leucemia felina (FeLV) e o vírus da imunodeficiência felina (FIV). Os gatos domésticos geralmente estão mais adaptados ao FIV e podem permanecer como portadores sadios do vírus por vários anos. Por outro lado, gatos infectados pelo FeLV que apresentam uma infecção progressiva desenvolvem doenças mais severas e têm um período de sobrevivência menor. Atualmente, o FeLV representa um desafio na clínica uma vez que pouco se sabe sobre a biologia do vírus, patogênese e a interação vírus-hospedeiro. Os mecanismos da resposta imunológica frente ao vírus são pouco compreendidos, e a apresentação dos diferentes tipos de infecção carecem de uma explicação clara.

A tese apresenta dois trabalhos com FeLV: o primeiro é um estudo *in silico* onde foi desenvolvido um novo método de classificação baseado nas características genéticas do vírus. O segundo, é um estudo *in vitro* sobre a interação dos fatores de restrição SERINC5 felino e humano com FeLV, FIV e HIV-1. A revisão bibliográfica está focada principalmente no FeLV, embora o FIV e o HIV-1 sejam brevemente mencionados, bem como uma explicação sobre fatores de restrição e o sistema de vetores retrovirais.

2. RETROVÍRUS

2.1 Histórico

A retrovirologia historicamente tem acompanhado o desenvolvimento do estudo da biologia desde o nível de organismo até o nível molecular. Inicialmente, doenças de animais relacionadas a retrovírus foram descritas há várias décadas desde que sinais clínicos associados à anemia infecciosa equina, leucose bovina e adenomatose pulmonar dos ovinos foram reconhecidos em meados de 1800. No início do século XX, veterinários demonstraram que a anemia infecciosa dos equinos era transmitida por um filtrado celular, assim também foi descrita a leucose das galinhas, uma forma de leucemia e linfoma (ELLERMANN; BANG, 1908). Posteriormente, foi relatada a transmissão do sarcoma das galinhas também

por um filtrado celular (ROUS, 1911). Estes agentes foram subsequentemente confirmados como os primeiros retrovírus descritos na história (LAIRMORE, 2011). Posteriormente, outros retrovírus associados a doenças neoplásicas em camundongos, gatos, bovinos e macacos foram identificados (COFFIN; HUGHES; VARMUS, 1997).

Em 1964 o vírus da leucemia felina (FeLV) foi o primeiro retrovírus felino reportado e descrito como agente responsável por quase metade dos casos de leucemia e linfoma em felinos domésticos (JARRETT, W. F. H. *et al.*, 1964). A descoberta do FeLV foi uma das mais importantes na área da oncovirologia daquela época. No entanto, estudos sobre a patobiologia do FeLV diminuíram com a redução da incidência da doença (por esquemas de quarentena e vacinação) e com as descobertas posteriores do vírus da imunodeficiência dos símios (SIV) (LETVIN *et al.*, 1985) e do vírus da imunodeficiência felina (FIV) (PEDERSEN *et al.*, 1987). Tanto o SIV quanto o FIV começaram a ser utilizados como modelos de estudo mais próximos ao vírus da imunodeficiência humana (HIV) (CHIU; HOOVER; VANDEWOUDE, 2018). Atualmente, o FIV é considerado um importante modelo de estudo para o HIV (HATZIIOANNOU; EVANS, 2012) e o FeLV é um modelo potencial das interações entre retrovírus endógenos e exógenos (CHIU; HOOVER; VANDEWOUDE, 2018).

2.2 Retrovírus exógenos e endógenos

Os retrovírus podem estar presentes de duas maneiras nos hospedeiros vertebrados: como retrovírus exógenos e como retrovírus endógenos. A infecção por retrovírus exógenos associa-se com importantes doenças na medicina veterinária e humana (síndromes imunosupressivas ou imunomediadas e cânceres) e são transmitidos horizontalmente (LAIRMORE, 2011; STOYE *et al.*, 2012). Os retrovírus endógenos são provírus (DNA) de origem retroviral que atingiram a linha germinativa do hospedeiro durante alguma infecção ocorrida milhões de anos atrás, se tornaram elementos estáveis e são transmitidos por herança mendeliana. Os retrovírus endógenos são conhecidos também como “fósseis virais” (por exemplo o vírus da leucemia felina endógeno ou enFeLV) (GRANDI; TRAMONTANO, 2018; NELSON *et al.*, 2003). Até 2006 acreditava-se que o processo de endogenização acontecia unicamente com os retrovírus simples (Figura 1) (WEISS, 2006). No entanto, recentemente foram caracterizados dois lentivirus endógenos: um em coelhos (*Rabbit endogenous lentivirus type K,* RELIK) e um em lêmures (*Prosimian immunodeficiency vírus*, PSIV). A descoberta do PSIV é importante para entender a origem dos lentivírus em primatas:

o vírus da imunodeficiência dos símios e o vírus da imunodeficiência humana (SIV e HIV) (GIFFORD, 2012; GIFFORD *et al.*, 2008; GILBERT *et al.*, 2009; KATZOURAKIS *et al.*, 2007; WEISS, 2006). O genoma dos gatos também contém sequencias retrovirais endógenas como o RD-114, Mac-1 e o enFeLV (MIYAZKAYUKI, 2002).

2.3 Classificação taxonômica e genoma

Os retrovírus pertencem à família *Retroviridae* que, por sua vez, está classificada em duas subfamílias (*Orthoretrovirinae* e *Spumaretrovirinae)* e sete gêneros (*Alpharetrovirus, Betaretrovirus, Gammaretrovirus, Deltaretrovirus, Epsilonretrovirus, Lentivirus* e *Spumavirus*). A Figura 1 apresenta a taxonomia dos retrovírus e em vermelho, os vírus estudados na tese.

Os retrovírus são vírus envelopados que contem duas fitas simples (entre 7 a 10kb) de RNA de polaridade positiva (ssRNA+) não complementares, conhecido como "pseudodiploide" já que apenas uma das cópias é integrada no genoma do hospedeiro. A partir do RNA é sintetizado um DNA de dupla fita que posteriormente é integrado no genoma do hospedeiro. O DNA integrado se conhece como provírus. A integração é irreversível e assim, o provírus se mantém durante toda a vida da célula, garantindo uma infecção persistente (STOYE *et al.*, 2012).

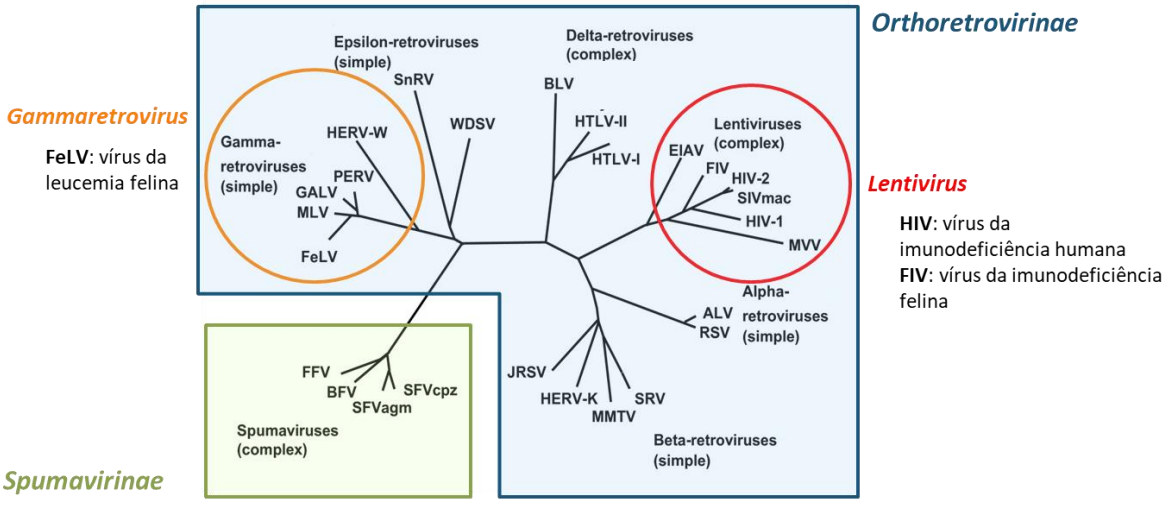

Figura –1- Árvore filogenética não enraizada das subfamílias retrovirais. Em um círculo estão indicados os vírus referidos na tese e o gênero ao qual pertencem: HIV-1 e FIV (lentivírus) em vermelho e, e FeLV (gammaretrovírus) em laranja. Adaptado de Weiss, 2006.

Dependendo da composição do genoma, os retrovírus já foram classificados em simples e complexos (Fig. 1 e Fig. 2) (CULLEN, 1991). O genoma dos retrovírus simples

(*Alpharetrovirus*, *Betaretrovirus*, *Gammaretrovirus* e *Epsilonretrovirus*) está conformado pelos genes principais *gag* (pelas siglas em inglês de *group specific antigen*), *pol* (*polimerase*) e *env* (envelope). Os retrovírus complexos (*Lentivirus*, *Deltavirus* e *Spumavirus*) possuem, além dos genes principais, alguns genes acessórios ou auxiliares. (STOYE *et al.*, 2012; WEISS, 2006) (Fig. 2). O *gag* codifica proteínas estruturais de matriz (MA), capsídeo (CA) e nucleocapsídeo (NC), *pol* codifica as enzimas virais: protease (PR), transcriptase reversa (RT), ribonuclease H (RH), integrase (IN) e, em alguns casos, dUTPase (DU). O gene *env* codifica as duas proteínas de envelope: de superfície e transmembrana (SU e TM). (AFFRANCHINO; GONZÁLEZ, 2014; LECOLLINET; RICHARDSON, 2008; PANCINO *et al.*, 1993). Um exemplo de retrovírus simples é o vírus da leucemia felina (FeLV) e de retrovírus complexos são o vírus da imunodeficiência humana 1 (HIV-1) e o vírus da imunodeficiência felina (FIV).

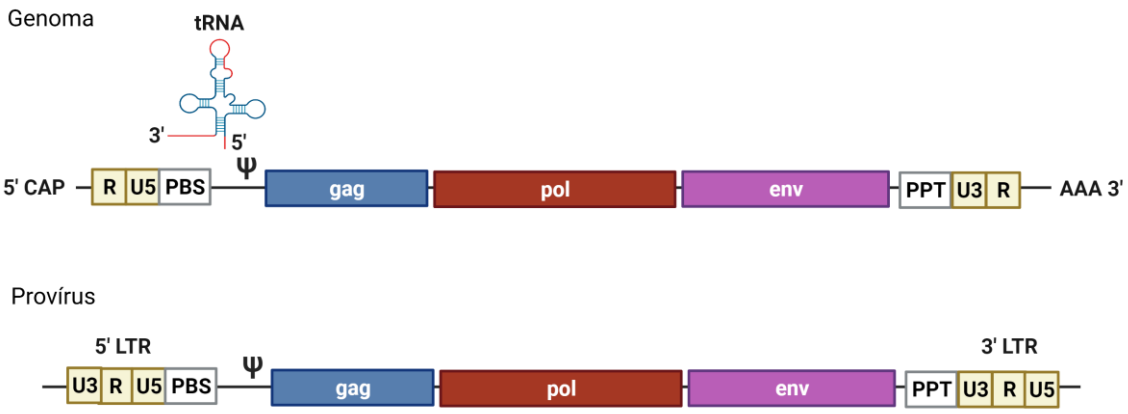

Figura –2. Organização genômica de um retrovírus simples: representação do genoma e o provírus indicando os genes principais e as regiões não codificantes. R: região repetida, U5: região única 5', PBS: *primer binding site*, ψ: *packaging signal*, PPT: *polypurine tract*, U3: região única 3', AAA 3': cauda poli A. Fonte: a própria autora.

Cada fita de RNA viral possui uma estrutura cap na região 5' e é poliadenilada (poli A) na extremidade 3'. Duas regiões curtas de sequências repetidas (R) se localizam na extremidade 5' logo após o cap, e na extremidade 3' exatamente antes da cauda de poli A (DELVIKS-FRANKENBERRY *et al.*, 2011; FLINT *et al.*, 2015; HU; HUGHES, 2012). Regiões únicas encontram-se imediatamente depois da 5'-R e imediatamente antes da 3'-R (U3 e U5, respectivamente). Em seguida, a U5 localiza-se na região PBS (*Primer Binding Site*) associada por complementariedade a uma molécula de RNA transportador (tRNA). A PBS é seguida pela sequência de empacotamento "*Packaging Signal* ψ" (psi,). Uma região rica em purinas "*polypurine tract"* (PPT) está localizada no final da região genômica, antes da U3 As regiões *leader* e codificante localizam-se entre as regiões únicas (STOYE *et al.*, 2012).

2.4 Ciclo replicativo

A replicação dos retrovírus se divide em adsorção e entrada, desnudamento, transcrição reversa, integração do provírus, síntese de proteínas e de genomas virais, montagem e brotamento (Fig. 3). A replicação inicia quando o vírus entra em contato com o receptor celular através de uma região de ligação ao receptor (RBS ou *Receptor Binding Site*) presente na glicoproteína de superfície do vírus. Isto gera uma mudança conformacional na glicoproteína viral e no receptor que permite a fusão do envelope viral com a membrana celular (PASERVAL, 2004). O vírus é internalizado e desnudado, liberando o genoma viral no citoplasma para iniciar a transcrição reversa (FLINT *et al.*, 2015).

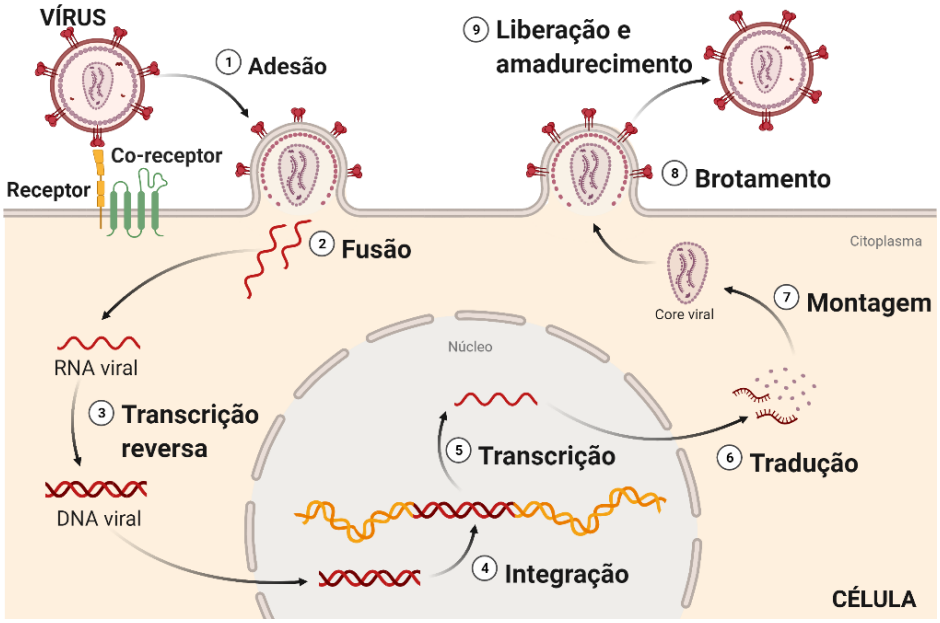

Figura –3- Ciclo replicativo dos retrovírus. Fonte: Adaptado de FLINT et al., (2015).

Durante o processo de transcrição reversa, um dsDNA é sintetizado a partir do RNA genômico viral por ação da RT (Fig. 4). A RT possui atividade de DNA polimerase e pode utilizar tanto um molde de DNA quanto de RNA. A RT também tem atividade RNase H, que degrada o RNA unicamente quando se encontra hibridizado com DNA. Inicialmente, a RT sintetiza um DNA complementar (cDNA), que é fita simples negativa (ssDNA-), utilizando como iniciador um RNAt celular ligado por complementaridade na região PBS do genoma viral. O primeiro cDNA sintetizado corresponde às regiões U5 e R na extremidade 5', região conhecida como *Strong Stop DNA*. Sendo uma dupla RNA/DNA; a atividade RNAse H degrada o RNA hibridizado e expõe o cDNA recém-formado (HU; HUGHES, 2012).

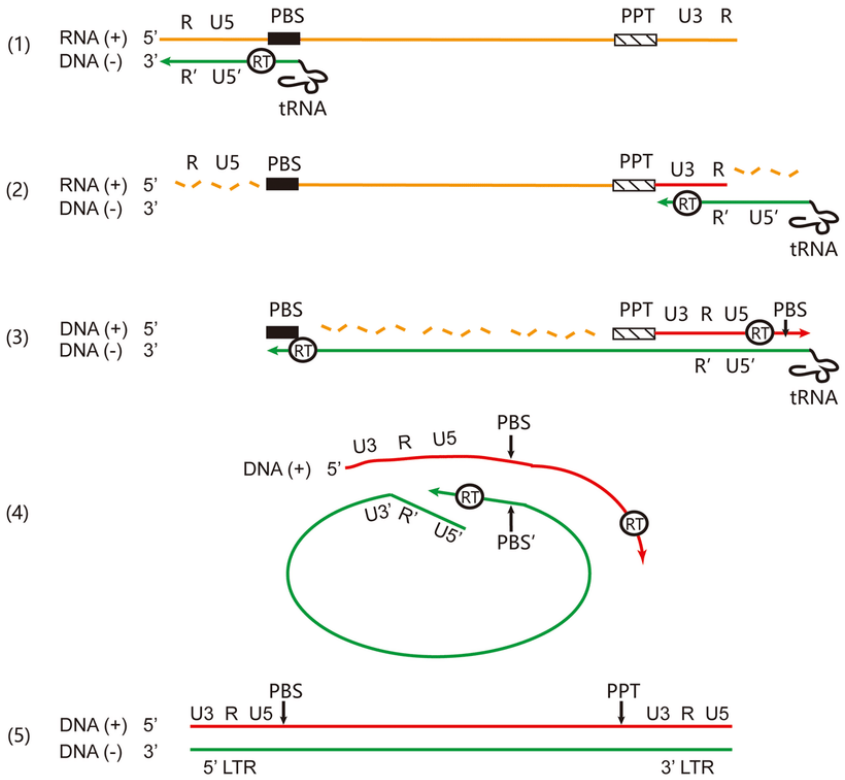

Figura –4. Transcrição reversa de um retrovírus. A atividade RNase H da RT está indicada por "RNase H", a linha pontilhada indica a hidrólise do RNA. Fonte: (ZHANG *et al*., 2018)

Devido às regiões R serem idênticas nas extremidades 5' e 3', o cDNA na região R da extremidade 5' hibridiza por complementariedade na região R da extremidade 3', mecanismo conhecido como primeira transferência da RT. Após esta transferência, a síntese do cDNA continua ao longo do genoma, junto com a degradação do RNA molde correspondente. No entanto, a região PPT é resistente a esta degradação, e em consequência serve como iniciador para a síntese da fita positiva de DNA (ssDNA+). Nessa etapa a RT copia também os primeiros nucleotídeos do tRNA, sendo substrato da RNAse H. À medida em que a síntese do cDNA se aproxima da extremidade 5', a região PBS é copiada. A extremidade 3' do ssDNA+ contém também uma região PBS produto do molde de tRNA. Estas duas hibridizam por complementaridade (segunda transferência da RT) e a síntese de DNA estende-se nas duas fitas cDNA (ssDNA-) e ssDNA+ (HU; HUGHES, 2012).

O processo de transcrição reversa gera um DNA proviral maior do que o RNA viral, uma vez que as duas extremidades do DNA viral contêm sequências tanto da extremidade 3' quanto da 5' do RNA (U3, R, U5). Estas regiões que flanqueiam o provírus são conhecidas como regiões terminais longas (LTR ou *Long Terminal Repeats)* e são indispensáveis no processo da integração no genoma do hospedeiro. A integração é mediada pela enzima

integrase (IN), a qual se associa com o DNA viral e com nucleoproteínas para ser transportado como um complexo ao núcleo (complexo de pré-integração) (HU; HUGHES, 2012). A integração acontece em duas etapas, a primeira é o processamento em 3', quando são removidos dois nucleotídeos na extremidade 3' do DNA viral em uma região CA (citosina-adenina) altamente conservada. A segunda etapa é conhecida como transferência da fita de DNA, na qual o DNA cromossomal é clivado e o DNA viral é inserido no lugar da clivagem (CRAIGIE; BUSHMAN, 2012).

Uma vez integrado, o genoma proviral é replicado junto com o DNA do hospedeiro durante os ciclos de divisão celular. Esse provírus é um molde para a transcrição do RNA viral, que irá gerar tanto o RNA genômico, quanto o RNA mensageiro (RNAm). Uma parte desses RNAm serão traduzidos nos precursores poliproteicos *gag* e *gag-pol*, enquanto a outra parte deles será processada no núcleo para formar o RNAm da poliproteína do envelope (Env). Essa será traduzida pelos ribossomos presentes no retículo endoplasmático. As proteínas são transportadas pelo aparelho de Golgi, onde são glicosiladas e clivadas por enzimas celulares para formar finalmente o complexo maduro TM-SU que se localizará na membrana celular. Os componentes do vírion (duas fitas de RNA, os precursores poliproteicos *gag* e *gag-pol*, e as proteínas SU-TM) são montados no interior da célula para serem liberados por brotamento do vírion. A protease viral PR cliva os precursores poliproteicos para produzir proteínas maduras e finalmente formar a partícula viral infecciosa (FLINT *et al.*, 2015).

3 VÍRUS DA LEUCEMIA FELINA (FELV)

3.1 FeLV endógeno e exógeno

Os ancestrais do gênero atual *Felis* spp que inclui *Felis chaus, Felis margarita, Felis sylvestris* e *Felis nigripes*), divergiram dos ancestrais *Felidae* há 6,2 milhões de anos. Acredita-se que após a divergência, houve uma infecção inter-espécies de um *Gammaretrovirus* de roedores (vírus da leucemia murina ou MLV) que originou um FeLV ancestral. Atualmente, os gatos domésticos (*Felis catus*) e alguns outros membros desse gênero (*Felis chaus, Felis margarita,* e *Felis sylvestris*) apresentam no seu genoma sequências provirais de FeLV ancestrais indicando um processo de endogenização viral, essas sequências se conhecem como FeLV endógeno (enFeLV) (WILLETT; HOSIE, 2013). O FeLV exógeno é um vírus amplamente distribuído em gatos domésticos que pode causar

mielosupressão, anemia, leucemias e linfomas (HARTMANN, 2012). O vírus foi também detectado em outras espécies selvagens (*Puma concolor, Lynx pardinus,* e *Puma yaggouaroundi*), demonstrando uma segunda transmissão desta vez, inter-gêneros (BROWN *et al.*, 2008; CUNNINGHAM *et al.*, 2011; LUACES *et al.*, 2008).

3.2 Classificação taxonômica e subgrupos

O FeLV pertence ao gênero *Gammaretrovirus* e são conhecidos seis subgrupos diferentes: FeLV-A, FeLV-B, FeLV-C, FeLV-D, FeLV-E, FeLV-T. Os primeiros três subgrupos (FeLV-A, FeLV-B e FeLV-C) foram identificados por testes de interferência que permitem avaliar qualitativamente a capacidade de um primeiro isolado em limitar a infecção de um segundo vírus, uma vez que cada subgrupo utiliza receptores diferentes (Tabela 2) (CHIU; HOOVER; VANDEWOUDE, 2018; SARMA; LOG, 1971).

Subgrupo	**Receptor**	**Referência**
A	Fethtr1	Donahue, et al., (1988) Mendoza, et al., (2006)
B	FePit1 FePit2	Anderson et al., (2001) Takeuchi et al., (1992)
C	FLVCR	Quigley et al., (2000; 2004) Tailor et al, (1999)
T	FePit1 FeLIX (enFeLV)	Cheng et al., (2007) Gwynn et al., (2000) Rohn et al., (ROHN, J L *et al.*, 1998; 1994)
D	?	Anai et al., (2012)
E	?	Miyake et al., (2016)

Tabela –1 Receptores celulares envolvidos na infecção por FeLV. Fonte: a própria autora.

Todos os animais que apresentam FeLV-B ou FeLV-C estão co-infectados com FeLV-A. Até hoje foi demonstrado que o FeLV-A é o único transmitido horizontalmente entre gatos (POWERS *et al.*, 2018), além de ser o menos patogênico (BOLIN; LEVY, 2011; CHIU; HOOVER; VANDEWOUDE, 2018; WILLETT; HOSIE, 2013). De acordo com a literatura, o FeLV-B se origina por recombinação entre o FeLV-A e o enFeLV durante o processo de transcrição reversa. A presença do FeLV-B foi associada com a aparição de tumores (CHIU; HOOVER; VANDEWOUDE, 2018; JARRETT, W. F. H. *et al.*, 1964; STEWART *et al.*, 1986). O FeLV-C é menos frequente em felinos domésticos; se origina por mutações no gene *SU* do FeLV-A e associa-se com anemia não regenerativa (JARRETT, O. *et al.*, 1978). O FeLV-T é um vírus T-citopático isolado inicialmente de um linfoma tímico e induz uma

síndrome de imunodeficiência adquirida felina fatal em gatos (FeLV-FAIDS). O FeLV-T possui inserções e deleções que o diferenciam do FeLV-A, e usa um receptor diferente para a entrada nas células (DONAHUE, Peter R *et al.*, 1991; HOOVER *et al.*, 2018). O FeLV-E é um subgrupo recentemente descrito e isolado de linfoma tímico, mas se desconhece o receptor celular usado (MIYAKE *et al.*, 2016). Finamente, o FeLV-D foi descoberto juntamente com um novo retrovírus endógeno de gatos domésticos (ERV-DC), distinto do enFeLV. Este grupo é pouco estudado (ANAI *et al.*, 2012).

3.3 Patogênese e curso da doença

A transmissão do FeLV ocorre por contato com gatos virêmicos. O vírus é excretado principalmente em saliva, secreção nasal, fezes, leite e urina (HARTMANN, 2012). Os animais podem se infectar por via oro-nasal e especialmente através de feridas por mordida. Posteriormente o vírus pode ser encontrado no tecido linfoide local e se dissemina através de monócitos e linfócitos gerando uma viremia primária. Nesta fase o vírus pode infectar a medula óssea e, à continuação se produz uma viremia secundária com leucócitos e plaquetas infectados (LITTLE *et al.*, 2020).

