UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

DETECÇÃO E CARACTERIZAÇÃO DE PAPILOMAVÍRUS EM CANINOS E BOVINOS

TESE DE DOUTORADO

CHRISTIAN DINIZ BEDUSCHI TRAVASSOS ALVES

Porto Alegre
2019

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Tese apresentada como requisito parcial para a obtenção do grau de doutor em Ciências Veterinárias

Orientador: Cláudio Wageck Canal
Coorientador: Samuel Paulo Cibulski

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Aprovada em 21 fevereiro 2019.

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Membro da Comissão

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Abreviatura	**Significado**
µL	Microlitro
°C	Graus Celsius
BPV	Papilomavírus bovino
DNA	Ácido desoxirribonucleico
ds	*Double strand* (fita dupla)
dNTP	Desoxinucleosídeo trifosfato
E	*Early* (precoce)
EcPV	Equs caballus papilomavírus
EDTA	Ácido etilenodiaminotetracético sal sódico
HPV	Papillomavirus Humano
HTS	*High Throughput Sequencing* (Sequenciamento de Alto Desempenho)
L	*Late* (tardio)
LCR	*Long control region* (Região longa de controle)
Min	Minutos
mL	Mililitro
ML	Modelo de inferência Maximum Likehood
mM	Milimolar
NGS	*Next generation sequencing* (Sequenciamento de última geração)
nt	Nucleotídeo
ORF	*Open reading frame* (Fase aberta de leitura)
pA	Sítio de poliadenilação
pAE	Sítio de poliadenilação precoce
pAL	Sítio de poliadenilação tardio
PaVE	Papillomavirus episteme
pb	Pares de bases

PBS	Solução salina tamponada com fosfatos
PCR	Reação em cadeia da polimerase
PDGFβR	Receptores β do fator de crescimento derivado de plaquetas
PV	Papilomavírus
RB	Proteína supressora de tumores de retinoblastoma
RCA	*Rolling circle amplification* (Amplificação por círculo rolante)
UV	Ultravioleta

RESUMO

A papilomatose é uma doença causada por um grande grupo de vírus epiteliotrópicos pertencentes a família *Papillomaviridae* que infectam virtualmente todos os animais amniotas. Os papilomavírus são compostos por um capsídeo formado pelas proteínas L1 e L2, do qual abriga uma molécula de DNA dupla fita e circular em seu interior, não contendo envelope lipoproteico. Eles são denominados vírus oncogênicos por causarem lesões benignas e malignas na epiderme e mucosas de seus hospedeiros. Ao longo dos últimos anos, há uma crescente identificação de diversos tipos de papilomavírus causando ampla variedade de lesões tanto em animais domésticos, quanto em animais selvagens. Isso se deve a difusão e maior facilidade ao acesso às ferramentas de sequenciamento convencional e de alto desempenho das quais permitem a identificação, a diferenciação e a quantificação viral. Grande parte das lesões papilomatosas podem ter significativo impacto na saúde animal, e também importantes perdas econômicas diretas e indiretas na pecuária. Esse estudo tem por objetivo compreender a diversidade genética de papilomavírus encontrados em cães e em bovinos leiteiros, provendo a comunidade científica e veterinária uma concisa atualização sobre este campo. Dessa maneira, foi realizada a detecção dos papilomavírus presentes em lesões associadas a papilomas, utilizando análise patológica, ferramentas de biologia molecular, sequenciamento pelo método Sanger e de alto desempenho, permitindo a classificação e a inferência filogenética das sequências obtidas. Este trabalho está dividido em dois capítulos. O primeiro capítulo apresenta o relato de um caso clínico de uma cadela com lesões papilomatosas incomuns que progrediram para uma neoplasia maligna. Foi possível descrever através da caracterização patológica das lesões, utilizando imunohistoquímica e hibridização *in situ*, revelando fortes sinais de associação entre o CPV16 com a neoplasia maligna. Foi possível recuperar o genoma viral presente na lesão, identificar o envolvimento do CPV16, construir uma inferência filogenética e comparar suas oncoproteínas. No segundo capítulo, descrevemos o projeto que teve como objetivo utilizar a estratégia de sequenciamento de alto desempenho afim de identificar coinfecções, possibilitar a montagem completa dos genomas virais, a caracterização e inferência filogenética dos tipos de papilomavírus bovino envolvidos nas lesões de teto de vacas leiteiras. Foi observado 23,5% de coinfecções, destacando a técnica de PCR convencional seguida por sequenciamento SANGER não demonstra a real totalidade de BPVs contidos na lesão. Além disso, foram caracterizados 17 novos prováveis novos tipos de BPVs, dentre eles um novo gênero e uma nova espécie. Devido a diversidade de BPVs encontrada nas lesões não se pode associar algum tipo a determinada localização anatômica. Esse estudo destaca a importância da oncogênese induzida pelo CPV e a diversidade de tipos de BPVs encontrada em tetos de vacas leiteiras, expandindo o atual conhecimento genético da família *Papillomaviridae*.

Palavras-chave: papilomavírus bovino; papilomavírus canino; sequenciamento; caracterização; detecção

ABSTRACT

The papillomatosis is a disease caused by a large group epitheliotropic viruses belonging to the family Papillomaviridae that infect virtually all amniote animals. They are non-enveloped viruses composed of a capsid that is structured by the L1 and L2 proteins, which harbor a circular double-stranded DNA molecule. PVs are oncogenic viruses that cause benign and malignant lesions in the epidermis and mucosa of their hosts. Over the last few years, there is increasing identification of various types of papillomavirus causing wide range of lesions in both domestic and wild animals. This is due to the diffusion and easier access to the conventional and high throughput sequencing tools that allow identification, differentiation and viral quantification. Much of the papillomatous lesions can have a significant impact on animal health, as well as significant direct and indirect economic losses on livestock. This study aims to understand the genetic diversity of papillomavirus found in dogs and dairy cattle, providing the scientific and veterinary community a concise update on this field. Hence, the detection of papillomavirus present in papillomatous-like lesions was performed using molecular biology tools, Sanger and high yield sequencing, allowing the classification and phylogenetic inference of the sequences obtained. This work is divided into two chapters. The first chapter report a clinical case of a female dog with uncommon papillomatous lesions that progressed to a malignant neoplasm. Among them, we were able to describe the associations of a CPV16 strain with invasive SCC, build a phylogenetic inference, identify and compare the oncoprotein genes of the CPV16 strain. In the second chapter, is was applied an unbiased molecular tool for the detection and characterization of BPV in samples regarding teats warts lesion. We observed 23.5% of coinfections, highlighting that PCR followed by sanger sequencing cannot represet the totality of BPVs presents in the sample. Moreover, we described fifteen putative new BPV tipes, among them one new genus and one new species. Due to the diversity of BPVs found in the lesions, no type can be associated with a specific anatomical location. This study highlights the importance of CPV-induced oncogenesis and the diversity of BPV types found in dairy cow teats warts, expanding the current genetic knowledge of the Papillomaviridae family.

Keyworlds: bovine papilomavirus; canine papilomavirus; sequencing; characterization; detection

SUMÁRIO

INTRODUÇÃO

A papilomatose é uma doença infectocontagiosa causada por um grande grupo de vírus epiteliotrópicos altamente diversos que ocorre na maioria dos vertebrados, incluindo as aves, os peixes, répteis e os mamíferos (CAMPO, 1997, 2006; DE VILLIERS et al., 2004; LÓPEZ-BUENO et al., 2016; RECTOR; VAN RANST, 2013). Os papilomavírus (PVs) são transmitidos por contato direto ou indireto através de fômites, devido a propriedade do agente de se manter infectivo no meio ambiente (MUNDAY; THOMSON; LUFF, 2017) A apresentação clínica do PV em seus hospedeiros varia de lesões proliferativas benignas na epiderme, conhecidas popularmente como verrugas, que regridem espontaneamente a partir de uma resposta imune eficiente do hospedeiro, a lesões neoplásicas malignas, as quais geralmente acometem as mucosas, acarretando risco à vida do animal (GIL DA COSTA et al., 2017; MUNDAY; KIUPEL, 2010; SILVESTRE et al., 2009).

As infecções por papilomavírus canino (CPV) costumam ser inaparentes, porém verrugas orais e cutâneas são as manifestações clínicas mais frequentes em cães. A infecção normalmente não resulta em hiperplasia epitelial clinicamente visível, devido a eficiente resposta do sistema imune que evita que as proteínas virais promovam alterações na regulação normal das células epiteliais (EGAWA; DOORBAR, 2017). No entanto, alterações do hospedeiro associadas ao seu estado imunitário, ainda não compreendidas em sua totalidade, podem permitir a maior expressão de proteínas virais. A imunodepressão parece ser um fator importante na predisposição do animal a desenvolver lesões associadas ao CPV e o aumento na frequência de casos associados a placas de pigmentação em cães sugere que fatores genéticos podem influenciar na evolução das lesões papilomatosas (MUNDAY; THOMSON; LUFF, 2017).

Diversos CPVs recentemente identificados e caracterizados estão sendo associados à indução de placas pigmentadas, que podem subsequentemente progredir para carcinomas *in situ* ou invasivos, através de uma imunodepressão causada por fármacos em sua maioria (GOLDSCHMIDT et al., 2006; LUFF et al., 2016; MUNDAY; KIUPEL, 2010). A frequente identificação de novos tipos de CPV e a observação de que alguns tipos virais podem de fato estar associados a lesões malignas, continuam alimentando esta discussão (GIL DA COSTA et al., 2017).

Da mesma forma, o papilomavírus bovino (BPV) causa uma enfermidade crônica, de caráter tumoral que afeta rebanhos bovinos de leite e de corte em todo o mundo, sendo associado a diversas formas de tumores benignos e malignos (BORZACCHIELLO; ROPERTO, 2008). As lesões ocasionadas pela infecção com BPV determinam prejuízos econômicos consideráveis à bovinocultura tanto por perdas diretas, causadas por mortes dos animais, quanto indiretas, representadas por reduções na produtividade e no valor comercial dos animais e subprodutos, como o leite (ALFIERI; LUNARDI; ALFIERI, 2012).

Especialmente a papilomatose de teto, a qual afeta vacas leiteiras do mundo todo, é um problema de saúde negligenciado na produção, resultando em perdas econômicas. A ordenha pode ser dificultada quando os animais apresentam numerosas verrugas no teto, e a ocorrência de ulceração e as rupturas de lesões cutâneas estabelecidas podem predispor à mastite e distorções nos ductos do teto (LUNARDI et al., 2016), além de causar desconforto, dor e sofrimento ao animal. A manutenção de animais com alterações na conformação da glândula mamária, assim como rebanhos com alto número de animais infectados pode resultar em redução do lucros na produção leiteira (BORZACCHIELLO; ROPERTO, 2008; CAMPO, 2002, 2003). Ademais, dentre os PVs que infectam animais de produção, os BPVs são os mais estudados, tanto pela sua relevância clínica, quanto pela utilização como modelo comparativo no estudo da patogênese das lesões induzidas pelos papilomavírus em geral, especialmente os PVs humanos (HPVs) (GIL DA COSTA; MEDEIROS, 2014).

Atualmente, mais de 200 tipos de HPVs foram totalmente sequenciados, caracterizados e catalogados, contrastando com o pequeno número de papilomavírus encontrados em bovinos e caninos (http://pave.niaid.nih.gov). Apesar de ser um agente etiológico importante, a detecção e a caracterização de PVs em animais é ainda deficiente (MUNDAY et al., 2007; RECTOR; VAN RANST, 2013). Esse fato se deve, em parte, às técnicas de detecção moleculares, que utilizam-se de testes baseados no conhecimento prévio de sequências genômicas (FREITAS et al., 2013) e ao pequeno número de estudos em papilomavírus não-humanos.

As técnicas de biologia molecular permitem a detecção, a diferenciação e a quantificação viral, além de identificar variantes virais e novos papilomavírus (BRUM; WEIBLEN, 2012). Além disso, com a difusão e maior facilidade ao acesso a tecnologias de sequenciamento convencional e de última geração (HARISMENDY et al., 2009;

SCHUSTER, 2008), estudos de caracterização genética dos tipos virais podem elucidar questões sobre as lesões induzidas pelo PV, auxiliando no controle e tratamento da papilomatose (DOORBAR et al., 2016).

O presente estudo teve por objetivo contribuir acerca do compreendimento da diversidade genética de papilomavírus encontrados em cães e em bovinos leiteiros. Através de métodos moleculares e patológicos foi possível detectar, caracterizar e inferir filogeneticamente os papilomavírus presentes nas lesões desses animais. Baseado na perspectiva tanto do bem-estar animal, quanto da produção animal, o estudo teve o intuito de prover novos conhecimentos para estabelecimento de estratégias de controle e tratamento desta enfermidade.

1 REVISÃO BIBLIOGRÁFICA

1.1 Histórico

A ocorrência de papilomas em humanos é descrita há séculos e está presente em relatos de origem grega e romana. Evidentemente, de acordo com o conhecimento científico da época, não se sabia a etiologia das lesões, fato é que verrugas genitais eram consideradas como apresentações distintas de sífilis e/ou gonorreia. A origem viral das lesões verrucosas foi demonstrada no começo do século XX por Ciuffo (1907), ao inocular um filtrado de células retiradas diretamente da lesão verrucosa em outro indivíduo, havendo assim a reprodução da doença.

Em 1898, M'Fadycan e Hobday relataram a etiologia infecciosa do papilomavírus oral canino (COPV) (apud ROUS; BEARD, 1935). No entanto, o primeiro papilomavírus em animal foi identificado nos anos 30, por Richard Shope, ao caracterizar a natureza da transmissão do papiloma cutâneo em uma espécie de coelho selvagem (do inglês *cottontail rabbit*, gênero *Sylvilagus*). Portanto, o *cottontail rabbit papillomavirus* (CRPV) foi o primeiro vírus DNA oncogênico identificado (apud NICHOLLS; STANLEY, 2000) e um importante modelo para estudos pioneiros sobre oncogênese viral. Porém, esse modelo de estudo oncogênico foi substituído pela descoberta dos poliomavírus, que poderiam ser replicados em cultivos de monocamada celular e ainda induziam transformações

morfológicas *in vitro*, em contraste ao CRPV cujos estudos somente poderiam ser feitos em cobaias (HOWLEY; SCHILLER; LOWY, 2013). Os estudos com os PVs foram menos intensos nas décadas de 50 e 60, no entanto, o advento da tecnologia do DNA recombinante e clonagem gênica na década de 70, permitiu aos pesquisadores "contornar" a impossibilidade de replicar os PVs *in vitro* (HOWLEY; SCHILLER; LOWY, 2013).

Historicamente, os poliomavírus e PVs formavam uma única família denominada *Papovaviridae*, cujo nome é derivado das iniciais de seus três membros (*Papillomavirus, Polyomavirus e Simian Vacuolating Agent – SV40*). Essa classificação se deu devido a semelhanças nas propriedades estruturais como tamanho e forma do vírion, ausência de envelope e genoma constituído por DNA fita dupla circular. A família *Papoviridae* incluía dois gêneros, distintos apenas com base no diâmetro médio dos vírions: os *Polyomavirus*, com as espécies poliomavírus e o SV-40, e o *Papillomavirus*. No entanto, o 7° Comitê Internacional de Taxonomia Viral (ICTV) dividiu a família *Papovaridae* nas famílias *Papillomaviridae* e *Polyomaviridae*, de acordo com estudos moleculares comparativos que indicaram diferenças fundamentais entre eles, destacando-se o tamanho e a organização genômica, na qual, praticamente, não são observadas similaridades na sequência de nucleotídeos entre estas famílias (VAN REGERNMORTEL et al., 2000).

1.2 Estrutura dos virions

Os papilomavírus são vírus pequenos, com 55 a 60 nm de diâmetro, desprovidos de envelope lipoprotéico, apresentando simetria icosaédrica (BORZACCHIELLO; ROPERTO, 2008) (Figura 1). Cada capsômero é composto por duas proteínas denominadas *late protein 1* e *late protein 2* (L1 e L2, respectivamente) (PFISTER; ZUR HAUSEN, 1978).

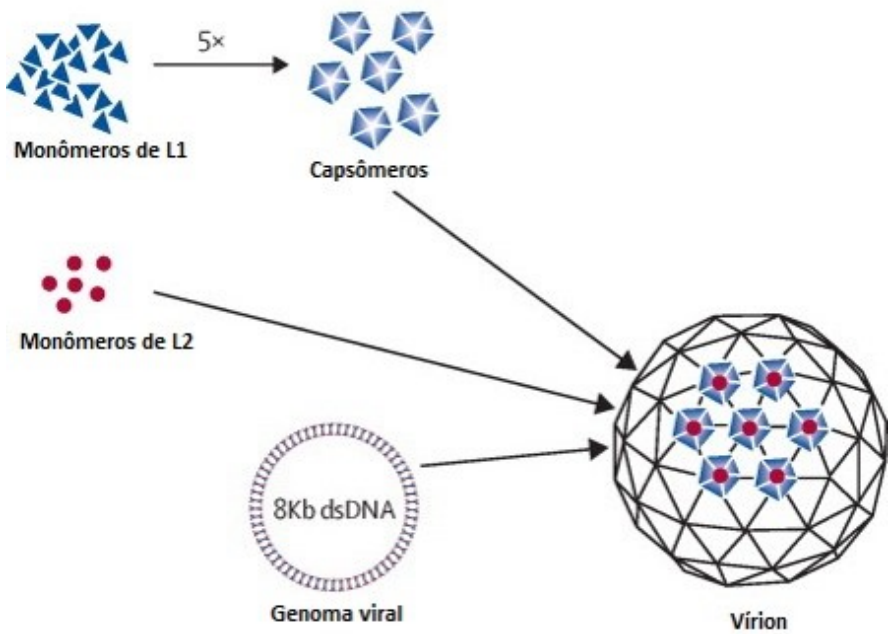

Figura 1. Esquema da organização do capsídeo viral e suas proteínas. Adaptado de Schiller e Müller (2015).

O capsídeo é composto por 72 capsômeros originando o aspecto arredondado do vírion observado por microscopia eletrônica (ME) (Figura 2). A partícula viral é resistente às condições ambientais e a solventes lipídicos, fator esse importante na transmissão do vírus.

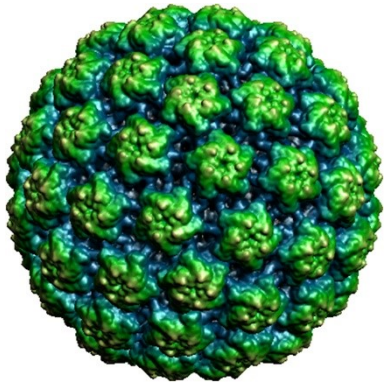

Figura 2. Microscopia eletrônica da superfície a partícula viral de um papilomavírus bovino tipo 1 (WOLF et al., 2010).

1.3 Organização genômica

O genoma dos PVs é composto por uma molécula de DNA fita dupla circular, de aproximadamente 7,3 a 8 kpb (RECTOR; VAN RANST, 2013). Contido dentro do capsídeo está o genoma dos PVs conjugado com histonas, formando um complexo semelhante à cromatina (DE VILLIERS et al., 2004). Todos os genótipos de PV seguem um mesmo padrão de organização genômica, com somente uma fita codificante e apresentam fases abertas de leitura (*open reading frame* - ORFs) sobrepostas e aninhadas (ALFIERI; LUNARDI; ALFIERI, 2012; LAZARCZYK et al., 2009). A arquitetura do genoma dos PVs é dividida em três diferentes regiões: a região longa de controle (*long control region* - LCR), que contém os elementos necessários para replicação e transcrição do DNA viral, e duas regiões que contém as ORFs, que correspondem aos genes precoces (*early* - E) e tardios (*late* - L) (BORZACCHIELLO; ROPERTO, 2008; VAN DOORSLAER et al., 2013). As três regiões são separadas por dois sítios de poliadenilação (pA), o sítio da região precoce (pA_E) e o sítio da região tardia (pA_L) (ZENG e BAKER, 2006).

A arquitetura genômica típica dos PVs utiliza o BPV1 como protótipo (RECTOR; VAN RANST, 2013). O BPV1 contém no segmento E (*early genes*) até seis ORFs, e o segmento L (*late genes*) contém duas ORFs. Os genes precoces (*E1, E2, E4, E5, E6 e E7*) codificam as proteínas (que recebem os mesmos nomes dos genes) envolvidas tanto na replicação e transcrição do DNA viral quanto na transformação celular (CORTEGGIO et al., 2013; LAMBERT, 1991; VENUTI et al., 2011); por outro lado, os genes tardios (*L1* e *L2*) codificam as proteínas do capsídeo viral L1 e L2. Há um elevado grau de conservação entre os genomas, porém estudos apontam organizações genômicas divergentes entre os membros da família *Papillomaviridae*, e somente o *core* das ORFs E1, E2, L2 e L1 está presente em todos seus membros já descritos (RECTOR; VAN RANST, 2013). A Figura 3 mostra um esquema da organização do genoma do BPV1.

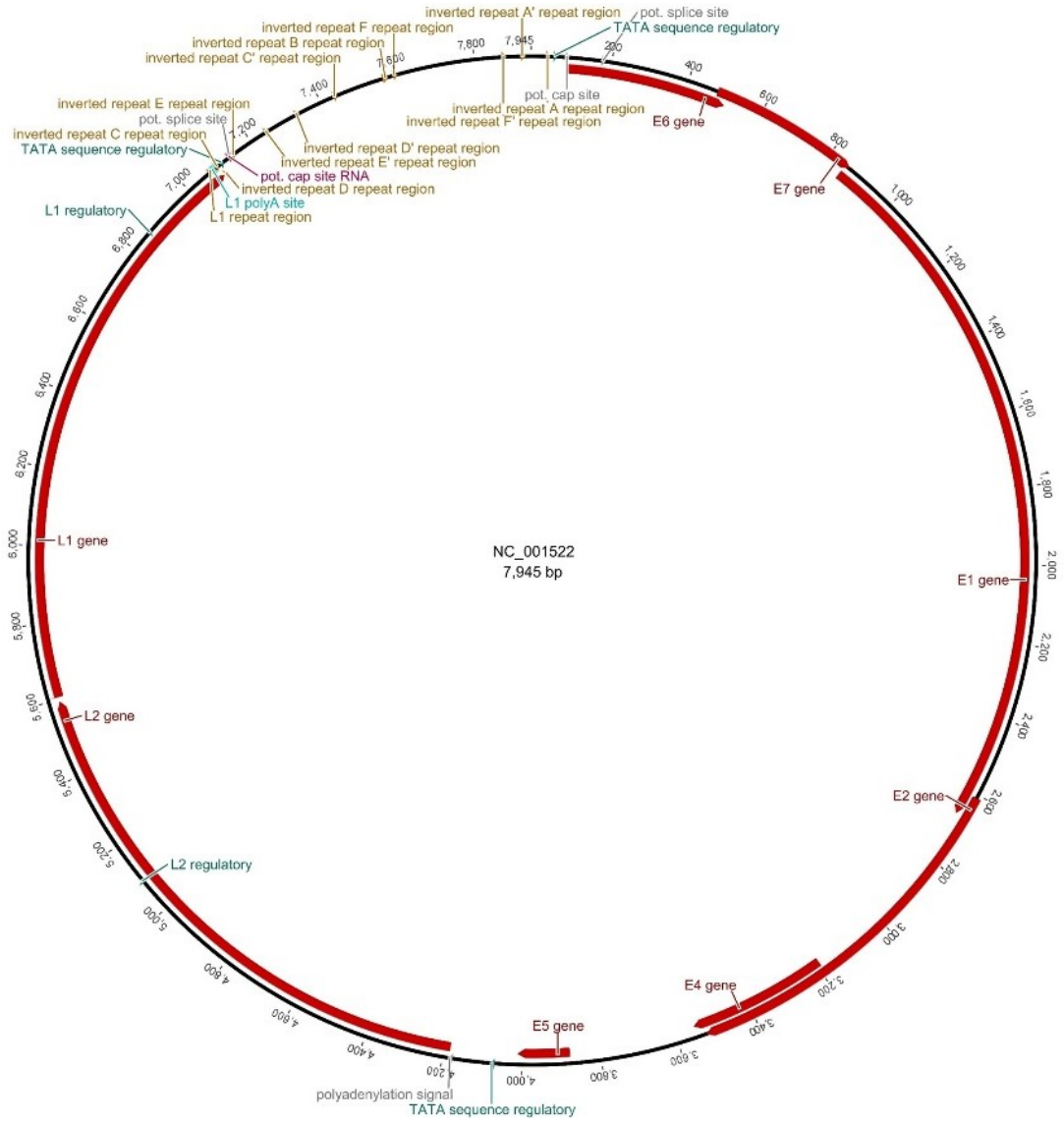

Figura 3. Representação esquemática da organização do genoma do BPV1. A figura da organização genômica foi montada com o programa Geneious (versão 8.1) (número de acesso no GenBank NC_001522).