O curso da infecção é variável e recentemente foram definidos três tipos: infecção progressiva, regressiva e abortiva (última atualização em Little et al., 2020). Uma vez que o gato se infecta, a **infecção progressiva** se desenvolve quando o vírus não é contido durante a etapa inicial e tem lugar uma elevada replicação viral nos tecidos linfoides, na medula óssea e posteriormente nas mucosas e nos tecidos glandulares epiteliais, o que se associa com uma elevada excreção de vírus infectantes na saliva e outras secreções. Os gatos com uma infecção progressiva apresentam uma pobre resposta imune ao vírus, com baixos níveis de anticorpos neutralizantes. Quando o paciente tem um sistema imune competente, após a infecção o vírus pode ser contido mas não eliminado, o que se conhece como uma **infecção regressiva**. Neste caso a excreção viral também é reduzida ou ausente e os animais apresentam elevados títulos de anticorpos neutralizantes. No entanto, os animais regressores (que apresentam apenas DNA proviral) podem infectar outros animais quando são utilizados como doadores de sangue (NESINA *et al.*, 2015). Os animais regressores podem reativar a infecção por estresse e/ou altas doses de corticosteróides e desenvolver uma infecção progressiva. Por último, a **infecção abortiva** é detectável apenas pela presença de anticorpos. Os animais não apresentam RNA viral nem DNA proviral (HARTMANN, 2012; HELFER-HUNGERBUEHLER *et al.*, 2015; HOFMANN-LEHMANN *et al.*, 2008; LITTLE *et al.*, 2020; POWERS *et al.*, 2018).

3.4 Diagnóstico e prevenção

Os testes rápidos e os moleculares permitem a classificação dos animais infectados em progressores e regressores (DUDA *et al.*, 2020; HOFMANN-LEHMANN *et al.*, 2008). Conhecer se um animal é regressor é de grande importância porque o paciente pode desenvolver uma infecção progressiva no futuro e infectar outros gatos. Animais regressores por exemplo não podem ser doadores de sangue porque o provírus é suficiente para causar infecção e doença em gatos receptores (NESINA *et al.*, 2015). A imunocromatografia rápida e o ELISA são testes de rotina comuns para realizar o diagnóstico de FeLV baseado na detecção do antígeno p27 no sangue e permitem a identificação de animais progressores. Os animais regressores não tem viremia, mas uma vez que o vírus conseguiu se integrar no genoma, o DNA proviral pode ser identificado por PCR quantitativa (qPCR). O resultado negativo de um teste de alta sensibilidade é confiável no caso de um animal sadio com baixo risco de infecção. No entanto, um resultado falso negativo pode acontecer em animais recentemente infectados antes da fase de antigenemia (< 30 dias pos-infecção) (KRECIC *et al.*, 2018; LITTLE *et al.*, 2020). A detecção de RNA viral em saliva é uma alternativa quando não é possível a coleta do sangue e em grupos grandes de gatos, mas a sensibilidade é menor. No entanto, a presença de RNA viral na saliva reflete a viremia e a excreção viral. Atualmente testes imunofluorescência (IFA) do sangue ou medula óssea também estão disponíveis comercialmente e detectam viremia secundária após da infecção da medula óssea. A prevenção de novas infecções se faz principalmente pela identificação de animais positivos e vacinação (LITTLE *et al.*, 2020).

3.5 Genoma

A estrutura genômica do FeLV corresponderia a um retrovírus simples, uma vez que contém os três genes principais *gag, pol* e *env*. No entanto o genoma também contém um códon de início prematuro anterior ao início do gene *gag* (CHIN *et al.*, 2020; DONAHUE, P R *et al.*, 1988; MIYAZKAYUKI, 2002). A glycoGag (glycosylated Gag) é uma proteína acessória expressa pelo vírus da leucemia murina (MLV) a tradução começa em um *start codon* ineficiente 88 aminoácidos antes do início da Gag. O resultado é uma proteína que abrange a sequência completa da Gag e 88 aminoácidos a mais na região 5’ (LI *et al.*, 2018; PRATS *et al.*, 1989; RENNER *et al.*, 2018). Esta proteína é clivada posteriormente em uma

proteína associada à membrana e uma proteína secretada (Fig. 5) (FUJISAWA *et al.*, 1997; LI *et al.*, 2018).

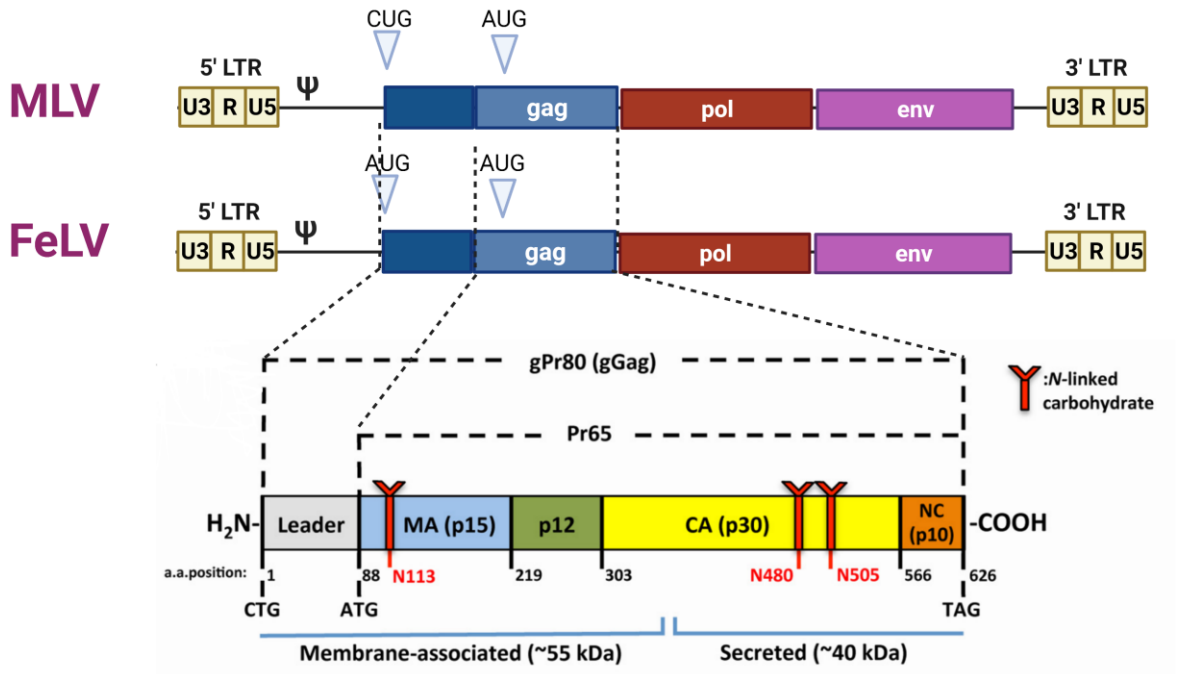

Figura –5 Representação da glycoGag. O start códon está indicado para vírus da leucemia murina (MLV) e para o vírus da leucemia felina (FeLV). Os produtos da clivagem porteolítica também são ilustrados. Adaptado de: (RENNER *et al.*, 2018).

4 VÍRUS DA IMUNODEFICIÊNCIA FELINA

O vírus da imunodeficiência felina (FIV) é um importante patógeno de distribuição mundial que acomete diversas espécies de felídeos (*Felis catus, Panthera leo, Panthera pardus, Panthera tigres, Felis concolor, Panthera onca, Acinonix jubatus, Lynx rufus*) e hienídeos (*Crocuta crocuta)* (TROYER, J. L. *et al.*, 2005). Foi isolado pela primeira vez em 1986 em Petaluma, Califórnia, em animais negativos para o FeLV e que apresentaram sintomatologia similar à imunodeficiência observada em humanos causada pelo HIV (PEDERSEN *et al.*, 1987). O FIV pertence ao gênero *Lentivirus* e é classificado em sete subtipos (A, B, C, D, E, F, U-NZenv) e diversos vírus recombinantes já foram descritos (CANO-ORTIZ, *et al.*, 2017; DUARTE *et al.*, 2006; HAYWARD; RODRIGO, 2010; HAYWARD; TAYLOR; RODRIGO, 2007; PECORARO *et al.*, 1996). A classificação está baseada em análises filogenéticas das regiões variáveis V3 e V5 do gene SU, sendo os subtipos A, B e C mais amplamente distribuídos (HAYWARD; RODRIGO, 2010).

A infecção causa uma síndrome de imunodeficiência adquirida similar ao HIV-1 em humanos. As fases da infecção são: aguda, assintomática e terminal; essas fases podem ser

observadas experimentalmente, mas em animais naturalmente infectados nem sempre são evidentes. Os sinais clínicos mais evidentes e comuns estão relacionados com infecções oportunistas, doença neurológica e neoplasias (HARTMANN, 2012). Durante a fase inicial da infecção, os animais manifestam sintomatologia leve e transitória, tais como: febre, linfadenopatia generalizada, letargia, enterite e gengivite-estomatite, dermatite, conjuntivite, doença do trato respiratório e perda de peso. Esse período é caracterizado por uma carga viral elevada e neutropenia (HARTMANN, 2012).

A resposta imune celular e humoral desenvolvida não é suficiente para eliminar o vírus do organismo, mas reduz a carga viral plasmática, marcando o início da fase assintomática. Esta etapa pode durar vários anos dependendo da patogenicidade da cepa, exposição a patógenos secundários e idade do animal no momento da infecção. No entanto, a diminuição gradual das células $CD4^+$ e a inversão na relação $CD4^+$:$CD8^+$ levam ao desenvolvimento paulatino da imunodeficiência (HARTMANN, 1998; HOSIE *et al.*, 2009; LECOLLINET; RICHARDSON, 2008; TANIWAKI; FIGUEIREDO; ARAUJO, 2013). A fase terminal caracteriza-se pela síndrome da imunodeficiência felina, que cursa com uma severa imunossupressão, diminuição dos anticorpos circulantes e aumento da carga viral. Esta etapa cursa com linfadenopatia, definhamento e infecções secundárias crônicas. Ocasionalmente ocorrem neoplasias e doença neurológica (LECOLLINET; RICHARDSON, 2008; TANIWAKI; FIGUEIREDO; ARAUJO, 2013). Mesmo apresentando tais condições de saúde, os animais nesta fase podem retornar a uma fase assintomática na possibilidade de tratamento e cuidado apropriado. No entanto, mais de 50 % dos gatos infectados não manifestam uma imunodeficiência severa e, com cuidados apropriados, podem sobreviver vários anos (HARTMANN, 2012).

A organização genômica do FIV é similar à do HIV-1 (Fig. 6). O genoma apresenta os três genes principais (*gag*, *pol* e *env*) e três genes acessórios: *orfA*, *vif* e *rev* (STICKNEY; DUNOWSKA; CAVE, 2013; TROYER, R. M. *et al.*, 2013). A pol codifica também para a dUTPase (DU). Entre os lentivirus, apenas o FIV e o HIV em humanos causam a síndrome de imunodeficiciência adquirida no hospedeiro.

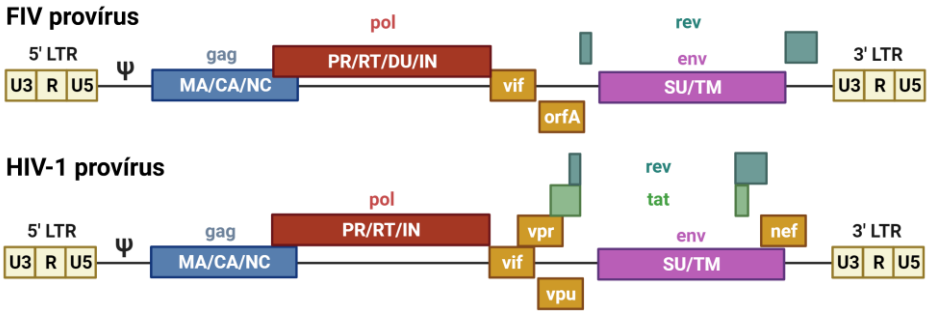

Figura –6 Estrutura genômica do vírus da imunodeficiência felina (FIV) e do vírus da imunodeficiência humana – 1 (HIV-1).

5. VÍRUS DA IMUNODEFICIÊNCIA HUMANA (HIV)

Os lentivírus de primatas como o HIV e o vírus da imunodeficiência dos símios (SIV) utilizam o receptor celular CD4 e um co-receptor para entrar nas células hospedeiras. A infecção por HIV em humanos resulta na síndrome de imunodeficiência adquirida (AIDS). O HIV destrói o sistema imunológico do hospedeiro e causa finalmente, infecções oportunistas e câncer. O HIV infecta células T $CD4^+$ humanas, macrófagos e células dendríticas. A infecção leva ao esgotamento das células T $CD4^+$ por piroptose, apoptose, e outros mecanismos e leva a uma dramática redução das mesmas. Os macrófagos e as células dendríticas não morrem com a infecção mas podem servir como células que contribuem para o reservatório viral latente. A terapia antiretroviral (ART) é capaz de controlar a replicação do HIV, embora os pacientes precisem da terapia pelo resto da sua vida. A interrupção da ART leva a uma recuperação da replicação do HIV, e facilita a ocorrência de vírus resistentes aos medicamentos, causando também o fracasso da terapia (Wensing et al., 2017).

À medida que a infecção progride para a fase da imunodeficiência (síndrome de imunodeficiência adquirida-AIDS), o sistema imunológico fica gravemente danificado e não pode controlar as infecções. Além disso, a incidência de tumores aumenta durante o declínio do sistema imune. Acredita-se que o HIV (HIV-1 e HIV-2) tiveram origem e evoluíram do SIV (SIVcpz em chimpanzés e SIVsmm em *sootye mangabeys*) e provavelmente foram transmitidos aos humanos através do contato sanguíneo com primatas durante atividades de caça (Sharp e Hahn, 2011). O HIV é transmitido principalmente através do contato sexual humano a humano, ou através da utilização de seringas contaminadas e transfusões de contaminadas. A AIDS é ainda uma importante questão de saúde global com mortes estimadas em mais de 25 milhões ao longo das últimas três décadas. De acordo com o último inquérito realizado pela World Health Organização (http://www.who.int/gho/hiv/en/), em

2020, havia 37,7 milhões de pessoas convivendo com com o HIV, com 1,5 milhões de novas infecções.

A infecção causada pelo vírus da imunodeficiência felina (FIV) em gatos é similar à causada pelo HIV-1 em humanos. Os pacientes também apresentam três fases durante a infecção retroviral (aguda, assintomática e terminal). Em gatos estas fases são observadas experimentalmente. Entre as espécies que podem ser infectadas por lentivirus, somente os humanos e os gatos desenvolvem uma síndrome de imunodeficiência adquirida durante a fase terminal.

6. FATORES DE RESTRIÇÃO – SERINC5

Algumas proteínas celulares hospedeiras atuam como a primeira linha de defesa contra a infecções retrovirais, bloqueando diretamente diferentes etapas do ciclo de replicação viral como está representado na figura 7.

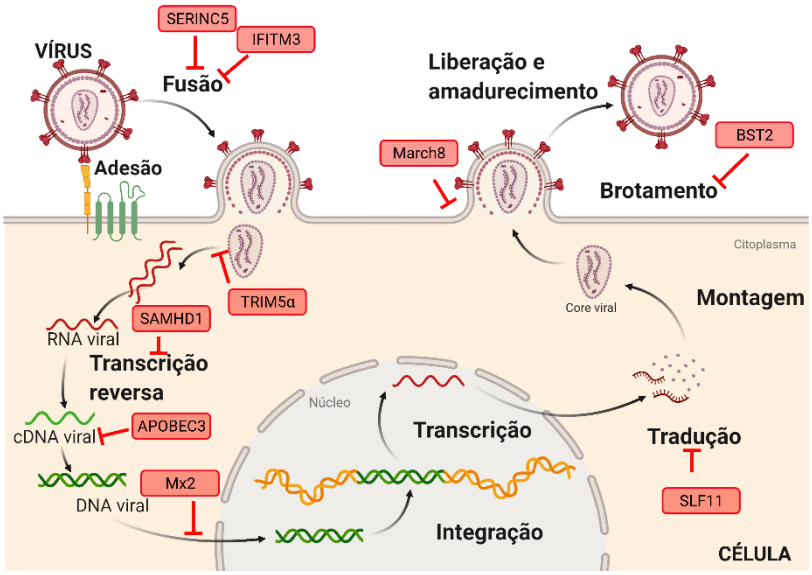

Figura –7 Fatores de restrição na célula infectada pelo HIV-1. Fonte: a própria autora. (BOSO; KOZAK, 2020).

Estas proteínas do hospedeiro, também chamadas fatores de restrição, fazem parte do sistema imune inato. Algumas que já foram bem descritas contra HIV-1 são membros da família das citidinas desaminases APOBEC3, Teterina/BST2, SAMHD1 e TRIM5α. Recentemente foram descritas a MX2, SERINC3/5, IFITM3, SLNF11 e MARCH2/8. Esta seção trata sobre o fator de restrição SERINC5.

SER1-5 pertencem a uma família de proteínas de membrana altamente conservadas em eucariotos cuja estrutura contem entre 9-11 domínios de transmembrana (INUZUKA; HAYAKAWA; INGI, 2005; PYE *et al.*, 2020). Entre elas, SER3 e 5 foram recentemente reconhecidas como fatores de restrição em humanos (ROSA *et al.*, 2015; SOOD *et al.*, 2017). Quando o HIV-1 não expressa Nef (HIV-1 Δ*nef*), SER é incorporada na partícula viral na célula produtora e restringe o processo de fusão do vírus com a membrana citoplasmática na célula alvo, alterando a conformação da proteína Env do HIV-1 e, como resultado, a capacidade de transferência do conteúdo viral para o citoplasma, assim fica comprometida a infectividade do vírus (CHEN *et al.*, 2020; ROSA *et al.*, 2015; SOOD *et al.*, 2017; USAMI; WU; GÖTTLINGE, 2015) (Fig. 7). Além da Nef, a glycoGag do MLV e a proteína S2 do vírus da anemia infecciosa equina (EIAV) contra-atacam o dito fator de restrição.

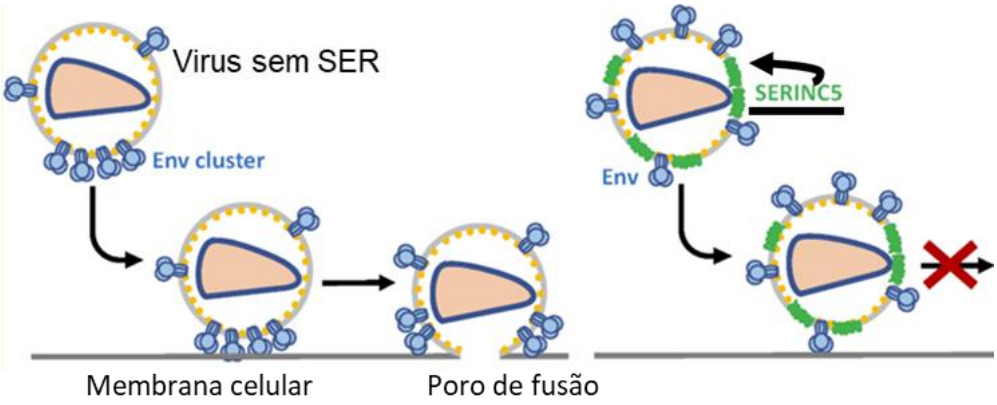

Figura –8 Mecanismo de ação da SERINC5. O vírus que não incorpora a SER5 consegue infectar a célula uma vez que o poro de fusão é formado, se o vírus incorpora a SER o processo de fusão dependente da Env não acontece e a infectividade é limitada. Adaptado de: (CHEN *et al.*, 2020)

7. VETORES RETROVIRAIS E TESTES DE INFECTIVIDADE VIRAL

O sistema de vetores retrovirais foi usado no segundo trabalho para avaliar o comportamento *in vitro* do FeLV, FIV e HIV-1 na presença e ausência dos fatores de restrição SERINC5 humano e felino. Esta seção introduz aos vetores retrovirais como ferramenta molecular.

Os vetores virais são uma ferramenta utilizada para introduzir genomas ou genes em células tanto *in vivo* quanto *in vitro. In vivo*, os vetores virais que se utilizam em terapia gênica precisam ser inócuos, não produzir toxicidade e ser estáveis. Atualmente existem em estudo vários vetores virais baseados em retrovírus, lentivirus, adenovírus e vírus adeno-associados (BULCHA *et al.*, 2021). O primeiro vetor lentiviral desenhado está baseado em

HIV-1 e o primeiro vetor lentiviral não-primata está baseado em FIV (PAROLIN; SODROSKI, 1995; POESCHLA; WONG-STAAL; LOONEY, 2000).

O sistema de vetores lentivirais de terceira geração baseado em HIV-1 inclui quatro componentes: o vetor de transferência (que inclui o gene de interesse para introduzir na célula) o vetor de empacotamento (que expressa Gag e Pol do vírus), um vetor que expressa Rev e um envelope viral que pode ser do mesmo vírus ou de um vírus diferente. O sistema de vetores para FIV é similar mas a Rev está contida dentro do vetor de empacotamento (PAROLIN; SODROSKI, 1995; POESCHLA; WONG-STAAL; LOONEY, 2000).

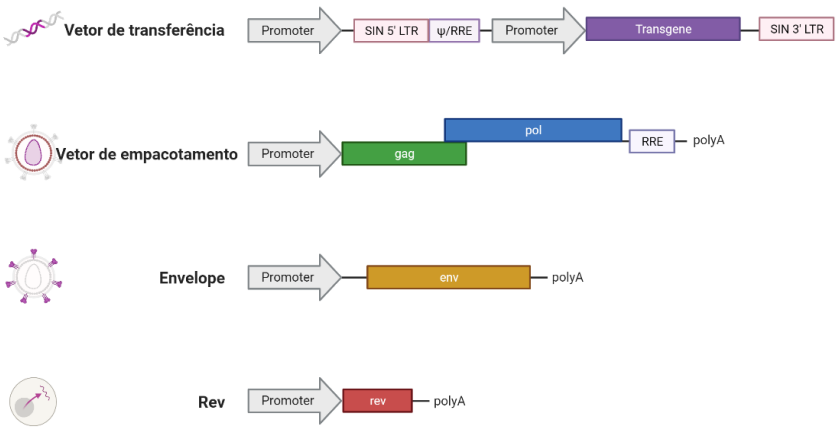

Figura –9 Esquema do sistema de vetores retroviras. O vetor de transferência usado expressa Luciferase e/ou GFP e contém o sinal de empacotamento. O vetor de empacotamento contem gag, pol. As partículas virais podem ser pseudtipadas com diferentes envolepes virais, por exemplo VSV-G. No sistema de vetores baseados em HIV-1 um vetor por separado expressa Rev.

Ambos os sistemas foram utilizados no segundo trabalho para avaliar o comportamento dos vírus na presença ou ausência de SERINC5 humana e felina. Para avaliar a infectividade do vírus foram usados como vetor de transferência plasmídeos repórteres que expressam luciferase e contém o sinal de empacotamento específico para cada vírus Atualmente não existe um sistema de vetores retrovirais com FeLV, portanto foram construídos vetores que permitiram trabalhar com este vírus de um jeito similar. Todos os plasmídeos e os métodos usados estão descritos detalhadamente no artigo. Para os experimentos os plasmídeos virais e os plasmídeos que expressam os fatores de restrição são co-transfectados em células HEK293T. As partículas virais são coletadas 48 h depois e os vírus são normalizados por um teste de transcriptase reversa. A mesma quantidade de vírus (baseado no valo da RT) é usada para infectar células finalmente a infectividade é avaliada medindo a atividade de luciferase intracelular.

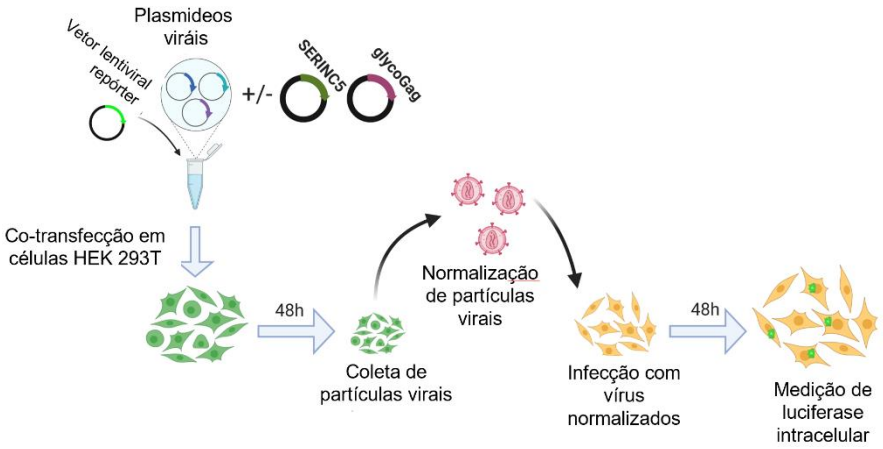

Figura –10 Produção de partículas virais e avaliação da infectividade viral.Os plasmídeos virais e o plasmídeos codificando a proteína de interesse são co-transfectados junto com um vetor reportero. Dois dias depois o sobrenadante é coletado, o vírus é normalizado por uma quantificação da RT e a mesma quantidade de vírus é usada para infectar novas células. A infectividade se avalia por um teste de Luciferase.

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A continuação são apresentados os artigos científicos:

1. COULD PHYLOGENETIC ANALYSIS BE USED FOR FELINE LEUKEMIA VIRUS (FELV) CLASSIFICATION? Publicado na revista *Viruses.* Fator de impacto: 5.08

2. FELINE LEUKEMIA VIRUS-B ENVELOPE TOGETHER WITH ITS GLYCOGAG AND HUMAN IMMUNODEFICIENCY VIRUS-1 NEF Publicado na revista *Journal of Molecular Biology*. Fator de impacto fator de impacto: 5.47

9. COULD PHYLOGENETIC ANALYSIS BE USED FOR FELINE LEUKEMIA VIRUS (FELV) CLASSIFICATION?

viruses

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Article

Could phylogenetic analysis be used for feline leukemia virus (FeLV) classification?

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Abstract: The surface envelope (SU) protein determines the cell tropism and consequently the pathogenesis of the feline leukemia virus (FeLV) in felids. Recombination of exogenous FeLV (exFeLV) with endogenous retroviruses (enFeLV) allows the emergence of more pathogenic variants. Currently, phenotypic testing through interference assays is the only method to distinguish among subgroups, named FeLV-A, -B, -C, -E and -T. This study proposes a new method for FeLV classification based on molecular analysis of the *SU* gene. Four hundred and four publicly available SU sequences were used to reconstruct a maximum likelihood tree. However, only 63 of these sequences had available information about phenotypic tests or subgroup assignment. Two major clusters were observed: a) clade FeLV-A, which includes FeLV-A, FeLV-C, FeLV-E and FeLV-T sequences, and b) clade enFeLV, which includes FeLV-B and enFeLV strains. We found that FeLV-B, FeLV-C, FeLV-E and FeLV-T SU sequences share similarities to FeLV-A viruses and most likely arose independently through mutation or recombination from this strain. FeLV-B and FeLV-C arose from recombination between FeLV-A and enFeLV viruses and FeLV-T is a monophyletic subgroup that has probably originated from FeLV-A through combined events of deletions and insertions. Unfortunately, this study couldn't identify polymorphisms that are specifically linked to FeLV-E subgroup. We propose that phylogenetic and recombination analysis together can explain the current phenotypic classification of FeLV viruses.

Keywords: FeLV, enFeLV, phylogenetics, recombination.