A LCR é uma região não-codificante do genoma, com aproximadamente 500-1000 nucleotídeos (nt), localizada a montante da região de transcrição precoce e compreende aproximadamente 10 a 15% do genoma viral (LAZARCZYK et al., 2009). Esta possui elementos regulatórios para a replicação viral e controla os genes de transcrição e transformação celular nos PVs. Genericamente as LCRs possuem organizações similares: uma região promotora, uma região potencializadora e um ou mais sítios de ligação altamente conservados para a proteína E2 (E2 *binding-sites* - E2BS) (DESAINTES; DEMERET, 1996;

ZHENG; BAKER, 2006). As posições dos E2BS nos diferentes genomas podem influenciar nas características da regulação da expressão do genoma viral (LAZARCZYK et al., 2009).

1.4 Proteínas virais

As proteínas dos BPVs são divididas em tardias (L1 e L2) e precoces (E1, E2, E4, E5, E6, E7 e E8). As proteínas L1 e L2 são estruturais, compõem o capsídeo viral e as oncoproteínas E5, E6 e E7, modulam o processo de transformação celular (MÜNGER; HOWLEY, 2002). Teoricamente, as proteínas E1, E2, L1 e L2 poderiam realizar sozinhas as tarefas básicas de replicação, regulação, estabilização e empacotamento do DNA viral, levando à liberação dos vírions (BRAVO; FÉLEZ-SÁNCHEZ, 2015).

As proteínas E1 e E2 são expressas logo após a infecção pelo PV, uma vez que suas funções são essenciais para a replicação viral (TEREZA et al., 2011). A ORF correspondente à proteína E1 é a segunda mais conservada dentre as demais, muito pelo seu papel essencial na replicação do PV. Para isso, três domínios funcionais atuam de forma a iniciar o processo induzindo a fosforilação da proteína quinase CdK2 (domínio N-terminal), promover a abertura da dupla hélice do DNA viral (domínio C-terminal) e formar o complexo proteico E1/E2 (WALLACE; GALLOWAY, 2014). Por sua vez o complexo proteico E1/E2 recruta proteínas responsáveis pela replicação viral, tais como topoisomerase I, DNA polimerase I e proteína de replicação A ao se ligar ao domínio presente na LCR denominado *ori* (ENEMARK et al., 2000; SCHUCK; STENLUND, 2015).

A proteína E2 é considerada uma importante moduladora da expressão proteica celular e viral (conhecida como reguladora epigenética), por causa de sua função inibitória no reparo a danos do DNA e de sua função regulatória da expressão das proteínas ditas oncogênicas dos PVs (E6 e E7) (ARALDI et al., 2017). Em altos níveis de expressão, a E2 inibe a ligação da RNA polimerase II, reprimindo a transcrição das proteínas E6 e E7 (CAI et al., 2013); em baixos níveis de expressão, a E2 recruta fatores de transcrição, formando um livre complexo de transcrição (GARCÍA-VALLVÉ; ALONSO; BRAVO, 2005). O domínio C-terminal da proteína E2 tem como função a distribuição equitativa das cópias virais após a citocinese, por interagir com resíduos de lisina das histonas acetiladas (WALLACE; GALLOWAY, 2014). A acetilação dos resíduos de lisina propicia o acesso de

fatores de transcrição e da RNA polimerase II a região codificadora por promover menor afinidade entre o DNA e as histonas, removendo as cargas positivas da porção N-terminal do componente proteico das histonas. Consequentemente, a acetilação estimula a transcrição; enquanto a deacetilação a inibe.

A proteína BPV E5 é capaz de ligar-se aos receptores β do fator de crescimento derivado de plaquetas (PDGFβR) e conduzir a transformação das células (BORZACCHIELLO et al., 2006). A proteína expressa pelo gene E7 associa-se fortemente à proteína supressora de tumores de retinoblastoma (RB), inativando-a e, por fim, estimulando o ciclo celular, sendo a E7 a principal proteína oncogênica viral (SHAI et al., 2007; ZUR HAUSEN, 2009). Adicionalmente, a oncoproteína E6 promove a transformação neoplásica por dois mecanismos: ativando a telomerase e inativando a proteína 53 (p53), desregulando o ciclo celular (SHAI et al., 2007; ZUR HAUSEN, 2009). Essas diferenças são, por um lado, críticas para a compreensão das especificidades das lesões induzidas pelo PVs e, por outro lado, uma oportunidade para obter conhecimentos sobre a fisiologia celular em geral (GIL DA COSTA et al., 2017). Em resumo a figura 4 apresenta a expressão diferencial das proteínas.

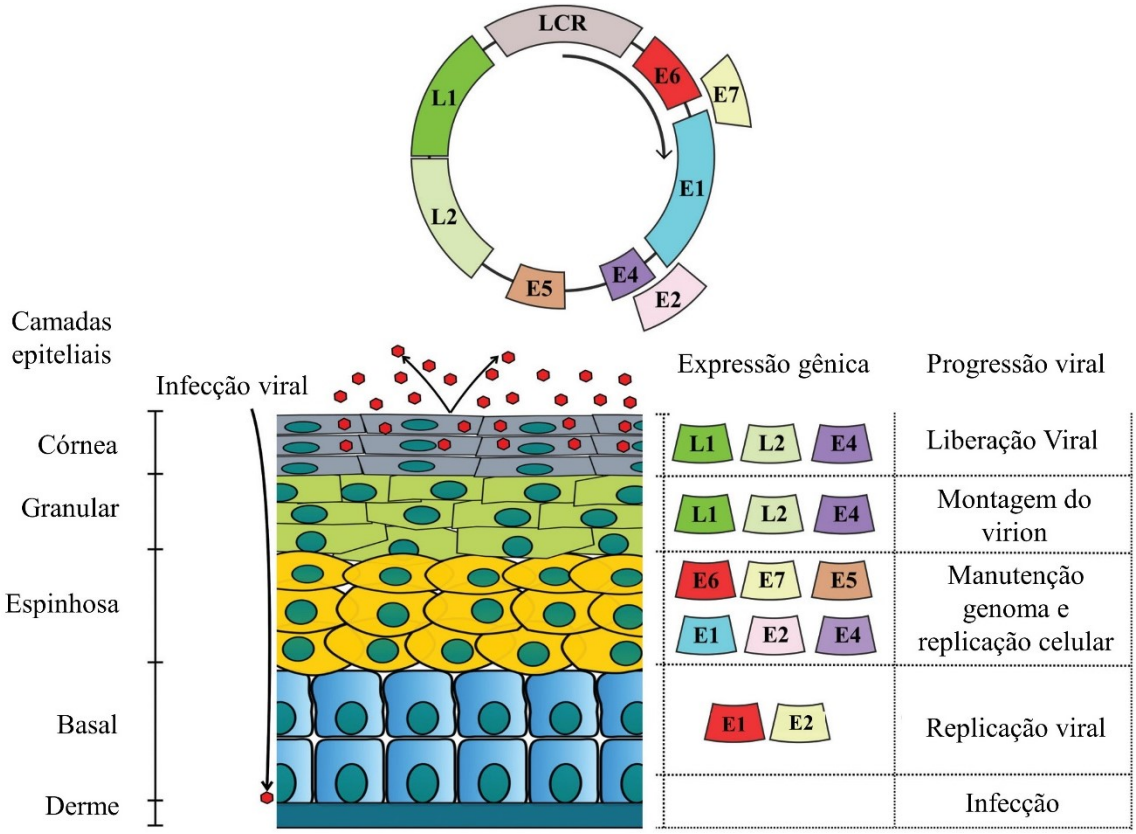

Figura 4. Organização do genoma de BPV e expressão diferencial de proteínas: o genoma DNA circular de cadeia dupla é dividido em região precoce (E), tardia (L) e na região longa de controle (LCR). A região inicial codifica as proteínas de replicação e oncoproteínas (E1, E2, E4, E5, E6 e E7). Essas proteínas são expressas da camada basal para a camada cornificada, estando envolvidas com a replicação viral e liberação de vírion (E4). A região tardia codifica as proteínas do capsídeo L1 e L2. Uma vez que essas proteínas estruturais estão envolvidas na montagem do vírus, elas são expressas nas camadas do epitélio mais diferenciadas (granular e cornea). Retirados de Araldi (2017).

1.5 Classificação

Esta grande família viral é formada por vírus filogeneticamente classificados em 49 gêneros e composta de diversas espécies, tipos, subtipos e variantes (http://ictvonline.org/index.asp) (Figura 5). Atualmente, a sequência de nucleotídeos do gene L1 é utilizada para a classificação dos PVs, por ser o gene mais conservado do genoma (BERNARD et al., 2010; DE VILLIERS et al., 2004). Um novo tipo de PV é considerado quando o seu genoma completo é totalmente sequenciado e a ORF L1 difere mais de 10% dos tipos de PVs já descritos. Mesmas espécies de PV compartilham entre 71% e 89% de

identidade de nucleotídeos nesta mesma ORF. As similaridades menores que 60% definem um novo gênero. Um subtipo é definido quando a diferença entre a ORF L1 é entre 2% e 10%, e uma variante viral ocorre quando esta diferença é menor do que 2% (BERNARD et al., 2010; DE VILLIERS et al., 2004).

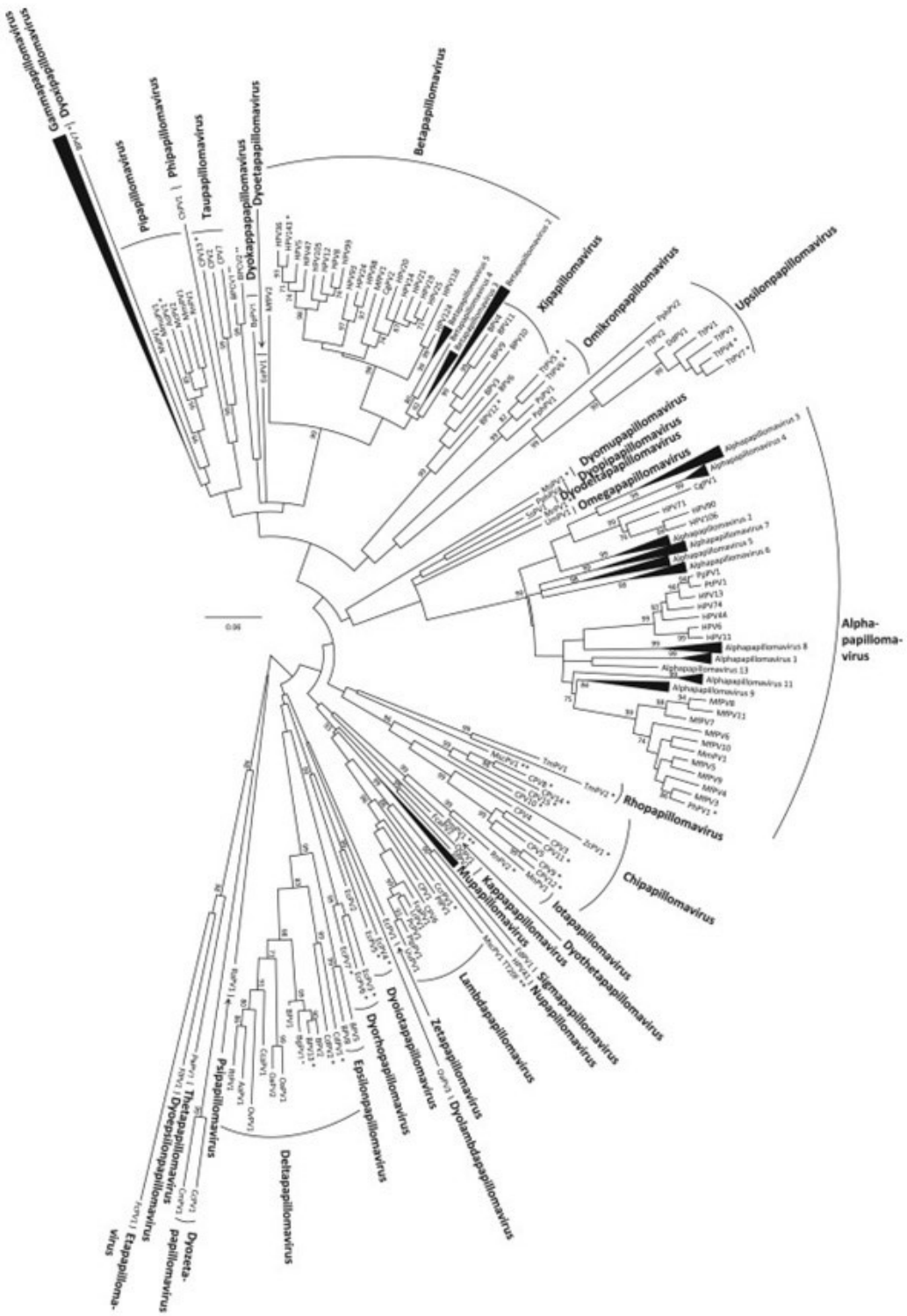

Figura 5. Relações filogenéticas entre todos os tipos de papilomavírus estabelecidos, demonstrando sua classificação. A árvore filogenética é baseada num alinhamento de sequência de nucleótideos L1 de todos os tipos de papilomavírus caracterizados (os 112 papilomavírus que não infectam humano). Retirado de Rector e Van Ranst (2013).

1.5.1 Papilomavírus bovino

Atualmente, os BPVs estão classificados em cinco gêneros, cinco espécies e 24 tipos. O gênero *Deltapapillomavirus* é constituído pela espécie *Deltapapillomavirus 4* (compreendendo o BPV1, 2, 13 e 14); *Epsilonpapillomavirus* pela espécie *Epsilonapapillomavirus 1* (BPV5 e BPV8); *Dyoxipapillomavirus* pela espécie *Dyoxipapillomavirus 1* (BPV7); o gênero *Xipapillomavirus* pelas espécies *Xipapillomavirus 1* (BPV3, 4, 6, 9, 10, 11, e 15), *Xipapillomavirus 2* (BPV12) e pelos BPVs tipo 17, 20, 23 e 24; por fim, o gênero *Dyokapapillomavirus* formado pelos tipos BPV16, 18 e 22. Os BPVs 19 e 21, recentemente descobertos, ainda não foram classificados (http://pave.niaid.nih.gov) (Tabela 1). A Figura 6 mostra a atual disposição dos grupos de BPV dentre os gêneros de papilomavírus descritos. Além disso, novos tipos de PVs estão sendo descobertos através do emprego de técnicas moleculares que facilitam a detecção e caracterização filogenética da diversidade viral encontrada entre os papilomavírus que infectam bovinos (DA SILVA et al., 2016).

Tabela 1. Esquema da classificação das espécies e tipos de papilomavírus bovino dentro dos respectivos gêneros. * Representa os tipos ainda não classificados dentro de uma espécie.

Gênero	**Espécie**	**Tipos de BPV**
Deltapapillomavirus	*Deltapapillomavirus 4*	1, 2, 13 e 14
Xipapillomavirus	*Xipapillomavirus 1*	3, 4, 6, 9, 10, 11 e 15
	Xipapillomavirus 2	12
	Xipapillomavirus *	17, 20, 23 e 24
Epsilonpapillomavirus	*Epsilonpapillomavirus 1*	5, 8
Dyokapapillomavirus	*Dyokapapillomavirus**	16, 18 e 22
Dyoxipapillomavirus	*Dyoxipapillomavirus 1*	7
*	*	19 e 21

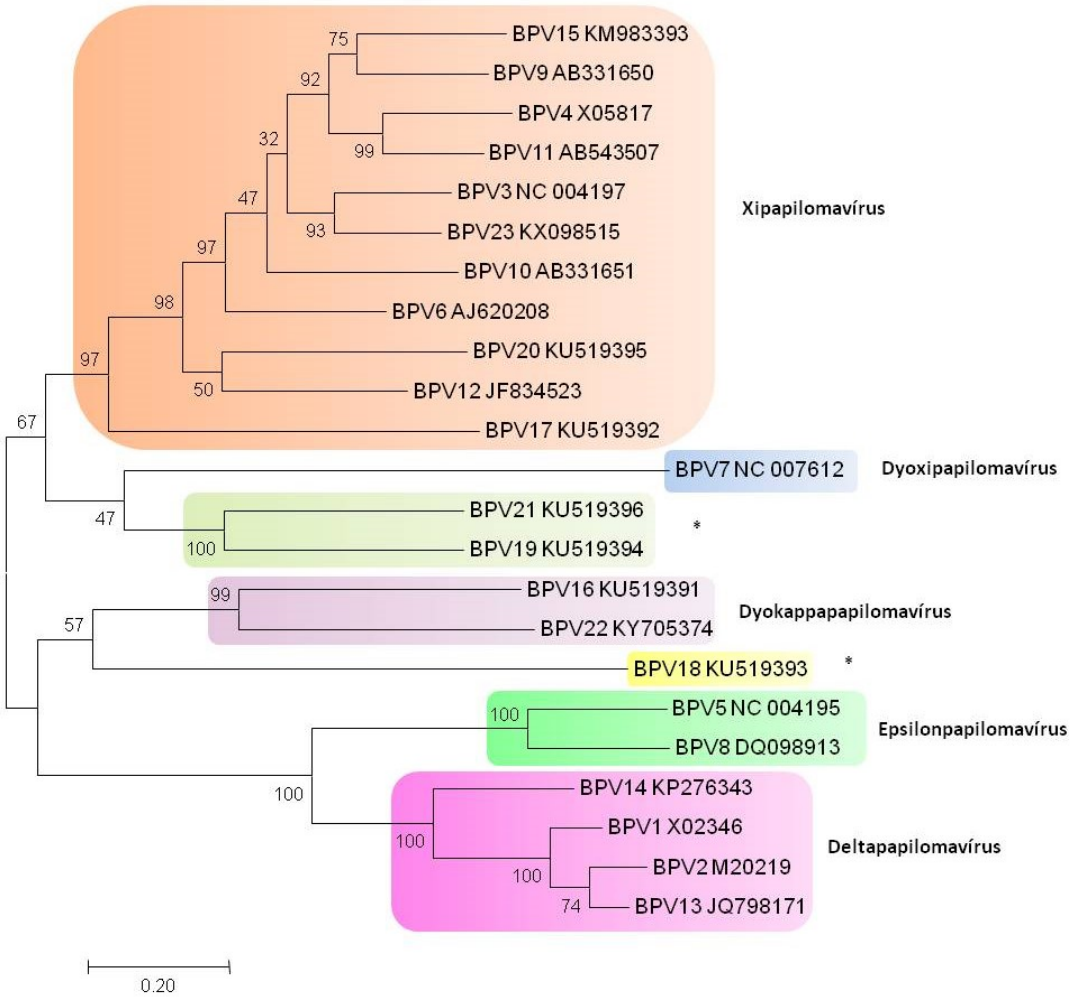

Figura 6. Disposição filogenética dos grupos de papilomavírus bovino utilizando a ORF L1 completa de cada representante de BPV. Gêneros ainda não classificados estão representados por *. Retirado de Chaves (2017)

1.5.2 Papilomavírus canino

Atualmente, 20 papilomavírus caninos (CPVs) já foram reconhecidos de acordo com a base de dados *Papillomavirus* Episteme (PaVE) (https://pave.niaid.nih.gov). Os CPVs são organizados entre três gêneros distintos (Figura 7). Os tipos 1 e 6 são classificados dentro do gênero *Lambdapapillomavirus*; CPV 2, 7, 13, 17 e 19 dentro do *Taupapillomavirus*; e o gênero *Chipapillomavirus* é composto pelos demais tipos (CPVs 3, 4, 5, 8, 9, 10, 11, 12, 14, 15, 16, 18 e 20) (Tabela 2).

Tabela 2. Esquema da classificação das espécies e tipos de papilomavírus canino dentro dos respectivos gêneros. * Representa os tipos ainda não classificados dentro de uma espécie.

Gênero	Espécie	Tipos de CPV
Lambdapapillomavirus	*Lambdapapillomavirus 2*	1
	Lambdapapillomavirus 3	6
Taupapillomavirus	*Taupapillomavirus **	17 e 19
	Taupapillomavirus 1	2 e 7
	Taupapillomavirus 2	13
Chipapillomavirus	*Chipapillomavirus **	18 e 20
	Chipapillomavirus 1	3, 5, 9, 11 e 12
	Chipapillomavirus 2	4 e 16
	Chipapillomavirus 3	8, 10, 14 e 15

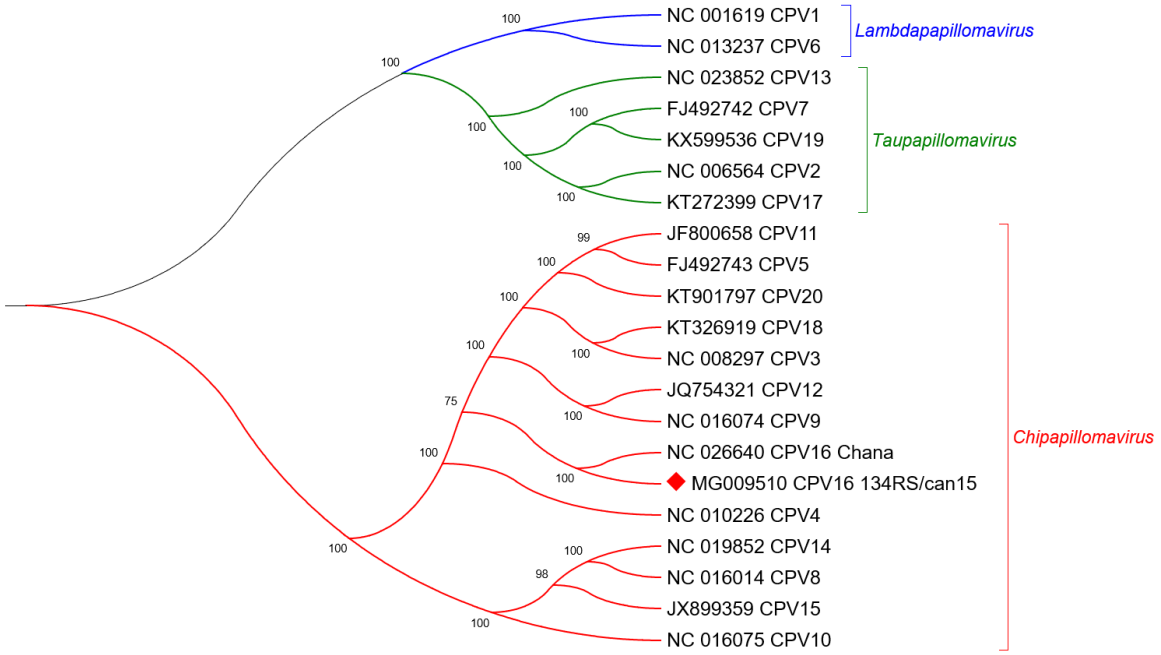

Figura 7. Disposição filogenética dos grupos de papilomavírus canino utilizando a ORF L1 completa de cada representante de CPV. Retirado de Alves colaboradores (em publicação).

1.6 Epidemiologia

As papilomatoses tem distribuição mundial e, embora a mortalidade seja baixa, lesões de caráter maligno podem levar o animal a óbito. O vírus se dissemina por contato direto ou indireto, sexual, fômites (agulhas, brincadores e outros aparelhos contaminados), instalações e, possivelmente, insetos (CAMPO et al., 1994; FINLAY et al., 2009; REID et al., 1994).

Diversos tipos de papilomavírus caninos foram relatados e associados a uma série de lesões de mucosas e/ou cutâneas. No entanto, existem dúvidas quanto a gama de lesões (principalmente benignas) atribuíveis a cada tipo viral (GIL DA COSTA et al., 2017). Em geral, acredita-se que o CPV1 é associado a papilomatoses orais (PORCELLATO et al., 2014; SANCAK et al., 2015) (Figura 8), em conjunto com o CPV13 (LANGE et al., 2009) que está presente em uma gama de lesões conjuntivais (BRANDES et al., 2009). Porém, a maioria dos papilomas orais não progridem para tumores malignos e raramente seu DNA é encontrado em carcinomas, tipo de câncer comum na epiderme (GIL DA COSTA et al., 2017).

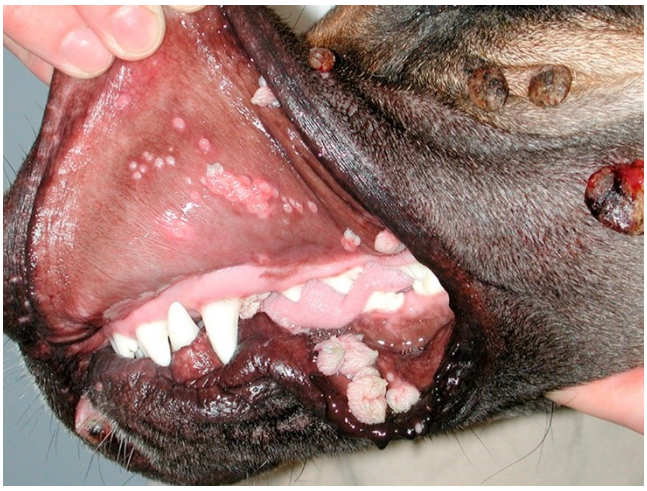

Figura 8. Papilomatose oral em um cão. Esta doença é caracterizada pela presença de numerosos crescimentos vegetativos exofíticos envolvendo os lábios e a boca. Este cão também tem papilomas cutâneos envolvendo a pele ao redor da boca (fotografia gentilmente cedida pelo Dr. Stephen White, da University of California Davis, Califórnia, EUA). Retirado de Munday, Thomson and Luff (2017).

Os CPVs pertencentes ao gênero *Chi* são largamente associados a placas pigmentadas, porém poucos tipos são identificados em lesões malignas (LUFF et al., 2015, 2016; TOBLER et al., 2006). Especula-se que algumas raças possam ser predispostas à formação de placas pigmentadas (Figura 9), causada pela infecção do CPV (NAGATA et al., 1995; TOBLER et al., 2008), enquanto o desenvolvimento da mesma lesão em outras raças tem sido restrita a cães imunodeprimidos (CALLAN; PREZIOSI; MAULDIN, 2005; STOKKING et al., 2004). Além disso, os recentemente identificados CPV12, 16 e 18 (LUFF et al., 2016), como também três dos quatros CPVs pertencentes ao gênero *Tau,* e por fim, o CPV2 foram reportados em casos de placas de pigmentação que se transformaram em lesões malignas (GOLDSCHMIDT et al., 2006; YUAN et al., 2007). Devido à escassez de artigos identificando associações entre os CPVs e as lesões cancerígenas, não se pode determinar quais tipos seriam mais prevalentes e, ainda, quais tipos causam quais lesões (GIL DA COSTA et al., 2017).

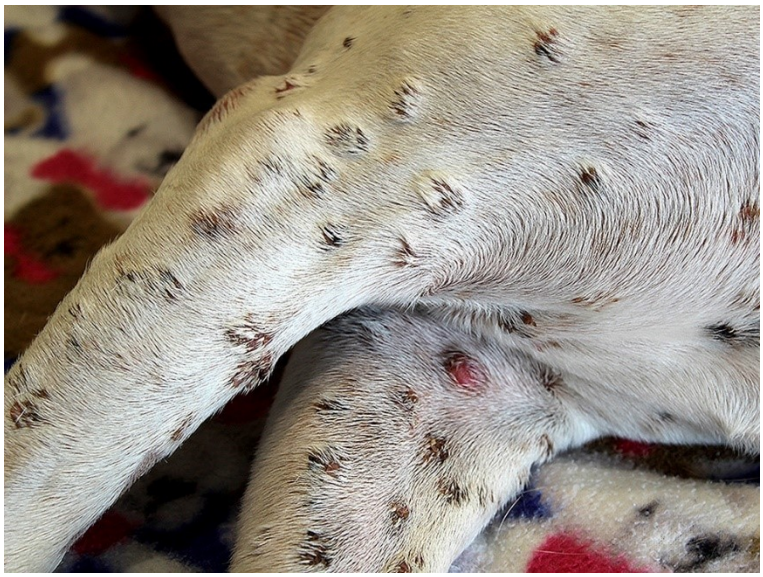

Figura 9. Placas pigmentadas em um cão. As placas são escuras e estão cobertas por uma camada de queratina (fotografia cortesia do Dr. Mark Turnwald, Clínica Veterinária Belmont, North Shore City, New Zealand). Retirado de Munday, Thomson and Luff (2017)

Em bovinos, os papilomavírus bovinos induzem lesões benignas em diversas localizações anatômicas da pele, verrugas no úbere, prepúcio e vulva e lesões malignas na mucosa da bexiga urinária e do tubo digestivo superior (CAMPO, 2002; ELZEIN et al., 1991; WOSIACKI et al., 2006) (Figura 10). Especificamente na bovinocultura leiteira, a ordenha pode tornar-se difícil devido às lesões papilomatosas no teto e manter as vacas afetadas nos rebanhos pode diminuir o lucro econômico na indústria de laticínios (LUNARDI et al., 2016) (Figura 11).

Figura 10. Papilomatose em um bovino. Lesões exofíticas nas regiões da cabeça, tabua do pescoço e cernelha. Retirado do banco de dados do site http://pave.niaid.nih.gov (BAKER, [s.d.]).

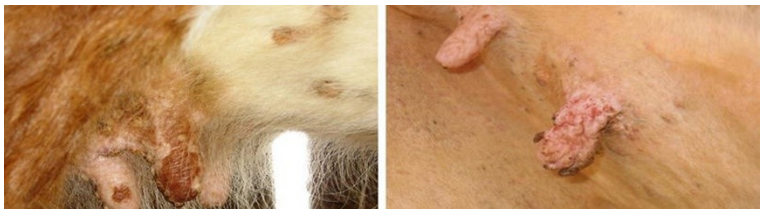

Figura 11. Papilomatose de teto em um bovino. Lesõe de padrão exofítico no teto uma vaca. Adaptado de Lunardi e colaboradores (2016).

Os tipos de BPV que estão classicamente relacionados com a infecção de tetos e úbere de vacas são BPV1, 5 e 6. No entanto, as investigações visando a detecção e genotipagem de novos tipos de BPV envolvidos como agentes causadores da papilomatose de teto revelaram uma alta diversidade de tipos virais associados a essas lesões (LUNARDI et al., 2016). Com base nestes achados epidemiológicos, pode-se concluir que estabelecer uma relação entre os tipos de BPV e o tropismo anatômico para verrugas cutâneas de bovinos é difícil e pouco

confiável, e que esses tipos virais podem não estar restritos à localização anatômica específica no gado (BATISTA et al., 2013; CLAUS et al., 2007, 2009; MAEDA et al., 2007; OGAWA et al., 2004).

1.7 Patogenia e sinais clínicos

1.7.1 Biologia da papilomatose

Atualmente, considera-se que, em geral, as infecções por PVs são limitadas ao epitélio estratificado mucocutâneo, sendo seu ciclo de replicação viral dependente e intimamente coordenado pela replicação e diferenciação de células da epiderme (DOORBAR, 2005; DOORBAR et al., 2012; MUNDAY, 2014). Acredita-se que, para a infecção ser estabelecida, é necessária a ocorrência de um microtrauma ou erosão da epiderme para que o vírus possa infectar o epitélio basal (DOORBAR et al., 2012; SCHILLER; DAY; KINES, 2010).

Uma vez que o PV infecta as células basais, o genoma viral é incorporado ao núcleo celular. Após a célula completar um ciclo mitótico, o vírus inicia a expressão de proteínas E1 e E2, resultando na produção de 10 a 200 cópias do genoma viral, que permanecem no núcleo celular como um cromossomo episssomal, não integrado ao genoma celular (BRAVO; FÉLEZ-SÁNCHEZ, 2015; DOORBAR et al., 2012). A replicação das células basais mantém a infecção, contudo, a diferenciação terminal e a queratinização das células infectadas é necessária para uma infecção produtiva (DOORBAR, 2006). A diferenciação celular desencadeia a expressão das proteínas virais precoces, que impedem as células suprabasais de deixar o ciclo celular (o que resultaria em degeneração nuclear) e, ao invés disso, faz com que as células reentrem na fase S do ciclo celular e produzam numerosas cópias virais (DOORBAR et al., 2012). As proteínas E6, E7 e, predominantemente, a proteína E5, no caso dos delta-BPVs, parecem mediar esse processo de proliferação das células das camadas basais e parabasais (BOHL; HULL; VANDE POL, 2001; DOORBAR et al., 2012; SILVA et al., 2013). A liberação do vírion ocorre durante a descamação das células infectadas da superfície das lesões (DOORBAR, 2006). Um esquema da infecção por HPV é ilustrado na figura 12.

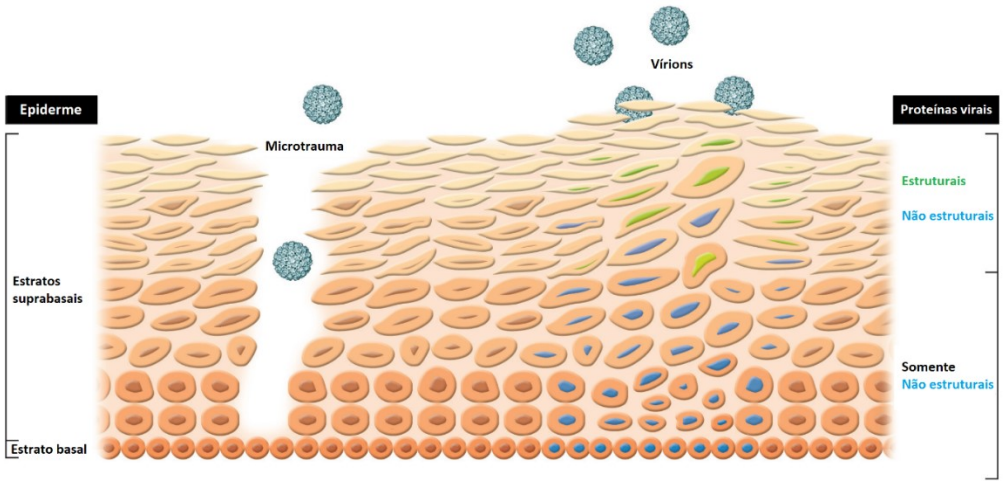

Figura 12. Representação esquemática do ciclo replicativo do papilomavírus. Adaptado de Lowry and Schiller (2006)

1.7.2 Papilomatose de teto bovino

Os agentes etiológicos das lesões de papiloma encontradas nos tetos e úbere consistem nos diversos tipos de BPVs, porém o BPV1, 5 e 6 têm sido frequentemente identificados nessas localizações anatômicas. As lesões cutâneas se caracterizam por serem exofíticas e histologicamente classificadas como neoplasias de células escamosas benignas (papiloma cutâneo) e fibropapilomas, independentemente do tipo viral identificado (Figuras 13 e 14) (CAMPO, 2002; LINDHOLM et al., 1984).

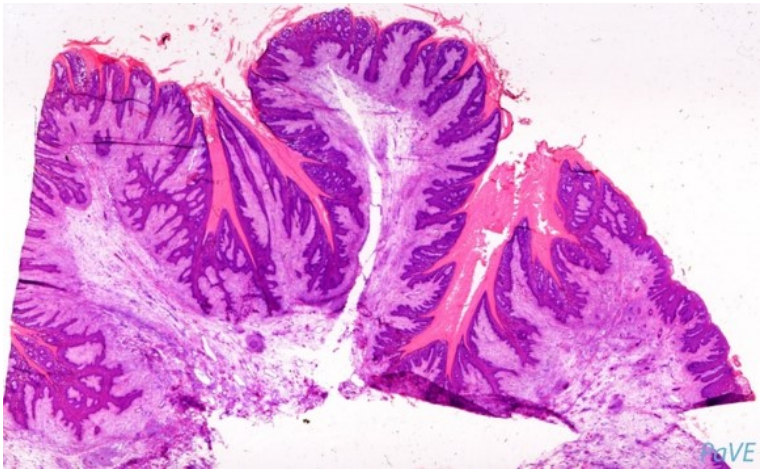

Figura 13. Corte histológico de um fibropapiloma bovino, coloração HE. Vírus do gênero delta, como o BPV1, causam fibropapilomas em ungulados. O vírus infecta e replica em fibroblastos dérmicos causando extensa proliferação e um grande fibroma subjacente ao epitélio. O epitélio apresenta acantose, coilocitose, paraqueratose e hiperqueratose. Retirado do banco de dados do site http://pave.niaid.nih.gov (BAKER, [s.d.]).

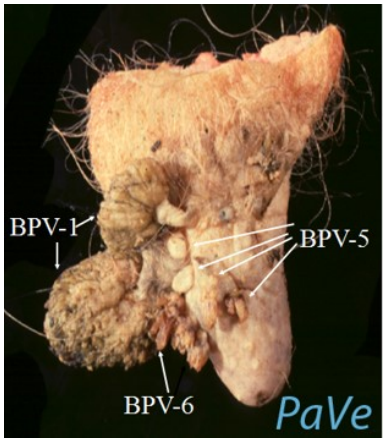

Figura 14. Papilomavírus de teto em uma vaca. As maiores lesões frontais de fibropapiloma são causadas por BPV1; pequenos fibropapilomas ovais ('grão de arroz') por BPV5 e pequenos papilomas epiteliais por BPV6. Retirado do banco de dados do site http://pave.niaid.nih.gov (BAKER, [s.d.]).

Microscopicamente, os tumores apresentam similaridades histológicas, caracterizadas por graus variados de hiperqueratose ou paraqueratose, e proliferações alongadas do epitélio escamoso (Figura 15A). Em todas as lesões avaliadas por Tozato et al. (2013), a maioria dos queratinócitos dentro do estrato espinhoso demonstrou halo perinuclear claro, núcleos picnóticos (caracterizados como coilócitos) e discreta degeneração; em algumas áreas, duas ou mais células degeneradas adjacentes fundidas produzindo microvesículas (Figura 15B-D). Além disso, neste mesmo trabalho, observou-se acantose,

focos de apoptose no epitélio escamoso e acumulações severas de grânulos irregulares queratohialinos dentro das células do estrato granuloso.

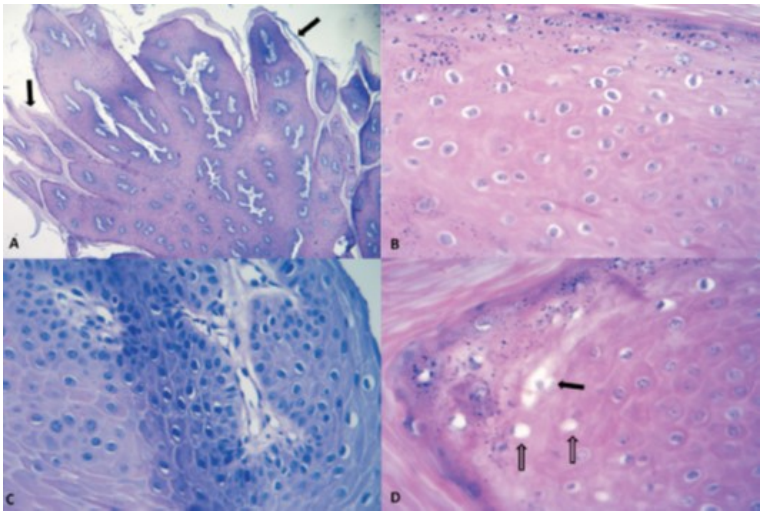

Figura 15. Caracterização histológica da lesão papilomatosa no teto. Caracterização histológica da lesão papilomatosa no teto. Existência proliferativa do tipo digital do epitélio escamoso com hiperqueratose (setas) do estrato córneo (A). Observa-se que os núcleos da maioria dos queratinócitos estão rodeados por um halo claro (B) e núcleo picnótico de coloração basofílica dos coilócitos. Degeneração de diversos queratinócitos (setas abertas) e observa-se também algumas células inchadas adjacentes das quais são coalescidas em uma microvesícula maior (seta fechada) (D). (A, Hematoxilina e eosina, 4 x Obj., B-D, Hematoxilina e eosina, 40 x Obj.). Adaptado de Tozato e colaboradores (2013).

1.7.3 Papilomatose associada a neoplasias cutâneas em cães

As lesões cutâneas em cães causadas por infecção pelo CPV podem ser subdivididas em papilomas e placas virais pigmentadas. Os CPVs cutâneos demonstram expressiva proliferação epidérmica e os tipos de CPVs associados a infecção são o papilomavírus canino oral (COPV ou CPV1), CPV2, CPV6 e CPV7 (TEIFKE, 1998). Em sua maioria, as lesões de papilomas cutâneos resolvem-se espontaneamente, no entanto, transformações malignas já foram relatadas em cães imunocomprometidos (GOLDSCHMIDT et al., 2006).

As placas virais caninas exibem aumento da espessura da camada córnea com presença de queratinócitos anucleados (ortoqueratose) e estão associadas com CPV3, CPV4 e CPV5 (LANGE et al., 2012; TOBLER et al., 2006, 2008). Em geral, as placas virais pigmentadas se mantêm pequenas e de pouca significância clínica, porém a lesão gerada não têm resolução espontânea (GROSS, T. L., IHRKE, P. J., WALDER, E. J. AND AFFOLTER,

2005). Atualmente, transformações malignas dessas placas de pigmentação têm sido frequentemente relatadas (LUFF et al., 2016; MUNDAY; O'CONNOR; SMITS, 2011; NAGATA et al., 1995; STOKKING et al., 2004; TOBLER et al., 2008).

Comumente encontradas no ventre, virilha e axilas de cães, as placas virais pigmentadas caracterizam-se macroscopicamente por serem elevações irregulares, de coloração escura, medindo em torno de 1 a 3 cm de diâmetro, entretanto, é comum placas alcançando diâmetros maiores (Figura 16) (MUNDAY; O'CONNOR; SMITS, 2011). O exame histológico da placa viral pigmentada revela espessamento papilar da epiderme, hiperqueratose, hipermelanose em toda a epiderme e derme superficial, e a presença de grandes grânulos queratohialinos (Figura 17) (NAGATA et al., 1995).

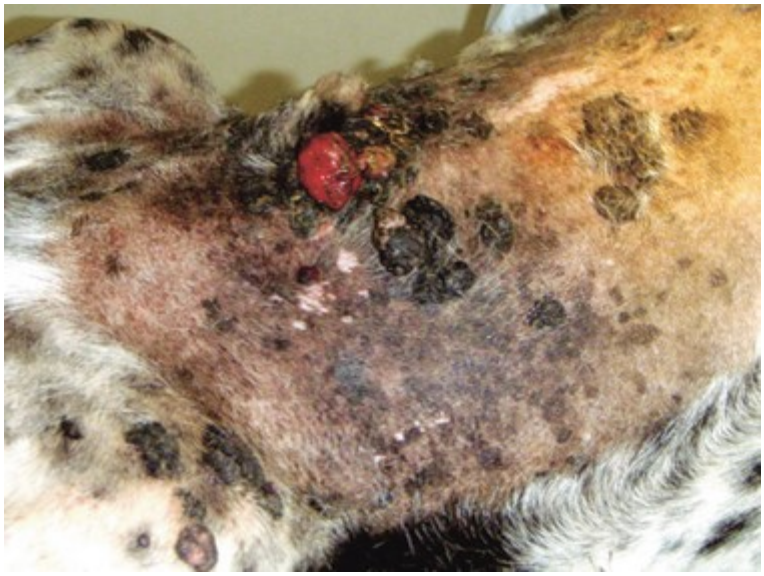

Figura 16. Múltiplas placas virais pigmentadas no ventre de um cão senil causadas pelo CPV. Adaptado de Munday e colaboradores (2011).

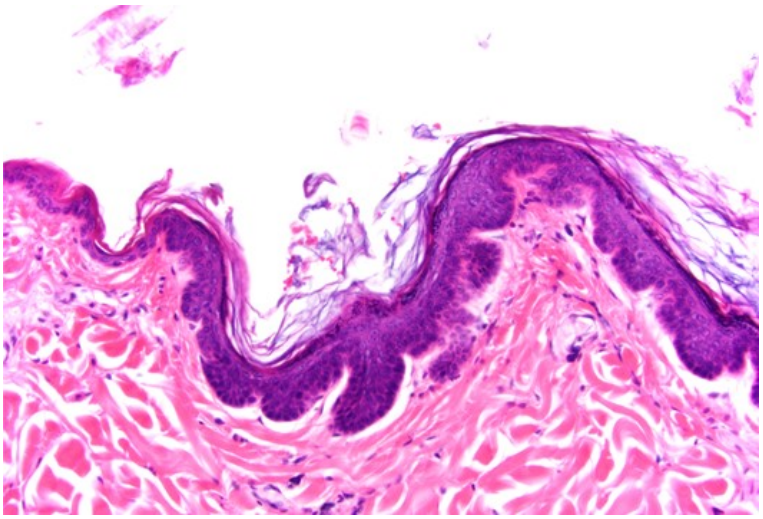

Figura 17. Placa viral pigmentada, pele de um cão, coloração H & E. Caracterizada por hiperplasia epitelial, hiperqueratose ortoqueratótica e hipergranulose. Adaptado de Luff e colaboradores (2016).