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1. Introduction

Feline leukemia virus (FeLV) is an exogenous gammaretrovirus horizontally transmitted among domestic and wild felids and is known to cause a range of diseases, including lymphadenopathy, anemia, bone marrow suppression, immune suppression, lymphoma, leukemia, and ultimately death [1–4]. FeLV is shed in saliva, nasal secretions, urine, feces, and milk of infected cats and is mainly transmitted horizontally through saliva, blood, and other body fluids by close contact between cats. Transmission can also take place from an infected mother cat to her kittens, either before they are born or while they are nursing {Formatting Citation}. FeLV has the highest case fatality rate in domestic cats of all major feline viruses and remains one of the few retroviral diseases for which there is an effective vaccine [6,7]. The incidence of the virus has been reduced in countries where the vaccination is efficient, however, FeLV is one of the most com-

mon viral agents that infects domestic cats in countries where the vaccination is not a common practice [8].

enFeLV is the endogenous counterpart of exogenous FeLV and are generally non-functional elements, naturally present in the genome of the genus *Felis* with different number of copies [4,9,10]. These endogenous elements invaded the feline genome prior to the speciation of cats and are transmitted from parent to offspring as integral components of chromosomes [11]. Despite not forming infectious particles or inducing disease in the host, expression of enFeLV transcripts has been observed in many tissue types of healthy or FeLV-positive cats [12,13]. In addition, enFeLV seem to act on the biology of exogenous retroviral infection either increasing or decreasing susceptibility to FeLV infection [14–16]. FeLV and enFeLV are approximately 86% similar at the nucleotide level and can readily recombine to generate new FeLV variants either infectious or not [17].

The entry of FeLV into the host cell is mediated by the binding of the envelope glycoprotein (SU or gp70) to host receptors which vary depending on the FeLV subgroup. Currently, FeLV classification is primarily based on phenotypic tests to evaluate the receptor usage, such as viral interference assays (IA), and six different groups have been described so far: FeLV-A, FeLV-B, FeLV-C, FeLV-D, FeLV-E, and FeLV-T [18,19]. Variations in SU protein affect the affinity to the cell receptor and ultimately can influence the clinical manifestation [20]. FeLV-A is the most commonly described subgroup of FeLV and has been reported to be less pathogenic than other FeLV subgroups [6]. FeLV-B arises through recombination events between the envelope (*env*) gene of FeLV-A and enFeLV transcripts [21]. Recombination occurs during retroviral transcriptase-directed DNA synthesis following copackaging of endogenous and exogenous viral genomes and might allow for a change in cellular tropism and a shift in disease progression [22]. The emergence of FeLV-B following FeLV-A infection is considered to result in higher morbidity and mortality and is rarely associated with horizontal transmission unless it is transmitted with FeLV-A [4,23,24]. FeLV-C is a less common subgroup that arises from *de novo* mutations in the *env* gene of FeLV-A and has been associated with the development of aplastic anemia [6,25]. FeLV-D is the result of recombination between exogenous viruses and domestic cat endogenous retrovirus (ERV-DC) that are divergent from enFeLV [26]. FeLV-E is a recently described subgroup which was isolated from natural thymic lymphoma in cats [27] and FeLV-T is a T-cytopathic virus, isolated from a cat with FeLV feline acquired immune deficiency syndrome (FeLV-FAIDS) that have emerged *via* the mutation of FeLV-A strains [27]. Although these recombination events and mutation in env typically have been associated with increased pathogenicity, the FeLV variants are associated with decreased ability for horizontal transmission and require FeLV-A as a helper virus [1,21,26]

Viral interference assays have initially determined the virus biological activity and co-infection profiles of FeLV viruses [6]. However, even with the advent of sequencing technologies classical techniques continue to be the main method to identify historic and novel subgroups [19]. There is a lack of evidence for the relationship between the current classification of FeLV samples based on phenotypic tests and their phylogenetic history and only a few studies aimed to characterize FeLV subgroups using a phylogenetic approach [3,6,19]. The objective of this study was to investigate the phylogenetic relationship underlying the FeLV subgroups and explore deeply the recombination process driving FeLV evolution.

2. Materials and Methods

2.1 Dataset:

Partial and complete FeLV SU sequences (march, 2021) were selected from the Genbank (http://www.ncbi.nlm.nih.gov/nucleotide/). To ensure the selection of high-quality data,

sequences were chosen only when meeting the following criteria: a) one sequence per host, b) minimum length of 1,050 base pairs (bp), and c) absence of premature stop codons. Nucleotide alignments were generated with MUSCLE software [28] and visualized in Aliview [29].

2.2 Recombination Analysis:

Recombinant sequences were detected using seven different methods (RDP, GENECONV, Chimaera, MaxChi, Bootscan, SiScan and 3Seq) in RDP4 v4.85 [30]. Default parameters were used, and recombination events were only accepted if three or more of these methods detected breakpoints with significant P-values ($P < 0.05$). Sequences were also imported into SplitsTree4 [31] and tested for recombination using the PHI test [32]. PHI test calculates the pairwise homoplasy index (PHI) as the mean of the refined incompatibility scores obtained for nearby nucleotide sites along the sequences.

Recombinant sequences were submitted to bootscanning analysis in Simplot 3.5.1 [33] to detect putative recombination among subgroups. The Kimura 2-parameter model was used as a distance model on a sliding window of 200 nucleotides (nt) by increments of 20 nt. Recombinant samples identified by RDP4 were used as query sequences in Simplot. For the bootscan, non-recombinant sequences were used as references.

2.3 Phylogenetic Reconstruction:

A maximum likelihood (ML) tree incorporating the best-fitted nucleotide substitution model (GTR+F+R5) was reconstructed in IQtree web-server [34]. The robustness of the resultant tree was evaluated by rapid bootstrapping (UFB) with 1000 pseudoreplicate datasets. Phylogenetic tree was visualized in FigTree v1.4.4 [35] and draw using R [36].

3. Results

3.1 Data set curation

Four hundred and fifteen FeLV SU sequences met our criteria for data quality and were downloaded from the GenBank (1,368 base pairs, Supplementary Table S1). New complete FeLV SU sequences were also deposited in GenBank (accession MW762576 - MW762583). Only 63 of these sequences had available information about phenotypic tests or subgroup assignment (39 FeLV-A, 5 FeLV-B, 1 FeLV-C, 7 FeLV-D, 1 FeLV-E, 2 FeLV-T, and 4 enFeLV). The extent of the differences between FeLV-D samples and other FeLV/enFeLV sequences prevents any analysis of this subgroup in the same phylogenetic context and, therefore, 7 FeLV-D sequences were excluded from the following analyses. Although it does not preclude phylogenetic analysis, the reduced number of sequences with subgroup characterization might interfere with the extent of the conclusions regarding FeLV evolution.

3.2 Different patterns of recombination were identified

The results for the PHI test support that FeLV evolution might be modeled by recombination ($P > 0.01$). To detect putative recombinant sequences and minimize the disruptive impact that recombination can have on phylogenetic interpretation [30,37], the dataset was analyzed in RDP4. Fifty-five FeLV SU sequences presented at least one of 24 different recombination patterns and were treated as recombinant sequences, including 1 FeLV-A, 4 FeLV-B and the unique FeLV-C sequence (Figure 1, Supplementary Figure 1 and Supplementary Table S1). After excluding those 55 recombinant sequences from the alignment, no significant recombination signal could be detected using PHI test ($P =$

0.8244), suggesting that all recombinant sequences were effectively identified. Simplot analysis allowed the inference of the most likely origin of each recombinant fragment.

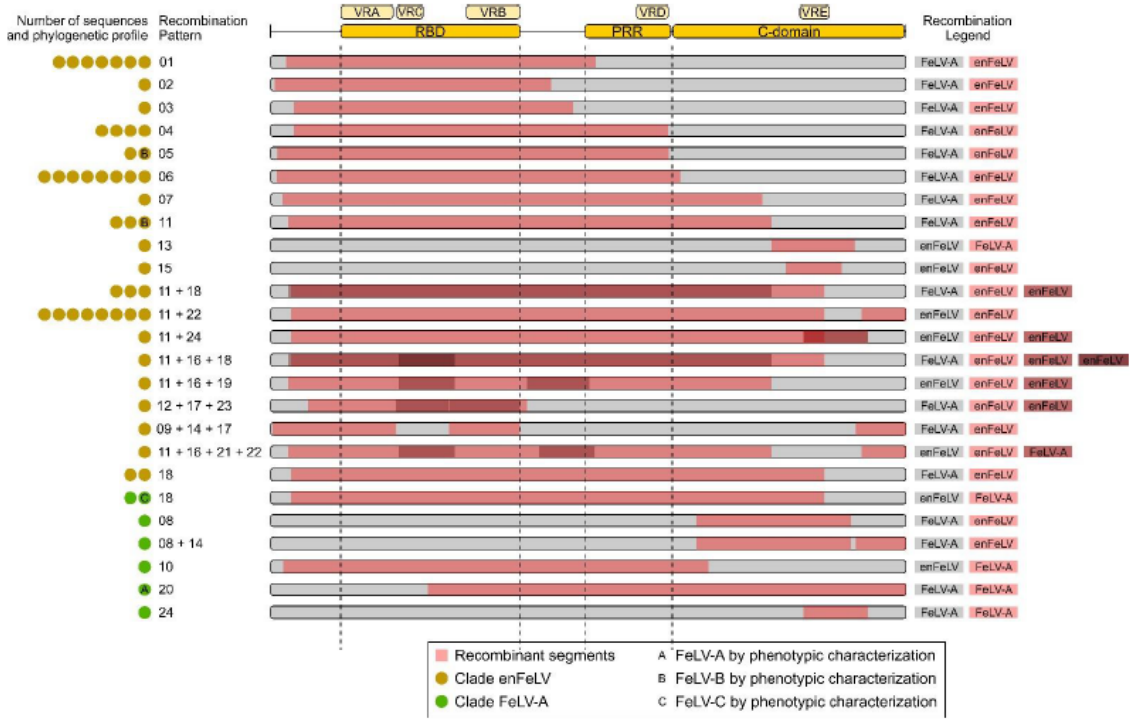

Figure 1. Scheme of the recombinant patterns. The number of sequences presenting each recombinant pattern are indicated by dots. Sequences belonging to Clade enFeLV are indicated in yellow and sequences belonging to Clade FeLV-A in green. The recombination segments are shown for each pattern.

3.3 FeLV SU gene can be divided into two big clades

Phylogenetic reconstruction was performed using all FeLV samples included in this study (Figure 2). The incorporation of recombinant sequences in phylogenetic analysis aims to identify the impact of recombination for FeLV subgroups, in addition to clarifying the evolutionary relationship between these samples. The phylogenetic tree displayed the subdivision of FeLV SU sequences into two well-supported (UFB > 90) main clades (Figure 2): a) clade FeLV-A, which includes all FeLV-A sequences intermingled with FeLV-C, FeLV-E and FeLV-T; and b) clade enFeLV, which groups all FeLV-B and enFeLV sequences included in this study. The split of the dataset into two different clades is corroborated by the analysis of the SU gene structure (Figure 2).

3.4 The SU envelope gene possess five variable regions, the pattern of the variable regions is conserved intra clade

Clade enFeLV contains 89 FeLV SU sequences, including 4 enFeLV (4/4 publicly available), 5 FeLV-B (5/5 publicly available) and 80 sequences without information about subgroup assignment (Figure 2). Four FeLV-B and other 44 samples without subgroup assignment presented evidence for recombination, displaying 19 different patterns of recombination. Despite being recombinants, most of the sequences (96%) preserved the RBD region derived from endogenous strains (absence of VRA and VRC but presence of VRB). In addition, most of these samples (78%) exhibits an enFeLV profile at the PRR region (Supplementary Table S1). Together, these results suggest that recombination seem to be an important factor for the evolution of FeLV-B and enFeLV samples, since 54% of the samples within this clade are recombinant sequences. Moreover, specific signatures inside RBD and PRR regions might be critical for these viruses as almost 80% of them presented conserved motifs at the gene level.

3.7 Geographical distribution of FeLV-A clade

A second tree was constructed using only non-recombinant FeLV sequences (Table S1) in order to understand the diversity of FeLV strains. Here we also included endogenous sequences as outgroup. The phylogeny shows three different clades (Figure 3): the basal clade comprises only sequences belonging to the enFeLV clade in the former phylogeny (Figure 2), but this time only without recombination. Two more clades which belong to the Clade FeLV-A, are clearly separated by the geographical origin. The bigger clade is mostly conformed by sequences from Japan and the smaller by sequences from USA, Brazil, United Kingdom and NA. All the Chinese sequences clustered in the enFeLV clade and there are not recombinants (Figure 3, Supplementary Table 1).

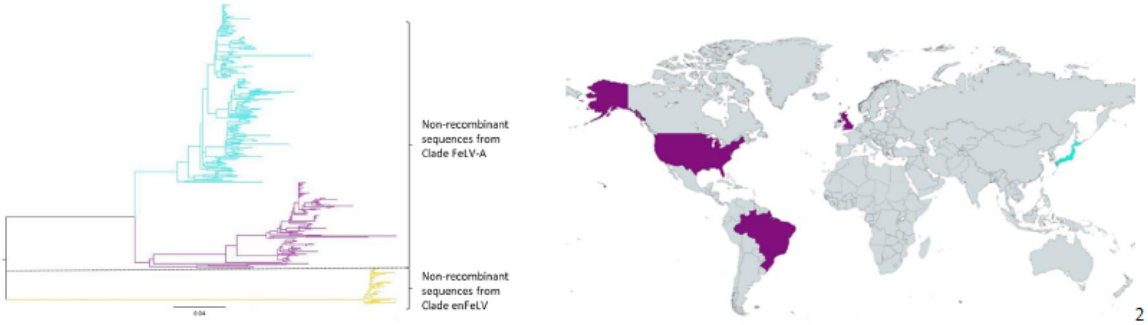

Figure 3. Phylogenetic reconstruction of the non-recombinant FeLV SU sequences and geographical distribution of the Clade FeLV-A. Maximum likelihood tree constructed by incorporating the best-fitted nucleotide substitution model (GTR+F+R5) in Iqtree web-server. The robustness of the resultant tree was evaluated by rapid bootstrapping (UFB) with 1000 pseudoreplicate datasets. Phylogenetic tree was visualized in FigTree v1.4.4 [35] and draw using R [36]. Three main clades are presented. The yellow clade corresponds to non-recombinant sequences from Clade enFeLV and the blue and purple to the non-recombinant sequences from the Clade A (see Figure 1).

4. Discussion

In the last forty years, viral interference assays have been the only method used to distinguish and define FeLV subgroups [19,42]. Analysis of the superinfection interference properties of FeLV variants has led to the identification of four interference types among FeLV subgroups eventually associated with specific clinical phenotypes: FeLV-A uses the feline thiamine transport protein 1 (feThTr1) [43] and it has been associated with

macrocytic anemia, immunosuppression, and lymphoma [2]; FeLV-B uses the inorganic phosphate-sodium symporter fePit1 (SLC20A1) and the closely related fePit2 (SLC20A2) [40,44,45] and is tumorigenic [2]; FeLV-C uses a heme export protein called FLVCR [46,47] and has been associated with the development of aplastic anemia [48]; and FeLV-T requires Pit1 and a soluble cofactor expressed from endogenous FeLV-related sequences (FeLIX) [49,50] and has been associated to fatal immunodeficiency [51]. FeLV-E was recently described, and its receptor remains unidentified [27]. Phenotypic tests are, however, laborious, and time-consuming which in turn creates a technical barrier and restricts extensive FeLV classification ultimately interfering in molecular epidemiology or evolutionary studies. With the advent of the next-generation sequencing, genetic tests have become cheaper, faster, more reliable, and more precise than phenotypic characterization. However, despite the advantages of sequencing technologies, only a few studies up to now have tried to correlate phenotypic classification to phylogenetic reconstruction for FeLV viruses [3,19,52,53]. Here, we used molecular data to characterize FeLV subgroups and outline a proposition for a new classification system based on phylogenetic analysis.

Despite being classified into different subgroups, FeLV-A, FeLV-C, FeLV-E and FeLV-T SU sequences grouped within the same clade in the phylogenetic tree (clade FeLV-A, Figure 2). All sequences within this clade presented similar gene organization sharing an insertion of 30 bp in VRA, 15 bp in VRC in addition to a 69 bp deletion in VRB and a 27 bp deletion in VRD (Figure 2). Since FeLV-A sequences are the most abundant strains in this cluster and were found scattered through the whole clade it is reasonable to assume that the most recent common ancestor of clade FeLV-A presented a gene organization related to FeLV-A and used feThTr1 as its host receptor. The inclusion of other FeLV subgroups with different host receptors, but similar gene organization within this clade may suggest that FeLV-C, FeLV-E and FeLV-T arose in separate monophyletic events from FeLV-A viruses most likely through mutation and/or recombination.

Apparently, FeLV-C has arose by means of recombination (Figure 1). Our analysis suggests that FeLV-C is the result of a recombination event that included mostly FeLV-A SU gene and only a small fragment of endogenous strains (Figure 1). The recombination breakpoint in the C-domain might be the explanation to the fact that FeLV-C has a different receptor (FLVCR) than the one used by most samples grouped within the clade FeLV-A (feThTr1) [43,46]. A previous study investigating the role of the C-domain region of FeLV-C strain proposes that this region is critical for efficient SU binding and may function as a second receptor-binding domain, which in addition to RBD, interacts with the host receptor to initiate virus infection [54]. The phenotypic results for FeLV-C seem to corroborate the molecular analyzes performed in this study, suggesting that recombination may be the main evolutionary event defining the receptor change in this variant.

FeLV-T, on the contrary, may have arisen from the accumulation of mutations in FeLV-A virions allowing the change of the host receptor from FETHTR1 to Pit1. Both FeLV-T samples included in this study grouped in a monophyletic clade and, unlike all sequences inside clade FeLV-A, exhibited an 18 bp deletion in VRA and an 18 bp insertion in the VRE region. The deletion of 18 bp in VRA is smaller but lies in the same position of the 30 bp deletion presented by FeLV-B viruses which could explain the use of the same receptor (Pit1) for these strains. Intriguingly, Shojima *et al.* (2006) in an *ex-vivo* model using different cell lines proposed that the host ranges of FeLV-T and FeLV-B were not exactly the same and suggested a different Pit1 usage at the post-binding level for the two strains [41]. Another 11 sequences with no information about subgroup also presented the 18 bp deletion in VRE but only one of these sequences (M87886) has the 18 bp insertion in the VRA and grouped in the same monophyletic clade as FeLV-T. This unclassified sequence most likely represents another strain of FeLV-T. Together, these results

support the idea that polymorphisms in VRA are notoriously more important to define the host receptor for strains grouping within clade FeLV-A than polymorphisms in VRE.

Unfortunately, this study was not able to find genetic polymorphisms or evolutionary mechanisms in FeLV-E strain that could justify its receptor usage. We used entropy analysis to compare FeLV-E with all FeLV-A sequences and no amino acid sites could be specifically associated to this strain ($p > 0.05$, data not shown). In addition, no recombination breakpoint was detected in this sequence. The fact that only one sample of this subgroup has been sampled so far may have prevented any conclusions about the relationship between phenotypic testing and phylogenetic clustering.

Clade enFelV grouped FeLV-B, enFeLV and most of the recombinant sequences identified in this study (87%). The similarity of the gene structure seems to be the main factor driving the clustering of these sequences together. In general, these samples presented a 30 bp deletion in the VRA (100% of the sequences in the clade), a 15 bp deletion in VRC (95%) and two insertions of 13 and 14 bp in the VRD (88%). Despite sharing a similar structure, enFeLV and FeLV-B sequences were found segregated within the enFeLV clade in our phylogenetic analysis. Notably, FeLV-B and all the recombinant sequences without subgroup assignment were basal to a monophyletic clade that includes, among other sequences, all 4 enFeLV strains.

Corroborating previous studies, our analysis shows that FeLV-B arose several times through recombination between FeLV-A and endogenous strains most likely after co-packaging of expressed transcripts into a single virion [55,56]. These results suggest that FeLV-B strains must include the RBD and PRR regions derived from endogenous viruses along with a short and variable length fragment derived from exogenous strains at the 3′ end of the SU gene (Figure 1). Pandey *et al.* (1991) examining exogenous/endogenous recombination during *in vitro* infections has revealed that replication efficiency and cellular tropism depends on the length and region of the enFeLV sequence incorporated into the FeLV-B recombinant [57]. In addition, this study shows that amino acid changes inside VRA and VRB are the main factors for the ability of FeLV-B to bind receptor Pit1 and/or Pit2. Sample JF957363 was identified as the only non-recombinant FeLV-B sample in our analysis, however, in the search for details we found that this isolate is an enFeLV strain that presents long terminal repeats (LTR) from exogenous viruses explaining the relationship with the enFeLV sequences in the phylogenetic tree [58].

Frequent recombination has generated FeLV strains mixing endogenous and exogenous viruses. Only 7 recombinant sequences grouped within clade FeLV-A and all of them have maintained the RBD and PRR regions from FeLV-A strains. The C-domain is, however, variable and can apparently be either derived from exogenous or endogenous viruses (Figure 1 and Supplementary Table S1). Once the RBD is completely exogenous, these recombinants probably display the same interference pattern of the exogenous FeLV-A viruses. On the other hand, clade enFeLV clustered a total of 48 recombinant sequences and most of them present the RBD and the PRR regions derived from endogenous strains. Following the pattern observed for recombinant sequences within clade FeLV-A, the C-domain region in these sequences were found to be derived from exogenous or endogenous viruses. It is interesting to highlight that the farther the sequence is from the root of the enFeLV clade in the phylogenetic tree, the greater the size of the fragment derived from exogenous viruses in these recombinant sequences (Supplementary Figure 1).

Here we used genetic and evolutionary approaches to explain the current phenotypic classification of FeLV viruses. We found that FeLV-B, FeLV-C, FeLV-E and FeLV-T SU gene sequences share similarities to FeLV-A viruses and most likely arose independently through mutation or recombination from this strain. FeLV-B is a classical product of recombination between FeLV-A and enFeLV strains that presents the RBD and PRR regions derived from endogenous viruses. Despite arising from recombination events as

well, FeLV-C has otherwise incorporated both RBD and PRR regions from FeLV-A strains. FeLV-T is a monophyletic subgroup that has probably originated from FeLV-A through combined events of deletions in the RBD region and insertions in the C-domain of the SU gene. Unfortunately, this study couldn't identify polymorphism that are specifically linked to FeLV-E subgroup and explain its phenotypic results for receptor usage. Furthermore, we propose that the use of phylogenetic analysis can be efficiently used to classify FeLV subgroups.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1, Figure S1.

Author Contributions: LCO: conceptualization, methodology, investigation, validation, formal analysis, investigation, data curation, writing – original draft, visualization. CT: methodology, formal analysis, investigation. PMR: writing review and editing, funding acquisition, ACF: investigation, writing – review and editing, supervision, funding acquisition. DMJ: conceptualization, methodology, investigation, validation, formal analysis, investigation, data curation, writing – original draft, writing – review and editing, visualization, supervision.

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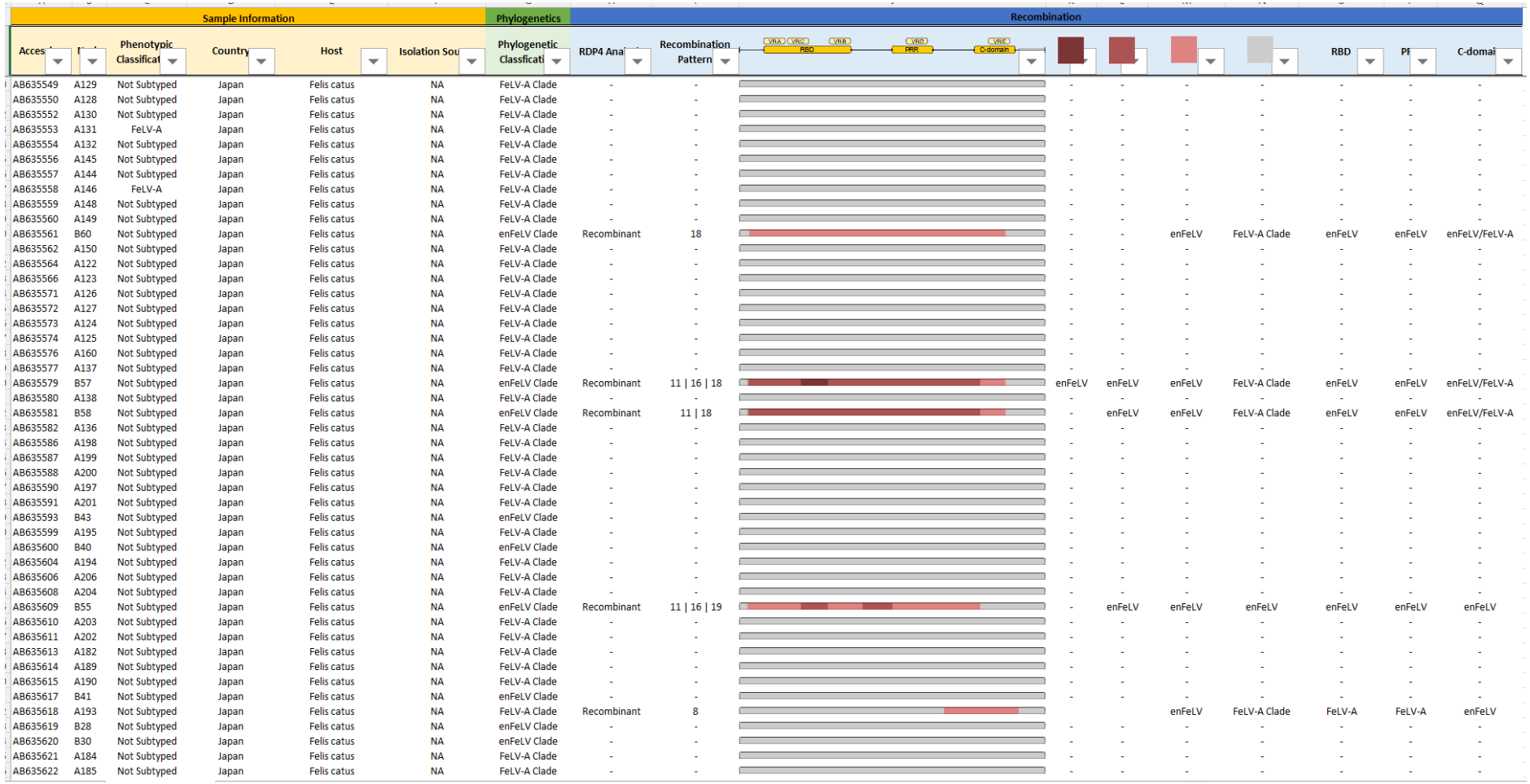
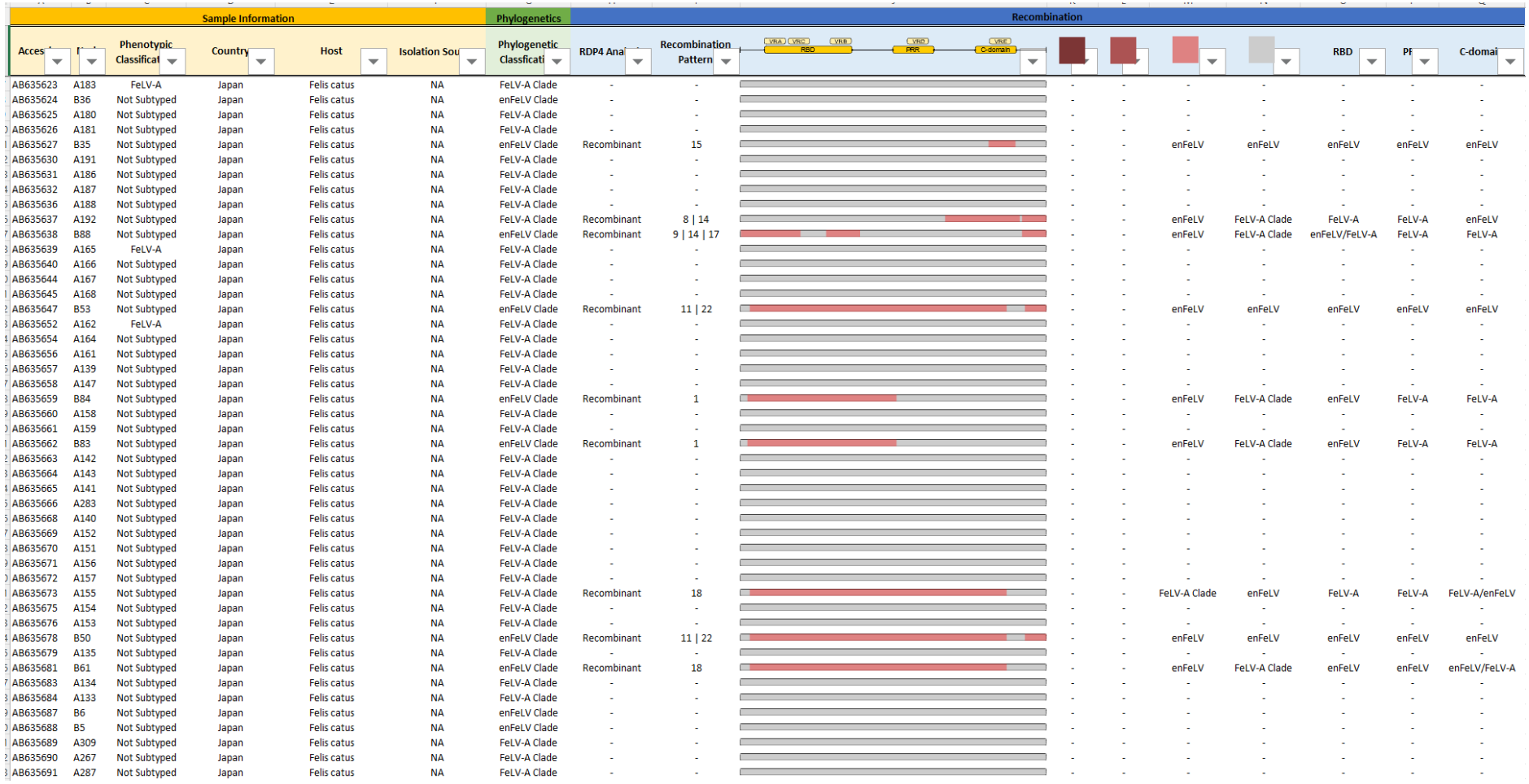
Supl. Table. 1.

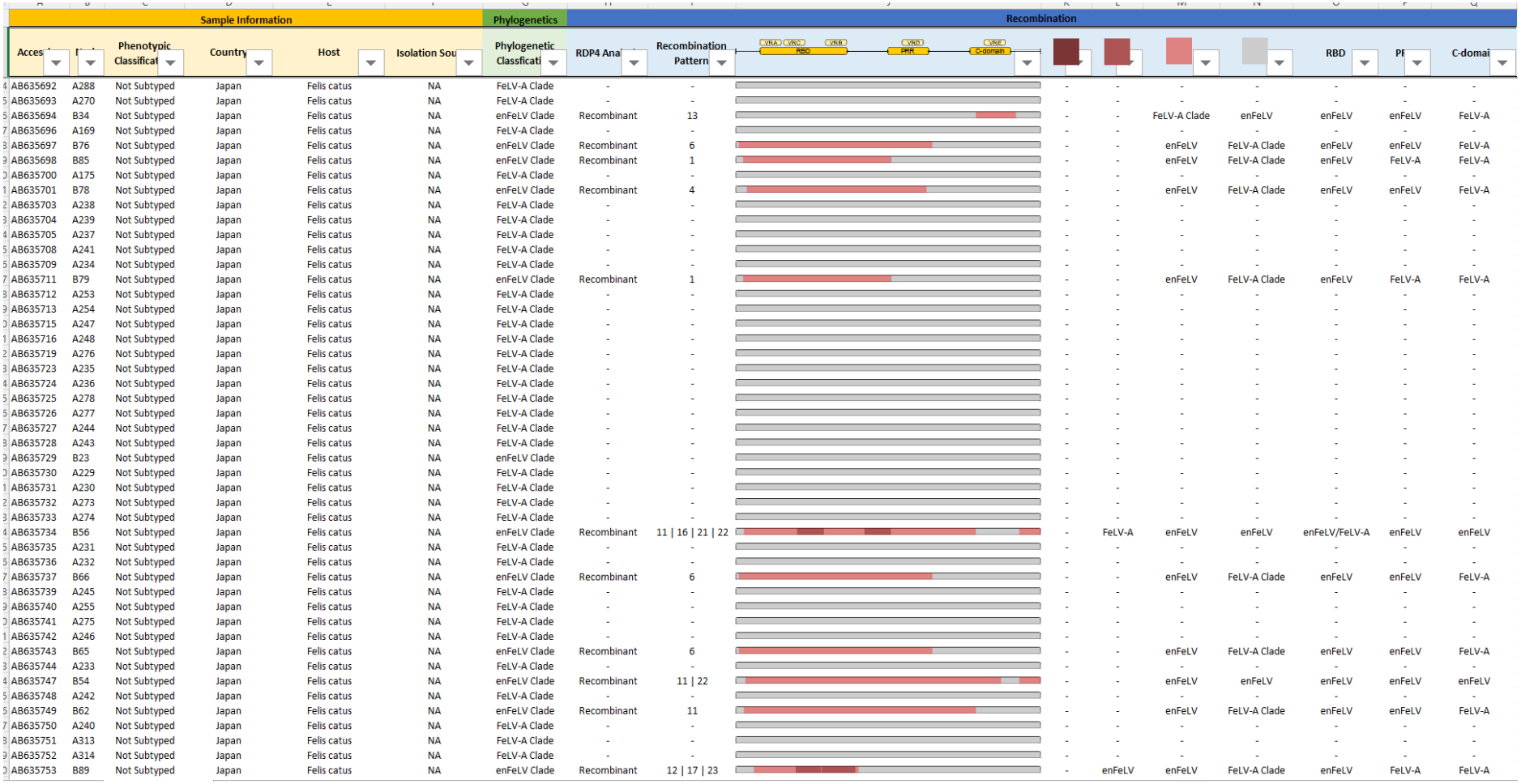
Sample Information						Phylogenetics	Recombination										
Acces		Phenotypic Classificat	Country	Host	Isolation Sou	Phylogenetic Classficati	RDP4 Ana	Recombination Pattern	VRA VRC VRB VRD VRE RBD PRR C-domain					RBD	PI	C-domai
AB060732	A196	Not Subtyped	NA	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635483	A82	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635485	B63	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	7		-	-	enFeLV	FeLV-A	enFeLV	enFeLV	FeLV-A
AB635486	A100	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635490	B51	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	11 | 22		-	-	enFeLV	enFeLV	enFeLV	enFeLV	enFeLV
AB635491	A101	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635492	B64	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	11		-	-	enFeLV	FeLV-A Clade	enFeLV	enFeLV	FeLV-A
AB635493	A102	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	Recombinant	24		-	-	FeLV-A Clade	FeLV-A Clade	FeLV-A	FeLV-A	FeLV-A
AB635494	B47	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	11 | 24		-	enFeLV	enFeLV	enFeLV	enFeLV	enFeLV	enFeLV
AB635495	A103	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635499	B80	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	1		-	-	enFeLV	FeLV-A Clade	enFeLV	FeLV-A	FeLV-A
AB635500	A99	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635501	A98	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635502	B81	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	1		-	-	enFeLV	FeLV-A Clade	enFeLV	FeLV-A	FeLV-A
AB635503	A104	FeLV-A	Japan	Felis catus	NA	FeLV-A Clade	Recombinant	20		-	-	FeLV-A Clade	FeLV-A Clade	FeLV-A	FeLV-A	FeLV-A
AB635504	A97	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635506	B46	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	11 | 22		-	-	enFeLV	enFeLV	enFeLV	enFeLV	enFeLV
AB635507	A106	FeLV-A	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635510	A105	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635511	A107	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635512	B75	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	4		-	-	enFeLV	FeLV-A Clade	enFeLV	enFeLV	FeLV-A
AB635513	A108	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635514	A111	FeLV-A	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635516	A110	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635517	B72	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	6		-	-	enFeLV	FeLV-A Clade	enFeLV	enFeLV	FeLV-A
AB635519	A109	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635520	B48	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	11 | 22		-	-	enFeLV	enFeLV	enFeLV	enFeLV	enFeLV
AB635521	A112	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635522	A113	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635524	A116	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635525	A117	FeLV-A	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635526	B71	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	6		-	-	enFeLV	FeLV-A Clade	enFeLV	enFeLV	FeLV-A
AB635527	A115	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635529	A118	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635532	A119	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635533	A120	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635535	A121	FeLV-A	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635536	B82	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	1		-	-	enFeLV	FeLV-A Clade	enFeLV	FeLV-A	FeLV-A
AB635538	A172	FeLV-A	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635539	A176	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635540	A173	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635541	A171	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635542	A170	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635543	A174	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635545	A179	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635547	A177	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635548	A178	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-



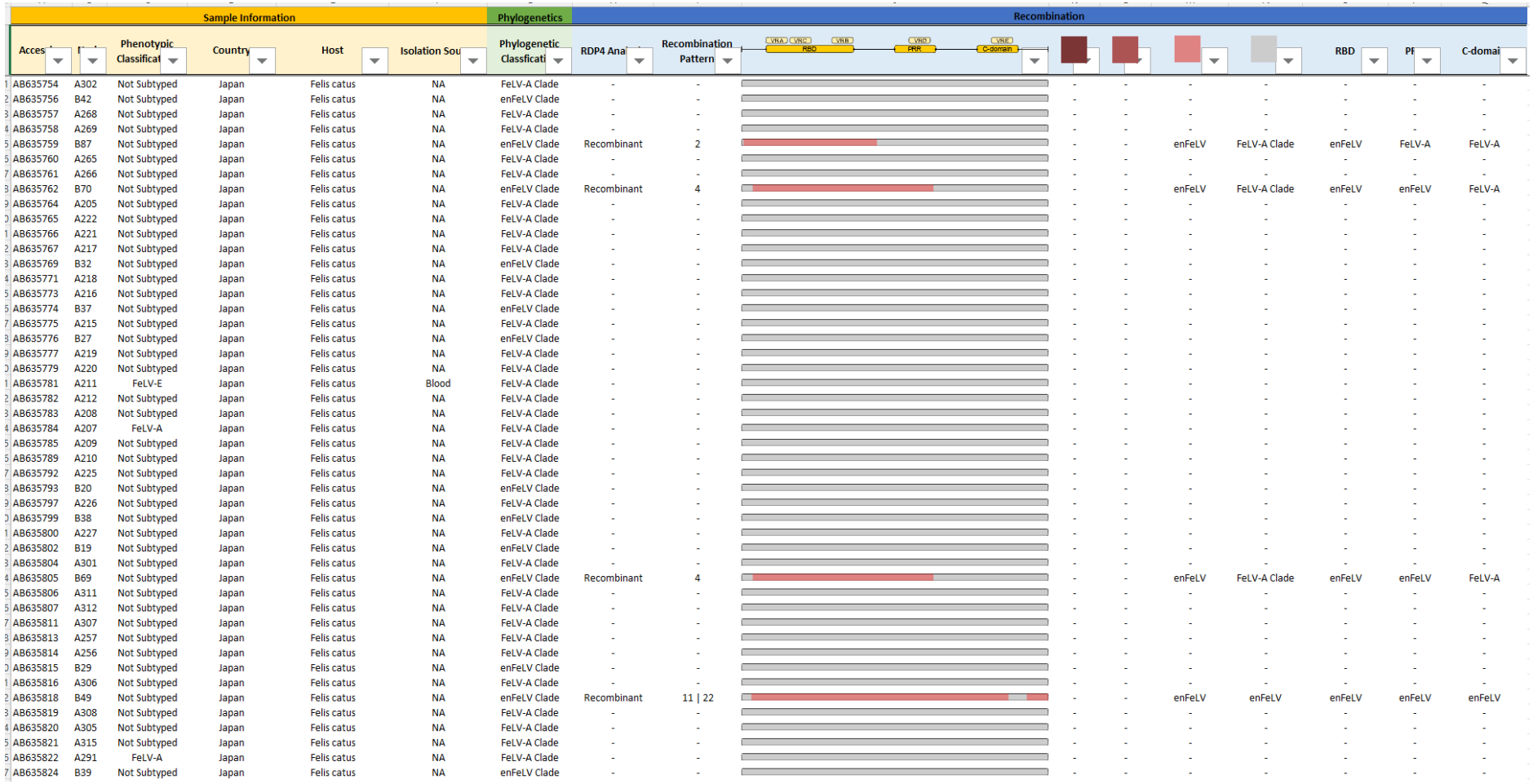
Sample Information						Phylogenetics	Recombination									
Acces		Phenotypic Classificat	Country	Host	Isolation Sou	Phylogenetic Classficati	RDP4 Ana	Recombination Pattern	VRA VRC VRB VRD VRE RBD PRR C-domain					RBD	PI	C-domai
AB635549	A129	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635550	A128	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635552	A130	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635553	A131	FeLV-A	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635554	A132	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635556	A145	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635557	A144	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635558	A146	FeLV-A	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635559	A148	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635560	A149	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635561	B60	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	18		-	-	enFeLV	FeLV-A Clade	enFeLV	enFeLV	enFeLV/FeLV-A
AB635562	A150	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635564	A122	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635566	A123	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635571	A126	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635572	A127	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635573	A124	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635574	A125	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635576	A160	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635577	A137	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635579	B57	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	11 | 16 | 18		enFeLV	enFeLV	enFeLV	FeLV-A Clade	enFeLV	enFeLV	enFeLV/FeLV-A
AB635580	A138	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635581	B58	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	11 | 18		-	enFeLV	enFeLV	FeLV-A Clade	enFeLV	enFeLV	enFeLV/FeLV-A
AB635582	A136	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635586	A198	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635587	A199	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635588	A200	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635590	A197	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635591	A201	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635593	B43	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-
AB635599	A195	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635600	B40	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-
AB635604	A194	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635606	A206	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635608	A204	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635609	B55	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	11 | 16 | 19		-	enFeLV	enFeLV	enFeLV	enFeLV	enFeLV	enFeLV
AB635610	A203	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635611	A202	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635613	A182	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635614	A189	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635615	A190	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635617	B41	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-
AB635618	A193	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	Recombinant	8				enFeLV	FeLV-A Clade	FeLV-A	FeLV-A	enFeLV
AB635619	B28	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-
AB635620	B30	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-
AB635621	A184	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635622	A185	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-



	Sample Information						Phylogenetics	Recombination										
	Acces		Phenotypic Classificat	Country	Host	Isolation Sou	Phylogenetic Classficati	RDP4 Ana	Recombination Pattern	VRA VRC VRB VRD VRE RBD PRR C-domain					RBD	PF	C-domai
	AB635623	A183	FeLV-A	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635624	B36	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-
	AB635625	A180	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635626	A181	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635627	B35	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	15		-	-	enFeLV	enFeLV	enFeLV	enFeLV	enFeLV
	AB635630	A191	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635631	A186	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635632	A187	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635636	A188	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635637	A192	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	Recombinant	8 | 14		-	-	enFeLV	FeLV-A Clade	FeLV-A	FeLV-A	enFeLV
	AB635638	B88	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	9 | 14 | 17		-	-	enFeLV	FeLV-A Clade	enFeLV/FeLV-A	FeLV-A	FeLV-A
	AB635639	A165	FeLV-A	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635640	A166	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635644	A167	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635645	A168	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635647	B53	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	11 | 22		-	-	enFeLV	enFeLV	enFeLV	enFeLV	enFeLV
	AB635652	A162	FeLV-A	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635654	A164	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635656	A161	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635657	A139	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635658	A147	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635659	B84	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	1		-	-	enFeLV	FeLV-A Clade	enFeLV	FeLV-A	FeLV-A
	AB635660	A158	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635661	A159	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635662	B83	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	1		-	-	enFeLV	FeLV-A Clade	enFeLV	FeLV-A	FeLV-A
	AB635663	A142	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635664	A143	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635665	A141	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635666	A283	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635668	A140	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635669	A152	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635670	A151	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635671	A156	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635672	A157	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635673	A155	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	Recombinant	18		-	-	FeLV-A Clade	enFeLV	FeLV-A	FeLV-A	FeLV-A/enFeLV
	AB635675	A154	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635676	A153	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635678	B50	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	11 | 22		-	-	enFeLV	enFeLV	enFeLV	enFeLV	enFeLV
	AB635679	A135	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635681	B61	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	18		-	-	enFeLV	FeLV-A Clade	enFeLV	enFeLV	enFeLV/FeLV-A
	AB635683	A134	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635684	A133	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635687	B6	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-
	AB635688	B5	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-
	AB635689	A309	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635690	A267	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
	AB635691	A287	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-

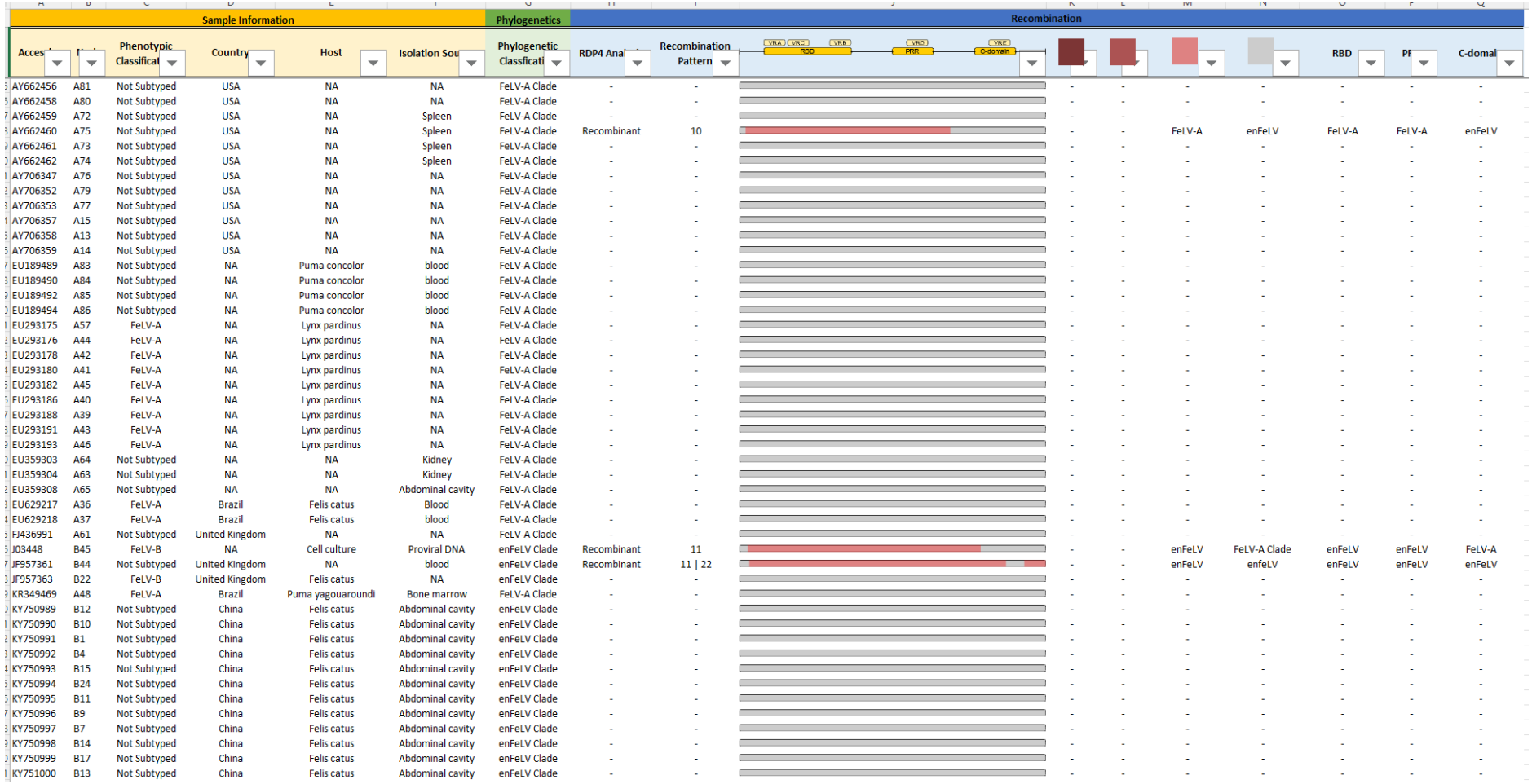


	Sample Information						Phylogenetics	Recombination											
	Acces		Phenotypic Classificat	Country	Host	Isolation Sou	Phylogenetic Classficati	RDP4 Ana	Recombination Pattern	VRA VRC VRB RBD VRD PRR VRE C-domain						RBD	PI	C-domai
4	AB635692	A288	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
5	AB635693	A270	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
5	AB635694	B34	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	13		-	-	FeLV-A Clade	enFeLV	enFeLV	enFeLV	enFeLV	FeLV-A
7	AB635696	A169	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
3	AB635697	B76	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	6		-	-	enFeLV	FeLV-A Clade	enFeLV	enFeLV	enFeLV	FeLV-A
9	AB635698	B85	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	1		-	-	enFeLV	FeLV-A Clade	enFeLV	FeLV-A	FeLV-A	FeLV-A
0	AB635700	A175	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
1	AB635701	B78	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	4		-	-	enFeLV	FeLV-A Clade	enFeLV	enFeLV	enFeLV	FeLV-A
2	AB635703	A238	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
3	AB635704	A239	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
4	AB635705	A237	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
5	AB635708	A241	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
6	AB635709	A234	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
7	AB635711	B79	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	1		-	-	enFeLV	FeLV-A Clade	enFeLV	FeLV-A	FeLV-A	
8	AB635712	A253	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
9	AB635713	A254	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
0	AB635715	A247	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
1	AB635716	A248	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
2	AB635719	A276	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
3	AB635723	A235	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
4	AB635724	A236	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
5	AB635725	A278	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
6	AB635726	A277	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
7	AB635727	A244	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
8	AB635728	A243	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
9	AB635729	B23	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
0	AB635730	A229	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
1	AB635731	A230	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
2	AB635732	A273	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
3	AB635733	A274	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
4	AB635734	B56	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	11 | 16 | 21 | 22		-	FeLV-A	enFeLV	enFeLV	enFeLV/FeLV-A	enFeLV	enFeLV
5	AB635735	A231	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
6	AB635736	A232	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
7	AB635737	B66	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	6		-	-	enFeLV	FeLV-A Clade	enFeLV	enFeLV	FeLV-A	
8	AB635739	A245	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
9	AB635740	A255	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
0	AB635741	A275	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
1	AB635742	A246	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
2	AB635743	B65	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	6		-	-	enFeLV	FeLV-A Clade	enFeLV	enFeLV	FeLV-A	
3	AB635744	A233	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
4	AB635747	B54	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	11 | 22		-	-	enFeLV	enFeLV	enFeLV	enFeLV	enFeLV	
5	AB635748	A242	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
6	AB635749	B62	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	11		-	-	enFeLV	FeLV-A Clade	enFeLV	enFeLV	FeLV-A	
7	AB635750	A240	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
8	AB635751	A313	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
9	AB635752	A314	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
0	AB635753	B89	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	12 | 17 | 23		-	enFeLV	enFeLV	FeLV-A Clade	enFeLV	FeLV-A	FeLV-A	



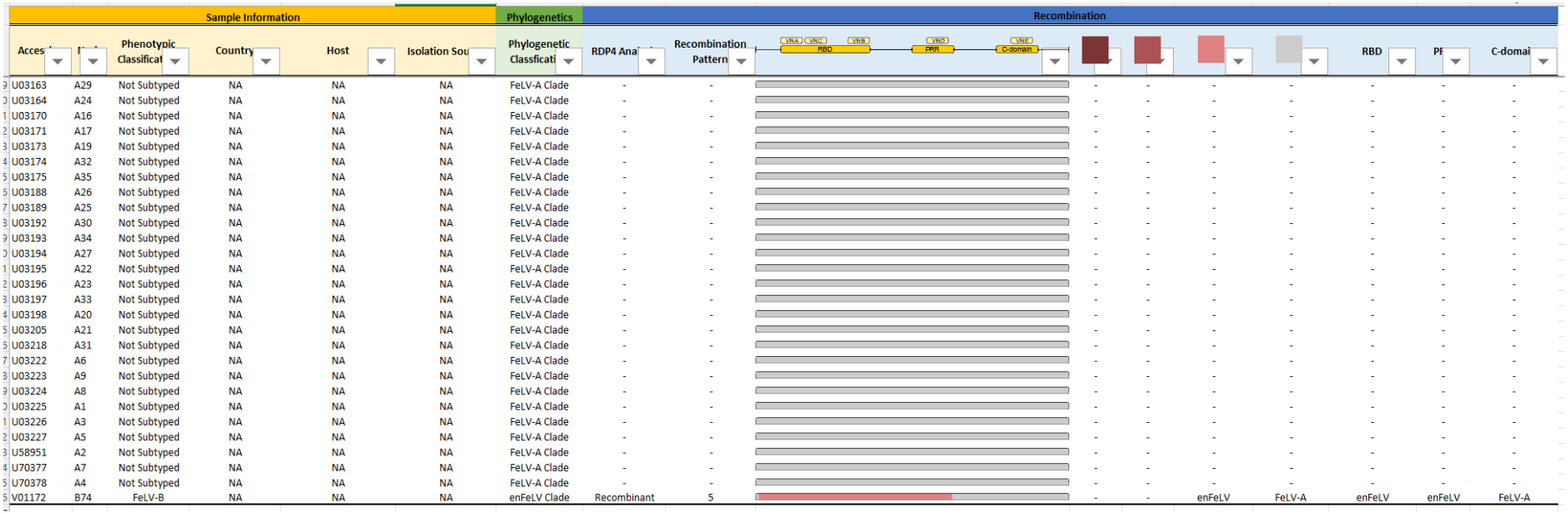
	Sample Information						Phylogenetics	Recombination											
	Acces		Phenotypic Classificat	Country	Host	Isolation Sou	Phylogenetic Classificati	RDP4 Ana	Recombination Pattern								RBD	PF	C-domai
1	AB635754	A302	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
2	AB635756	B42	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-	-	
3	AB635757	A268	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
4	AB635758	A269	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
5	AB635759	B87	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	2		-	-	enFeLV	FeLV-A Clade	enFeLV	FeLV-A	FeLV-A		
6	AB635760	A265	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
7	AB635761	A266	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
8	AB635762	B70	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	4		-	-	enFeLV	FeLV-A Clade	enFeLV	enFeLV	FeLV-A		
9	AB635764	A205	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
10	AB635765	A222	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
11	AB635766	A221	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
12	AB635767	A217	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
13	AB635769	B32	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-	-	
14	AB635771	A218	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
15	AB635773	A216	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
16	AB635774	B37	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-	-	
17	AB635775	A215	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
18	AB635776	B27	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-	-	
19	AB635777	A219	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
20	AB635779	A220	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
21	AB635781	A211	FeLV-E	Japan	Felis catus	Blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
22	AB635782	A212	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
23	AB635783	A208	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
24	AB635784	A207	FeLV-A	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
25	AB635785	A209	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
26	AB635789	A210	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
27	AB635792	A225	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
28	AB635793	B20	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-	-	
29	AB635797	A226	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
30	AB635799	B38	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-	-	
31	AB635800	A227	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
32	AB635802	B19	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-	-	
33	AB635804	A301	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
34	AB635805	B69	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	4		-	-	enFeLV	FeLV-A Clade	enFeLV	enFeLV	FeLV-A		
35	AB635806	A311	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
36	AB635807	A312	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
37	AB635811	A307	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
38	AB635813	A257	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
39	AB635814	A256	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
40	AB635815	B29	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-	-	
41	AB635816	A306	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
42	AB635818	B49	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	11 | 22		-	-	enFeLV	enFeLV	enFeLV	enFeLV	enFeLV		
43	AB635819	A308	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
44	AB635820	A305	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
45	AB635821	A315	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
46	AB635822	A291	FeLV-A	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-	
47	AB635824	B39	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-	-	