A evidência histológica da infecção viral tem sido demonstrada nas placas virais pigmentadas (CALLAN; PREZIOSI; MAULDIN, 2005; LUFF et al., 2016; NAGATA et al., 1995; STOKKING et al., 2004) em casos que existiu a progressão para carcinoma *in situ* (SCC *in situ*), embora a transformação neoplásica seja rara (Figura 18). Essa, por sua vez, caracteriza-se por ser uma neoplasia maligna da qual ainda não houve a invasão do estroma adjacente, portanto o crescimento está restrito à área de origem. Todavia, como são menos aderidas entre si, essas células cancerígenas possuem grandes chances de movimentar-se e infiltrar-se no estroma e nos tecidos adjacentes. O termo *in situ* refere-se ao padrão não infiltrativo, assim preservando a membrana basal (MORTON; BIRNIE; EEDY, 2014).

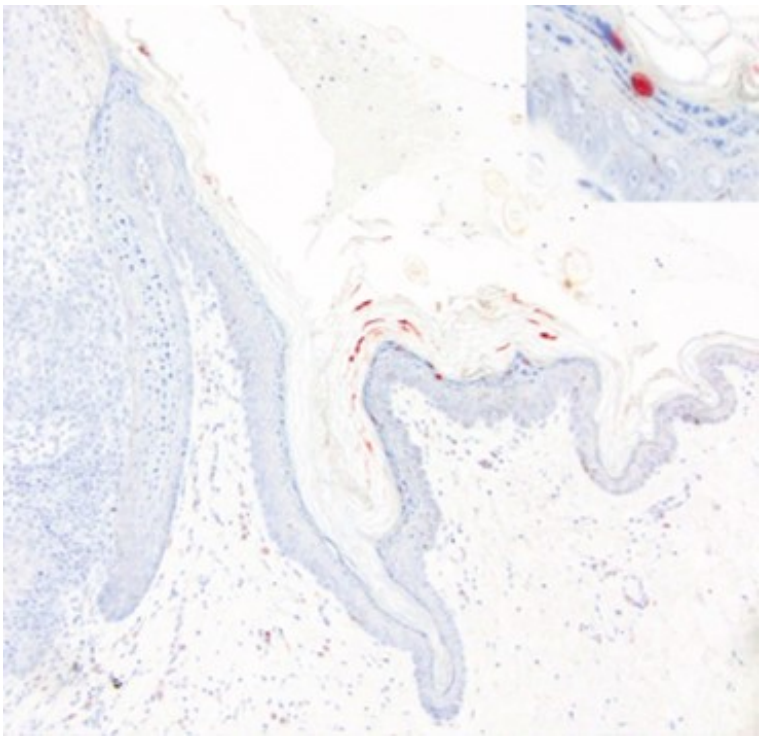

Figura 18. Placa viral pigmentada, pele de um cão, IHC para papilomavírus. Imunorreatividade nuclear forte para o antígeno do papilomavírus dentro dos queratinócitos hiperplásticos da placa viral pigmentada. *In box*: maior amplificação para demonstrar imunoreatividade nuclear forte para o antígeno do papilomavírus. Adaptado de Luff e colaboradores (2016).

1.8 Diagnóstico e tratamento

O diagnóstico clínico é comum em alterações na epiderme do animal, lesões essas denominadas verrugas. O clínico pode concluir o diagnóstico de acordo com as características macroscópicas das lesões em conjunto com a coleta de dados e histórico do animal ou do rebanho (MUNDAY; THOMSON; LUFF, 2017). Porém, quando é necessário lançar mão de diagnósticos complementares, diversos métodos (de aplicabilidade clínica e experimental) têm sido empregados a fim de identificar o vírus, alguns exemplos são: histopatologia, imuno-histoquímica, hibridização *in situ* e PCR (ARALDI et al., 2017).

Através da biópsia do tecido lesionado pode-se observar microscopicamente em cortes histológicos as alterações histológicas provocadas pelo vírus. Uma vez que o ciclo viral dos PVs promove a proliferação epitelial, os achados histopatológicos revela um espessamento do epitélio. Alterações nas células epiteliais também são visíveis e incluem o aumento do tamanho celular, associado a atrofia do nucléolo envolto por halo citoplasmático

(coilócitos). O exame histopatológico revela moderada acantose da epiderme, hiperqueratose e proeminente pigmentação melânica (MUNDAY; THOMSON; LUFF, 2017).

O exame de imuno-histoquímica tem como alvo detectar a produção da proteína L1 do PV no tecido lesionado. A marcação imunológica confirma a presença do vírus, porém é restrita a lesões que contenham infecção ativa (LONGWORTH; LAIMINS, 2004). Já o teste de hibridização *in situ* utiliza sondas específicas que tem como alvo genes precoces e tardios, representando mais uma estratégia de detecção dos PVs.

O diagnóstico viral também é realizado através da PCR. Esta é uma técnica que vem sendo largamente utilizada para identificação de PV devido ao alto grau de especificidade e sensibilidade (FORSLUND et al., 1999). No entanto, a diversidade genética apresentada pela família *Papillomaviridae* é uma desvantagem na elaboração de um par de oligonucleotídeos capaz de detectar todos os tipos existentes. Resultados negativos de detecção estão relacionados a ineficiente homologia da região 3' de um ou ambos os iniciadores (FORSLUND et al., 1999). Como também, o teste pode ser afetado por fatores primários como a concentração e a pureza da amostra de DNA (OGAWA et al., 2004). Portanto, a coleta e armazenamento adequados da amostra, assim como o cuidado na extração do DNA, são passos importantes e que devem ser levados em consideração para termos resultados confiáveis na biologia molecular.

A vacina autógena é o tratamento mais comumente utilizado para a papilomatose bovina (SCHUCH, 2001). É feito um macerado com os papilomas do animal afetado e o vírus é inativado (AIELLO; ASA, 2001; SCHUCH, 2001), porém os resultados dependem do tipo de papiloma, da preparação da vacina e do estágio de evolução das lesões (SCHUCH, 2001). No Brasil, a vacina autógena tem sido utilizada como tratamento terapêutico para animais extensamente atingidos por papilomatose, no entanto apresenta resultados discutíveis de recuperação. Além disso, este tratamento possui custo elevado ao produtor (SILVA et al., 2004).

Os papilomas pequenos podem ser removidos cirurgicamente, podendo-se usar criocirurgia em papilomas grandes, porém, muitos regridem espontaneamente dentro de alguns meses sem o tratamento (SMITH, 2006). Uma das formas de tratamento consiste na utilização de uma ou duas doses de clorobutanol, na dose de 50 mg/Kg, em solução alcoólica via subcutânea (SCHUCH, 2001).

1.9 Prevenção e controle

Acredita-se que os papilomas em cães se desenvolvam quando um cão não infectado é infectado pela primeira vez por um tipo específico de PV. O desenvolvimento de um papiloma coincide com o liberação de um grande número de virions, portanto, é aconselhável impedir o contato entre um cão afetado e um cão que nunca teve papilomas (SANCAK et al., 2015). Porém, como as partículas virais dos PVs são resistentes ao meio ambiente, infecções podem acontecer sem o contato direto entre os animais (RODEN; LOWY; SCHILLER, 1997).

A vacinação profilática de bovinos com partículas semelhantes a vírus (*virus like particles* – VLPs) ou com vírus purificados induz uma alta proteção somente ao tipo viral homólogo e não confere nenhum efeito terapêutico em tumores estabelecidos. Além disso, as vacinas compostas de VLPs apresentam limitações devido ao custo e restrições em relação à sua produção em sistemas de cultivo celular eucariótico (RIBEIRO-MULLER; MULLER, 2014). Por outro lado, a proteína menor do capsídeo viral (L2) produzida em sistema procarioto se mostrou eficaz em tratamentos profiláticos e terapêuticos em bovinos, e a massiva infiltração de linfócitos sugere que este peptídeo contenha epítopos específicos que estimulem células T (JARRETT et al., 1991).

Estudos posteriores *in vitro* e *in vivo*, demonstraram que a porção terminal da proteína L2 do capsídeo viral induz uma resposta imune heteróloga contra um grande número de PV, incluindo o BPV1 (RUBIO et al., 2011). Grande parte de estudos sobre PV, como a indução de tumores malignos e o conhecimento do papel dos oncogenes, foram conduzidos utilizando o BPV e o bovino como modelos de estudo (CORTEGGIO et al., 2013). Portanto, o bovino é atualmente o animal mais indicado para a condução de testes vacinais, uma vez que ele vem contribuindo de maneira significativa para a elucidação da transformação celular em tumores de ocorrência natural, assim como para o estudo do papel dos genes precoces na transformação celular (BORZACCHIELLO et al., 2009).

A elaboração de uma vacina parte da dificuldade de que vários tipos de PV estarem envolvidos nas lesões. Segundo, para que a vacina previna a infecção é necessário que ela seja administrada antes de uma primeira exposição ao PV e, por último, ela deve ser economicamente viável mesmo tendo a necessidade de protocolos vacinais diferentes,

quando o objetivo é prevenir ou tratar CPVs comuns ou raros (MUNDAY; THOMSON; LUFF, 2017).

2 OBJETIVOS

2.1 Geral

Compreender a diversidade genética de papilomavírus encontrados em bovinos e caninos.

2.2 Específicos

- Investigar os tipos de BPV presentes em lesões papilomatosas de bovinos de leite do Rio Grande do Sul, através de sequenciamento de alta eficiência;
- Sequenciar os genomas completos de prováveis novos tipos virais encontrados neste estudo;
- Detectar os casos de coinfecções em amostras de papilomas de teto;
- Associar à transformação maligna de placas de pigmentação em carcinoma de células escamosas (SCC);

3 CAPÍTULO 1

Papilomavírus canino tipo 16 (CPV16) associado ao carcinoma de células escamosas em um cão: achados patológicos e virológicos.

O presente experimento já foi concluído e um artigo científico foi submetido no periódico *Veterinary Pathology*. O artigo científico será apresentado a seguir, tal qual foi submetido.

Brief Communication

Canine papillomavirus type 16 (CPV16) associated to squamous cell carcinoma in a dog: pathological and virological findings

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Abstract

Papillomavirus (PV) is a circular double-stranded DNA virus belonging to *Papillomaviridae* family. During the infection cycle, PV translate proteins that can influence cell growth and differentiation, leading to hyperplastic papillomas (warts) or neoplasia. *Canis familiaris* papillomaviruses (CPVs) have been associated with different lesions, such as oral and cutaneous papillomatosis, pigmented plaques, and squamous cell carcinomas (SCC). Here, we reported a clinical case of a female dog with uncommon papillomatous-like lesions induced by the CPV16 (*Chipapillomavirus 2*) that progressed to SCC. In addition, we characterized pathological lesions caused by this virus, using immunohistochemistry and *in situ* hybridization, which revealed strong signals within the neoplastic tissue. The full genome of the CPV16 recovered directly from the lesion was characterized, and the phylogenetic relationships were determined. The identification of oncogenic protein genes (E5, E6, and E7) by high throughput sequencing and (HTS) their expected domains are suggestive of the malignant transformation conducted by CPV16.

Keywords: CPV16; canine papillomavirus; oncogenesis; squamous cell carcinoma.

Papillomaviruses (PV) are circular double-stranded DNA viruses belonging to the *Papillomaviridae* family(DE VILLIERS et al., 2004). Although the majority of PV infections do not develop visible lesions, some PVs can produce hyperplastic papilloma (warts) and induce neoplasia.(MUNDAY; THOMSON; LUFF, 2017) *Canis familiaris* papillomaviruses (CPV) have been associated to different lesions such as oral and cutaneous papillomatosis, pigmented plaques, and squamous cell carcinomas (SCC).(LUFF et al., 2016; MUNDAY; O'CONNOR; SMITS, 2011) However, CPV-associated malignant lesions are rare, and most cases of SCC have no CPV-induced etiology.(MUNDAY; THOMSON; LUFF, 2017) Pigmented plaques typically presents well-demarcated erythematous hyperkeratotic plaque with an irregular border, which can result in the development of multiple lesions or progress to invasive SCC. Here, we describe the features of a CPV16 induced malignant neoplasia in a female dog. The full genome of CPV16 was recovered directly from lesions, and their phylogenetic relationships and genome characterization were determined. In addition, we pathologically characterized the lesions caused by this virus, using immunohistochemistry and *in situ* hybridization.

The eight-year-old mongrel female dog exhibited proliferative lesions like scabs and sores on the plantar and palmar cushions, on both sides (S1).The abdominal skin presented inverted papilloma up to 2 cm in diameter with ulceration (S2). Numerous warts smaller than 1 cm in diameter and multiple pigmented plaques of several sizes (1 cm to 6 cm) were distributed in the abdomen and groin (S3), as well as an infiltrative subcutaneous tumor measuring up to 3 cm in diameter without ulceration (S4).Pigmented plaques had the standard distribution in the ventrum and axilas.(MUNDAY; KIUPEL, 2010) In contrast, the diameter reached 6 cm, bigger than the standard growing stabilization within 1 cm of diameter.(MUNDAY; KIUPEL, 2010) Beside the fact that the lesions were already in a high stage of maturation, it was not possible to evaluate the progress of this pigmented plaques, since the dog never returned for a follow up. Similar cases of dogs with pigmented plaques that developed subsequently into SCC were well documented.(GIL DA COSTA et al., 2017) Skin and subcutaneous tissue samples of the infiltrated subcutaneous tumor were collected and sent for histopathological analysis. Half sample was fixed with

formalin for histopathology, immunohistochemistry (IHC), and *in situ* hybridization (ISH), and the other half was stored at −20°C for DNA extraction and viral genomic analysis. The tissue samples were obtained after being removed for clinical purposes only. The removal of the tissues was not part of the study design. The animal and sampling activities described in this study were conducted under accordance with the standards of the Ethics Committee on the Use of Animals (CEUA) of the Federal University of Rio Grande do Sul (UFRGS) under the protocol number #24984. Its attributions and competences are defined according to the provisions of Law 11794/08 and resolutions of the National Council for the Control of Animal Experimentation (CONCEA).

According to the histological examination, the infiltrated subcutaneous tumor showed the absence of ordered pattern of cell maturation in the epidermis, cellular dysplasia, islands of tumor epithelial cells and transformation that evidenced the loss of the basal membrane compatible with invasive SCC (S5). The presence of the “keratin pearls” reveals the origin of the tumor from squamous layer. Also, the “halo cells” enclosed to the squamous layer (named koilocyte) were characterized by clear perinuclear area and condensed nuclei, which occurs because of the papillomavirus infection. Furthermore, neoplastic tissue was surrounded by epidermis that was markedly thickened, contained large numbers of large keratohyaline granules (S6). The keratinocytes in the affected epidermis exhibited variable anisokaryosis and anisocytosis, prominent nucleoli, and marked mitosis with in average 5 to 6 figures of mitosis per field (S7).

IHC was performed on fixed tissue fragments with the aid of monoclonal anti-human PV antibody against the major capsid protein (K1H8, DakoCytomation) for the detection of papillomavirus antigens. Antibodies against Ki67 and cytokeratin (type I and II) antigens (DakoCytomation) were used to detect the activity of cell differentiation and division. There was also multifocal marking anti-Ki67 in hyperplastic cells (S8). Anti-pancytokeratin exhibited diffuse immunoreactivity to the neoplastic epithelium (S9). These two antibodies are used as markers of tumor cell proliferation. ISH was performed according to a standardized protocol in order to detect CPVs using three pairs of oligonucleotides to target the open reading frames

(ORFs) E6 and E7, as well as the gene L1, as previously described.(TEIFKE, 1998) Neoplastic tissue revealed strong diffuse nuclear hybridization signals within the spinosum stratum of the epithelium layer (Fig. 1 and 2), suggesting the earlier steps of virus replication. There was also intense CPV capsid protein immunostaining in IHC along the margins of hyperkeratosis and within keratinocytes in the upper epithelial layers, where virion assembly occurs during the last steps of PV infection, before its release (Fig. 3 and 4). The two different locations confirm the virus cycle, and these findings strongly support a causative role for CPV in carcinogenesis. Since the CPV can be detected in normal skin, the results of IHC and ISH discarded the possibility of surface contamination.

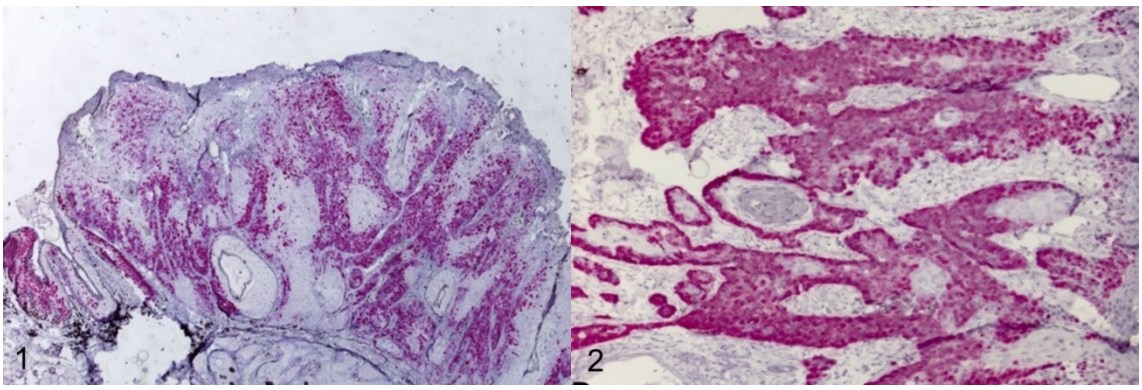

Figure 1-2. *In situ* hybridization assay, SCC; specific associated signal with the CPV16-specific probes in epithelial cells (500 µm and 100 µm, respectively).

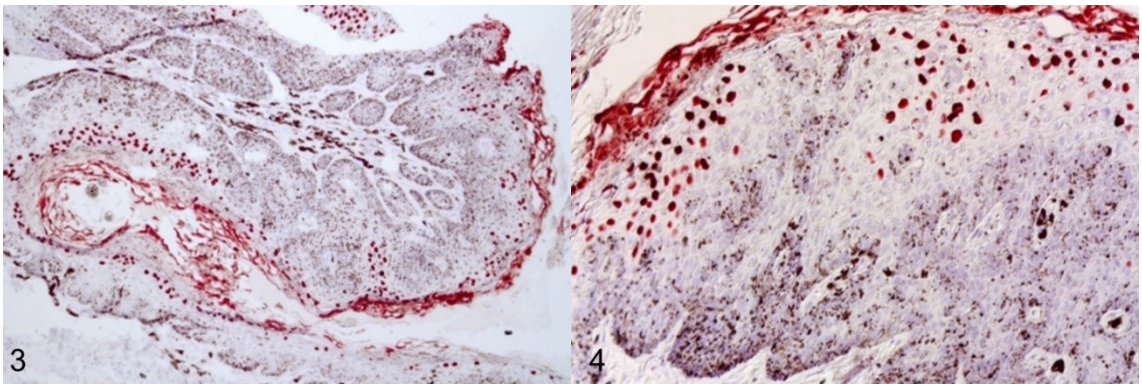

Figure 3-4. Immunochemistry assay anti-papillomavirus, SCC; marked cytoplasmic and nuclear immunoreactivity of a part of the neoplastic cells of the spinous and

spinous and granular layers and the cornified layer (500 μm and 100 μm, respectively).

Indeed, previous studies reported lesions reminiscent of human epidermo dysplasia verruciformis in dogs.(MUNDAY; THOMSON; LUFF, 2017) It is a genetic predisposition to develop flat warts, some of which subsequently undergo cancer transformation. The lesions could be limited to pigmented plaques and did not undergo malignant transformation.(MUNDAY; THOMSON; LUFF, 2017) Nevertheless, the larger pigmented plaques that contain greater histological changes may be predisposed to malignant transformation.(LUFF et al., 2016)There is no doubt about the histopathological changes that underwent in this process and the favorable environment provided by the pigmented plaques; but the viral factors that promoted this malignant transformation are still not clear.

The CPV was obtained directly from an invasive SCC by PCR using primer pairs FAP59/FAP64.(FORSLUND et al., 1999) In order to sequence the papillomavirus whole genome present in the invasive SCC, we proceeded with multiply-primed rolling-circle amplification (RCA) to enrich the sample followed by high throughput sequencing (HTS) as previously described.(DA SILVA et al., 2016) The HTS generated 153,620 paired-end high-quality reads (average read length of 140 nt). *De novo* assembling in SPAdes 3.6 software revealed a circular contig of 7,796 nt, sharing 99.7% nucleotide identity with the recently described CPV16 strain Chana (GenBank accession number NC_026640).(LUFF et al., 2015) The complete genome size of CPV16 (named 134RS/can15) has a GC content of 50.6%, and encodes eight proteins: six early genes E1, E2, E4, E5, E6, and E7; and two late genes L1 and L2 (S10).The phylogenetic tree was reconstructed with optimized alignments based on the nucleotide sequence of the L1 gene using Bayesian analysis.(HUELSENBECK; RONQUIST, 2001) Using a set of genus-representative sequences of CPV, CPV16 134RS/can15 strain grouped within the *Chipapillomavirus* genus, closest to the Chana strain (S11), which was also detected from pigmented skin plaques that progressed to squamous cell carcinoma in a female Basenji dog.(LUFF et al., 2015)

153 In cattle, the *Deltapapillomavirus* comprise the high-risk mucosal types that
154 can cause urinary bladder cancer in their natural hosts. The set of all three
155 oncoproteins E5/E6/E7 present in the *Deltapapillomavirus* and its distribution in the
156 genome appear to be essential for the progression of malignancy.(DAUDT et al.,
157 2018a) Similarly, we identified the putative oncoproteins E5, E6 and E7 by the
158 genomic characterization besides the pathological analysis. The presence of the
159 oncoprotein genes in the tissue suggests that the oncoproteins could be interfering
160 in the cellular cycle. Therefore, the replication of the oncogenic virus in the tissue
161 strongly suggests the malignant transformation conducted by CPV16. The putative
162 E6 of CPV16 134RS/can15 strain has two zinc-finger domains ($CX_2CX_{29}CX_2C$) that
163 are critical for the transformation activity of the viral oncoproteins, including the
164 product of the pRB gene.(MANTOVANI; BANKS, 2001) Moreover, the putative E7
165 contains one zinc-finger domain, as well as the conserved binding site for the
166 retinoblastoma protein (LXCXE) that is closely linked to malignant potential and cell
167 transformation.(DAHIYA et al., 2000; MÜNGER et al., 2001)
168 In order to correlate the presence or absence of the oncoprotein complex (E5,
169 E6 and E7) with malignant potential, the complete genomes of reference sequences
170 were aligned (Fig. 5). The comparison revealed that the oncoprotein complex is
171 absent in the *Chipapillomavirus* strains linked to malignant transformation (CPV9,
172 CPV12). However, CPV16 strains Chana and 134RS/can15 contain this complex
173 equally arranged in the genome, besides the process of pathological transformation
174 has similarities.

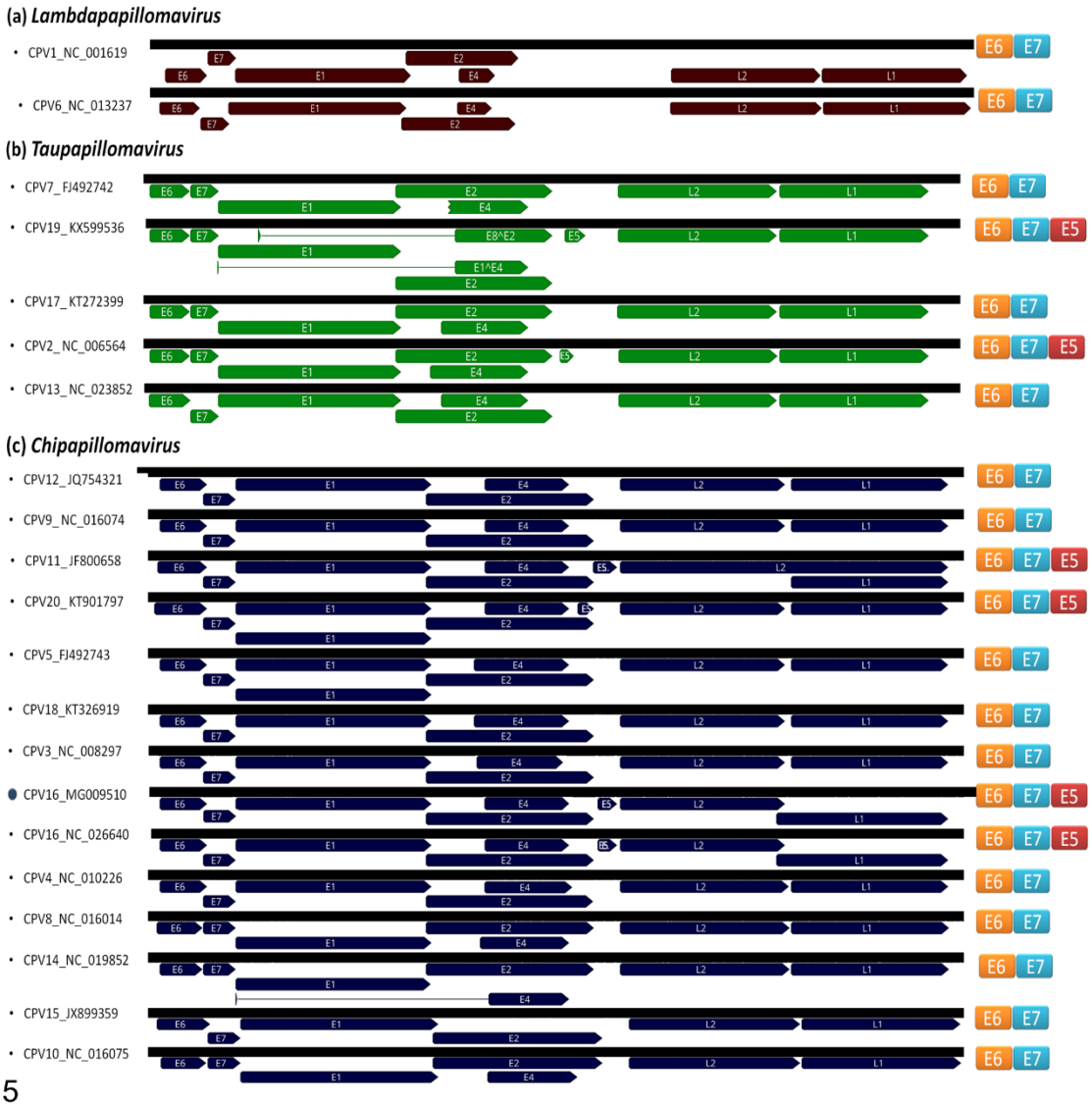

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Figure 5. The oncogenes comparison using complete genome sequences alignment. The E5 oncogene is present in two CPV19, CPV2 (*Taupapillomavirus* genus) and in CPV11, CP16 and CPV20 (*Chipapillomavirus*genus). CPV16 strains Chana and 134RS/can15 present this complex of oncoproteins equally arranged in the genome.

Contributing factors that cannot be disregarded are iatrogenic immunosuppression, breed susceptibility, systemic diseases, senility, genetic mutations, among others. In a study describing the occurrence of papillomavirus

185 infection in transplanted dogs with X-linked severe combined immunodeficiency
186 (XSCID), CPV2 was linked with a papilloma lesion that progressed to metastatic
187 SCC.(GOLDSCHMIDT et al., 2006) Interestingly, the mongrel female described in
188 this study did not present history of use of any immunosuppressive drugs. Currently,
189 there is no breed with a genetic predisposition to CPV-associated-malignant tumor
190 described in the literature.

191 In this work, we reported a mongrel female dog suffering from numerous
192 lesions related to CPV infection. Among them, we were able to describe the
193 association of one CPV16 strain with invasive SCC, build a phylogenetic inference,
194 identify and compare the oncoprotein genes of the CPV16 strain. Also, the study
195 highlights the importance of CPV-induced oncogenesis and expands the current
196 knowledge of the genetic background of the *Papillomaviridae* family.

197

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203

204 **Declaration of Conflicting Interests**

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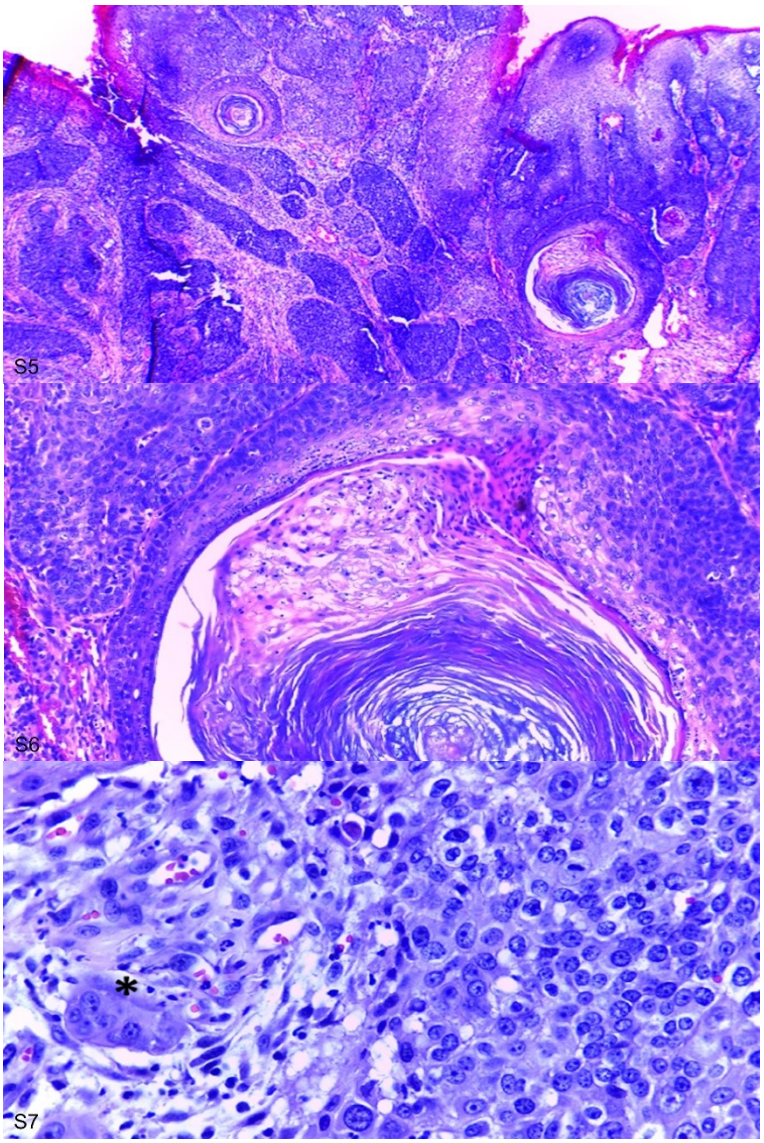
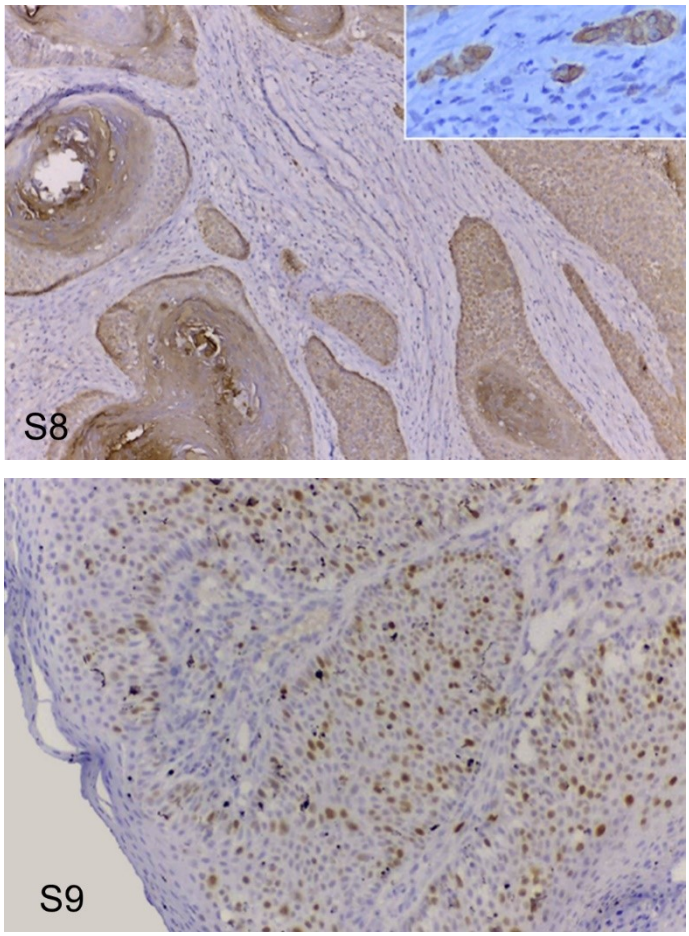
Supplemental figures legends

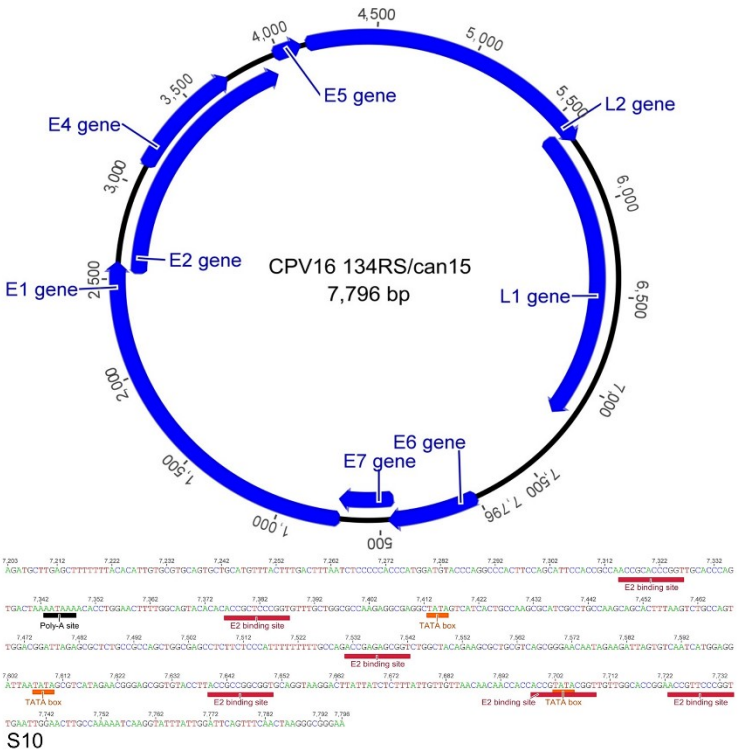
Supplemental Figures 1–4. Macroscopic resentation of lesions caused by CPV in a mongrel female dog. **S1.** Proliferative lesion with scabs and sores on the plantar cushion. **S2.** Inverted papilloma in the abdomen, with ulcerated and irregular surface. **S3.** Numerous pigmented plates in abdominal skin. **S4.** Infiltrative subcutaneous tumor near the mammary gland.



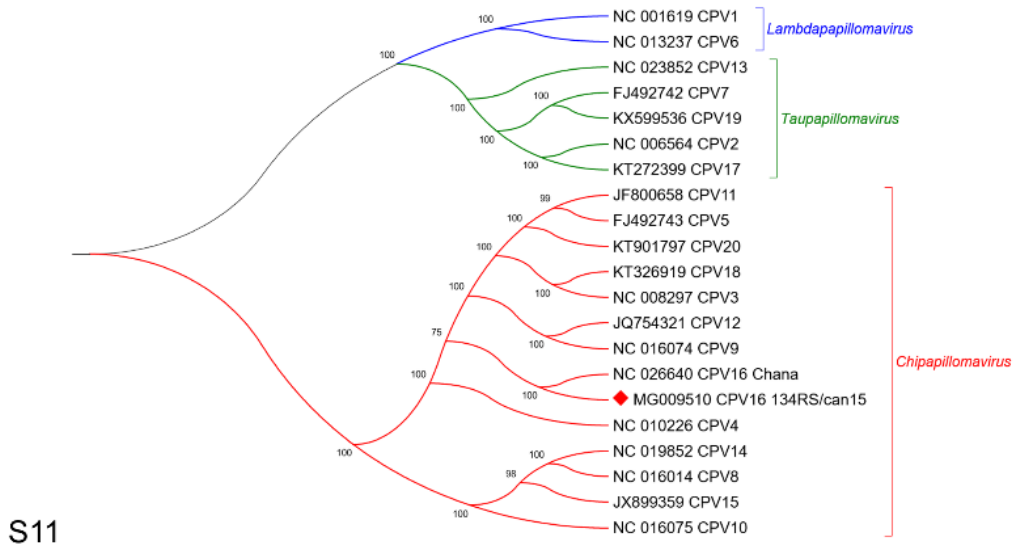
Supplemental Figure 5-7. The SCC *in situ* neoplastic tissue, epidermis, mongrel female dog. **S5** Formation of neoplastic nests in the dermis, HE. **S6** Accented dysplasia involving follicular infundibulum and marked anisocytosis and anisokaryosis, HE. **S7** In addition, the cells present cellular atypia such as increased nucleus-cytoplasm ratio, cellular pleomorphism, nuclei and increased nucleoli, several mitoses and the presence of atypical mitoses, HE [*].



Supplemental Figure 8-9. Immunochemistry assay, SCC *in situ*; **S8** anti-cytokeratin immunoreactivity exhibits the borderline between neoplastic tissue and connective tissue. Inset: strong cytoplasmic immunoreactivity signal. **S9** antibody anti-Ki67 with multifocal marking in neoplastic cells with intense cellular proliferation (700 µm and 100 µm, respectively).



Supplemental Figure 10. Genomic features of the CPV16 134RS/can15; **S10** Genomic map of CPV16. Identification of putative ORFs was made with the aid of ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder), and figure drawn was performed in Geneious software, 8.1.4 version.(KEARSE et al., 2012) Features of the CPV16 134RS/can15 non-coding region. Colored boxes (red) display the genomic locations of the E2 binding site (ACCN$_5$GGT), polyadenylation site (AATAAA) (black), and TATA box (TATAAA) (orange).



S11

Supplemental Figure 11. Phylogenetic analysis of the complete L1 of canine papillomavirus. L1 nucleotide sequence was compared to those of 20 canine papillomaviruses retrieved from GenBank. Posterior probability values are indicated above the branches. CPV16 134RS/can15 is labeled with a red diamond. Evolutionary analyses were conducted in MrBayes (v3.2.1).(HUELSENBECK; RONQUIST, 2001)

4 CAPÍTULO 2

Detecção de papilomavírus bovino em tetos de bovinos leiteiros.

O presente experimento já foi concluído e seu manuscrito está em fase final de elaboração, com a intenção de publicação no periódico *Scientific Reports*.

Scientific Reports

Title: Detection of 17 putative new bovine papillomavirus types in teat warts from dairy cattle

Running title: BPV new types in teat warts

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Abstract

Papillomavirus (PV) are viruses which affect virtually all amniotes, including humans. Bovine papillomaviruses (BPVs) cause benign warts in the skin, among them teat lesions that can difficult the milking and result in lower profits for the milk industry. In the past few years, genetic characterization of animal PVs has increased due to the availability of new techniques, which simplified the sequencing of complete genomes. Currently, 24 BPV are fully characterized and assigned in four genera, in contrast to the more than 200 types of the human papillomavirus (HPVs). The aim of this study was to identify the BPV types associated with teat warts, by conventional PCR, followed by Sanger sequencing. After, the samples were selected to perform rolling circle amplification (RCA) followed by high throughput sequencing (HTS) and phylogenetic analysis. Twenty-five complete BPV genomes were assembled from the 17 papillomatous-like teat warts displaying a 23.5% of coinfection rate. Moreover, we observed 17 new putative BPV types, including one new species and one new genus. It can be concluded that the etiology of papillomatosis in the teat of cows is much more complex than previously reported, that about one half of the lesions has more than one BPV type and that HTS is more appropriate for typing papillomavirus.

Introduction

Papillomaviruses (PVs) are small viruses whose comprise a highly diverse group that can produce epithelial proliferative lesion virtually found infecting all amniotes (RECTOR; VAN RANST, 2013). Bovine papillomavirus (BPV) is recognized as an etiological agent associated with several forms of benign tumors, among them the teat papillomatosis that are distributed worldwide in dairy cows (RECTOR; VAN RANST, 2013). The papillomatosis

in teat can result in lower profits for the milk industry. Moreover, it can cause ulceration and rupture of the wart and the lesions may predispose to mastitis and distortion of the milk ducts (BORZACCHIELLO; ROPERTO, 2008; CAMPO, 2003).

Currently, only 24 BPV types have been reported, in contrast to more than 200 types of the human papillomavirus (HPVs) (http://pave.niaid.nih.gov). The BPVs are assigned in four genera, although the types BPV19 and 21 have not been grouped within any genus. The genus *Deltapapillomavirus*, with one species the *Deltapapillomavirus 4*, comprising four types (BPV1, 2, 13 and 14); the genus *Epsilonpapillomavirus*, comprising the species *Epsilonpapillomavirus 1*, with two types (BPV5 and BPV8); *Dyoxipapillomavirus*, which comprises *Dyoxipapillomavirus 1* species (BPV7); *Dyokappapilomavirus*, comprising three types without species differentiation (BPV 16, 18, 22); the *Xipapillomavirus* genus, composed by the *Xipapillomavirus 1* (BPV3, 4, 6, 9, 10, 11 and 15), the *Xipapillomavirus 2* (BPV12) and the types BPV17, 20, 23, 24 that have not been classified in species demarcation (http://pave.niaid.nih.gov).

Despite the BPV1, 5 and 6 have been classically associated with udder and teat lesions (CAMPO, 2002; JARRETT et al., 1984; SAVERIA CAMPO et al., 1981) other BPV types have been detected in this anatomic region (CLAUS et al., 2008; LINDHOLM et al., 1984; MAEDA et al., 2007; OGAWA et al., 2004; TOZATO et al., 2013). The detection and classification of BPVs using conventional PCR carried out with primer pair FAP59 and FAP64 (FORSLUND et al., 1999) followed by Sanger sequencing is widely used. The applicability of the designed primers has been satisfactory for most of the papillomavirus types, but negatives results appears due to inefficient base pairing in the 3’ region between one or both primers and the templates (FORSLUND et al., 1999). Recently, the application of unbiased tools as rolling circle amplification (RCA) followed by high throughput

sequencing (HTS) for BPV identification revealed the presence of new BPVs (DA SILVA et al., 2016; DAUDT et al., 2016a, 2018b). Moreover, this technique allows the identification of BPV-coinfections in a single papilloma lesion (DAUDT et al., 2016a). Therefore, the present study aimed to detect coinfections, recover the complete the viral genome, characterized and perform the phylogenetic inferences based on the RCA followed by HTS methods in seventeen teat warts from dairy cattle.

Material and methods

Ethics Statement

Lesions were collected by veterinarians in commercial abattoirs with owner's permission. All procedures were performed in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Treaty Series—No. 170 revised 2005) and the procedures of the Brazilian College of Animal Experimentation (COBEA). It must be highlighted that this project was approved by Universidade Federal do Rio Grande do Sul Animal Ethics Committee (number 28460).

Sample collection

Teat warts of 76 specimens of dairy cows from tree different municipalities (Farroupilha, Passo Fundo and Dois Irmãos) of Rio Grande do Sul state, Southern Brazil, were collected. Each papilloma specimen represents one bovine carcass sent for slaughter. The teats with papillomatous lesions were kept in 10% formalin, routinely processed for histopathology,

and stained by hematoxylin and eosin (H&E). Other part of the papillomatous lesions were ground with sterile sand in 10 mL of phosphate buffered saline (PBS) (pH 7.4), centrifuged at 720 x g for 10 min and 1000 µL of the supernatant was stored at -20 °C for molecular analysis. Based on the histopathological assay and PCR(FORSLUND et al., 1999) followed by Sanger sequencing, the diagnoses of epidermal papillomatosis was confirmed in 63 specimens (these data belong to the previous study and are available on request). For this study, we selected seventeen specimens among the 63 positives papillomavirus specimens to procced the rolling-circle amplification (RCA) followed to high throughput sequencing (HTS). The criteria of the selection was based on three aspects: topology of the phylogenetic tree, quantity and quality of extracted DNA and nucleotide sequence similarity of the putative new PV types to their closest related PV.

Rolling-circle amplification (RCA) and high throughput sequencing (HTS)

The RCA assay was performed as previously described (DEZEN et al., 2010; RIJSEWIJK et al., 2011). Briefly, 100 ng of total DNA in a final solution of 10 µL from papillomatous tissue was denatured at 95°C for 5 minutes and immediately cooled on ice. It was prepared a solution containing 3.6 mM of each dNTP (Ludwig Biotec, Porto Alegre, RS, Brazil), 15.5 mM random exonuclease-resistant hexanucleotides (Thermo Fisher, Waltham, MASS, USA), 2 U of ϕ29 DNA polymerase (Thermo Fisher, Waltham, MASS, USA), 2X Bovine Serum Albumine and 2X of reaction buffer [50 mM Tris/HCl pH 7.5, 10 mM MgCl2, 10 mM (NH4)2SO4, 4 mM dithiothreitol] in a total volume of 10 µL. The amplification solution was incubated for 18 hours at 30°C, followed by 10 min at 65°C to inactivate the enzyme. The amplicon was electrophoresed in a 0.8% agarose gel and visualized on a UV light source

after ethidium bromide staining. The RCA products were purified with a commercial kit (GFX™ Purification Kit; Amersham Biosciences, Little Chalfont, Bucks, UK). The quality and quantity of the DNA were assessed through spectrophotometry and fluorometry performed with NanoDrop™ (Thermo Fisher Scientific) and Qubit™ (Thermo Fisher Scientific) respectively. DNA fragment libraries were further prepared with one ng of purified RCA DNA using a Nextera XT DNA sample preparation kit and sequenced using an Illumina MiSeq instrument (2× 150 paired-end reads with the Illumina v2 reagent kit). The reads quality was evaluated with FastQC, trimmed in Geneious software (version 9) and were *de novo* assembled into contigs using SPAdes (3.6 version) (BANKEVICH et al., 2012). The contigs were compared to known sequences in the GenBank nucleotide and protein databases using BLASTn/BLASTx (YE; MCGINNIS; MADDEN, 2006). Geneious software was used for alignments, open reading frame (ORF) prediction, genome annotations and similarity searches were performed with the NCBI BLAST server(YE; MCGINNIS; MADDEN, 2006).