Sample Information						Phylogenetics	Recombination										
Acces		Phenotypic Classificat	Country	Host	Isolation Sou	Phylogenetic Classficati	RDP4 Ana	Recombination Pattern	VRA VRC VRB VRD VRE RBD PRR C-domain					RBD	PI	C-domai
AB635825	A284	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635826	A285	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635827	A271	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635828	A272	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635829	A249	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635830	A250	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635831	A280	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635832	A279	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635839	B67	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	6		-	-	enFeLV	FeLV-A Clade	enFeLV	enFeLV	FeLV-A
AB635840	A251	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635841	A252	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635842	A281	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635844	B52	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	11 | 18		-	enFeLV	enFeLV	FeLV-A Clade	enFeLV	enFeLV	enFeLV/FeLV-A
AB635845	A224	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635846	A223	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635848	B77	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	6		-	-	enFeLV	FeLV-A Clade	enFeLV	enFeLV	FeLV-A
AB635849	A228	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635850	B21	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-
AB635851	A300	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635853	B86	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	3		-	-	enFeLV	FeLV-A Clade	enFeLV	FeLV-A	FeLV-A
AB635854	A282	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635856	A292	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635857	A304	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635859	A303	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB635860	B68	Not Subtyped	Japan	Felis catus	NA	enFeLV Clade	Recombinant	6		-	-	enFeLV	FeLV-A Clade	enFeLV	enFeLV	FeLV-A
AB635863	A290	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB672612	A114	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB970837	A259	Not Subtyped	Japan	Felis catus	blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB970838	A261	Not Subtyped	Japan	Felis catus	blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB970839	A264	Not Subtyped	Japan	Felis catus	blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB970840	A258	Not Subtyped	Japan	Felis catus	blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB970841	A263	Not Subtyped	Japan	Felis catus	blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB970842	A262	Not Subtyped	Japan	Felis catus	blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB970843	A310	Not Subtyped	Japan	Felis catus	blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB970844	A299	Not Subtyped	Japan	Felis catus	blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB970845	A289	Not Subtyped	Japan	Felis catus	blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AB970846	A260	Not Subtyped	Japan	Felis catus	blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AF052723	A59	FeLV-A	NA	Felis catus	Tumor	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AF403716	B73	FeLV-B	NA	Cell culture	Proviral DNA	enFeLV Clade	Recombinant	5		-	-	enFeLV	FeLV-A	enFeLV	enFeLV	FeLV-A
AY364318	B31	enFeLV	NA	Felis catus	Proviral DNA	enFeLV Clade	-	-		-	-	-	-	-	-	-
AY364319	B33	enFeLV	NA	Felis catus	Proviral DNA	enFeLV Clade	-	-		-	-	-	-	-	-	-
AY662447	A78	FeLV-A	USA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AY662449	A71	Not Subtyped	USA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AY662450	A70	Not Subtyped	USA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AY662453	A69	Not Subtyped	USA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AY662454	A68	Not Subtyped	USA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
AY662455	A67	Not Subtyped	USA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-



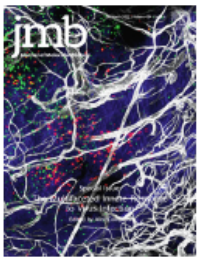
	Sample Information						Phylogenetics	Recombination											
	Acces		Phenotypic Classificat	Country	Host	Isolation Sou	Phylogenetic Classficati	RDP4 Ana	Recombination Pattern	VRA VRC VRB VRD VRE RBD PRR C-domain						RBD	PF	C-domai
5	AY662456	A81	Not Subtyped	USA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
6	AY662458	A80	Not Subtyped	USA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
7	AY662459	A72	Not Subtyped	USA	NA	Spleen	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
8	AY662460	A75	Not Subtyped	USA	NA	Spleen	FeLV-A Clade	Recombinant	10		-	-	FeLV-A	enFeLV	FeLV-A	FeLV-A	enFeLV
9	AY662461	A73	Not Subtyped	USA	NA	Spleen	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
10	AY662462	A74	Not Subtyped	USA	NA	Spleen	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
11	AY706347	A76	Not Subtyped	USA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
12	AY706352	A79	Not Subtyped	USA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
13	AY706353	A77	Not Subtyped	USA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
14	AY706357	A15	Not Subtyped	USA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
15	AY706358	A13	Not Subtyped	USA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
16	AY706359	A14	Not Subtyped	USA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
17	EU189489	A83	Not Subtyped	NA	Puma concolor	blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
18	EU189490	A84	Not Subtyped	NA	Puma concolor	blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
19	EU189492	A85	Not Subtyped	NA	Puma concolor	blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
20	EU189494	A86	Not Subtyped	NA	Puma concolor	blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
21	EU293175	A57	FeLV-A	NA	Lynx pardinus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
22	EU293176	A44	FeLV-A	NA	Lynx pardinus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
23	EU293178	A42	FeLV-A	NA	Lynx pardinus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
24	EU293180	A41	FeLV-A	NA	Lynx pardinus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
25	EU293182	A45	FeLV-A	NA	Lynx pardinus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
26	EU293186	A40	FeLV-A	NA	Lynx pardinus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
27	EU293188	A39	FeLV-A	NA	Lynx pardinus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
28	EU293191	A43	FeLV-A	NA	Lynx pardinus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
29	EU293193	A46	FeLV-A	NA	Lynx pardinus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
30	EU359303	A64	Not Subtyped	NA	NA	Kidney	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
31	EU359304	A63	Not Subtyped	NA	NA	Kidney	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
32	EU359308	A65	Not Subtyped	NA	NA	Abdominal cavity	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
33	EU629217	A36	FeLV-A	Brazil	Felis catus	Blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
34	EU629218	A37	FeLV-A	Brazil	Felis catus	blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
35	FJ436991	A61	Not Subtyped	United Kingdom	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
36	J03448	B45	FeLV-B	NA	Cell culture	Proviral DNA	enFeLV Clade	Recombinant	11		-	-	enFeLV	FeLV-A Clade	enFeLV	enFeLV	FeLV-A
37	JF957361	B44	Not Subtyped	United Kingdom	NA	blood	enFeLV Clade	Recombinant	11 | 22		-	-	enFeLV	enFeLV	enFeLV	enFeLV	enFeLV
38	JF957363	B22	FeLV-B	United Kingdom	Felis catus	NA	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
39	KR349469	A48	FeLV-A	Brazil	Puma yagouaroundi	Bone marrow	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
40	KY750989	B12	Not Subtyped	China	Felis catus	Abdominal cavity	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
41	KY750990	B10	Not Subtyped	China	Felis catus	Abdominal cavity	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
42	KY750991	B1	Not Subtyped	China	Felis catus	Abdominal cavity	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
43	KY750992	B4	Not Subtyped	China	Felis catus	Abdominal cavity	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
44	KY750993	B15	Not Subtyped	China	Felis catus	Abdominal cavity	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
45	KY750994	B24	Not Subtyped	China	Felis catus	Abdominal cavity	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
46	KY750995	B11	Not Subtyped	China	Felis catus	Abdominal cavity	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
47	KY750996	B9	Not Subtyped	China	Felis catus	Abdominal cavity	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
48	KY750997	B7	Not Subtyped	China	Felis catus	Abdominal cavity	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
49	KY750998	B14	Not Subtyped	China	Felis catus	Abdominal cavity	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
50	KY750999	B17	Not Subtyped	China	Felis catus	Abdominal cavity	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
51	KY751000	B13	Not Subtyped	China	Felis catus	Abdominal cavity	enFeLV Clade	-	-		-	-	-	-	-	-	-	-

Sample Information						Phylogenetics	Recombination											
Acces		Phenotypic Classificat	Country	Host	Isolation Sou	Phylogenetic Classficati	RDP4 Ana	Recombination Pattern	VRA VRC VRB RBD PRR VRD VRE C-domain						RBD	PI	C-domai
KY751001	B26	Not Subtyped	China	Felis catus	Abdominal cavity	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
KY751002	B16	Not Subtyped	China	Felis catus	Abdominal cavity	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
KY751003	B8	Not Subtyped	China	Felis catus	Abdominal cavity	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
KY751004	B25	Not Subtyped	China	Felis catus	Abdominal cavity	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
KY751006	B2	Not Subtyped	China	Felis catus	Abdominal cavity	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
LC029807	A214	FeLV-A	Japan	Felis catus	Blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
LC029808	A213	FeLV-A	Japan	Felis catus	Blood	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
LC144878	A286	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
LC144879	A295	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
LC144880	A293	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
LC144881	A294	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
LC144882	A298	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
LC144883	A296	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
LC144884	A297	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
LC196053	B18	enFeLV	Japan	Felis catus	Proviral DNA	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
LC485155	A163	Not Subtyped	Japan	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
M14331	A96	FeLV-C	NA	Cell culture	Proviral DNA	FeLV-A Clade	Recombinant	18		-	-	FeLV-A	enFeLV	FeLV-A	FeLV-A	FeLV-A/enFeLV
M18246	A12	FeLV-T	NA	Felis catus	Intestine	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
M18247	A18	FeLV-A	NA	Felis catus	Intestine	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
M18248	A58	FeLV-A	NA	Felis catus	Tumor	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
M23025	A95	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
M23026	A60	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
M25425	B3	enFeLV	NA	Felis catus	Placenta	enFeLV Clade	-	-		-	-	-	-	-	-	-	-
M87886	A11	Not Subtyped	NA	Felis catus	Intestine	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
M89997	A10	FeLV-T	NA	Felis catus	Bone marrow	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
M89998	A62	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
M89999	A66	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
MF681664	A94	FeLV-A	USA	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
MF681665	A88	FeLV-A	USA	Puma concolor	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
MF681666	A89	FeLV-A	USA	Puma concolor	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
MF681667	A92	FeLV-A	USA	Puma concolor	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
MF681668	A91	FeLV-A	USA	Puma concolor	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
MF681669	A90	FeLV-A	USA	Puma concolor	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
MF681670	A47	FeLV-A	USA	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
MF681671	B59	FeLV-B	USA	Puma concolor	NA	enFeLV Clade	Recombinant	11 | 18		-	enfeLV	enFeLV	FeLV-A	enFeLV	enFeLV	enFeLV/FeLV-A
MF681672	A87	FeLV-A	USA	Puma concolor	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
MH116004	A38	Not Subtyped	USA	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
MH116005	A93	Not Subtyped	USA	Felis catus	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
MW762576	A54	Not Subtyped	Brazil	NA	Proviral DNA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
MW762577	A53	Not Subtyped	Brazil	NA	Proviral DNA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
MW762578	A55	Not Subtyped	Brazil	NA	Proviral DNA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
MW762579	A56	Not Subtyped	Brazil	NA	Proviral DNA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
MW762580	A52	Not Subtyped	Brazil	NA	Proviral DNA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
MW762581	A51	Not Subtyped	Brazil	NA	Proviral DNA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
MW762582	A49	Not Subtyped	Brazil	NA	Proviral DNA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
MW762583	A50	Not Subtyped	Brazil	NA	Proviral DNA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-
U03162	A28	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-	-



	Sample Information						Phylogenetics	Recombination										
	Acces		Phenotypic Classificat	Country	Host	Isolation Sou	Phylogenetic Classficati	RDP4 Ana	Recombination Pattern	VRA VRC VRB RBD PRR VRD C-domain VRE					RBD	PI	C-domai
9	U03163	A29	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
0	U03164	A24	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
1	U03170	A16	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
2	U03171	A17	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
3	U03173	A19	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
4	U03174	A32	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
5	U03175	A35	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
6	U03188	A26	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
7	U03189	A25	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
8	U03192	A30	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
9	U03193	A34	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
0	U03194	A27	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
1	U03195	A22	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
2	U03196	A23	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
3	U03197	A33	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
4	U03198	A20	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
5	U03205	A21	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
6	U03218	A31	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
7	U03222	A6	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
8	U03223	A9	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
9	U03224	A8	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
0	U03225	A1	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
1	U03226	A3	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
2	U03227	A5	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
3	U58951	A2	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
4	U70377	A7	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
5	U70378	A4	Not Subtyped	NA	NA	NA	FeLV-A Clade	-	-		-	-	-	-	-	-	-
6	V01172	B74	FeLV-B	NA	NA	NA	enFeLV Clade	Recombinant	5		-	-	enFeLV	FeLV-A	enFeLV	enFeLV	FeLV-A

10. FELINE LEUKEMIA VIRUS-B ENVELOPE TOGETHER WITH ITS GLYCOGAG AND HUMAN IMMUNODEFICIENCY VIRUS-1 NEF MEDIATE RESISTANCE TO FELINE SERINC5



Feline Leukemia Virus-B Envelope Together With its GlycoGag and Human Immunodeficiency Virus-1 Nef Mediate Resistance to Feline SERINC5

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Abstract

Human SERINC5 (SER5) protein is a recently described restriction factor against human immunodeficiency virus-1 (HIV-1), which is antagonized by HIV-1 Nef protein. Other retroviral accessory proteins such as the glycosylated Gag (glycoGag) from the murine leukemia virus (MLV) can also antagonize SER5. In addition, some viruses escape SER5 restriction by expressing a SER5-insensitive envelope (Env) glycoprotein. Here, we studied the activity of human and feline SER5 on HIV-1 and on the two pathogenic retroviruses in cats, feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV). HIV-1 in absence of Nef is restricted by SER5 from domestic cats and protected by its Nef protein. The sensitivity of feline retroviruses FIV and FeLV to human and feline SER5 is considerably different: FIV is sensitive to feline and human SER5 and lacks an obvious mechanism to counteract SER5 activity, while FeLV is relatively resistant to SER5 inhibition. We speculated that similar to MLV, FeLV-A or FeLV-B express glycoGag proteins and investigated their function against human and feline SER5 in wild type and envelope deficient virus variants. We found that the endogenous FeLV recombinant virus, FeLV-B but not wild type exogenous FeLV-A envelope mediates a strong resistance against human and feline SER5. GlycoGag has an additional but moderate role to enhance viral infectivity in the presence of SER5 that seems to be dependent on the FeLV envelope. These findings may explain, why *in vivo* FeLV-B has a selective advantage and causes higher FeLV levels in infected cats compared to infections of FeLV-A only.

Introduction

The serine incorporator (SERINC, SER) protein family consists of transmembrane proteins highly conserved among eukaryotes with five distinct members (SER1-5) with incompletely defined function.[1–7] Structurally, SER proteins have two domains constituted by 10 transmembrane helices divided by a diagonal helix.[8] The human SER3 and SER5 (huSER3 and huSER5) were recently reported as restriction factors against the human immunodeficiency virus-1 (HIV-1).[2,6] SER3/5 also have an intracellular role by enhancing the production of type I interferon and nuclear factor kB (NF-kB) signaling.[9] SER3/5 are counteracted by HIV-1 Nef, glycosylated Gag (glycoGag) of murine leukemia virus (MLV) and S2 from the equine infectious anemia virus (EIAV).[2,6,10,11] When present, Nef, glycoGag and S2 block the incorporation of SER3/5 in the viral particle by inducing its downregulation from the cell membrane. The SER downregulation involves the cellular transport machinery, with polyubiquitination and degradation of SER via the endosome/lysosome system.[5,12,13] Nef internalizes SER5 into Rab5, Rab7 and LAMP1 endosomes through AP-2-dependent-endocytosis and thus excludes SER5 from the cell membrane, preventing incorporation in the viral particle during budding.[12,14] In the absence of Nef, viral membrane associated SER5 interferes with the envelope mediated virus-cell fusion, thus preventing infection of the target cell.[15] The antiviral mechanism is not fully characterized and additional Nef-mediated counteraction mechanisms against virus-associated SER5 were recently suggested.[16] When the virus incorporates SER5, the fusion pore formation is impaired which reduces its ability to fuse with the cell membrane.[17] SER5 restriction is also dependent upon the retroviral core[18] and the viral envelope glycoprotein.[18–21] HIV-1Δ*nef*Δ*env* pseudotyped with heterologous vesicular stomatitis virus G protein (VSV-G) or Ebola virus glycoprotein (EBOV GP) is resistant to SER5 restriction.[10] In contrast, such HIV-based virions pseudotyped with Env of amphotropic or xenotropic MLV are sensitive to SER5.[10,18] HIV-1 Envs are in general sensitive to SER5, however, some HIV-1 isolates express envelope proteins that escape its restriction.[19] The Env determinants of this insensitivity are located in the V1, V2 and V3 loops,[22] and truncation of the cytoplasmic tail also confers resistance to SER5.[23]

The glycoGag (glycosylated Gag) is an accessory protein encoded by some gammaretroviruses. The MLV-glycoGag (gPr80) translation starts at an inefficient CUG start codon upstream of the Gag AUG start codon and results in a protein that comprises the complete Gag sequence with 88 additional residues at the N-terminal end.[5,24] The mature protein is then cleaved in a membrane-associated protein of around 55-kDa (N-terminal) and a secreted protein around 40-kDa (C-terminal product).[5,25] MLV glycoGag counteracts SER5 in MLV and HIV-1Δ*nef* infections.[10,26,27] The Nef-like activity of the MLV-glycoGag is exerted by the cytoplasmic but not its extracellular domain.[26] In addition, MLV glycoGag is able to antagonize restriction by the IFITM3 protein.[28]

Domestic cats can be infected by three different retroviruses: pathogenic *feline immunodeficiency virus* (FIV, a lentivirus), *feline leukemia virus* (FeLV, a gammaretrovirus), and apathogenic *feline foamy virus* (FFV, a spumavirus). Similar to HIV-1, FIV causes immunodeficiency in domestic cats, which sometimes results in a moderate increase in lethality compared to FIV negative cats.[29] FIV has been classified into seven subtypes (A–F, U-NZenv) based on the V3 and V5 regions of the *env* gene and recombinants have been identified.[30,31] FIV uses the CD134 receptor together with the CXCR4 coreceptor to enter target cells and infects $CD4^+$ T lymphocytes, monocytes, and macrophages. FIV also exhibits tropism for $CD8^+$ T and B lymphocytes and microglial cells.[32,33] In contrast, the disease produced by FeLV is more aggressive, progresses more rapidly and reduces life expectancy.[34] FeLV disease is characterized by immunosuppression and myeloproliferation.[35] FeLV has been classified into six subgroups (A–E, T) by viral interference assays.[36,37] Each subgroup uses a specific receptor to enter the cells, *e.g.*, FeLV-A uses feline THTR1 and FeLV-B the feline SLC20A1 and SLC20A2 (formerly fePit1/2).[38] Importantly, FeLV-B arises from recombination between the exogenous virus (FeLV-A) and endogenous FeLV sequences present in the cat genome[36,39]. The presence of FeLV-B is associated with a more severe clinical manifestation and higher viral loads[40–42]. Interestingly, many different FeLV-B recombinants can be found in one unique host.[43] FeLV infects lymphocytes and monocytes during primary viremia. If infection progresses, the virus reaches the bone marrow and infects myelomonocytic precursor cells, neutrophils and platelets during secondary viremia.[29,44]

FIV and FeLV genomes encode for three primary retroviral proteins Gag, Pol, and Env. The FIV genome additionally encodes the accessory genes *vif, orfA* and *rev* that confers high replication rates to the virus.[45] FIV Vif counteracts feline APOBEC3s (A3s) restriction via degradation.[46–49] OrfA localizes to the nucleus, induces cell cycle arrest and downregulates the CD134 receptor from the cell membrane.[50,51] The FeLV genome encodes a predicted glycoGag open reading frame starting with an AUG[40,52–54] and a glycoGag-related protein (around 40-kDa) was found in cell-culture medium of FeLV infected cells.[55]

In this study, we evaluated whether feline SERINC5 has antiviral activity against HIV-1, FIV and FeLV to further elucidate host mechanisms of viral restriction.

Results

Feline and human SERINCs are highly conserved

The amino acid sequences of SER3 and SER5 from domestic cats and humans are highly conserved between species. SER5 exhibited a nucleotide identity of 90% (Figure 1(A)) and SER3 was 87% homologous (results not shown). Because no antibodies are available against SER5, expression constructs with tagged SER were generated in the widely used SERINC expression plasmid pBJ6.[6,11,56] We demonstrated that feSER5 and huSER5 are expressed at a similar level in the tissue culture system used in these studies (Figure 1(B)).

FIV is sensitive to SERINC5

In order to test whether SER5 is a restriction factor against FIV, FIV particles were produced in the presence or absence of SER5 using full-length FIV plasmids PRR and C36.[57] Both feSER5 and huSER5 reduced FIV infectivity 4- to 5-fold, while feSER3 was less antivirally active (Figure 2(A)). To specifically evaluate the envelope sensitivity, a FIV three-plasmid system expressing viral genes *gag-pol-rev* were used to produce viral particles pseudotyped either with FIV envelope (EE14)[58] or VSV-G. FIV pseudotyped with FIV envelope was sensitive to feSER5 and huSER5 inhibition. In contrast, FIV pseudotyped with VSV-G was not inhibited (Figure 2(B)). These data demonstrate that the SER5-insensitivity of VSV-G protein is not only seen with HIV but also FIV viral cores.[2,19] In addition, FIV-C36 mutants not expressing *vif, orfA* and double mutants, were analyzed in the presence or absence of increasing amounts of fe/huSER5 (0, 50, 100 or 200 ng). Interestingly, no difference were found between the FIV-C36 WT and the FIV-C36Δ*orfA*, however, both mutants lacking the *vif* gene lost some of their sensitivity to both fe/huSER5 (Figure 2(C)). In addition, we compared

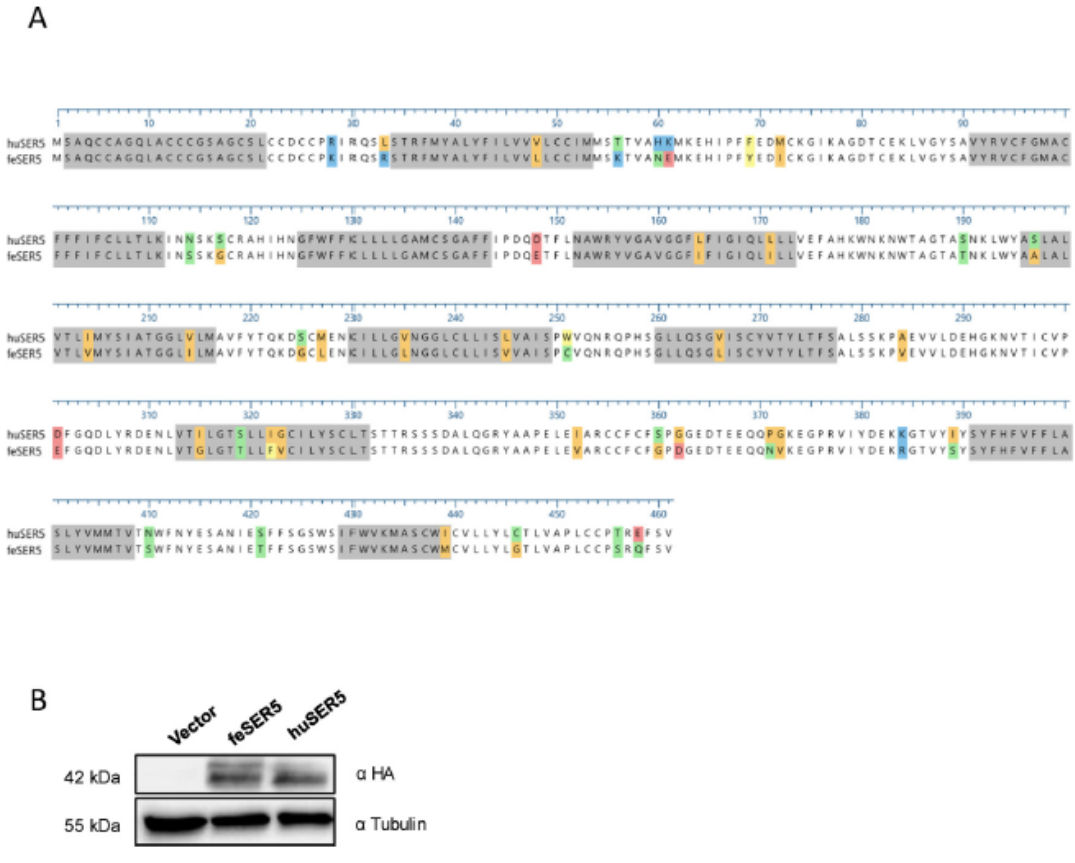


Figure 1. Feline and human SERINC5 are highly conserved and pBJ6 driven expression plasmids express similar levels of protein. (A) Amino acid alignment of feline and human SERINC5 (XM_011284262 and NM_001174072, respectively). The highlighted amino acids correspond to disagreements, which are colored according to their side chain chemistry. Grey boxes represent the transmembrane domains. (B) HEK293T cells were transfected with feline SERINC5 (feSER5) or human SERINC5 (huSER5) pBJ6 expression plasmids or empty plasmid. Cells were harvested and analyzed by immunoblotting with anti-HA and anti-tubulin antibodies.

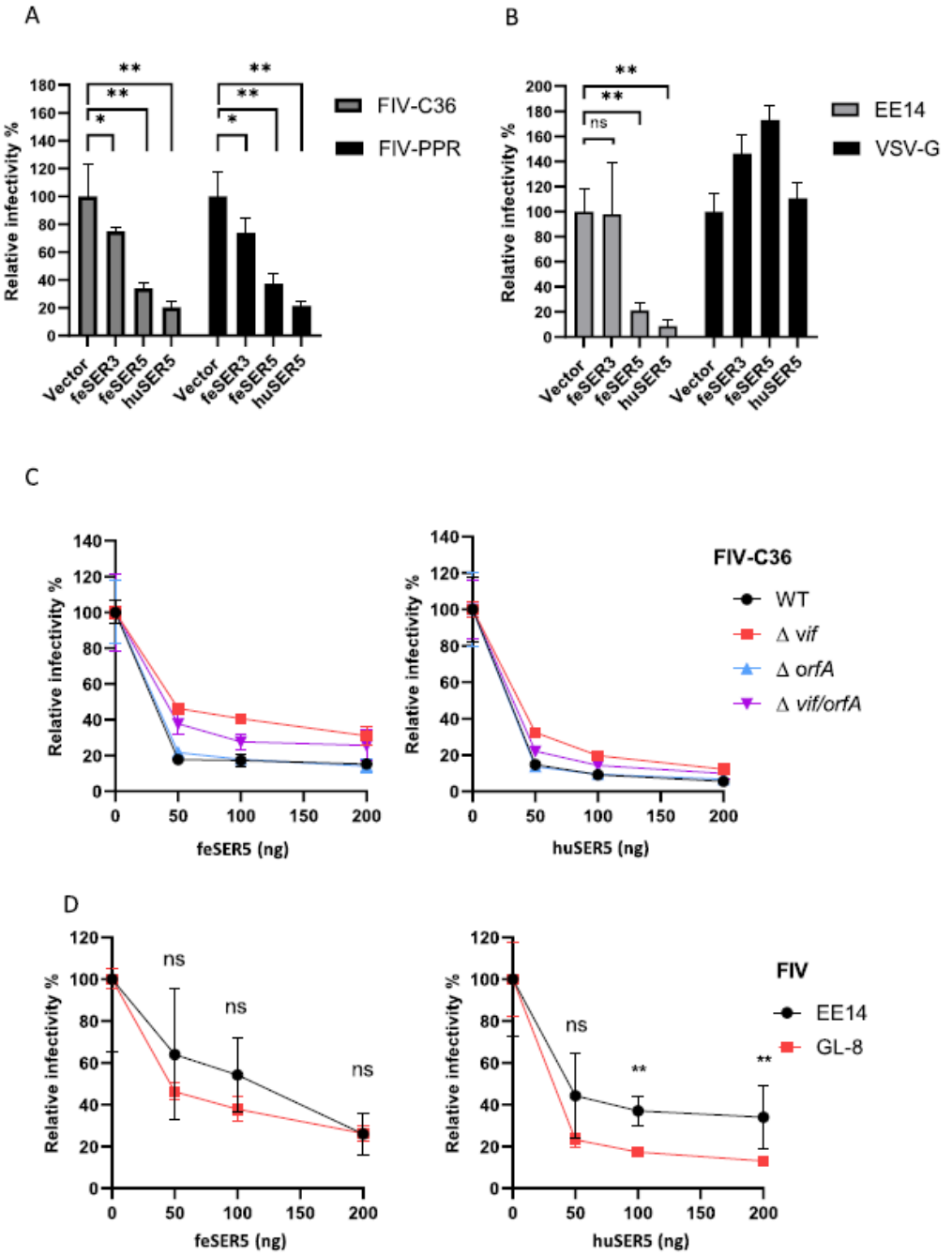


Figure 2. The anti-FIV activity of SERINC. (A) Full length FIV molecular clones C36 (highly pathogenic) (grey bars) or PPR (low pathogenic) (black bars) and a FIV-luciferase transfer vector were co-transfected with human SERINC5 (huSER5) or feline SERINC5 (feSER5) expression plasmids, pBJ6 empty vector was added as a control (vector). (B) Single-round FIV Env EE14 (grey bars) or VSV-G (black bars) pseudotyped FIV luciferase reporter virions (three plasmid system, no *vif*, no *orfA*) were produced in the presence of SER expression plasmids (feSER5, feSER3 or huSER5), pBJ6 empty vector was added as a control (vector). (C) FIV-C36 mutants Δ*vif*, Δ*orfA* or Δ*vif*/*orfA* were produced in the presence or absence of different amounts of fe/huSER5. (D) Single-round FIV three-plasmid system pseudotyped with two different FIV envelopes (EE14 or GL-8) were produced in increasing amounts of fe/huSER5. Viral infectivity was determined after normalization for reverse transcriptase activity by quantification of luciferase activity in CRFK-CD134$^+$ cells infected with FIV particles. Shown are means plus standard deviations (error bars) of a representative experiment performed three times in technical triplicate. Asterisks represent statistically significant differences: *P* value <0.001 extremely significant (***), 0.001–0.01 very significant (**), 0.01–0.05 significant (*), >0.05 not significant (ns).

two different FIV envelopes (EE14[58] and GL-8[59]) in a titration experiment of coexpressed feline or human SER5 in the FIV three-plasmid system. Both pseudotyped viruses were inhibited similarly, but GL-8 pseudotypes were more sensitive to huSER5 than EE14 pseudotyped vectors, while there were no significant differences in the experiments using feSER5 (Figure 2(D)). These results suggest that FIV is sensitive to SER5, but has no SER5-counteracter.

HIV-1 counteracts human and feline SERINC5 by its Nef protein

SER5 orthologs from non-human species have been reported to inhibit HIV-1.[27,60] The presence of the Nef protein in virus producer cells counteracted SER5 activity and rescued HIV-1 infectivity.[2,6,12,17] To test the effect of feSER5 on HIV-1 infectivity, full-length HIV-1 plasmids with and without Nef expression were produced in the presence of feSER3, feSER5 or huSER5. Our results demonstrate that wild-type HIV-1 was resistant to feSER5 and huSER5 while HIV-1 lacking Nef activity were sensitivity to both SER5 orthologues (Figure 3(A)). HIV-1 vectors produced with a three-plasmid system pseudotyped with HIV-1 BaL26 envelope, which do not express Nef, were more sensitive to feSER5 and huSER5 than feSER3 (Figure 3(B)) and SER5 decreased HIV-1 infectivity in a dose-dependent manner (Figure 3(C)). These data demonstrate that feSER5 is a strong antiviral protein that, similar to huSER5, is sensitive to HIV-1 Nef-mediated counteraction.

FeLV-A and FeLV-B express low levels of glycoGag

FeLV, similar to MLV, has an open reading frame that encodes a glycoGag protein. The predicted FeLV glycoGag start codon (ATG) is located 222 nucleotides (encoding 74 residues) before the start codon of *gag* in the plasmid pFGA-5 (FeLV-A) and 231 nucleotides upstream of the start codon in the plasmid pFGB (FeLV-B). For our experiments, we constructed FeLV expression plasmids with an inactivation mutation in *glycoGag*, an *env* deletion or a combination of both. pFGA-5Δ*env* and pFGBΔ*env* are envelope-deleted constructs but express glycoGag, pFGA-5X and pFGBX do not express glycoGag but express the envelope, and pFGA-5XΔ*env* and pFGXBΔ*env* are double mutant plasmids which do not express glycoGag and envelope (Figure 4(A)). The expression or the lack of expression of both viral factors was analyzed with immunoblots using

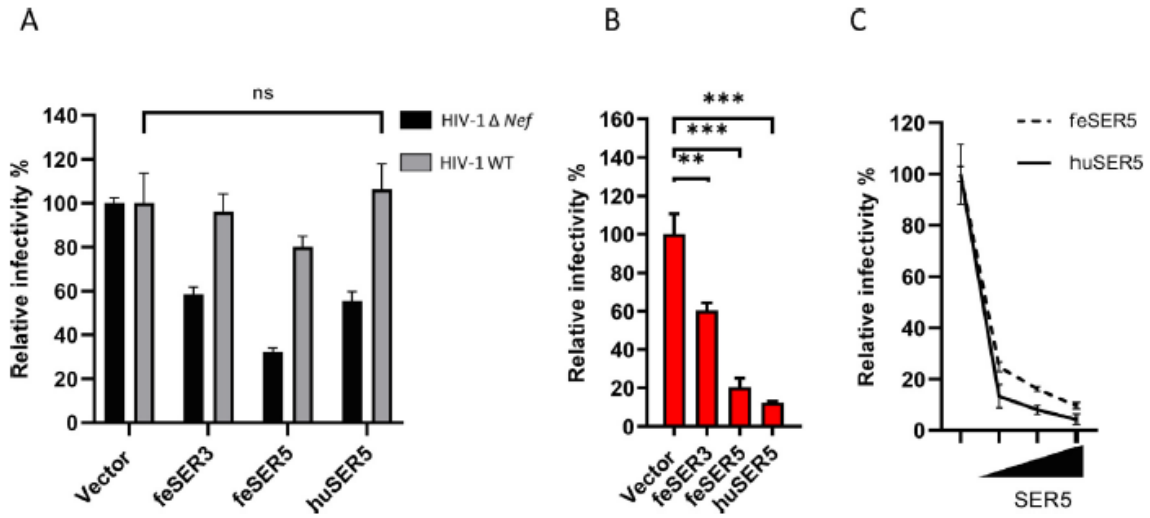
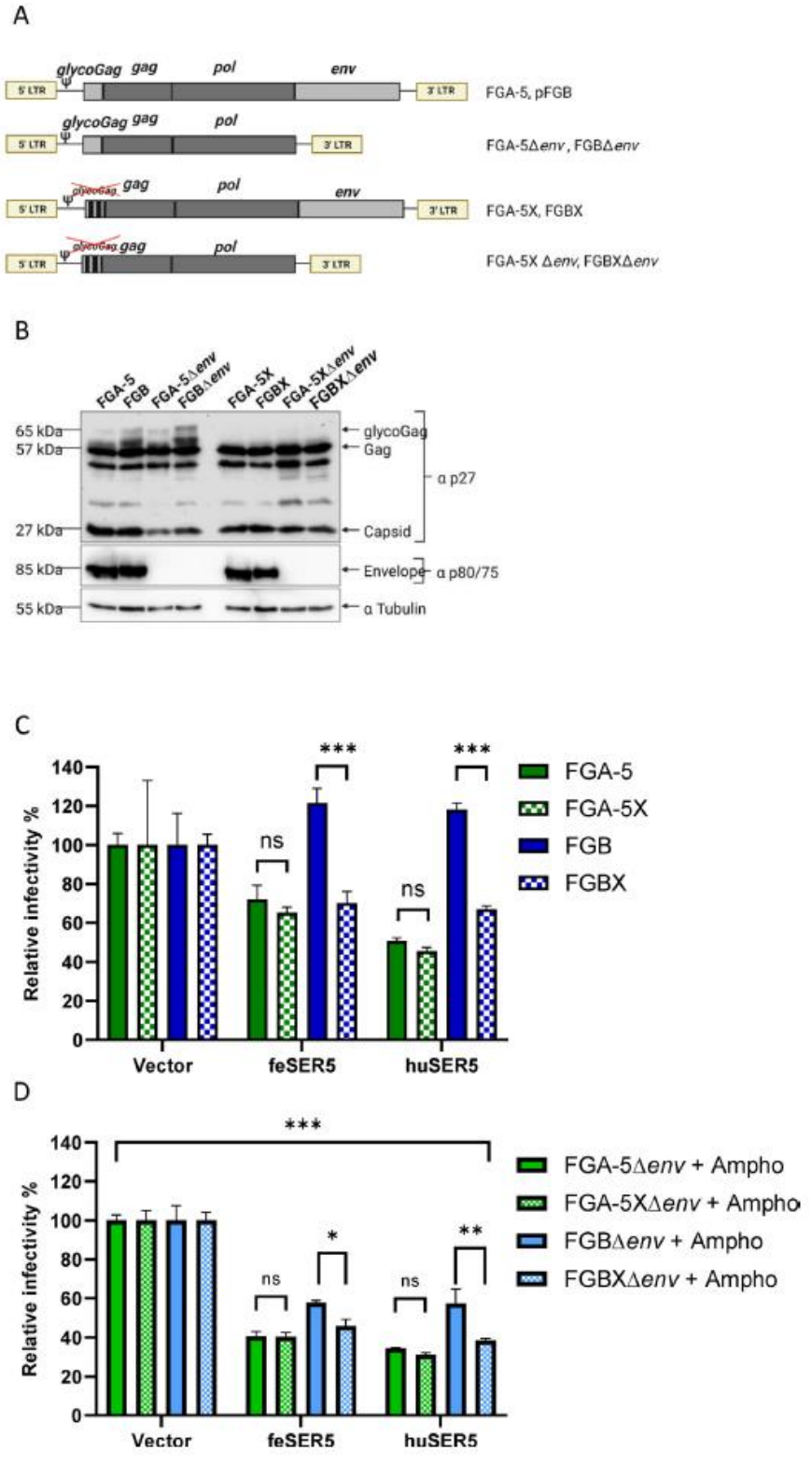


Figure 3. The anti-HIV-1 activity of SERINCs. (A) Full length molecular clones HIV-1 NL4-3 lacking Nef (HIV-1 NL4-3Δ*nef*) (black bars) or expressing Nef (HIV-1 NL4-3 SF2 *nef*) (grey bars) were transfected in the absence or presence of feSER3, feSER5 or huSER5). Relative infectivities were determined on TZMbl cells. (B) HIV-1-based lentiviral luciferase vectors produced with a three-plasmid system (which does not encodes Nef) and pseudotyped with HIV-1 BaL26 envelope were produced in the presence of SER (feSER3, feSER5 or huSER5) or pBJ6 empty plasmid as control. (C) HIV-1 pseudotyped vectors (no Nef) with BaL envelope were also used to produce viral particles in a titration assay with feSER5 and huSER5 with increasing amounts of each plasmid (0, 100, 200 or 400 ng). Particles were normalized by RT activity and HOS-CD4+CCR5+ cells were transduced. The infectivity was determined by quantification of luciferase activity. Shown are means plus standard deviations (error bars) of a representative experiment performed three times in triplicate. Asterisks represent statistically significant differences: *P* value <0.001 extremely significant (***), 0.001–0.01 very significant (**), 0.01 to 0.05 significant (*), >0.05 not significant (ns).

anti-p27, (which detects Gag and glycoGag), or anti gp80/75 (which detects the Env). In addition to the full-length Gag protein (57 kDa), an additional protein of around 65 kDa was observed, which corresponds to the size of the predicted full-length glycoGag (Figure 4(B)). The level of expression of



the glycoGag was lower than Gag proteins. FeLV-B plasmids (pFGB) expressed slightly more glycoGag than FeLV-A plasmids (pFGA-5) and in addition another Gag protein larger than 57 kDa but smaller than 65 kDa, which is possible a processed version of the full-length glycoGag (Figure 4(B)).

FeLV-B is resistant to SERINC5

To evaluate the effect of SER5 on FeLV-A (FGA-5) and FeLV-B (FGB), wild type or the glycoGag deficient viruses were generated in the presence feSER5, huSER5 or empty pBJ6 vector as control. Wild type FeLV-A was moderately sensitive to feSER5 or huSER5 and the glycoGag deficiency had no significant impact to the antiviral activity of SER5 (Figure 4(C)). Interestingly, FeLV-B was not only resistant to feSER5 and huSER5, but showed moderately higher infectivity when SER5 was co-expressed compared to vector controls (Figure 4(C)). The loss of glycoGag expression in FeLV-B (FGBX) resulted in an enhanced sensitivity to restriction by SER5 (Figure 4(C)). FeLV-A and FeLV-B differ mainly in the *env* gene, while *gag*, *pol* and LTRs in the pFGA-5Δ*env* and pFGBΔ*env* plasmids are more than 95% identical (data not shown). To understand whether FeLV Env is important for SER5 resistance, envelope deficient viruses were pseudotyped with amphotropic-MLV envelope. Amphotropic MLV envelope was demonstrated to be sensitive to SER5.[10,61] FeLV-AΔ*env* pseudotyped with amphotropic Env showed a further enhanced sensitivity to human and feline SER5 compared to WT virus (Figure 4(D)). Pseudotyping FeLV-B with amphotropic Env also resulted in an increased sensitivity to feSER5 or huSER5 (Figure 4 (D)). The envelope gene of FeLV-A confers some resistance to SER5, while the Env gene of FeLV-B allows a greater degree of resistance to SER5. Furthermore, glycoGag from FeLV-B confers an additional level of protection from SER5, while glycoGag deficiency in FeLV-AΔ*env* did not increase viral sensitivity (Figure 4(D)). Together, these data suggest that FeLV-B is mostly protected from SER5 by its envelope and that its glycoGag contributes to resistance. In FeLV-A, the envelope appears to be more resistant to SER5 than amphotropic Env but more sensitive than FeLV-B envelope, while the glycoGag expression was irrelevant.

FeLV-B envelope can protect HIV-1 vectors against SERINC5

Our findings suggest that the envelope of FeLV-B is an important determinant of FeLV-B's SER5 resistance. To further assess the effect of FeLV envelopes on the antiviral activity of feSER5 or huSER5, HIV-1 reporter particles were produced using the three-plasmid system pseudotyped with different envelopes: BaL26 (an HIV-1 envelope), amphotropic MLV envelope and FeLV-A or FeLV-B envelopes in the presence of increasing concentrations of SER5. The observed dose dependent inhibition of HIV was greatest for HIV-1 Env and lowest for FeLV-B Env (Figure 5(A, B)). Our results indicate that FeLV-B but not FeLV-A Env protected HIV-1 vectors against antiviral activity of feline and human SER5s (Figure 5(A, B)). Immunoblots of cells co-expressing FeLV-B Env and SER5 ruled out that this Env protein induces degradation of SER5 (Figure 5(C)).

Two unique FeLV glycoGag clades are identified across isolates

GlycoGag FeLV nucleotide sequences (n = 90) were downloaded from GenBank (November, 2021). After translation, sequences with identical residues were removed to construct an alignment, which contains 37 unique sequences. Our alignment includes 17 FeLV-A glycoGag sequences (including FGA-5), three FeLV-B glycoGags (including FGB), one endogenous

Figure 4. The activity of SERINC against FeLV. (A) Schematic representation of FeLV–A and FeLV-B wild type and mutants. The plasmids pFGA-5 (FeLV-A) and pFGB (FeLV-B) were used to construct mutant FeLV variants: pFGA-5Δ*env* and pFGBΔ*env* are envelope deleted constructs, glycoGag mutants pFGA-5X and pFGBX have frame shift mutations in the 5′ reading frame before the *gag* ATG codon to prevent expression of glycoGag, pFGA-5XΔ*env* and pFGXBΔ*env* carry inactivated glycoGags and deleted envelope genes. (B) Detection of Gag (p57), glycoGag (p65), Capsid (p27) and Env (p85/p70) protein by immunoblotting of FeLV-A and FeLV-B expression plasmids. HEK293T cells were transfected with WT or mutant FeLV expression plasmids. Cells were harvested and analyzed by immunoblotting with anti-p85/70, anti-p27 and anti-tubulin antibodies. (C) FeLV particles were produced by using the full-length molecular clones pFGA-5, pFGB (expressing glycoGag), pFGA-5X or pFGBX (not expressing glycoGag), or (D) FeLVΔ*env* particles pseudotyped with amphotropic envelope: pFGA-5Δ*env*, pFGBΔ*env* (expressing glycoGag) or pFGA-5XΔ*env*, pFGBXΔ*env* (not expressing glycoGag). Plasmids were transfected together with SER (feSER5 or huSER5) expression plasmids and a luciferase transfer vector. pBJ6 empty vector was added as a control (vector). Viral infectivity was determined after normalization for reverse transcriptase activity by quantification of luciferase activity. Shown are means plus standard deviations (error bars) of a representative experiment performed three times in triplicate. Asterisks represent statistically significant differences: P value <0.001 extremely significant (***), 0.001–0.01 very significant (**), 0.01–0.05 significant (*), >0.05 not significant (ns).

FeLV (enFeLV) glycoGag and 16 unclassified FeLV glycoGag sequences (Suppl. Figure 1(A)). The main variation between sequences is a three amino acid gap occurring at residue 20 (Suppl. Figure 1(A)). Two well-supported clades were identified. The primary glycoGag clade contains 22 sequences and is formed by 16 FeLV-A sequences (including FGA-5), one FeLV-B and five unclassified FeLVs. The second glycoGag clade includes 15 sequences including enFeLV, one FeLV-A, two FeLV-B (including FGB) and 11 non-subtyped FeLVs (Suppl. Figure 1(B)). The presence of an intact glycoGag reading-frame in all full-length FeLV sequences, including enFeLV, suggests strong selection for the expression of glycoGag in FeLV and a presence of glycoGag in FeLV ancestor viruses.

FeLV glycoGag overexpression can moderately increase resistance of HIV vectors to SERINC5

MLV glycoGag is known to counteract the inhibitory effect of SER5 against MLV and HIV-1.[2,6,26,27] We also tested the glycoGag MLV plasmid with the HIV-1 three-plasmid system. This plasmid encodes the first 88 amino acids of glycoGag, which counteracts huSER5 in a dose dependent manner (Suppl. Figure 2). The glycoGag proteins of FGA-5 and FGB share about 91% identity (Figure 6 (A)). To evaluate the ability of FeLV-glycoGag to counteract SER5s in the context of HIV-1, expression plasmids for HA-tagged glycoGags were generated that encode the first 74 or 77 residues of either FeLV-A or FeLV-B glycoGag (Figure 6(A, B)). Expression was verified using anti-HA antibody (Figure 6(C)). HIV-1 reporter particles pseudotyped with the HIV-1 envelope BaL were produced in the presence or absence of SER5s and in the presence or absence of each FeLV glycoGag. Under these experimental conditions, we found that overexpression of both glycoGags moderately increased the resistance of HIV-1 Env to SER5s (Figure 7(A)). To exclude unphysiological results by "cell-free" infections, we tested glycoGag expression in an HIV vector system where infections are mediated largely by cell–cell contacts.[62] HIV-1 virions were pseudotyped with BaL envelope and applied the transfer vector UCHRinLuc which contains a luciferase reporter gene expressed post reverse transcription of the spliced vector RNA.[62] After co-culture of virus-producer cells with target cells, both human and feline SER5 inhibited the HIV-1 envelope dependent fusion and similar to "cell-free" infections, and FeLV glycoGag slightly increased the resistance to SER5 (Figure 7(B)).

FIV pseudotyped with FeLV-B envelope is resistant to SERINC5

We speculated that FIV replication in SER5 expressing cells *in vivo* might benefit from endogenous or exogenous FeLV proteins, thereby decreasing FIV sensitivity to SER5. To test this hypothesis, FIV particles (3-plasmid system) were co-transfected with SER5 plasmids and FeLV glycoGag expression plasmids. FeLV-glycoGag proteins failed to counteract the inhibition of both feSER5 and huSER5 in the context of FIV (Figure 8). In strong contrast, FIV particles pseudotyped with FeLV-B envelope were resistant to both SER5s and even showed enhanced infectivity (Figure 8). For unknown reasons, we were not able to generate infectious FIV vectors pseudotyped with FeLV-A envelope. These data may suggest that FIV *in vivo* could increase its resistance to SER5 by including non-FIV envelope proteins into its viral membrane.

Cat cells express a moderate level of SERINC5

As the full length FIV is sensitive to SER5, we evaluated the expression of SER5 in cat cells or in HEK293T cells transfected with the feSER5 expression plasmids. We used peripheral blood mononuclear cell (PBMC) and macrophages obtained from one cat. The expression of SER5 was also evaluated in feline CRFK cell lines. The data are presented as ΔCt values, where a low average indicates a high expression value. The ΔCt results showed that cat cells have a moderate expression level of feSER5 (Figure 9(A)).

The surface expression of feSER5 is slightly reduced in the presence of FeLV-A/B glycoGag or FeLV-A/B envelope

As it was described that HIV-1 Nef downregulates 30 to 60% of huSER5 from the cell surface,[12–14] we tested by FACS whether FeLV glycoGag or FeLV envelope have a similar activity and reduce the feSER5 expression from the cell surface. Since both 5′ and 3′ termini of the SER5 protein are intracellular, a new plasmid was constructed with a HA tag located in the fourth extracellular loop, similar to previously published huSER5-iHA constructs.[2,16] For the FACS analysis, HEK293T cells were co-transfected with feSER5 and FeLV-A/B glycoGags, FeLV-A/B envelopes or an expression plasmid for Nef (HIV-1 SF2) or MLV glycoGag. The steady-state expression of surface feSER5 was reduced by around 70% with the Nef protein, 30% with MLV glycoGag, and 20% with FeLV-A/B glycoGag or FeLV envelopes (Figure 9(B, C)). These findings support that FeLV glycoGags have a moderate activity on feSER5 counteraction. Unexpectedly, both FeLV envelope proteins also showed a moderate capacity to reduce the feSER5 surface expression.