Phylogenetic inferences

Local sequence alignments were constructed to determine the sequence identity with BLASTn (YE; MCGINNIS; MADDEN, 2006). Representative sequences of the ruminants PV sequences were retrieved from GenBank. Nucleotide alignments were performed using MUSCLE software (EDGAR, 2004). The best selection model to generate the phylogenetic trees was selected with the MEGA6 software (TAMURA et al., 2013). A phylogenetic tree with 1,000 bootstrap resamples of the alignment data sets was generated using the Maximum Likelihood method in MEGA6 (TAMURA et al., 2013). The taxonomy criteria of the BPV

samples was conducted based on the L1 gene(BERNARD et al., 2010). The entire L1 gene sequence must be different more than 10% of nucleotide pairwise identity of the closest know type to be considered a new type. The PV types within a species share between 71% and 89% nucleotide identity and members of the same genera share until 60%.

Results

HTS and data analysis

The HTS using Ilumina MiSeq System generated 68,976 to 3 papillomavirus-related reads of the seventeen specimens. The paired-end reads were *de novo* assembled in SPAdes 3.6 and revealed circular contigs, sharing highest identities with the *Papillomaviridae* family members. Overall were recovered 25 complete *papillomavirus* genomes, and contigs (fragments) relative for *papillomavirus* members. Based on the L1 nucleotide sequence among the 25 complete genomes there are eight that can be classified as BPV type 7, BPV8, BPV9 or BPV12. Moreover, seventeen of them displayed identity consistent with putative new types, including one that can be a new species and another can represent a new genus (Table 2). The sequence 4160/3 can considered a new genus because form a distinct cluster between members of *Epsilonpapillomavirus* and *Dyokappapapillomavirus* and was more closely related to *Rusa timorensis papillomavirus type 2* (RtPV2) with 65.4% sequence identity. The sequence 4160/1 likely represents a new species, grouped in the limits of the cluster representative of the genus *Dyokappapapillomavirus* and most closely related to BPV7 (68.2% nucleotide identity). Among the new types there are nine sequences belonging to the species *Xipapillomavirus 1*, four belonging to the *Xipapillomavirus 2* and two

sequences clustered within *Epsilonpapillomavirus 1* species (Figure 1). Two or more papillomavirus sequences were observed in 23.5% of the papilloma samples (04/17) (3895-16, 3896-16, 4144-16, 4160-16). The new type of XiPV1 was the most frequently found BPV type since it was detected in six different samples, while other BPVs ranged from one to two detections. The details and the GenBank accession numbers about these genomes are described in Table 2.

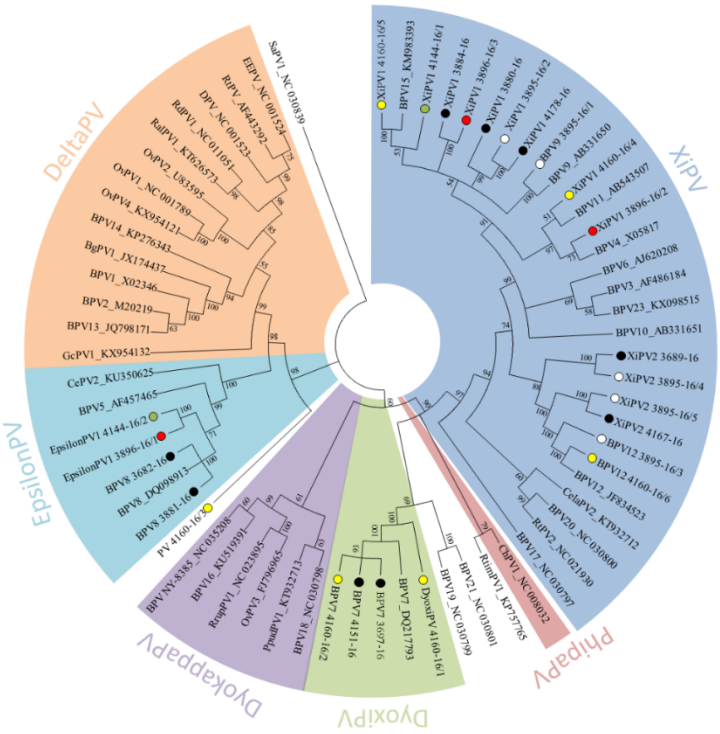

Figure 1. Phylogenetic tree of the papillomaviruses based on the complete sequences of the L1 ORF. Sequence belonging to single infection are indicated with black dots and sequences that are from the same sample have the same dot color. All the 67 PV types described to infect ruminants were analyzed. Accession numbers for the sequences are included and abbreviations are used according to PAVE.

Sample	Description	Classification (L1)	Best Blastn Hit	GenBank Accession Number	Parwise Identity (%)
3682-16	Complete Genome	BPV8	BPV8	DQ098913	99.9%
3689-16	Complete Genome	New Type (XiPV2)	BPV12	JF834523	71.4%
3697-16	Complete Genome	BPV7	BPV7	DQ217793	98.8%
3880-16	Complete Genome	New Type (XiPV1)	BPV11	AB543507	75.6%
3881-16	Complete Genome	BPV8	BPV8	DQ098913	100.0%
3884-16	Complete Genome	New Type (XiPV1)	BPV Aks-2	KM983393	75.5%
3895-16	Complete Genome 1	BPV9	BPV9	AB331650	99.6%
	Complete Genome 2	New Type (XiPV1)	BPV15	KM983393	74.6%
	Complete Genome 3	BPV12	BPV12	JF834524	93.4%
	Complete Genome 4	New Type (XiPV2)	BPV12	JF834524	71.2%
	Complete Genome 5	New Type (XiPV2)	BPV12	JF834524	78.3%
3896-16	Complete Genome 1	New Type (EpsilonPV1)	BPV5	AF457465	76.3%
	Complete Genome 2	New Type (XiPV1)	BPV24	MG602223	80.1%
	Complete Genome 3	New Type (XiPV1)	BPV11	AF486184	75.3%
4144-16	Complete Genome 1	New Type (XiPV1)	BPV3	AF486184	71.9%
	Complete Genome 2	New Type (EpsilonPV1)	BPV5	AF457465	76.3%
4151-16	Complete Genome	BPV7	BPV7	DQ217793	99.3%
4152-16	Fragment L2	Unclassified			
4160-16	Complete Genome 1	New Species (DyoxiPV)	BPV7	KM096429	68.2%
	Complete Genome 2	BPV7	BPV7	DQ217793	99.7%
	Complete Genome 3	New Genus	RtPV2	KT852571	65.4%
	Complete Genome 4	New Type (XiPV1)	BPV11	AB543507	81.4%
	Complete Genome 5	New Type (XiPV1)	BPV Ask-2	KM983393	86.2%
	Complete Genome 6	BPV12	BPV12	JF834523	96.9%
4163-16	Fragment L1/L2	Putative New Type (EpsilonPV1)	BPV 8	DQ098917	74.5%
4167-16	Complete Genome	New Type (XiPV2)	BPV12	JF834523	78.2%
4178-16	Complete Genome	New Type (XiPV1)	BPV3	AF486184	73.0%
4826-16	Fragment E1/L1	Putative New Type (XiPV1)	BPV 9	AB331650	74.0%
4833-16	Fragment L1	BPV 7	BPV7	DQ217793	98.8%

Table 2. The description of pairwise identity percentage between complete genome sequences from the BPV samples compared with sequences available in GenBank. Genomes recovered from t sample are numbered sequentially after the sample name, thus identifying cases of coinfection.

Discussion

In the present study, seventeen samples of BPV teat warts were tested by a combination of RCA followed by HTS which allowed (i) to detect multiple coinfections, (ii) enable the complete assembly of the viral genome, (iii) its characterization and (iv) its phylogenetic inferences. Classically, BPV was believed to have a tropism for specific anatomic locations (CAMPO, 2002; JARRETT et al., 1984; SAVERIA CAMPO et al., 1981). However, recent studies have suggested that BPV types do not distinguish between beef and dairy cattle, and no tropism by anatomical location has been confirmed (BATISTA et al., 2013; LUNARDI et al., 2016; MAEDA et al., 2007; OGAWA et al., 2004). Although there is a tendency to design a tropism by anatomical regions for each type of BPV in order to better understand its biology, it seems that BPV infection is guided mainly by its tropism by mucous and cutaneous epithelia (DAUDT et al., 2018a). Based on our results, we detected classical BPV types 7, 8, 9 and 12, but there were 68% of new BPVs involved in the teats warts. Overall, the most detected BPV genus among the specimens was *Xipapillomavirus* with 68% of total detections (Figure 1).

PVs are usually characterized by conventional PCR followed by Sanger sequencing, using degenerate oligonucleotide pairs FAP59/FAP64 that amplify a relatively conserved L1 gene fragment virtually from all known PV types and species (FORSLUND et al., 1999). The applicability of the designed oligonucleotides has been satisfactory for most of the papillomavirus types, but negatives results appears due to lower homology of the base pairing in the 3' region of primer biding sites (FORSLUND et al., 1999). Moreover, direct sequencing of the amplicons might be unsuccessful, yielding overlapping peak patterns, since the presence of more than one type of *Papillomavirus* template in each sample (FORSLUND et al., 1999). In our study, 25 complete genomes were recovered directly from seventeen

lesions of teat warts. The conventional PCR followed by Sanger sequencing is a weak predictive instrument since it may not reflect of the actual variability of BPVs found in the samples, due to lower homology of oligonucleotide annealing sites, low coverage compared to HTS and the impossibility of detecting coinfections.

Currently, the occurrence of coinfections by different types of BPV in cattle has been reported in several regions (DAUDT et al., 2018a). These findings reflect the finding of HPV cutaneous infections, where coinfections with more than 10 different types of HPV can be detected (ANTONSSON et al., 2000). However, BPV coinfections comprising up to seven known PV types are rarely reported (DAUDT et al., 2016a). Although most of the work still employs conventional PCR methods with different oligonucleotide combinations for the detection and characterization of probable new viral types, such protocols have important limitations (DAUDT et al., 2016a). Here in, the RCA-HTS method allowed the detection of 23.5% of coinfection within seventeen teat wart samples with up to six BPV types involved, better characterization e phylogenetic inference of the BPV types involved in coinfection than conventional PCR followed by Sanger sequencing.

Human PV (HPV) encompasses more than 200 types that are fully sequenced, characterized and cataloged, in contrast to the low number of BPV, which comprises only 24 types (http://pave.niaid.nih.gov). The use of random oligonucleotides in RCA-HTS offers the possibility to amplify and detect any circular DNA that is present in a non-specificity sample, thus allowing a large overview of unknown PVs. This methodology can increase the sensibility of detection of PVs because there is a greater quantity and quality of recovery of the viral sequences present in each sample, making possible the understanding of the natural history of the infection by different types of PV. Moreover, the increasing in application of

RCA-HTS in BPV lesions allowed the characterization of new BPV types recently (DA SILVA et al., 2015; DAUDT et al., 2016b, 2018b).

In the present study, it was applied an unbiased molecular tool for detection and characterization of BPV in samples regarding teat warts lesion that are important infection disease in the dairy cattle herds around the world. We observed 23.5%% of coinfections, highlighting that PCR followed by Sanger sequencing cannot represent the totally of BPVs present in the sample. Moreover, we described one BPV classified in new genus, one BPV classified in a new species and, fifteen putative new types. It was also possible to verify that BPVs types apparently are not associated with specific anatomic presence. Our work reinforces that RCA followed by HTS can yield bias in BPV diversity in different samples.

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5 CONCLUSÕES

Em resumo, os dois trabalhos que compõem a presente tese de doutorado levaram às seguintes conclusões descritas abaixo:

Capítulo 1:

- O genoma completo do vírus foi recuperado diretamente da lesão e sua caracterização, bem com sua inferência filogenética foi determinada e classificado como CPV16.
- A provável associação entre CPV16 com a neoplasia maligna foi determinada caracterização das alterações patológicas utilizando imuno-histoquímica e hibridização *in situ*, aonde pode se observar os marcadores oncogênicos e uma infecção viral ativa na lesão.
- O estudo destacou a importância da oncogênese induzida pelo CPV e expande o conhecimento atual do *background* genético da família *Papillomaviridae*.

Capítulo 2:

- O sequenciamento de alto desempenho possibilitou a utilização de ferramentas moleculares para a detecção e caracterização de sequencias de BPV extraídas de lesões de teto de vacas leiterias.
- Foi possível detectar em 23,5% das amostras de BPV de teto mais de uma cepa de BPV.
- A PCR seguida do sequenciamento Sanger não é uma boa ferramenta preditiva da totalidade dos BPVs encontrados em uma amostra.
- O sequenciamento do gene L1 de 17 amostras de lesões de teto revelou a presença dos tipos conhecidos BPV7, BPV8, BPV9 e BPV12. Também foram descritos um provável novo gênero e uma provável nova espécie pertencente ao gênero *Dyokappapapillomavirus*. Entre os novos tipos de BPV, 9 são pertencentes a espécie *Xipapillomavirus 1*, 4 pertencentes ao *Xipapillomavirus 2* e 2 ao *Epsilonpapillomavirus 1.* Desta forma, 17 prováveis novos tipos de BPV foram adicionados aos 24 já descritos.

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ANEXOS

Anexo A: Coautoria de artigos científicos relacionados ao tema da tese

Artigo de revisão

Experimento concluído e artigo publicado na revista *Transboundary and Emerging Diseases*.

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REVIEW ARTICLE

WILEY

Papillomaviruses in ruminants: An update

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Summary

Papillomaviruses (PVs) are complex viruses which infect the skin or mucosae of a broad range of amniotes worldwide. They cause benign or malignant lesions depending on environmental factors, virus oncogenicity and the location of infection. Bovine papillomaviruses (BPVs) are the second most studied PVs beyond human PVs. In the past few years, genetic characterization of animal PVs has increased due to the availability of new techniques, which simplified the sequencing of entire genomes. Therefore, this review aims to provide an update of the current epidemiology, classification and genome features of ruminant PVs (mainly BPVs) affecting animals worldwide. The review also aimed to clarify the key differences between the high-risk *Delta* papillomaviruses and the seemingly low-risk *Xi*, *Epsilon*, *Dyoxi* and *Dyokappapillomavirus* as well as the recently described PVs BPV18, 19, 21 and PpuPV1 that belongs to an unclassified genus.

KEYWORDS
bovine papillomavirus, molecular epidemiology, papillomavirus, phylogeny, Ruminantia

1 | INTRODUCTION

Papillomaviruses (PVs) have been associated with cutaneous and mucosal warts, and neoplasia in a variety of animal species (Borzacchiello & Roperto, 2008; Rector & Van Ranst, 2013). Members of this viral family are described in a diversity of mammal genera, but they have also been described in birds, reptiles and fish, and probably infect all amniotes (López-Bueno et al., 2016; Rector & Van Ranst, 2013). PVs are strictly species- and tissue-specific although some established (Lunardi, Alfieri, et al., 2013; Lunardi, De Alcântara, et al., 2013; Nasir & Campo, 2008) and unusual cross-infections have been reported (Munday, Hanlon, Howe, Squires, & French, 2007).

Within the large group of ungulates (diverse group of mammals that includes odd-toed and even-toed ungulates), bovine papillomavirus (BPV) plays a role in a variety of diseases in domestic and wild ruminants (Bam, Kumar, Leishangthem, Saikia, & Somvanshi, 2013; Borzacchiello et al., 2003; Kumar, Nagarajan, Saikumar, Arya, & Somvanshi, 2013; Lunardi, Alfieri, et al., 2013; Tomita, Literak, Ogawa, Jin, & Shirasawa, 2007; Van Dyk et al., 2012), causing considerable morbidity to the affected animals. The suborder Ruminantia comprises a large and relevant group of terrestrial herbivorous mammals which embrace the Tragulidae, Giraffidae, Antilo-capridae, Moschidae, Cervidae and Bovidae families that contain at least 200 extant species (Fernández & Vrba, 2005). Within these families, PVs

have been detected and characterized into Bovidae, Cervidae and Giraffidae families.

This scenario can lead to economic losses mainly in countries that have vast amount of cattle herds, as BPV induces chronic, tumoral and contagious diseases that affects dairy and beef cattle herds worldwide and is associated with a variety of benign and malignant tumours (Araibi, Marchetti, Ashrafi, & Campo, 2004; Campo, 1997; Campo, Jarrett, Barron, Neil, & Smith, 1992; Da Silva et al., 2015; Jarrett, Campo, Neil, Laird, & Coggins, 1994; Martano et al., 2013; Ogawa et al., 2004). BPV is also highly important as an in vivo model for human papillomavirus (HPV) studies beyond veterinary interest (Campo, 1997; Gil da Costa & Medeiros, 2014).

Due to the increscent detection number of PV types that mainly affect large ruminants, this review aimed to provide an update of the current epidemiology, classification and genomic features of ruminant PVs (infecting Giraffidae, Bovidae and Cervidae hosts), focusing on BPVs. The review also aimed to clarify the key differences between the apparently high-risk *Delta* papillomaviruses and the seemingly low-risk *Xi*, *Epsilon*, *Dyoxi* and *Dyokappa-PVs* as well as the recently described BPVs 19 and 21 that belong to an unclassified genera.

2 | CLASSIFICATION OF PAPILLOMAVIRUSES: OLD AND NEW CONSIDERATIONS

After being classified into the *Papovaviridae* family, PVs were discriminated as a separate family, the *Papillomaviridae* (King, Adams, Carsten, & Lefkowitz, 2012). Traditionally, PVs are classified into genera, species, types, subtypes and variants. The type classification is substantial due to its great clinical relevance.

Currently, PV classification is based on nucleotide sequence identity of L1 open reading frame (ORF), a relatively conserved region that can be aligned for all known PVs for the construction of phylogenetic trees (Bernard et al., 2010; de Villiers, Fauquet, Broker, Bernard, & zur Hausen, 2004). The type of PV is determined when the entire genome sequenced, and the L1 ORF differs by more than 10% in comparison with any other PV type. New PV genera are proposed when the identities are lower than 60%. ORF L1 differences between 2% and 10% determine a new subtype, and a variant is defined when the difference is <2% (Bernard et al., 2010; de Villiers et al., 2004) (Figure 1). Nevertheless, not just the percentage of identity should be taken into account to define the PV's classification. The interpretation of the phylogenetic position, genome organization, biology and pathogenicity should also be considered to define the PV's classification (Bernard et al., 2010).

Among the suborder Ruminantia, PVs have been described mainly in Bovidae family, followed by Cervidae and Giraffidae families (Table 1). The Cervidae PVs are represented by PV members of *Xi*, *Phipa*, *Epsilon*, *Delta* and *Dyokappa* genus and the Bovidae PVs include the genera *Dyoxipapillomavirus*. The Giraffidae PV (GcPV1) is the only PV representative species described in this host species, *Giraffa camelopardalis*, and belongs to the *Delta* genera.

Bovine papillomaviruses are a crescent group of epitheliotropic viruses that recognize *Bos taurus* as its classical host, although there are some well-established cross-infections involving BPV1 and BPV2. Currently, bovine papillomaviruses (BPVs) consist of 23 types (https://pave.niaid.nih.gov/), two of them (BPV22 and 23) recently described (Bauermann et al., 2017; Da Silva et al., 2016). BPVs are distributed into five known genera and one unclassified genera (https://pave.niaid.nih.gov/). The *Xipapillomavirus* genus comprises two species, *Xipapillomavirus 1* (BPV3, BPV4, BPV6, BPV9, BPV10, BPV11, BPV15) and *2* (BPV12), and two recently described types

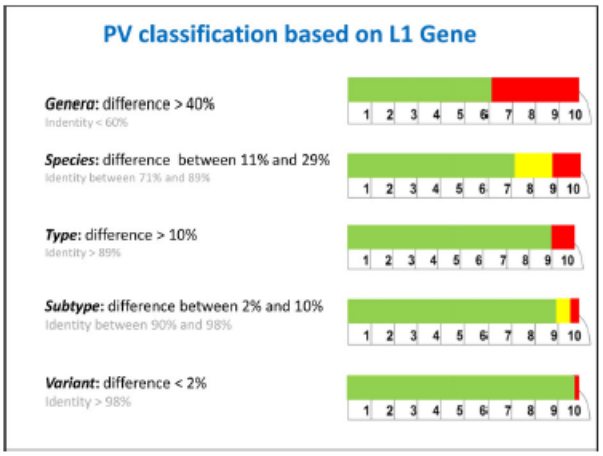

FIGURE 1 Classification scheme of Papillomaviruses (PVs) based on L1 nucleotide sequence. The green colour represents the identity. The red colour represents the differences, and the yellow colour represents the variation percentage in the classification

TABLE 1 Distinct BPV types and their geographical distribution and association with different lesions in large ruminants' species

PV type	Country[a]	Biological sample/lesion[b]	Body site	Animal family	Genera	Species
AaPV1	SE	CP, PF	Lung	Cervidae	*Delta*	*Delta 1*
BgPV1	CN	FP		Bovidae	*Delta*	*Delta 4*
BPV 1	BR, JP, UK, IT, IN, MA	CP, FP, Flies, UB, Semen, Blood, HS	Body, UBT, Teat	Bovidae	*Delta*	*Delta 4*
BPV 2	DE, BR, NZ, JP, IN, IT, TR, KOR	CP, FP, UB, Semen, Blood, Milk, Urine	Body, UBT, Udder	Bovidae	*Delta*	*Delta 4*
BPV 3	DE, JP, BR, CN	CP, FP	Body, Teat, Udder	Bovidae	*Xi*	*Xi 1*
BPV 4	UK, BR	CP, UDT, Milk, Urine, Blood	Body, UDT	Bovidae	*Xi*	*Xi 1*
BPV 5	JP, BR, IN	CP, HS, UDT	Teat	Bovidae	*Epsilon*	*Epsilon 1*
BPV 6	UK, JP, BR	CP, HS, SP, HS	Body, Teat	Bovidae	*Xi*	*Xi 1*
BPV 7	JP, BR, IT	CP, SP	Body, Teat	Bovidae	*Dyoxi*	*Dyoxi 1*
BPV 8	JP, BR	CP, FP, Blood	Body, Teat	Bovidae	*Epsilon*	*Epsilon 1*
BPV 9	JP, BR	CP, FP, SP	Body, Teat, Udder	Bovidae	*Xi*	*Xi 1*
BPV 10	JP, BR	CP, FP, SP, Blood	Body, Teat, Udder	Bovidae	*Xi*	*Xi 1*
BPV 11	JP, BR	CP, Blood, HS		Bovidae	*Xi*	*Xi 1*
BPV 12	SE, JP, BR	EP, HS	Tongue	Bovidae	*Xi*	*Xi 2*
BPV 13	BR, CN, IT	CP, UB		Bovidae	*Delta*	*Delta 4*
BPV 14	US, NZ, BR	CP, FP, Feline sarcoids		Bovidae	*Delta*	*Delta 4*
BPV15	CN	Bovine "sarcoid"		Bovidae	*Xi*	*Xi 1*
BPV16	BR	CP	Body	Bovidae	*Dyokappa*	UNC[c]
BPV17	BR	CP	Body	Bovidae	*Xi*	UNC
BPV18	BR	CP	Body	Bovidae	*Dyokappa*	UNC
BPV19	BR	CP	Body	Bovidae	UNC	UNC
BPV20	BR	CP	Body	Bovidae	*Xi*	UNC
BPV21	BR	CP	Body	Bovidae	UNC	UNC
BPV22	US	Vulvovaginitis	Vagina	Bovidae	*Dyokappa*	UNC
BPV23	BR	CP	Body	Bovidae	*Xi*	*Xi* 1
CcaPV1	HU	FP		Cervidae	*Delta*	*Delta 5*
CePV1	IT	FP		Cervidae	*Epsilon*	UNC
CePV2	NZ	CP		Cervidae	*Xi*	UNC
ChPV1	BE	HS		Bovidae	*Phippa*	*Phippa 1*
GcPV1	DK	Skin lesion		Giraffidae	*Delta*	UNC
OvPV1	US	FP		Cervidae	*Delta*	*Delta 2*
OaPV1	AU	MI[d]		Bovidae	*Delta*	*Delta 3*
OaPV2	AU	MI		Bovidae	*Delta*	*Delta 3*
OaPV3	IT	SCC		Bovidae	*Dyokappa*	*Dyokappa 1*
OaPV4	IT	FP	Scrotum	Bovidae	*Delta*	*Delta 3*
PpuPV1	DE	Hair follicles		Cervidae	UNC	UNC
RalPV1	UK	CP		Cervidae	*Delta*	UNC
RrPV1	IT	Nasolabial neoplasia		Cervidae	*Dyokappa*	*Dyokappa 2*
RtPV1	US	MI		Cervidae	*Delta*	*Delta 1*
RtPV2	NO	Eye swab		Cervidae	*Xi*	*Xi 3*
RtiPV1	DE	Hair follicles		Cervidae	UNC	UNC

PV, Papillomaviruses.

[a]Countries: Australia (AU); Brazil (BR); China (CN); Denmark (DK); Germany (DE); Italy (IT); India (IN); Japan (JP); New Zealand (NZ); Norway (NO); Sweden (SE); United States of America (US); Belgium (BE); Hungary (HU); Turkey (TR); United Kingdom (UK); South Korea (KOR).

[b]Biological samples/lesion: cutaneous papillomas (CP), pulmonary fibromatosis (PF); epithelial papilloma (EP); fibropapilloma (FP); urinary bladder (UB), healthy skin (HS); upper digestive tract (UDT); sarcoid (SAR); squamous cell carcinoma (SCC); squamous papilloma (SP); urinary bladder tumours (UBT).

[c]UNC (unclassified).

[d]MI (missing information).

that are still not classified as species (BPV17 and BPV20). The *Deltapapillomavirus* and *Epsilonpapillomavirus* genera comprise one single species each, *Deltapapillomavirus 4* (BPV1, BPV2, BPV13 and BPV14) and *Epsilonpapillomavirus 1* (BPV5 and BPV8), respectively. The BPV7 also comprises a single species, the *Dyoxypapillomavirus 1* species.

Lately, two new BPV types named BPV16 and BPV18 were classified into the *Dyokappapapillomavirus* genus. Other two new BPV types (BPV19 and BPV21) recently described are representatives of an unclassified genus. Further, two more of the recently described new types (BPV22 and BPV23) cluster in the *Dyokappa* (Bauermann et al., 2017) and *Xipapillomavirus* genus (Da Silva et al., 2016), respectively. The current classification (https://pave.niaid.nih.gov/) of Ruminantia PVs and some classification suggestions are summarized in Table 2 (see also Figure 5 and Figure 6).

The genetic characterization by PCR using the degenerated primer pairs originally designed for HPV detection (FAP59/FAP64 or MY09/MY11), which amplifies a highly conserved region of PV L1 gene (Forslund, Antonsson, Nordin, Stenquist, & Hansson, 1999; Snijders et al., 1990), has enabled the identification and characterization of several PV types in almost all affected PV species (Antonsson & Hansson, 2002; Gottschling, Wibbelt, Wittstatt, Stockfleth, & Nindl, 2008; Munday, Dunowska, Hills, & Laurie, 2013; Rector et al., 2004), as well as in new BPV types in both dairy and beef cattle from distinct geographical regions worldwide (Lunardi, De Alcântara, et al., 2013; Ogawa et al., 2004). Also, putative and new BPV types, partially or totally sequenced by Sanger or NGS, have been published recently (Da Silva et al., 2015; Daudt, da Silva, Streck, et al., 2016; Lunardi et al., 2016). However, the development of NGS has enabled the discovery of new BPV types that were not amplified using FAP or MY primer pairs (Da Silva et al., 2016; Daudt, da Silva, Streck, et al., 2016).

TABLE 2 Classification scheme of Ruminantia PV genus, species and types

Genus	Species	Ruminantia PV types
Deltapapillomavirus	*Deltapapillomavirus 1*	AaPV1, RtPV1
	Deltapapillomavirus 2	OvPV1
	Deltapapillomavirus 3	OaPV1, 2 and 4
	Deltapapillomavirus 4	BPV1, 2, 13 and 14
		BgPV1
	Deltapapillomavirus 5	CcaPV1, RalPV1
	[a]	GdPV1
Xipapillomavirus	*Xipapillomavirus 1*	BPV3, 4, 6, 9, 10, 11, 15 and 23
	Xipapillomavirus 2	BPV12
	Xipapillomavirus 3	RtPV2
	[a]	BPV20 and 17 and CePV2
Epsilonpapillomavirus	*Epsilonpapillomavirus 1*	BPV5 and 8
	[a]	CePV1
Dyoxipapillomavirus	*Dyoxipapillomavirus 1*	BPV7
Dyokappapapillomavirus	*Dyokappapapillomavirus 1*	OaPV3
	Dyokappapapillomavirus 2	RrPV1
	[a]	BPV16 and 22
Phipapillomavirus	[a]	ChPV1, RtiPV1
[a]	[a]	BPV18, PpuPV1
[a]	[a]	BPV19 and 21

PV, Papillomaviruses.
[a]Indicate types not yet assigned into a species or genera.

3 | VIRUS STRUCTURE AND GENOME ARCHITECTURE: A COMPARISON OF LARGE RUMINANT PV GENOMES

The PV virion shell is composed of the major L1 and minor L2 capsid proteins, which constitute an icosahedral structure devoid of lipoprotein envelope. The capsid shell contains 72 capsomers (pentamers) of L1 protein and possesses approximately 600 Å in diameter (Modis, Trus, & Harrison, 2002).

The genome of these viruses is double-stranded and circular DNA of approximately 8,000 base pairs (Bernard et al., 2010; de Villiers et al., 2004; Modis et al., 2002; Rector & Van Ranst, 2013), and in the mature virion, the viral DNA is associated with host cell histone proteins H2a, H2b, H3 and H4 in a chromatin-like complex (King et al., 2012). Additionally, it is organized within the virion (and infected cells) like cellular DNA in a minichromosomal form (episomal) (King et al., 2012). Genomes of oncogenic PVs may also be integrated into the host cell chromosomes, an event commonly linked with enhanced expression of oncogenes and consequent cell transformation (Doorbar et al., 2012).

Their genome organization comprises five or six early (E) ORFs and two late (L) ORFs (Bernard et al., 2010). The early ORFs coding for two proteins (E1 and E2), which are involved in replication and transcription; three oncoproteins (E5, E6 and E7), which are involved in cell transformation; and the E4 protein, which contributes to virion production and actually exhibits an expression pattern closer to the late proteins. The late proteins L1(major) and L2 (minor) are the structural components of the viral capsid (Doorbar et al., 2012).

4 | *DELTAPAPILLOMAVIRUS* AND ONCOGENICITY: HIGH-RISK PVS?

In humans, most mucosal HPV types cause benign lesions, but there are some types which are considered oncogenic or high-risk PVs that lead to the development of malignant lesions (De Martel et al., 2012). The high-risk HPV types (mainly *Alpha* genus) are considered a necessary cause of cervical cancer, and its prevalence in penile, vulvar, vaginal and anal cancers is of major relevance (De Martel et al., 2012). Similarly, in cattle, the *Delta-PVs* comprise the high-risk mucosal types that can cause urinary bladder cancer in their natural

hosts. Both BPV1 and BPV2 can cause single infection on epithelial and/or mesenchymal cells but cross-infections are also frequent (Bergvall, 2013; Bocaneti et al., 2016; Munday et al., 2015).

Considering the ruminant PV genomes, the *Delta PVs* are more often involved in malignant lesions hitherto (Borzacchiello & Roperto, 2008; Martano et al., 2013; Roperto, Munday, Corrado, Goria, & Roperto, 2016). In cattle, the BPVs 1 and 2 are the most frequently reported in urothelial bladder tumours worldwide (Borzacchiello & Roperto, 2008; Martano et al., 2013; Roperto, Munday, et al., 2016; Roperto, Russo, et al., 2016). Recently, the involvement of BPVs 13 and 14 as causative agents of urinary bladder tumours in cattle grazing on pasture containing bracken fern has been suggested through detection of their genome in single- and co-infections or evidence of their E5 expression in affected cattle from Italy (Roperto, Munday, et al., 2016; Roperto, Russo, et al., 2016; Roperto, Russo, Ozkul, Corteggio, et al., 2013). Accordingly, all the *Delta PVs* encode the E5 ORF (Figure 2) following the E2/E4 ORF. Moreover, these genomes contain the E6 and E7 ORFs following the long control region (LCR). The set of all three E6/E7/E5 oncogenes are clearly present in the *Delta-PVs* while the other genomes encompass even two of these ORFs. The only exception observed is the BPV8 (*Epsilon-PV*) that shares all these properties but was never reported in malignant lesions (Table 1).

4.1 | Ruminantia PV oncoproteins: a comparison

4.1.1 | E5 protein

Despite its small size (only 40–85 amino acids (aa)), the E5 protein is the major BPV oncoprotein (DiMaio & Petti, 2013). The E5 protein shows high leucine content. This very hydrophobic protein is localized in the cell endomembrane compartments, particularly the Golgi apparatus (Nasir & Campo, 2008). This type II transmembrane protein is usually localized in basal keratinocytes (DiMaio & Petti, 2013), showing strong transforming activity and is highly expressed in cancer cells (International Agency for Research on Cancer, 2007). However, the role of E5 in productive infection is poorly defined (DiMaio & Petti, 2013).

Studies in cattle have detected the E5 expression only in cancer cells but not in normal ones (Borzacchiello & Roperto, 2008). The E5 protein interacts with the platelet-derived growth factor receptors (PDGF-R) (involved in cancer development) in both epithelial and vascular tumours of the urinary bladder in cattle (Borzacchiello & Roperto, 2008). The comparison between all Ruminantia PV genomes shows that E5 oncogene is present in all *Delta-PVs* (high-risk PVs), one *Epsilon-PV* (which has not been related to cancer up till now) and in a variety of *Xi-PVs* (BPV9, BPV10 and BPV15), not including BPV4, which is the only *Xi-PV* related to malignancy so far (Table 1, Figures 2 and S1). Usually, *Delta* genomes encode the E5 gene at the 3′ end of the early genome region and is expressed from a spliced mRNA that initiates upstream of the E2 gene (DiMaio & Petti, 2013). Also, cofactors such as bracken fern ingestion, which contains mutagenic chemicals and immunosuppressants, are substantial for the occurrence of cancer (International Agency for Research on Cancer, 2007).

Interestingly, when *Xi* genomes contain E5 genes, they are substituting E6/E8 genes (first early region ORF). Despite its distinct location, the protein size is almost the same in length (42 aa) when compared to *Delta* E5 (41–53 aa).

The analysis of E5 predicted aa sequences from the Ruminantia PVs showed that the *Deltapapillomavirus* 4 species has great similarity among the PVs analysed (Figure S2). In the *Delta* 4 species, the most dissonant E5 aa sequence is from BPV14, which was recently detected in urothelial bladder tumours in cattle from southern Italy (Munday et al., 2015; Roperto, Munday, et al., 2016). Also, the *Xi-PVs*, which are not involved with cancer so far, showed the most variable E5 (Figure S2).

Interestingly, the high-risk BPVs (BPV1, BPV2 and BPV13) and the BgPV1 are almost identical in its E5 aa sequence. Most differences observed between BPV13 and BgPV1 E5 are located at the

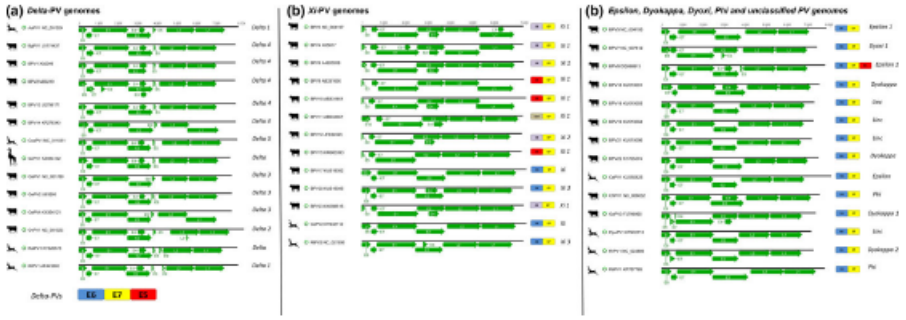

FIGURE 2 Ruminant genomes and their putative open reading frames (ORFs). All *Delta* members contain the E6, E7 and E5 oncogenic ORFs

C-terminal protein. BPV13 presents a conservative aa shift (E to D) at 46th position (Lunardi, De Alcântara, et al., 2013), and the BgPV1 contains a non-conservative change (G to S) at 51st aa position. Additionally, BPV14, as GcPV1, contains non-conservative changes at these positions. Moreover, the BPV1, BPV2, BPV13 and BgPV1 consist of a peculiar conserved region from 11th to 15th aa position, prior to the leucine rich region.

This conserved region among the *Delta 4 PVs* comprises a sequence of non-polar and polar aa followed by three non-polar aa. However, BPV14 comprises a polar and basic aa followed by three non-polar aa, which also occurs in the *Delta 3 PVs* (OaPV1, OaPV2 and OaPV4) and *Delta 5* (CcaPV1). The *Epsilon* 1 representative consists of a basic, polar, neutral and polar aa sequence in this region. Therefore, this conservative region among the high-risk *Delta-PVs* could play an important role in malignancy cell transformation (Figure S2).

4.1.2 | E6 protein

The proliferation of the basal and parabasal cells is mediated by E6 and E7 oncoproteins, especially when the epithelium is infected by the high-risk HPV types (Doorbar et al., 2012). The E6 oncoprotein promotes neoplastic transformation as E6 is a transcriptional activator. This oncoprotein targets the tumour suppressor protein p53 for degradation via ubiquitination. The E6 oncoprotein inhibits the p53 tumour suppressor by discontinuing the transcription of the coativator CBO/p300 that may be necessary, but not sufficient, for cell transformation (Zimmermann et al., 2017). The CBO/p300 proteins are involved in transcription and cell transformation and participate in a variety of cellular functions such as in the complex biological processes that affect cell growth, transformation and development (Goodman & Smolik, 2000).

The most oncogenic BPVs reported (BPV1, BPV2, BPV13) and BgPV1 contain an acid aa residue at 70th position (E), a non-conservative change, while the other Ruminantia contains a majority of non-polar, polar or basic aa. All *Delta 4* also contain an acid aa residue at 80th position (D), as *Delta 5* (CcaPV1 and RalPV1) and the *Dyokappa* member BPV22 and the unclassified BPV18. *Dyaxi 1* (BPV7) contains four more aa (CXX-X33-CXX) that are polar, non-polar followed by two basic aa in the first zinc finger prediction.

All *Delta 4 PVs* consist of an extra polar aa, polar aa (N) in the second zinc finger prediction (CXX-X30-CXX) (aa position 168). Interestingly, following this extra polar aa, the *Delta 4* consists of two non-polar aa residues (II) (BPV14 contains an aa conservative change IV). The *Delta* GcPV1 (GV), the *Xi* 3 RtPV1 (GW) and the undefined genera components, RtiPV1 (AW) and ChPV1 (WW), also contain non-polar aa residues at the same position while other Ruminantia consist of variations. However, all Ruminantia E6 ends this section with a non-polar aa residue, except CePV1 (KY), which is composed of an undefined genera related to the *Epsilon* genera (Figure 3).

Moreover, albeit it is not commonly reported, five E6 predicted proteins have one putative retinoblastoma protein-binding site (pRB-binding domain LXCXE) overlapping one of the two zinc-binding domains. Also, although the zinc finger does not fold properly, it, somehow, could also play a role in targeting the retinoblastoma protein (Figure 3). Interestingly, these sequences belong to *Delta* genus (*Delta* 4, BPV1 and BPV13—aa position 62–66; *Delta* 3, OaPV1, OaPV2 and the *Delta* member OaPV4—aa positions 99–103), which are the most common genera linked to malignant cell transformation (Borzacchiello & Roperto, 2008). The Ruminantia E6 sequences lack a PDZ-binding motif (ETQL) in its C-terminus. Although there are divergences amidst the predicted E6 protein in the Ruminantia PVs, there are some global conservative regions beyond the zinc finger domains that could be essential to its transforming functions (see positions 72, 109, 139, 151 and 152 in Figure 3).

4.1.3 | E7 protein

E7 oncoproteins are thought to interfere with the host cell cycle by targeting the cell cycle regulator pRB, leading to proliferation of the basal and parabasal cells (Doorbar et al., 2012). High expressions of E6 and E7 in cells at the lower layers interfere directly with the cell cycle, stimulate uncontrolled division and are directly related to the increasing severity of neoplasia in humans (Doorbar et al., 2012).

The pRB-binding domain is present in 23 of 41 E7 Ruminantia encoded proteins. While all Ruminantia *Xi*, *Dyokappa* and *Phi* genus lodges pBR, *Delta*, *Epsilon* and *Dyaxi* members lack the canonical pRb-binding domain LXCXE. The absence of pRB-binding domain in its E7 proteins with the E5 presence in some Ruminantia PVs has been linked to fibropapilloma-associated viruses (Narechania et al., 2017). However, there are some *Delta* and *Epsilon PVs* related to fibropapilloma lesions (Table 1).

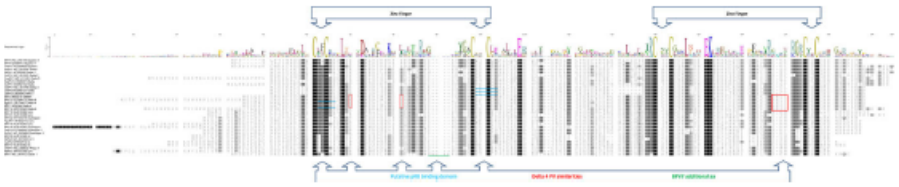

FIGURE 3 Alignment of E6 proteins from Ruminantia Papillomaviruses (PV) genomes

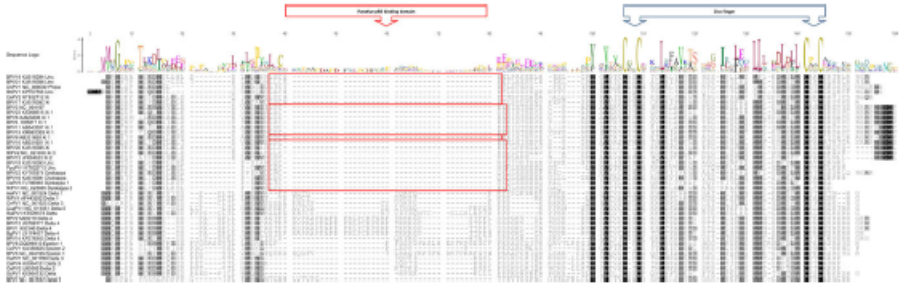

FIGURE 4 Alignment of E7 proteins from Ruminantia Papillomaviruses (PVs)

The majority of Ruminantia E7 oncoproteins consist of a zinc finger domain (CXXC-29X-CXXC). The exceptions are BPV6, CePV2, BPV8 and PpuPV1, which belong to *Xi*, *Epsilon* and unclassified genera, wherein the CXXC motifs are separated by 30 aa residues (Figure 4). The set of BPV oncoproteins, E5, E6 and E7, are known to cooperate in cell transformation (Nasir & Campo, 2008). Both the careful analysis of its predicted proteins and the biological sample histological classification are important to elucidate the real role of each oncoprotein in cell transformation amidst Ruminantia hosts.

5 | PAPILLOMAVIRUSES SPECIES IN RUMINANTS

Nowadays, there are at least 200 HPV types fully sequenced and characterized. In contrast, only 39 ruminant PV types are recognized up to now (https://pave.niaid.nih.gov/), which leads us to support the importance of improving the efforts to characterize animal PVs. Recent studies, some using high-throughput sequencing platforms have shown that animal PVs were underestimated and new types have been characterized (Bauermann et al., 2017; Da Silva et al., 2016; Daudt, da Silva, Streck, et al., 2016; López-Bueno et al., 2016; Tore et al., 2017).

Although numerous BPV types have been described lately (Bauermann et al., 2017; Da Silva et al., 2016), other ruminant species are less studied and comprise only 18 PV types in a total of 13 ruminant species (Table 1). After analysing the Ruminantia PVs, it can be noticed that all ruminant PVs belong to the same genera described for BPVs, with one exception, the ChPV1 (*Capra hircus papillomavirus 1*—domestic goat). Therefore, the genetic characterization of Ruminantia PVs could offer an important basic knowledge to understand the intraspecific and interspecific relationship between the PVs as well as between PVs and their distinct hosts.

Ruminantia PVs comprise six known genera: *Delta*, *Epsilon*, *Xi*, *Phi*, *Dyokappa* and *Dioxypapillomavirus* and one putative unclassified genus (https://pave.niaid.nih.gov/). Here, we suggest one new unclassified genus related to the *Dyokappa* genus (Figures 5, 6, S2C and S3). This clade is supported by BPV18 and PpuPV1, which diverge from *Dyokappa* by more than 55% and represents a diverse branch from *Dyokappa* members. Also, on exploring the identity and the PVs phylogenetic position, the same analysis comprising of the *Delta-PVs* shows that GcPV1 is the most unlike *Delta* genome and, probably, is a representative of a new species in this genus (Figure S2A). In the same way, the CePV1 could comprise the *Epsilonpapillomavirus* 2 in the *Epsilon-PVs* (Figure S2D), and BPV20 (more related to RtPV2) could be considered as a *Xipapillomavirus* 3 species (Figure S2B).

6 | EPIDEMIOLOGY

In *Bos taurus*, BPVs are widespread, besides the type distribution is not the same in the studied regions. They are present more frequently in young animals, especially in housing conditions, including dairy cows regarding the milking processes. Also, bad nutrition, lack of proper cleaning and inadequate installations are risk factors for the development of papillomatosis, as they can cause stress and, consequently, immunodepression, which influences the presence and the severity of this disease (Da Silva et al., 2015; Smith, 2006). Independent of technology level of livestock exploration, the papilloma lesions are particularly relevant in dairy cattle herds.

Due to the complex and the difficulty of tissue culture systems for the in vitro propagation of the PV (Bienkowska-haba et al., 2018), detailed genetic and molecular information has been accumulated, especially in human and bovine papillomavirus, using cloning and Sanger sequencing. Lately, the new technology of high efficiency sequencing has enabled the characterization of new and putative new BPVs (Da Silva et al., 2016; Daudt, da Silva, Streck, et al., 2016; Munday et al., 2015). As PVs are cosmopolitan viruses, they have been detected in many regions of almost all continents infecting a large range of amniote species (Rector & Van Ranst, 2013).