FeLV glycoGags increase the infectivity of some viruses

To evaluate whether the FeLV glycoGags could increase the infectivity of the tested viruses

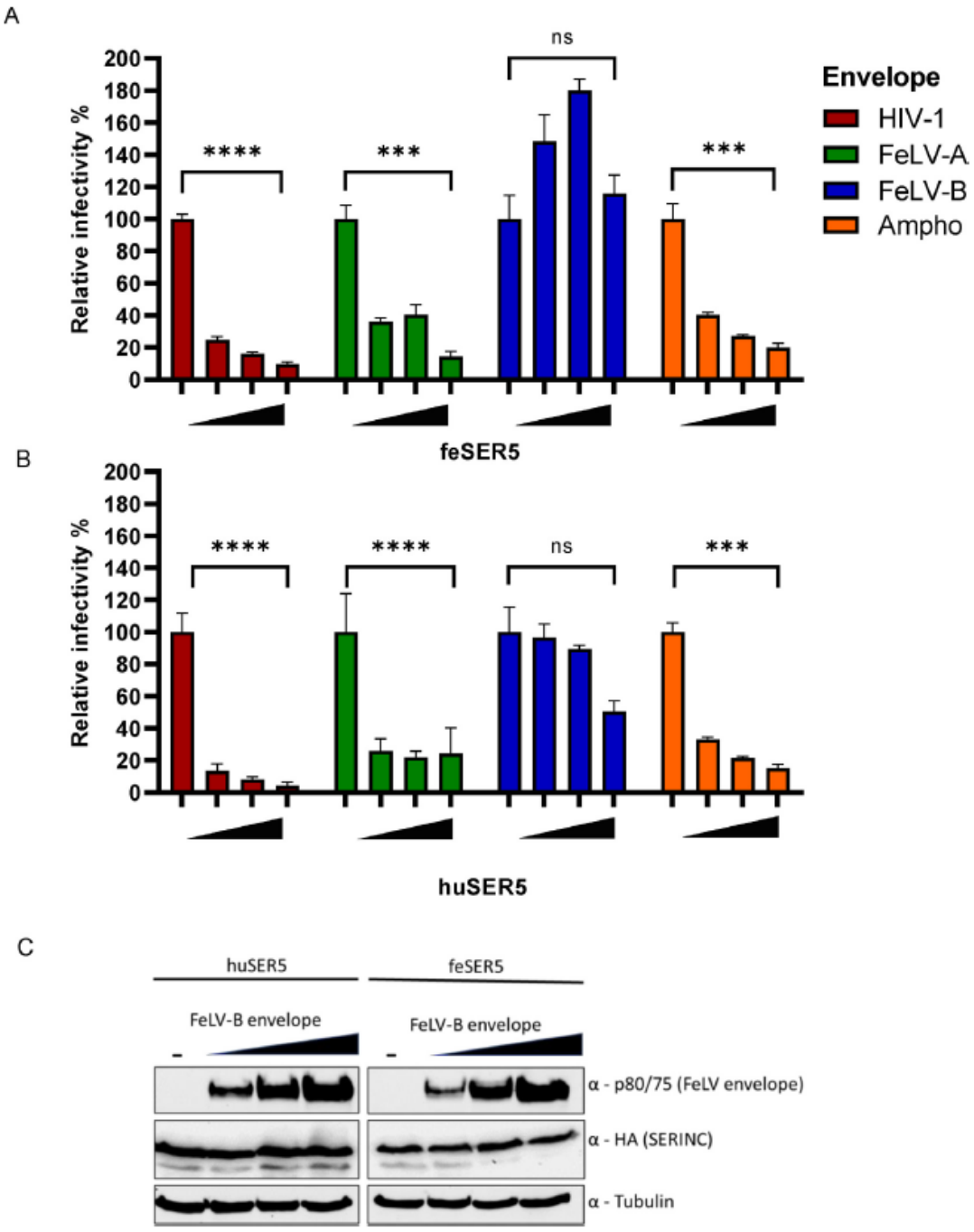


Figure 5. Effect of SER5 on HIV-1 particles pseudotyped with different envelopes (A) HIV-1 reporter vectors (3-plasmid system, no Nef) pseudotyped with envelopes of HIV-1 BaL (red), FeLV-A (green), FeLV-B (blue) or amphotropic (ampho) Env (orange) were produced in the presence of increasing amounts of feline SERINC5 (feSER5) or (B) human SERINC5 (huSER5) (0, 100, 200 or 400 ng). Viral infectivity was determined after normalization for reverse transcriptase activity by quantification of luciferase activity. Shown are means plus standard deviations (error bars) of a representative experiment performed three times in triplicate. Asterisks represent statistically significant differences: *P* value <0.001 extremely significant (***), 0.001 to 0.01 very significant (**), 0.01 to 0.05 significant (*), >0.05 not significant (ns). (C) SERINC5 stability in the presence of increasing amounts of FeLV-B envelope. HEK293T cells were transfected with fe/huSER5 and increasing amounts of FeLV-B envelope expression plasmids or empty vector as control. Cells were harvested and analyzed by immunoblotting using anti-p85/70, anti-HA and anti-tubulin antibodies. α p85/70 detects envelope (SU-TM p85) and SU only (p70), α HA detects HA-SER5.

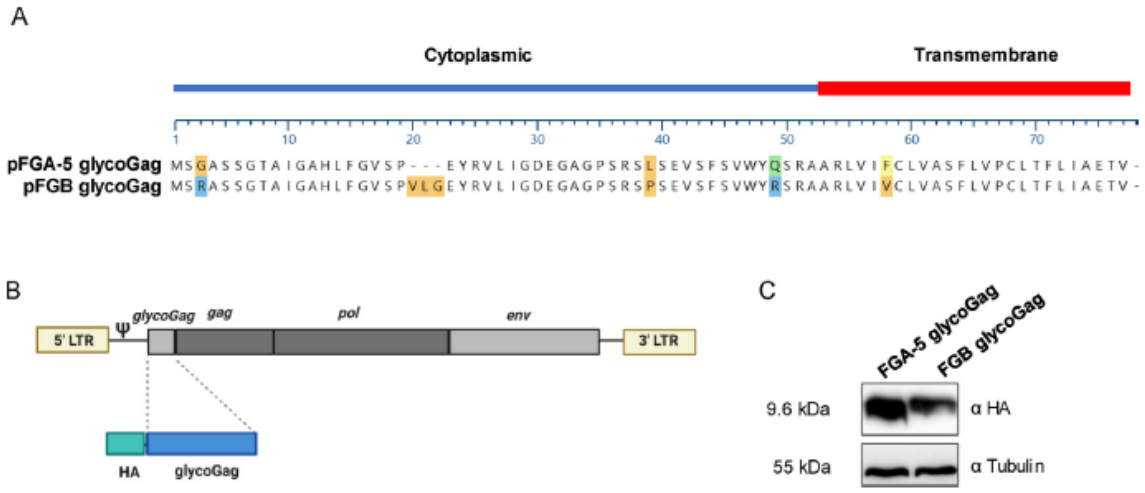


Figure 6. Generation of FeLV glycoGag expression constructs. (A) Sequences of FeLV-A and FeLV-B glycoGags. The highlighted amino acids correspond to disagreements, which are colored according to their side chain chemistry. Cytoplasmic and transmembrane domains are indicated. (B) Plasmids expressing the HA-tagged FeLV glycoGag were produced by amplifying the glycoGag from pFGA-5 and pFGB (FeLV-A and FeLV-B glycoGag respectively), yielding a 9.6 kDa protein. (C) HEK293T cells were transfected with the generated expression plasmids, harvested two days post-transfection and analyzed by immunoblotting using anti-HA and anti-tubulin antibodies.

without co-expression of SER5, viral particles were produced either with three-plasmid systems for HIV-1 or FIV or full-length FeLV-A or -B expressing or not the glycoGag or WT FIV together with FeLV glycoGag constructs. We observed that expression of glycoGag of FeLV-A and –B moderately increased the infectivity of HIV-1 and FIV vectors, and the FIV-PPR full-length virus. Only glycoGag of FeLV-B additionally enhanced FeLV-A or -B variants lacking glycoGag expression (Suppl. Figure 3). These data may suggest that FeLV glycoGag has a more general activity to enhance virus replication beyond the counteraction of SER5.

Discussion

FeLV disease during progressive infection in domestic cats is aggressive and reduces life expectancy. However, disease progression is only one possible disease scenario, and interestingly, some cats do not develop the disease and contain the viral replication (regressive infection).[42] What determines the outcome of the disease is not completely elucidated.[63] FeLV-B arises from recombination between the endogenous FeLV and the exogenous FeLV-A.[64,65] FeLV-B is also more virulent than FeLV-A[53,66] and the development of lymphoma and leukemia has been associated to the emergence of FeLV-B.[41,42] The reason for the enhanced replication of FeLV-B is unknown and has been postulated to be related to the wider repertoire of target cells FeLV-B can infect due to altered receptor usage.[67] Here, we demonstrated while FeLV-A is partially SER5 resistant, FeLV-B is strongly resistant to SER5, suggesting FeLV Env composition and restriction may mediated disease outcomes. The Env to a larger part as well as the glycoGag mediate FeLV-B SER5 resistance. FeLV-B Env does not directly destabilize SER5, and likely forms a viral envelope that escapes the antiviral activity of SER5 similar to some SER5 resistant HIV-1 and non-retroviral envelopes.[10,11,23] MLV glycoGag counteracts SER5[10,26,68] much more potently than FeLV glycoGag for unknown reasons and, the counteraction of SER5 by the MLV glycoGag is also glycoprotein-dependent[10,28] which we have also observed with FeLV-B envelope. MLV glycoGag contains a conserved YXXL motif found to be crucial for its viral restricting activity[10,26]; this YXXL motif is also contained in FeLV glycoGags.[26] In addition, GlycoGag of MLV protects MLV against APOBEC3s expressed in target cells during their infection and can also prevent the encapsidation of human APOBEC3A.[69–71] Whether the FeLV glycoGag has functions to counteract feline APOBEC3s is unknown. In addition, FeLV glycoGag may also enhance the infectivity of some FIVs in a SER5-independent way. The molecular mechanism of this SER5 independent virus enhancement is unknown, but possible involves some cellular inhibitory factor present in HEK293T cells.

In striking contrast to FeLV, FIV appears to be highly sensitive to feSER5 restriction. We found that FIV could escape the SER5 restriction by co-expression of heterologous Env, such as FeLV-B Env or VSV-G. For reasons unknown, the co-expression of FeLV glycoGag did not protect FIV against SER5. Thus, *in vivo* FIV may prevent

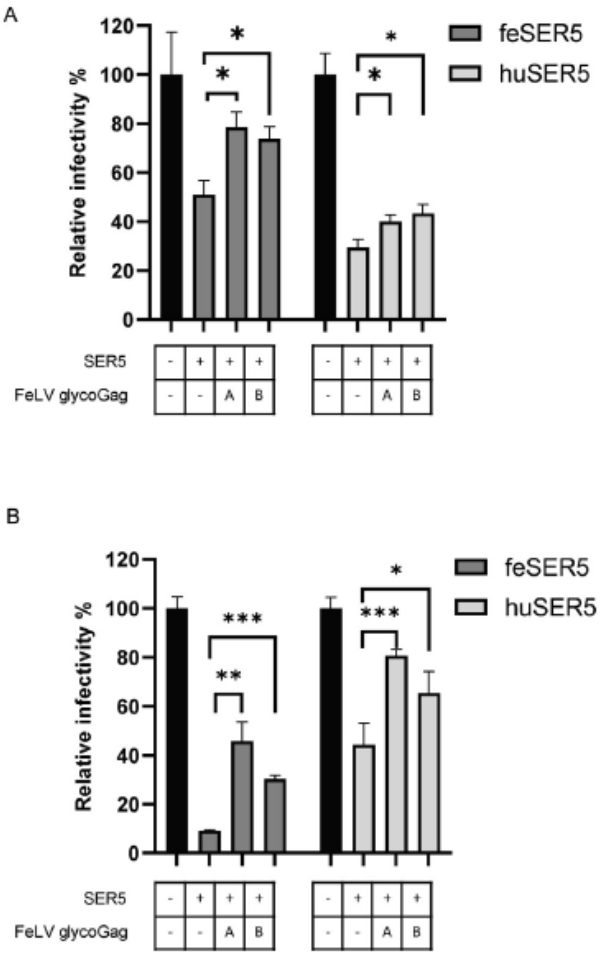


Figure 7. Effect of FeLV glycoGag on HIV-1 in the presence of SER5. (A) HIV-1 reporter vectors (3-plasmid system, no Nef) pseudotyped with HIV-1 BaL envelope were produced in the absence or presence of feline SERINC5 (feSER5) or human SERINC5 (huSER5) together with FeLV-A glycoGag, FeLV-B glycoGag or empty vector. Particles were normalized by RT activity and used for infections. The infectivity determined by quantification of luciferase activity. Shown are means plus standard deviations (error bars) of a representative experiment performed three times in triplicate. Asterisks represent statistically significant differences: *P* value <0.001 extremely significant (***), 0.001–0.01 very significant (**), 0.01–0.05 significant (*), >0.05 not significant (ns). (B) Effect of SERINC and glycoGag on HIV-1 cell-to-cell transmission. HIV-1 reporter vectors (3-plasmid system, no Nef) pseudotyped with HIV-1 envelope BaL were produced in the absence or presence of feline SERINC5 (feSER5) or human SERINC5 (huSER5) and FeLV-A or -B glycoGag. After transfection, virus producer HEK293T cells were co-cultured with HEK293T transfected with expression plasmids for human CD4 and human CCR5. The infectivity determined by quantification of luciferase activity of a vector that is detectable only after reverse transcription of the spliced luciferase gene. Shown are means plus standard deviations (error bars) of a representative experiment performed three times in triplicate. Asterisks represent statistically significant differences: *P* value <0.001 extremely significant (***), 0.001–0.01 very significant (**), 0.01–0.05 significant (*), >0.05 not significant (ns).

SER5 restriction by incorporating FeLV-B envelopes or similar endogenous envelope proteins into viral membranes. In addition, in cats FIV may preferentially replicate in cells or tissues with low SER5 restrictive activity. The level of mRNA of feSER5 in the evaluated feline cells could reflect a moderate level of expression of feSER5, however this level is lower than the mRNAs obtained of transfected HEK293T cells even in the lowest tested concentration (50 ng) which also reduced the infectivity of the FIV-C36 WT significantly (Figure 2(C)). Furthermore, we cannot rule out that *in vivo* FIV has a way to decrease the expression of feSER5 using a mechanism not observed in our cell culture system. FIV OrfA's precise function is unknown

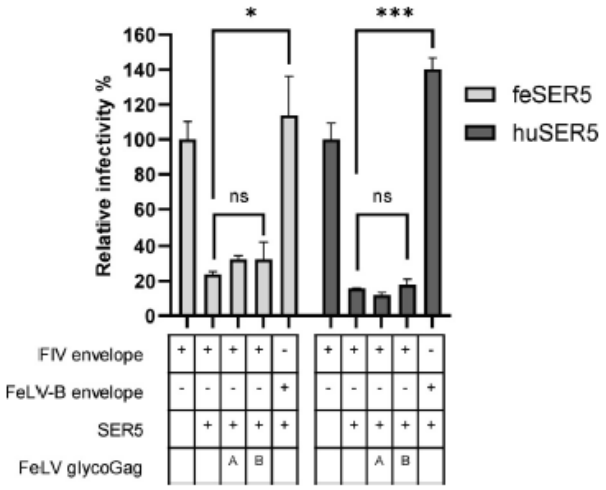


Figure 8. Effect of FeLV glycoGag or FeLV-B envelope on FIV in the presence of SER5. FIV particles (3-plasmid system) pseudotyped with FIV EE14 envelope were produced in the absence or presence of feline SERINC5 (feSER5) or human SERINC5 (huSER5) and FeLV glycoGag or in the absence of FIV EE14 envelope and glycoGag and pseudotyped by FeLV-B envelope. Particles were normalized by RT activity and used for infection. The infectivity determined by quantification of luciferase activity. Shown are means plus standard deviations (error bars) of a representative experiment performed three times in triplicate. Asterisks represent statistically significant differences: *P* value <0.001 extremely significant (***), 0.001–0.01 very significant (**), 0.01–0.05 significant (*), >0.05 not significant (ns).

and data suggests that OrfA contributes to the downregulation of the FIV receptor (CD134), E2 ubiquitin-conjugating enzymes (UBE2 C, D2, L3, N and V1) and one E3 ubiquitin-protein ligase (UBE3A).[50,72,73] Therefore, it is possible that OrfA contributes *in vivo* to SER5 counteraction.

We found that feline SER5, similar to many other non-human SER5s is sensitive to HIV-1 Nef[4,74] and is a potent inhibitor of Nef-deficient HIV-1. Thus, our data suggests that feline SER5 would not block HIV-1 replication in feline cells. We conclude that in addition to HIV-1, FeLV is resistant to feline SER5, with FeLV-B exhibiting greater resistance than FeLV-A. FeLV-B glycoGag contributed to FeLV counteraction of SER5, which mainly was determined by its envelope protein. To best of our knowledge, this is the first study that describes the expression and activity of FeLV glycoGag. How FIV *in vivo* circumvents a restriction by feline SER5 is a question for future research.

Methods

Feline and human SERINC5 alignment

Feline and human SERINC5 nucleotide sequences were obtained from Genbank (XM_011284262 and NM_001174072, respectively), and aligned using MUSCLE[75] on MEGA-X[76] and visualized on DNASTAR.

Cells

HEK293T (ATCC CRL3216) and CRFK (ATCC CCL-94) cells were maintained in Dulbecco's high-glucose modified Eagle's medium (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS) (PAN Biotech, Aidenbach, Germany) 2 mM L-glutamine (PAN Biotech), penicillin (100 U/ml), and streptomycin (100 μg/ml) (PAN Biotech). HOS cells expressing human CD4 and CCR5 (HOS $CD4^+CCR5^+$) and CRFK expressing feline CD134 (CRFK $CD134^+$) were maintained under puromycin 1 μg/mL and G-418 800 μg/mL respectively. Feline PBMC were isolated from EDTA-treated whole blood by Histopaque-1077 (Sigma, St. Louis, Mo.) density gradient centrifugation and stimulated with 5 μg/ml of concanavalin A (Thermo scientific) for three days. Feline PBMCs were cultured with RPMI 1640 (Gibco BRL, Grand Island, N.Y.), supplemented with 10% fetal bovine serum (FBS), 5×10^{-5} M 2-mercaptoethanol (Gibco BRL), penicillin (100 U/ml), and streptomycin (100 μg/ml) (PAN Biotech), 2 mM L-glutamine (PAN Biotech), and 100 U of human recombinant IL-2 (hrIL-2) (Collaborative Biomedical Products, Bedford, Mass.) per ml. Feline macrophages were isolated from whole blood and were maintained in RPMI 1640 containing 1,000 U/ml monocyte colony-stimulating factor (M-CSF). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

Plasmids

pCT-PPR and pCT-36[57] express full length FIV molecular clones (gifts of Eric M. Poeschla); pLinSin[46] is a FIV luciferase transfer vector; pFP93 is a FIV packaging construct (a gift from Eric

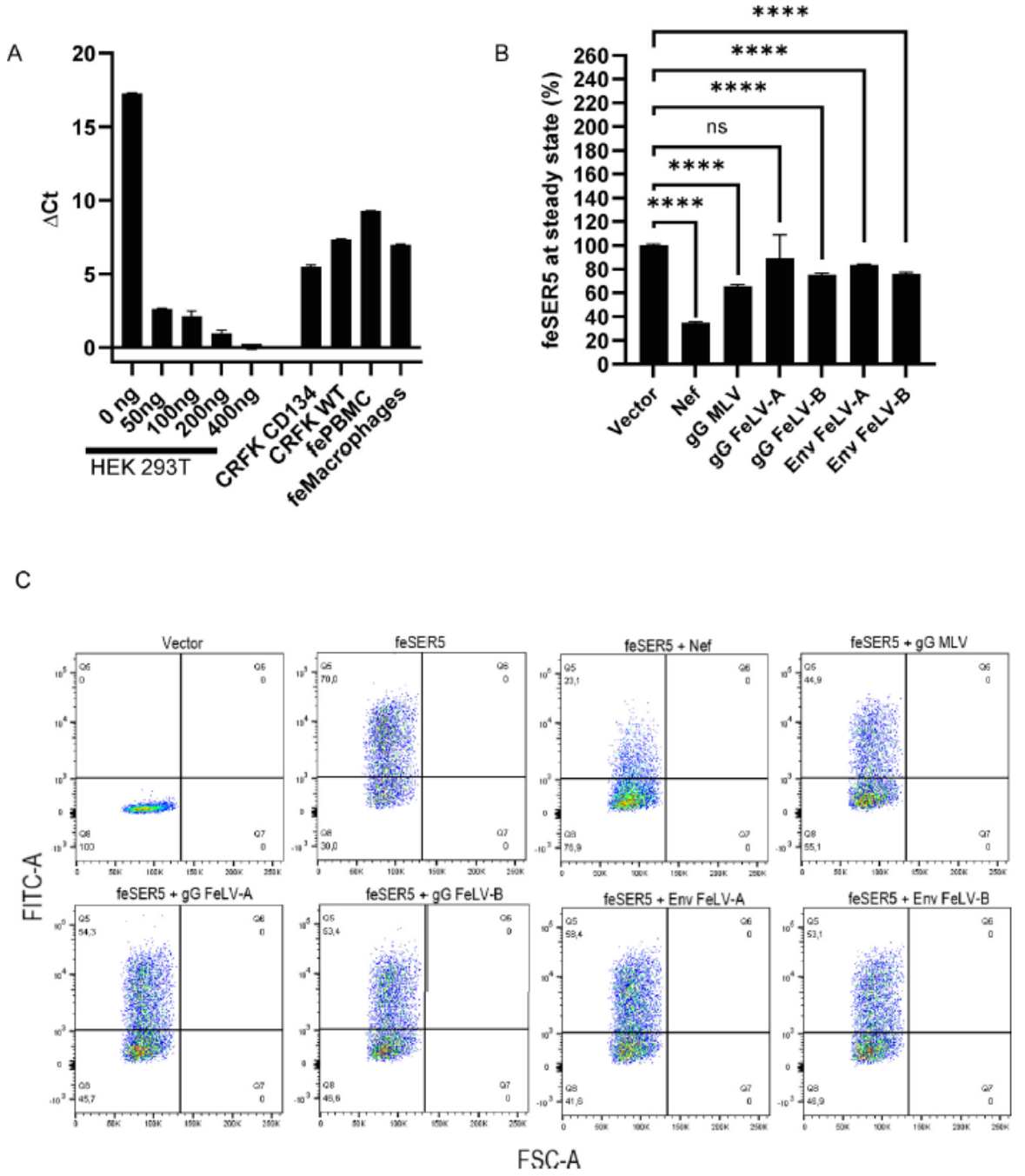


Figure 9. Comparison of delta Ct values of feSER5 in transfected HEK293T cells and in cat cells and surface expression of feSER5 in the presence of different FeLV proteins. (A) HEK293T cells were transfected with different amounts of feSER5 plasmid. The endogenous feSER5 were evaluated in feline CRFK cell lines and PBMC or macrophages from one cat. (B) HEK293T cells were cotransfected with feSER5-iHA and empty plasmid, Nef, MLV glycoGag , FeLV-A/B glycoGag or FeLV-A/B envelope. The percent of feSER5 positive cells was evaluated by flow cytometry analysis and the total of feSER5 positive cells were scaled to 100%. Shown are means plus standard deviations (error bars) of a representative experiment performed three times in triplicate. Asterisks represent statistically significant differences: *P* value <0.001 extremely significant (***), 0.001–0.01 very significant (**), 0.01–0.05 significant (*), >0.05 not significant (ns). (C) Representative plots of one of three experiments showing the surface expression of feSER5-iHA (HA-Alexa Fluor 488). Green fluorescence was quantified using the FITC-A channel vs. FSC-A (10,000 cells were counted).

M. Poeschla), which only expresses *gag*, *pol*, and *rev* (57); pEE14[58] (a gift of Mauro Pistello) and pGL-8[59] are FIV Env expression plasmids; pNL4-3 expresses full-length HIV-1,[77] HIV-1 NL4-3Δ*nef* does not express Nef,[78] HIV-1 NL4-3 SF2 Nef expresses SF2 Nef[79]; pUCHRinLuc (a gift of Dmitriy Mazurov) is a HIV-1-based reporter transfer vector, which contains an antisense-oriented expression cassette of the firefly luciferase gene interrupted by an in-sense intron[62]; pBABEpuro-hCCR5 expresses human CCR5 (a gift of Nathaniel Landau); pMX expresses human CD4,[80] VR1012 FeLV-B env (a gift of Brian Willet)[54] expresses the envelope of a FeLV-B strain; pHIT456 expresses the MLV amphotropic envelope,[81] pFGA-5 (FeLV-A Glasgow strain) and pFGB (FeLV-B Gardner-Arstein strain) are full-length wild-type FeLV infectious proviral clones partially described.[64,82] The viral coding sequence of the pFGB was deposited in GenBank (accession MZ964580). MP71-luc[83] is MLV luciferase reporter plasmid; HA-gg88 is a plasmid which express the amino-terminal amino acids of MLV glycoGag (M-MuLV) (a gift of Hung Y. Fan).[84] The following plasmids were constructed as described here: human or feline *SERINC3* or *SERINC5* genes were cloned from cDNA of Jurkat and CRFK cells, and the sequences were verified. The SERINC open reading frames were cloned into pcDNA3.1(+) and pBJ6[2,85] with a C-terminal HA tag by using EcoRI and NotI restriction enzymes. The same plasmid was used to construct a second feSER5 expression plasmid with the HA tag located in the fourth extracellular loop as reported previously for huSER5-iHA,[2,16] two fragments were produced and fused with a fusion PCR. The first fragment was produced with primers PF_feSER5_-NotI and PR_HA feSER5 Ex8 and the second fragment with primers PF_HA feSER5 Ex8 and Fe SERINC5 EcoRI-R. To construct FIV-36 mutants (Δ*vif,* Δ*orfA* or the double mutants Δ*vif*/Δ*orfA),* two stop codons were introduced after the start codon of each gene by overlapping PCRs. The first fragments of FIVs *vif* mutants (FIV-C36 mut *vif*) were produced with primers FIV-C36 F and FIV-C36 Vif Stop R. The second fragment were produced with primers FIV-C36 Vif Stop F and FIV-C36 R. For the fusion PCR, we used primers FIV-C36 F and FIV-C36 R and FIV-C36 R. Generation of FIV *orfA* mutants (FIV-C36 mut *orfA*) was done by overlapping PCR: For the first fragment we used primers FIV-C36 F and FIV-C36 OrfA Stop R. The second fragments were amplified with primers FIV-C36 OrfA Stop F and FIV-C36 R, FIV-PPR OrfA Stop F. Plasmids expressing FeLV glycoGag were cloned into pcDNA3.1(+) with N-terminal HA-tag by amplifying the glycoGag from pFGA-5 (pcFeLV-A_glycoGag) and pFGB (pcFeLV-B_glycoGag) with primers FW HA-FeLV-A/B GG EcoRI and RV GG FeLV-A NotI or RV GG FeLV-B NotI. FeLV-A envelope was amplified from pFGA-5 with primers Env_pFGA-5_PF_SalI and Env_pFGA-5_PR_NotI and cloned into VR1012 plasmid. FeLV pFGA-5 and pFBG plasmids were used to construct FeLV mutants, lacking the whole envelope gene, not expressing glycoGag or the double mutants. pFGA-5Δ*env* and pFGBΔ*env* are envelope deleted constructs produced by digesting both plasmids with DraIII and RsrII to remove a fragment of 1502 bp and 1860 pb, respectively. Each product was treated with T4 polymerase (New England Biolabs, United Kingdom) to create blunt ends and afterwards circularized with T4 ligase (New England Biolabs). To construct FeLV glycoGag mutants (full-length pFGA-5X and pFGBX or the double mutants with the envelope deleted pFGA-5XΔ*env* and pFGBXΔ*env*) a frame shift mutation was introduced in the 5′ reading frame before the *gag* ATG codon to prevent the expression of the glycoGag. For this, two PCR fragments were fused using overlapping PCRs. pFGA-5X and pFGA-5XΔ*env* were constructed using pFGA-5 and pFGA-5Δ*env* as template to amplify two fragments separately: the first fragment with primers PF1_bef_ClaI_pFGA-5 and PR1_XbaI_GG_pFGA-5 and the second fragment with PF2_XbaI_GG_pFGA-5 and PR2_BglII_pFGA-5. The fragments were joined through overlapping extension PCR with primers PF1_bef_ClaI_pFGA-5 and PR2_BglII_pFGA-5 and this fragment including the mutation was cloned into pFGA-5 or pFGA-5Δ*env* using restriction sites ClaI and BglII. The same process was followed for pFGBX and pFGBXΔ*env* with primers PF1_ClaI_pFGB and PR1_XbaI_GG_pFGB in the first PCR and the pair primers PF2_XbaI_GG_pFGB and PR2_BglII_pF GB in the second PCR. The fragments were fused through a third PCR with primers PF1_ClaI_pFGB and PR2_BglII_pF GB and cloned into pFGB or pFGBΔ*env* using restriction sites ClaI and BglII. All PCR products were amplified with Q5 High-Fidelity DNA Polymerase (New England BioLabs). All PCR primers are listed in the Table 1.

Flow cytometry analysis

To monitor efficient SER3/5 expression in the NL4-3 experiments, HEK293T cells used for virus production in the presence or absence of SERINCs were detached and fixed with 4% PFA. The cells were permeabilized using Perm Buffer II (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions and stained using 1:40 HA-FITC (Miltenyi Biotec, Bergisch Gladbach, Germany). The samples were analyzed by flow cytometry using a BD FACSVERSE (BD Biosciences). To evaluate a change in the surface level of feSER5 in the absence or presence of HIV-1 SF2 Nef,[86] FeLV-A or B glycoGag (Flag tagged) or FeLV-A or B envelopes, HEK293T cells were transfected with a plasmid expressing feSER5, HA tagged in the fourth extracellular

Table 1 PCR primers used in this study.

Primer name	Sequence 5′–3′
Hu + Fe SERINC5NotI-F	ATGCGGCCGCATGTCAGCTCAGTGCTGTGCGG
Hu SERINC5 EcoRI-R	ATGAATTCCTAAGCGTAATCTGGAACATCGTATGGATACACAGAGAACTCCCGGGTG
Fe SERINC5 EcoRI-R	ATGAATTCCTAAGCGTAATCTGGAACATCGTATGGATACACGGAGAACTGCCGAGAG
PF_feSER5_NotI	AAGCGGCCGCACCATGTCAGCGCAGTGCTGTGC
PR_HA feSER5 Ex8	AGCGTAATCTGGAACATCGTATGGGTACTCATCTAGAACTACTTCTA
PF_HA feSER5 Ex8	TACCCATACGATGTTCCAGATTACGCTCATGGGAAAAATGTTACAAT
FW HA-FELV-A/B GG EcoRI	TTGAATTCACCATGTATCCGTATGATGTGCCGGATTATGCGTCTGGAGCCTCTAGTGGGAC
RV GG FELV-A NotI	AAGCGGCCGCGACGGTTTCTGCAATTAAAAAG
RV GG FELV-B NotI	AAGCGGCCGCGACGGCTTCTGCAATTAAAAAG
RV GG-MA FELV-A/B NotI	AAGCGGCCGCGTAGAGGGAAGAGGTAAG
Env_pFGA-5_PF_SalI	AAGTCGACACCATGGAAAGTCCAACGCACCC
Env_pFGA-5_PR_NotI	ACGATCCGGACCGACCATGAGCGGCCGCAA
PF1_bef_ClaI_pFGA-5	ACTGACCACTGGAGACCTGA
PR1_XbaI_GG_pFGA-5	GCTGTCCCACTAGAGGCTCCATCTAGACATCAGACACCC
PF2_XbaI_GG_pFGA-5	GGGTGTCTGATGTCTAGATGGAGCCTCTAGTGGGACAGC
PR2_BglII_pFGA-5	CGAAGATCTTTTTTTCAACCTGGG
PF1_ClaI_pFGB	TTATCGATGATAAGCTGTCAAACATGA
PR1_XbaI_GG_pFGB	GCTGTCCCACTAGAGGCTCGATCTAGACATCAGACACCCGTGG
PF2_XbaI_GG_pFGB	CCACGGGTGTCTGATGTCTAGATCGAGCCTCTAGTGGGACAGC
PR2_BglII_pF GB	AAGATCTTCTTCTCAACCTGG
FIV-C36 F	AGCATTAACTAGAATGATGAGAGG
FIV-C36 OrfA Stop F	GAGCTATGAATGTGAGAGATAATTCCATTATGAAATAAGGCTACA
FIV-C36 OrfA Stop R	TGTAGCCTTATTTCATAATGGAATTATCTCTCACATTCATAGCTC
FIV-C36 R	GGTTCTGGTTACATCCTAATTCTTGC
FIV-C36 Vif Stop F	GGGATGAGTGAATAAGATTGGCAGGTAAGTTAAGGACTCTTTGCA
FIV-C36 Vif Stop R	TGCAAAGAGTCCTTAACTTACCTGCCAATCTTATTCACTCATCCC
feGAPDH_F	AAGGCTGAGAACGGGAAAC
feGAPDH_R	CATTTGATGTTGGCGGGATC
huGAPDH_F	CAACAGCGACACCCACTCCT
huGAPDH_R	CACCCTGTTGCTGTAGCCAAA

loop.[2,16] Cells were incubated with mouse anti-hemagglutinin (anti-HA) antibody (1:500 dilution, MMS-101P; Covance, Münster, Germany) and as secondary antibody donkey anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (1:1000 dilution Thermo Fisher Schwerte, Germany). After washing, the cells were resuspended in one ml of FACS buffer and finally green fluorescence was measured with a FACS Canto III flow cytometer (BD Biosciences). The cell population was defined based on their side scatter and forward scatter profile as to exclude cell debris and doublets. Green fluorescence was quantified using the FITC-A channel vs. FSC-A (10,000 cells were counted). FACS data were processed with the FlowJo software package (BD Biosciences).

Virus production

HIV was produced by transfecting HEK293T as previously described.[27] Here, 1.5 μg HIV-1 pNL4-3 plasmid[77] DNA lacking Nef (HIV-1 NL4-3Δ*nef*)[78] or expressing Nef (HIV-1 NL4-3 SF2 Nef)[79] and 0.5 μg SER3/5 expression plasmids were co-transfected using PEI (3:1 of total DNA). For VSV-G co-expression, 0.5 μg of VSV-G expression plasmid DNA (pMD2.G Addgene 12259) was added. The supernatant was collected 48 h after transfection and passed through 0.45 μm filters. HIV-1 particles were also produced with the 3-plasmid system using 0.2 μg pMDLg/pRRE packaging plasmid, 0.1 μg pRSV-Rev,[87] 0.2 μg GFP luciferase encoding transfer-vector pSIN.Luc (pSIN.PPT.CMV.Luc.IRES.GFP)[88] and 0.2 μg pBaL26 plasmid which encodes the HIV-1 envelope (HIV-1 strain BaL; pBaL.26).[89] To produce HIV-1 pseudotyped particles, 0.4 μg of plasmids expressing FeLV-A envelope, FeLV-B envelope[54] or amphotropic MLV envelope (pHIT456)[81] were used. For the SER titration experiments, 0.1, 0.2 or 0.4 μg of fe/huSER5 were co-transfected together with the viral plasmids or with the pBJ6 empty vector as a control. To evaluate the effect of MLV glycoGag, HIV particles were produced with the 3-plasmid system in the presence or absence of huSER5 (0.4 μg) and with increasing amounts of MLV glycoGag (0, 0.1, 0.3 or 0.5 μg). HIV particles were produced with the 3-plasmid system pseudotyped with different envelopes (0.5 μg of HIV pBaL, FeLV-A, FeLV-B or amphotropic envelope), in the presence or absence of SER5 (0.4 μg) and FeLV glycoGag (0.8 μg) (pcFeLV-A_glycoGag or pcFeLV-B_glycoGag). To produce FIV particles, HEK293T cells were co-transfected with 0.8 μg of the full length FIV molecular clones PPR, C36 (using plasmid pCT-PPR and pCR-36[57] or FIV mutants and 0.8 μg of FIV luciferase vector pLinSin[46] together with 400 ng of fe/

huSER5 or empty vector. FIV particles were also produced with a 3-plasmid system by co-transfecting 0.3 μg of the replication deficient packaging construct pFP9,[90] which only expresses *gag*, *pol*, and *rev*, 0.3 μg of the FIV luciferase vector pLin-Sin; 0.1 of μg VSV-G expression plasmid pMD.G or 0.2 μg of FIV Env expression plasmid pEE14[58] together with 0.3 μg of fe/huSER5 or the empty vector as a control. FeLV particles were produced by co-transfecting 0.8 μg of the full-length FeLV molecular clones (wild type or mutants) and 0.8 μg of the MLV luciferase reporter plasmid MP71-luc.[83] FeLV pseudotyped particles with amphotropic envelope were produced by co-transfecting 0.6 μg of the FeLVΔ*env* plasmids (lacking the whole envelope), 0.6 μg of MLV luciferase reporter plasmid and 0.6 μg of amphotropic MLV envelope together with 1 μg of fe/huSER5 or empty vector as control.

Infections

For full-length HIV-1 experiments the relative infectivity per unit reverse transcriptase (RT) activity was calculated by normalization of relative light units (RLU) and RT units measured by SG-PERT.[91] TZM-bl cells[92] were infected with the same units of RT of HIV-containing supernatant produced with the full-length HIV-1 plasmids. The RT activity of all other viruses and viral vectors, was quantified by using the Cavidi HS lenti RT kit (Cavidi Tech, Uppsala, Sweden). After normalizing for RT activity, the same amounts of viruses based on RT values were used for infections. One day before transduction 1×10^4 cells by well were seeded in 96-well plate. Forty-eight hours after infection, cells were lysed with cell culture lysis reagent (Promega) and Firefly luciferase activity was measured with a Berthold CentroXS^3 LB 960 luminometer (Berthold Detection Systems, Pforzheim, Germany), using the luciferase assay system from Promega according to the manufacturer's instructions. Each transduction was done in triplicates. Infections with viral HIV-1.BaL26 particles were performed in HOS CD4$^+$CCR5$^+$ cells. CRFK CD134$^+$ were transduced with FIV particles. FeLV-A particles or viruses pseudotyped with FeLV-A envelope were used to transduce CRFK cells. FeLV-B or pseudotyped viruses with FeLV-B or amphotropic envelopes were used to transduce HEK293T cells.

Cell-to-cell transmission

To evaluate the effect of the SER5 and FeLV glycoGag on the HIV-1 cell-to-cell infection, the pUCHRinLuc plasmid was used. First, HEK293T cells were seeded in a 6-well plate (700.000 cells/well). In order to produce viral particles, the cells were transfected with 0.4 μg of packaging plasmid pMDLg/pRRE, 0.15 of μg pBaL26 HIV envelope, 0.2 μg of pRSV-Rev and 0.4 μg of the transfer vector pUCHRinLuc, in the presence or absence of SER5 and FeLV glycoGag. In parallel, independent HEK293T cells were transfected with 0.3 μg of human CD4 and 0.3 μg of human CCR5 expression plasmids. 24 h later, HEK293T cells expressing CD4/CCR5 were collected and added (700.000 cells/well) to the plate with the virus producer cells. 36 h later, cells were collected and the infection was evaluated by luciferase assay.

Immunoblot analysis

48 h post-transfection HEK293T cells were lysed for 20 min on ice in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl [pH7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], protease inhibitor cocktail set III (Calbiochem, Darmstadt, Germany). The lysate was centrifuged at 21.000 g for 20 minutes at 4°C. The suspension was boiled at 95^{0}C for 5 min with Roti load reducing loading buffer (Carl Roth, Karlsruhe, Germany) and resolved on a SDS-PAGE gel. The expression of SERINCs were assessed after treating the cell lysate at RT for 5 min with Roti load reducing loading buffer. SERINCs were detected by mouse anti-hemagglutinin (anti-HA) antibody (1:7500 dilution, MMS-101P; Covance). The glycoGag expression in the FeLV plasmids was evaluated by using anti-FeLV p27 antibody (1:7500 dilution; PF12J-10A: sc-65623; Santa Cruz Biotechnology, Inc. Bergheimer Heidelberg. Germany) (which detects Gag and glycoGag). The envelope deleted version of the FeLV plasmids were confirmed by using the anti-FeLV gp85/gp70 (1:7500 dilution; C11D8; sc-65621; Santa Cruz). Tubulin was detected using mouse anti-tubulin antibody (1:7500, dilution, clone B5-1-2; Sigma-Aldrich, Taufkirchen, Germany). Anti-mouse conjugated to horseradish peroxidase was used as secondary antibody (α-mouse-IgG-HRP; GE Healthcare, Munich, Germany). Blots were developed with ECL chemiluminescence reagents (GE Healthcare).

Quantitative reverse transcription PCR (RT-qPCR) for feSER5

In order to determine the expression level of feSER5, we evaluate the ΔCt value between the endogenous feSER5 and the feline Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (housekeeping gene) in the feline CRFK lines, feline PBMC or feline macrophages. The ΔCt value was also determined in transfected HEK293T cells with feSER5 plasmids by using the human GAPDH as housekeeping gene. Transfections were made in duplicates with feSER5 in different concentrations (0, 50, 100, 200 or 400 ng). The RNA was extracted with RNeasy Mini Kit (Qiagen) and a treatment with DNase I RNase free (Thermo Scientific) was made for the HEK293T cells samples to remove the residual plasmid DNA. The cDNA was

synthesized with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher) and the qPCR was developed with SYBR Green Master Mix (Thermo Fisher). Each sample were run in triplicates. We used the primers qPCR-feSER5F (located in the 5′ terminal of the exon 6 and the 3′ terminal of exon 7 of the feSER5 gene) and qPCR-feSER5R (located in the fourth loop) to amplify a fragment of 221 nt. The housekeeping genes were amplified with primers feGAPDH_F and feGAPDH_R or huGAPDH_F and huGAPDH_R (Table 1). Samples were analyzed in 96-well plates using a ViiA 7 Real-Time PCR System and the ViiA™ 7 Software (Applied Biosystems, Waltham, Massachusetts, United States).

FeLV glycoGag phylogenetic reconstruction

We performed a phylogenetic analyses with the available FeLV glycoGag sequences in GenBank. After excluding duplicates, an alignment of 77 amino acids with of 37 sequences (16 FeLV-A, 3 FeLV-B, 1 enFeLV and 16 non-subtyped) were generated using MUSCLE[75] on MEGA-X.[76] A Maximum Likelihood (ML) phylogeny was inferred in PhyML[93] by using the SMS (Smart Model Selection in PhyML) which incorporates the best-fitted amino acid substitution model HIVb + G.[94] The approximate likelihood-ratio test (aLRT) based on Shimodaira-Hasegawa-like procedure were used to assess confidence in topology.[95] The maximum likelihood phylogenetic tree was drawn using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

Statistical analysis

Data are represented as the mean with SD in all bar diagrams. Statistically significant differences between two groups were analyzed using the unpaired Student's t test with GraphPad Prism version 9 (GraphPad software, San Diego, CA, USA). Validity of the null hypothesis was verified with significance level at α value = 0.05.

CRediT authorship contribution statement

Lucía Cano-Ortiz: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Qinyong Gu:** Methodology, Formal analysis, Investigation. **Patricia de Sousa-Pereira:** Methodology, Formal analysis, Investigation. **Zeli Zhang:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Catherina Chiapella:** Methodology, Formal analysis, Investigation. **Augustin Penda Twizerimana:** Methodology, Formal analysis, Investigation. **Chaohui Lin:** Methodology, Formal analysis, Investigation. **Ana Cláudia Franco:** Writing – review & editing. **Sue VandeWoude:** Writing – review & editing. **Tom Luedde:** Writing – review & editing. **Hanna-Mari Baldauf:** Investigation, Writing – review & editing, Supervision. **Carsten Münk:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2021.167421.

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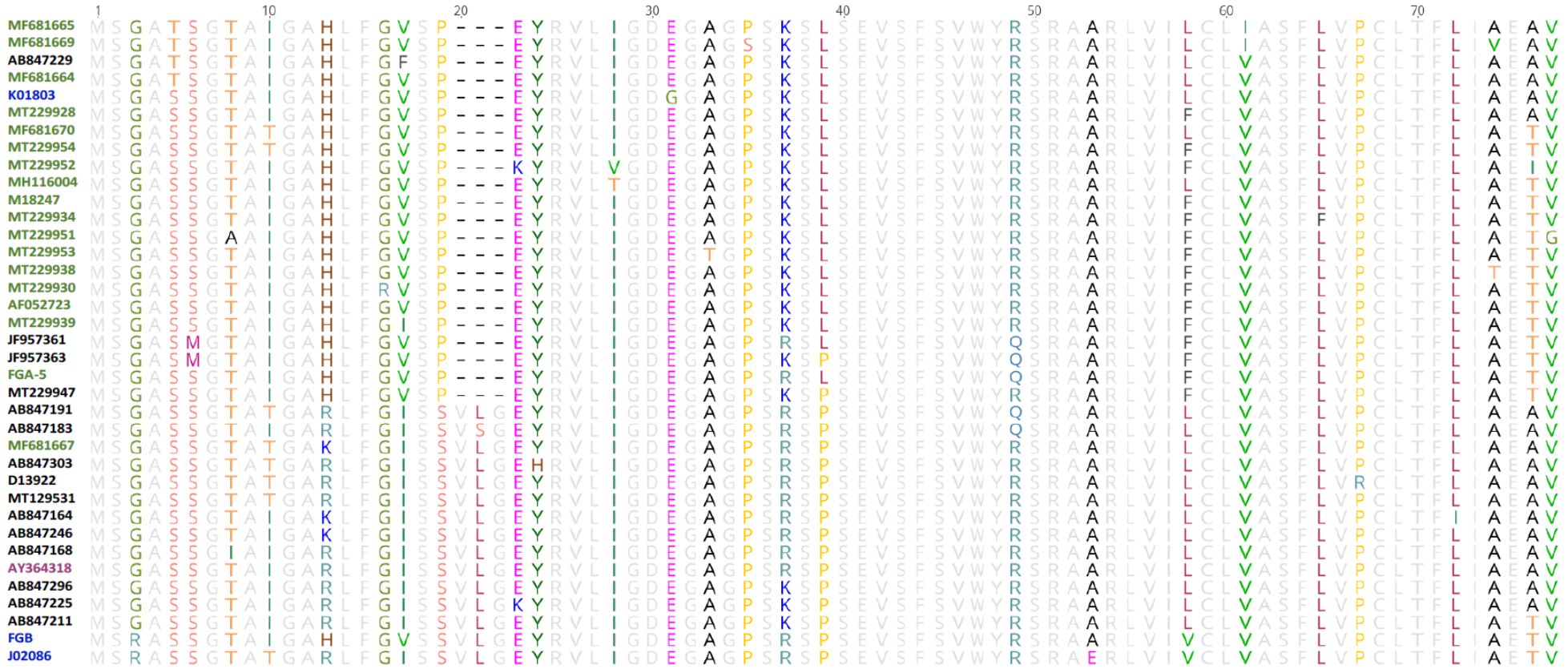
SUPPL. FIG. 1A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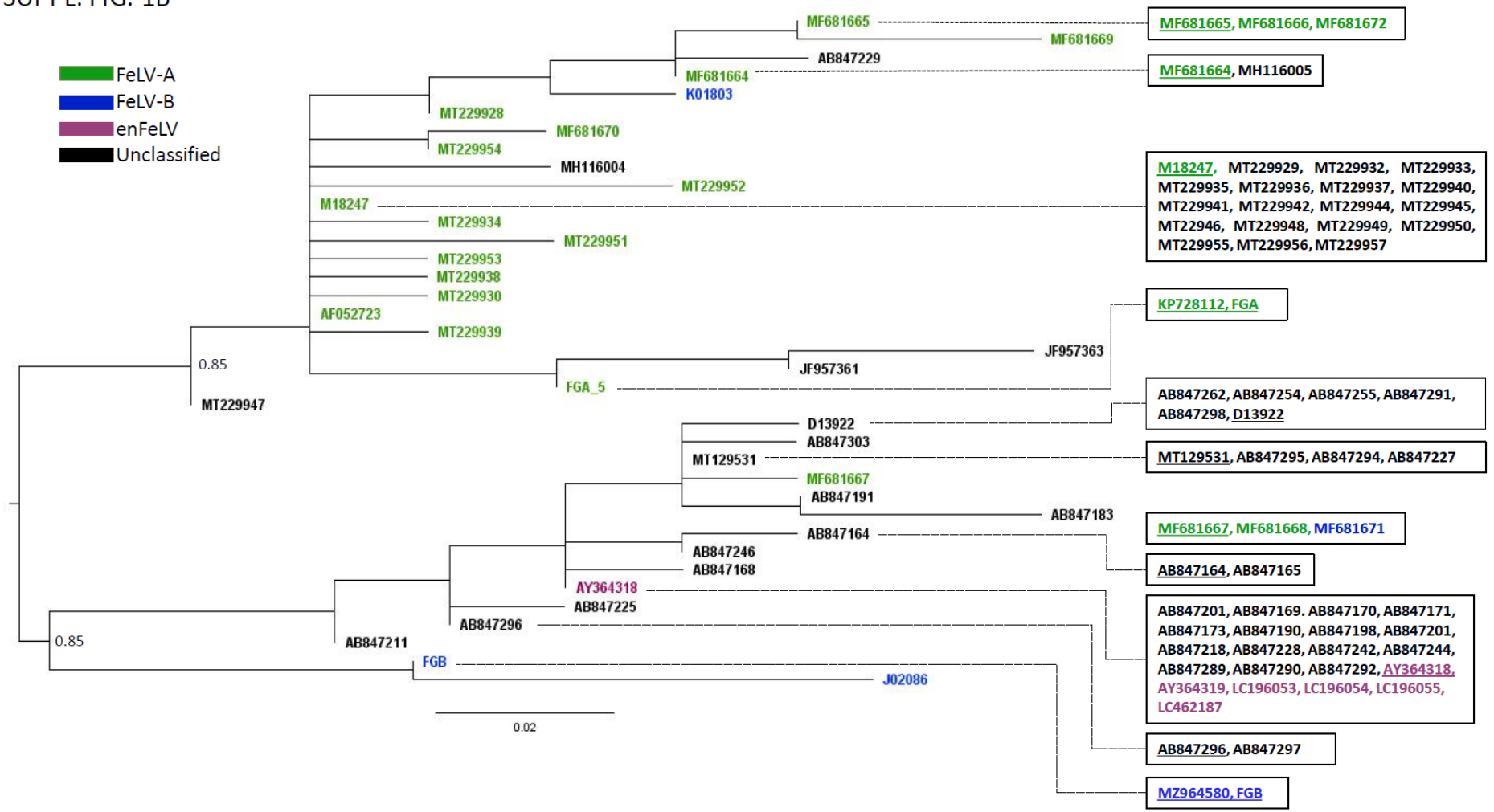
SUPPL. FIG. 1B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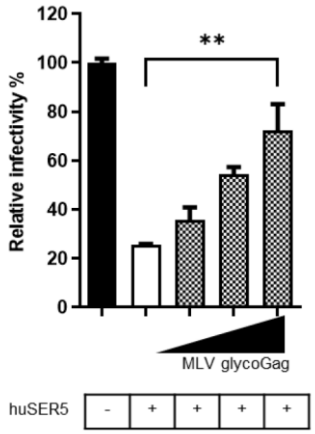
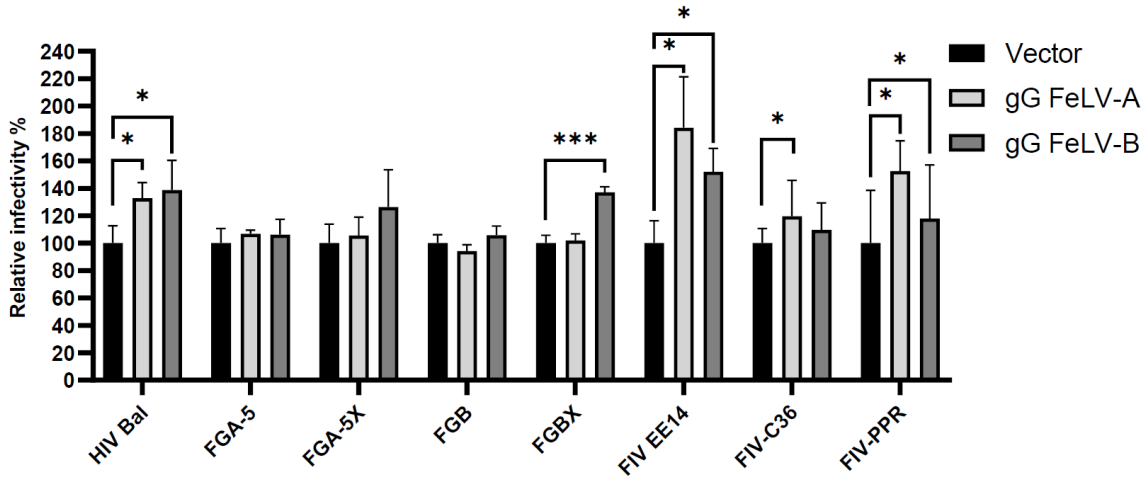
SUPPL. FIG. 2



SUPPL. FIG. 3



11. CONCLUSÕES

- ▷ Poucos grupos de pesquisa trabalham com retrovírus de felinos FIV e FeLV. Estes estudos podem contribuir não só para entender a patogenia dos mesmos, mas de outros retrovírus em humanos.
- ▷ A pesar do FeLV ter uma grande importância na clínica veterinária, os estudos de virologia molecular são limitados. A pouca informação dificulta o entendimento da doença e a sua patogênese.
- ▷ O desfecho clínico difere entre indivíduos (infeções progressivas, regressivas, abortivas). No entanto, ainda se desconhece que determina que uma doença seja severa ou leve. As diferentes apresentações da doença causada pelo FeLV poderiam ser explicadas tanto pela genética do vírus quanto do hospedeiro.
- ▷ Foi desenvolvido um novo método de classificação para FeLV baseado na própria genética do vírus. Este método é mais preciso e confiável e permitirá novos estudos focados nas variantes virais e a sua associação com o curso da doença.
- ▷ O novo método de classificação descrito poderá ser usado em trabalhos de epidemiologia molecular.
- ▷ Existem poucos trabalhos que estudam o efeito dos fatores de restrição para os retrovírus felinos. Este é o primeiro trabalho que descreve e demonstra o efeito de um fator de restrição sobre o FeLV.
- ▷ Foi demonstrado que o FeLV-B é resistente à inibição por SER5 e essa resistência é determinada em grande parte pelo envelope viral. Foi escrita e demonstrada pela primeira vez a presença da proteína glycoGag no FeLV.
- ▷ Diferentes recombinantes apresentam variação na sequência do envelope, isto pode estar relacionado com diferentes graus de resistência à SER5 assim como a outros fatores de restrição.
- ▷ Existem poucos reagentes para trabalhar com FeLV a diferença de HIV e até o próprio FIV. Neste trabalho vários plasmídeos foram construídos. Estes plasmídeos são novas ferramentas que podem ser usadas em biologia molecular em outros trabalhos de pesquisa.