Besides the high lesions frequency level, the PV genotyping is still sporadic. The majority of BPV type-specific prevalence data are from Japan and Brazil. Brazil is one of the largest beef and milk producer in the world, and 21 of 23 BPV types were detected and described here, as well as a great number of putative new types (Batista et al., 2013; Carvalho, Batista, Silva, Balbino, & Freitas, 2012; Da Silva et al., 2016,

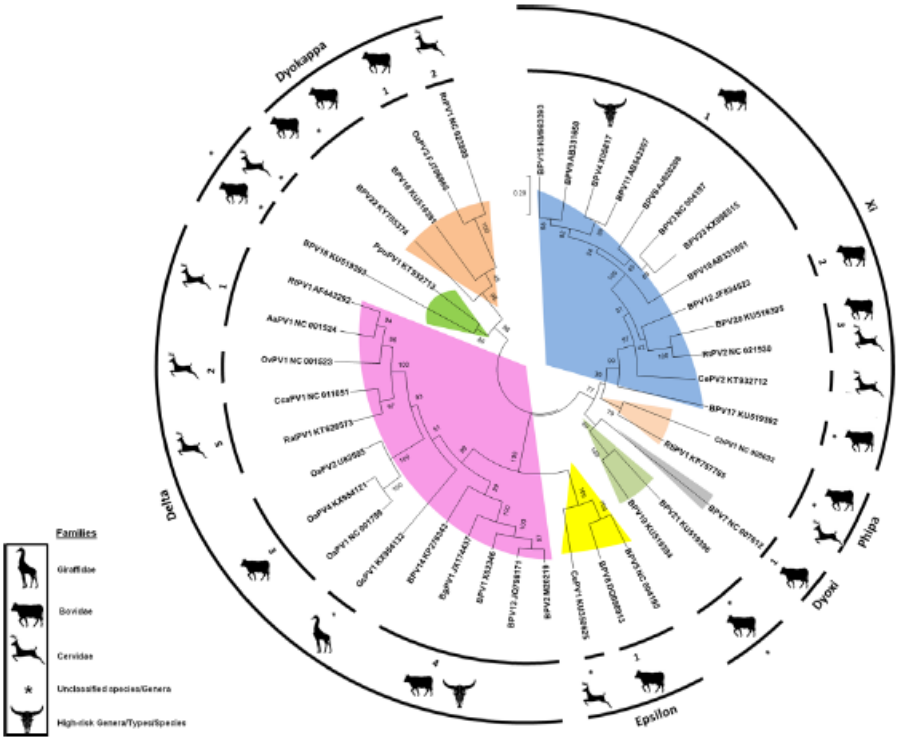

FIGURE 5 Evolutionary relationship between Ruminantia Papillomaviruses (PV) L1 complete gene. The evolutionary history was inferred using the maximum-likelihood method based on the general time-reversible model. The tree with the highest log likelihood (−38,092.55) is shown. Initial tree(s) for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.8349)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 9.10% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 41 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1,307 positions in the final data set. Evolutionary analyses were conducted in MEGA7 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013)

2017; Daudt, da Silva, Cibulski, et al., 2016; Daudt, da Silva, Streck, et al., 2016; Lunardi, Alfieri, et al., 2013; Lunardi et al., 2016). The BPV type-specific distribution and their association with specific lesions in cattle are summarized in Table 1.

The Bovidae family also consists of PVs that were characterized in three other host species: *Capra hircus* (ChPV1); *Ovis aries* (OaPV1, OaPV2, OaPV3 and the OaPV4 recently described (Tore et al., 2017); and *Bos grunniens* (BgPV1). The other Ruminantia PVs are sporadically diagnosed. Beyond the Bovidae family, the Cervidae family is the second most studied, comprising of 11 PV types, characterized into nine animal species nowadays https://pave.niaid.nih.gov/). The roe deer papillomavirus (CcPV1) infection has been identified as an endemic disease in roe deer population of the Carpathian Basin in Central Europe (Hungary, Austria and Croatia) (Erdélyi et al., 2009). The PV type-specific distribution and their lesion association in Ruminantia are summarized in Table 1. The distribution of different BPV types is shown in Figure S3.

7 | PAPILLOMAVIRUS DISEASES

7.1 | Cutaneous papillomas

The cutaneous lesions in cattle can display distinct morphologies and have been grossly classified into filiform, pedunculate and atypical

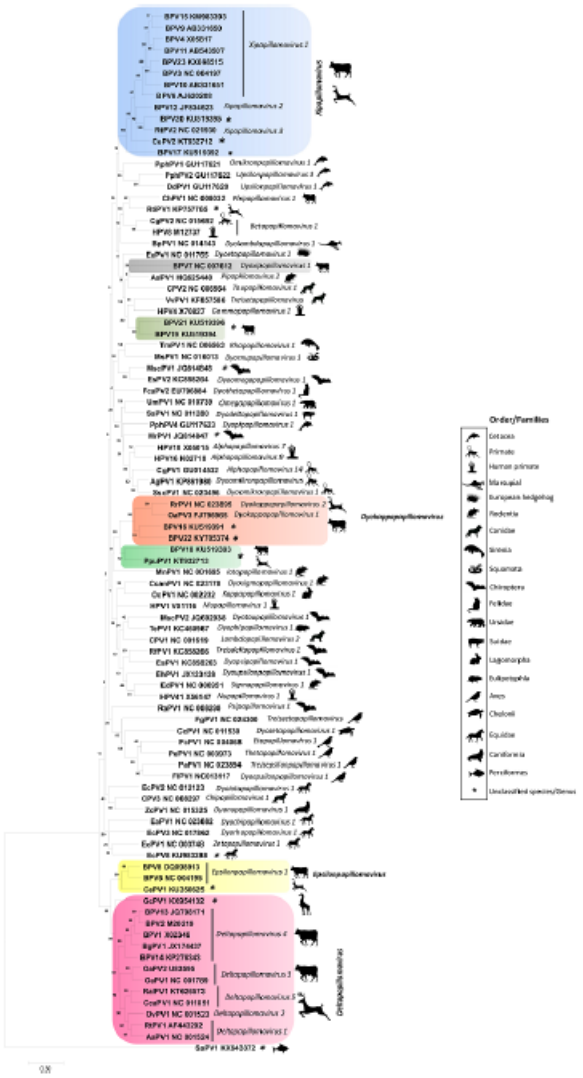

FIGURE 6 Evolutionary relationship between Ruminantia papillomaviruses L1 gene and representatives of each Papillomaviruses (PV) genera available in PaVE. The evolutionary history was inferred using the maximum-likelihood method based on the general time-reversible model. The tree with the highest log likelihood (−80,284.03) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 0.9786)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 4.28% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 92 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1,156 positions in the final data set. Evolutionary analyses were conducted in MEGA7 (Tamura et al., 2013)

forms, as planar shape or squamous papilloma (Da Silva et al., 2015; Grindatto et al., 2015; Lunardi et al., 2016). The typical pedunculate form presents verrucous aspect, known as cauliflower form. Papillomatous lesions are found in head, neck, dorse, abdomen, udder, teat and mucosa of digestive and genital tracts (Batista et al., 2013; Borzacchiello & Roperto, 2008; Claus, Vivian, Lunardi, Alfieri, & Alfieri, 2007; Lunardi et al., 2016) (Table 1). Virtually all BPVs have been detected in cutaneous lesions, even the PVs which are found in malignant lesions, as the *Delta-PVs* represented by BPVs 1, 2, 13 and 14 (Table 1).

Cutaneous HPV types cause asymptomatic or self-limited benign tumours and are usually classified as "low-risk" PV types (mainly from the *Gamma* and *Beta* genera) (De Martel et al., 2012). Similarly, cutaneous BPV types could be classified as "low risk" as their infection usually causes self-limited benign tumours (Table 1), as it happens in low-risk HPV types. These low-risk BPV viruses have been classified into all genera that BPV types belong to (Figure 1). Among Ruminantia members, cutaneous papillomas and fibropapillomas are the most frequently detected lesions induced by PVs (Table 1).

Commonly, diverse BPV genera are associated with distinct diseases. In general, *Xipapillomavirus* are classified as epitheliotropic PVs (BPV3, BPV4 and BPV6); *Deltapapillomavirus* are associated with fibropapillomas and cutaneous papillomas (BPV1, BPV2, BPV13 and BPV14) as well as the urinary bladder (BPVs 1, 2, 13 and 14) (Borzacchiello & Roperto, 2008; Lunardi, Alfieri, et al., 2013; Roperto, Russo, et al., 2016). The *Epsilonpapillomavirus* genus (BPV5 and BPV8) is linked to cutaneous papillomas (Claus et al., 2009; Da Silva et al., 2015, 2016; Tomita et al., 2007), and the *Dyoxipapillomavirus* genus was described in healthy skin swabs and has been linked to cutaneous papillomas in teat and body (Ogawa, Tomita, Okada, & Shirasawa, 2007; Savini, Gallina, Alberti, Müller, & Scagliarini, 2016; Tozato et al., 2013). BPV6, BPV7, BPV8, BPV9 and BPV10 are usually linked to benign squamous papilloma (showing varying degrees of hyperkeratosis or parakeratosis, with elongated digital-like proliferation of the squamous epithelium) and fibropapillomas (besides BPV7) (Lunardi et al., 2016; Tozato et al., 2013).

Although considerable efforts have been made to map the preferred body site for each BPV type and it is thought to be important to better understand its biology, it appears that the BPV infection is guided mainly by its tropism for mucosal and cutaneous epitheliums (Table 1). Also, as the animals harbour PVs in healthy skin (Campo, Jarrett, O'Neil, & Barron, 1994; Ogawa et al., 2004) and tumours appear at sites of damaged skin (Campo et al., 1994; Doorbar et al., 2012), there is a probability to find BPV types in sites where there is more abrasion, according to the farm management. In addition, there are evidences that they are not restricted to any anatomical site in cattle (Batista et al., 2013). PV infection regarding Cervidae, Giraffidae and other members of Bovidae family is poorly detected and studied, and it is difficult to speculate the frequent body site where it causes lesion. Therefore, more studies involving this issue are necessary to clarify the relevance of this aspect in PV ruminant infections.

7.2 | Teat papillomas

BPV6, BPV7, BPV8, BPV9 and BPV10 were identified in teat papillomas in dairy cows of Japan and southern and south-eastern Brazil through sequencing of FAP primer pair PCR products (Hatama, Nobumoto, & Kanno, 2008; Lunardi et al., 2016; Tozato et al., 2013). Recently, the putative new types BPV/BR-UEL6 and BPV/BR-UEL7, BAPV9 and a subtype of putative new type BAPV4 were also reported in teat papillomas in these Brazilian regions (Lunardi et al., 2016). Although the presence of teat and udder lesions could be associated with mastitis and the decrease in milk production (Campo, 2006), which leads to the cow's early disposal, there are few studies regarding teat papillomatous lesions.

7.3 | Bladder carcinomas

Bladder carcinomas are relatively common in cattle grazing on bracken fern in synergy with BPV1 and BPV2 (Roperto et al., 2010; Roperto, Russo, Ozkul, Corteggio, et al., 2013; Roperto, Russo, Sepici-Dincel, et al., 2013; Wosiacki, Reis, Alfieri, & Alfieri, 2002). They were also detected in buffaloes and yaks, although sporadically (Roperto, Russo, Ozkul, Corteggio, et al., 2013; Roperto, Russo, Sepici-Dincel, et al., 2013).

The clinical signs of chronic intoxication caused by the bracken fern are anorexia, inappetence, progressive weight loss, bloody diarrhoea, cough and dysphagia. The immunosuppression caused by bracken fern could promote haematogenous spread of BPV2 to the bladder and allow prolonged infection of the bladder by the PV, leading to haematuria and cystitis progression (Munday, 2014). The urinary bladder tumour incidence among cattle grazing on this pteridophyte is higher than 90%, and it is characterized by haemorrhagic and hyperplasic lesions from bladder mucosa, which frequently progress to neoplasm (Roperto et al., 2010).

7.4 | Upper GI carcinomas

The upper gastrointestinal carcinomas are mainly caused by BPV4 infection in synergism of bracken fern grazing (Borzacchiello & Roperto, 2008; Campo, 2006) but may even be due to some other factors such as infection with bovine viral diarrhoea virus (Borzacchiello & Roperto, 2008). The BPV4 infection leads to uncontrolled cell division, and the presence of bracken fern carcinogens added to the secondary genetic changes can lead to carcinomas (Doorbar, 2005).

Quercetin is a mutagenic flavonoid that is present in *Pteridium aquilinum*, which binds to the DNA, causing several DNA and chromosomal damages as the DNA breaks and rearranges and arrests normal proliferating cells in the G1 phase of the cell cycle (Beniston, Morgan, Brien, & Campo, 2001). Primary bovine cells (PalF) partially transformed by BPV4 activate its oncogenic transformation after a single exposure to quercetin (Beniston et al., 2001). This potent mutagen can activate a cis-acting element located at BPV4 LCR, which can lead to over transcription of E7 oncogene (Borzacchiello & Roperto, 2008).

7.5 | Papillomavirus cross-infections

Generally, PVs are strictly species- and tissue-specific, although equine sarcoids can be caused by BPV1, BPV2 and BPV13 (Lunardi, De Alcântara, et al., 2013; Nasir & Campo, 2008). Other examples of cross-species infections determined by *Delta-PVs* are the detection of BPV1 in cutaneous fibropapillomas of a giraffe and a sable antelope (Van Dyk et al., 2012), cutaneous warts from

buffalos infected with BPVs 1 and 2 (Pangty, Singh, Goswami, Saikumar, & Somvanshi, 2010), and the involvement of BPV2 in carcinogenesis of urinary bladder of buffalos grazing on pasture containing bracken fern (Roperto, Russo, Ozkul, Corteggio, et al., 2013). Also, BPV1 and BPV2 were reported in yaks associated with cutaneous papillomatosis in the north-east region of India (Bam et al., 2013).

Recently, the consistent identification of BPV14 DNA in mesenchymal neoplasms of domestic and exotic felids has suggested the role of BPV14 as the causative agent of feline sarcoids. As BPV14 was only found in association with lesions in cattle, cats and African lions, it seems that the host range for this viral type may be limited to bovids and felids (Da Silva et al., 2012; Munday & Knight, 2010; Munday, Knight, & Howe, 2010; Munday et al., 2015; Orbell, Young, & Munday, 2011; Roperto, Russo, et al., 2016).

7.6 | Mixed and co-infections

Occurrence of mixed and co-infections determined by different BPV types in cattle with cutaneous papillomatosis has been consistently documented by investigations involving affected animals from diverse geographical regions (Claus et al., 2009; Da Silva et al., 2012; Savini et al., 2016; Schmitt, Fiedler, & Müller, 2010). The high frequency of such infections has been demonstrated by molecular cloning of amplicons and sequencing of selected clones, PCR with specific primers, PCR-RFLP assay and multiplex-PCR in Luminex platform (Kawauchi, Takahashi, Ishihara, & Hatama, 2015; Lunardi et al., 2016; Schmitt et al., 2010; Silva et al., 2013).

More recently, the rolling circle amplification followed by next-generation sequencing strategy has demonstrated the high level of BPV co-infection, as well as the characterization of several new BPVs (Daudt, da Silva, Streck, et al., 2016). The implications of the high diversity of different BPV genotypes in a single lesion in cattle suffering from cutaneous papillomatosis are still unclear. Nevertheless, the frequent observation of co-infection in skin warts of cattle resembles the scenario shown for cutaneous HPVs where infections by more than 10 HPV types can be identified (Antonsson, Forslund, Ekberg, Sterner, & Hansson, 2000).

Curiously, examples of BPV co-infections have recently extended beyond skin lesions and are characterized by frequent presence of several *Delta-PVs* in individual urothelial tumours of cattle grazing on pasture containing bracken fern as well as identification of up to five viral types circulating in the blood from animals with and without cutaneous warts (Bocaneti et al., 2016; Roperto, Munday, et al., 2016; Roperto, Russo, et al., 2016; Santos et al., 2014). Some studies have shown co-infection of BPV13 and BPV2 in cattle urothelial bladder tumour cells, as well as BPV13 itself (Roperto, Munday, et al., 2016). Additionally, BPV1 and BPV2 were detected by qPCR in a wart collected from the reticulum of a buffalo from India (Kumar et al., 2013). In the case of urinary bladder and gastrointestinal tract cancer, prolonged ingestion of immunosuppressant compounds of bracken fern could explain the ease of multiple BPV types infecting their hosts (Roperto, Munday, et al., 2016; Roperto, Russo, et al., 2016).

7.7 | BPV infection in non-epithelial cells

Bovine papillomaviruses replication with virion production is known to occur in the epithelial component of associated lesions. However, detection of HPV DNA in peripheral blood mononuclear cells (PBMCs), plasma, serum, trophoblasts, umbilical cord blood as well as evidence of productive infection by identification of viral transcripts in PBMC in blood donors and urogenital infected patients reinforces the concept that PVs are not strictly epitheliotropic (Bodaghi et al., 2005; Sarkola, Rintala, Grénman, & Syrjänen, 2008; Widschwendter et al., 2003). The findings in HPV instigated investigations to confirm active infection of BPV in bovine non-epithelial cells.

In cattle, the presence of BPV DNA was demonstrated through partial amplification of viral segments in blood, urine, milk, semen, placenta, lymph nodes, and cells and tissues of reproductive female tract. Expression of main structural protein and/or oncoproteins was evidenced by RT-PCR, Western blotting, immunoprecipitation, immunohistochemistry and electron microscopy examination of placenta, sperm cells and PBMCs of healthy cattle or animals suffering from urinary bladder tumours (Cota, Peleteiro, Petti, Tavares, & Duarte, 2015; De Carvalho et al., 2003; Lindsey et al., 2009; Roperto et al., 2008, 2011, 2012; Santos et al., 2014), and these consistent remarks have confirmed that BPV replication is not restricted to epithelia. Besides, it has been suggested that active infection of $CD4^+$ and $CD8^+$ lymphocytes may represent the main circulating targets for BPV in blood, allowing for the spread of this pathogen to numerous organs at initial stages of infection (Corteggio, Florio, Roperto, & Borzacchiello, 2011; Roperto et al., 2011).

8 | DIAGNOSIS

Diagnosis of papillomatosis can be performed clinically once the alterations are well characterized as peduncle, or planar progressions of the epidermis can be observed (Schuch, 2001). Also, the histopathological findings and the DNA detection are indispensable for confirming the diagnosis (Betiol et al., 2012; Da Silva et al., 2015; Lunardi et al., 2016; Munday, 2014).

The PCR technique is an important diagnostic tool and is largely used for PV identification due to its high specificity and sensitivity (Forslund et al., 1999; Ogawa et al., 2004). However, PCR detection can be affected by primary factors such as DNA concentration and its purification (Ogawa et al., 2004). Type identification through PCR and sequencing, using tools such as BLAST (Basic Local Alignment Search Tool), is shown to be the most sensitive method for PV identification and characterization (Borzacchiello et al., 2003; Zhu et al., 2012).

Some techniques, such as *southern blot*, *dot blot* and immunohistochemistry (IHQ), can be used for PV diagnosis (Munday et al., 2007). The IHQ is a substantial tool that allows the identification of the virus proteins, which is important in viral activity observations (Nakamura, Mashima, Kameyama, Mukai, & Oguchi, 1997). The BPV identification through IHQ is generally carried out using commercial monoclonal antibodies (anti-L1 and anti-E7, which allows the

immune detection of the major capsid protein and the E7 oncoprotein, respectively).

Histologically, papillomas are epithelial neoplasms that present digitiform progression through the cellular surface. It is common to observe hyperkeratosis, acanthosis of the spinal layer and koilocytosis (Da Silva et al., 2015; Lunardi et al., 2016; Monteiro et al., 2008). Also, cutaneous papillomas in cattle show epidermis proliferation and kerato-hyaline granules (Campo, 2006; Grindatto et al., 2015; Lunardi et al., 2016). The presence of several islands of degenerated epithelial cells surrounded by a thick halo of hyperplastic epidermis can also be observed (Lunardi et al., 2016).

9 | CONCLUSION AND PERSPECTIVES

Papillomaviruses are ancient viruses spread worldwide and infect a considerable number of animal species, including the species that are not of commercial interest. The recent research focusing on bovines has discovered a broad range of new virus types and found that co-infections in animals are as common as the ones reported in human beings.

The analysis of Ruminantia PV oncoproteins has shown interesting similarities between the *Delta* 4 species, which could be related to their great potential for malignant cell transformation. Hence, it could be said that they are high-risk representatives. Also, their prediction in certain body parts is still unclear and, apparently, the majority of BPVs were detected in cutaneous papillomas hitherto. Even though BPVs are the major representative PVs in the Ruminantia Suborder, more studies are required to confirm these speculations. BPVs are detected virtually in all cattle herds that are studied. However, even wild ruminant research is important, both to understand viruses biology, intra- and interspecific relationship, and mainly its evolution, which is underrepresented.

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CONFLICT OF INTEREST

None of the authors of this manuscript has any conflict of interest. This manuscript has not been simultaneously submitted for publication in another journal, and all co-authors have been approved the final version.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Anexo B: Autoria e coautoria de artigos científicos desenvolvidos durante o doutorado

Artigo 1

Experimento concluído e artigo publicado na revista *Virology*.

Título: *Characterization of dog serum virome from Northeastern Brazil*

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Characterization of dog serum virome from Northeastern Brazil

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ABSTRACT

Domestic dogs share habitats with human, a fact that makes them a potential source of zoonotic viruses. Moreover, knowledge regarding possible bloodborne pathogens is important due to the increasing application of blood transfusion in dogs. In the present study, we evaluated the serum virome of 520 dogs using throughput sequencing (HTS). The serum samples were pooled and sequenced using an Illumina MiSeq platform. Our unbiased method identified prevalent canine pathogens as canine protoparvovirus 1 (canine parvovirus 2), undersenched agents as canine bocaparvovirus 1 (minute virus of canines) and canine circovirus, circular viruses closely related to viruses recently found in human samples, and new parvovirus and anelloviruses. The dog virome described in the present work furthers the knowledge concerning the viral population in domestic animals. The present data includes information regarding viral agents that are potentially transmitted through blood transfusion among dogs.

1. Introduction

Domestic dogs (*Canis lupus familiaris*) are the most popular pet worldwide and share extensive contact with humans. Dogs share their habitat with humans, other domestic animals, as well as wild animals, which renders them a potential risk factor for the transmission of zoonotic viruses, such as rabies virus (Lackay et al., 2008) and rotavirus (Tsugawa and Hoshino, 2008; Wu et al., 2012). Moreover, blood transfusions in veterinary medicine have become increasingly common and form an integral part of lifesaving and advanced treatment of the critically ill (Kisielewicz and Self, 2014; Langston et al., 2017). Although the screening for non-viral pathogens such as *Babesia* spp., *Leishmania* spp., *Ehrlichia* spp., *Anaplasma* spp., and *Brucella canis* is recommended (Reine, 2004; Wardrop et al., 2005),the knowledge and monitoring of viral agents through effective tools represents an important sanitary step.

Through virome analysis, it is possible to detect viruses whose propagation is difficult or impossible in cell culture and are not detectable by molecular detection tests since they contain no common gene, such as the ribosomal 16 S gene present in bacterial species (Delwart, 2007; Moreno et al., 2017). The enhanced availability and application of high throughput sequencing (HTS) technologies has facilitated the detection of known and unknown viruses (Goodwin et al., 2016; Kohl et al., 2015; Virgin, 2014). Moreover, metagenomic viral detection is "unbiased" as it uses non-specific primers to detect nucleic acid sequences (Toohey-Kurth et al., 2016; Virgin, 2014).

Previous studies concerning the dog virome addressed the viral components of the gastrointestinal flora (Li et al., 2011; Moreno et al., 2017) and characterized novel viruses as canine sapovirus and canine kobuvirus (Li et al., 2011). Despite the fact that serum virome was assessed with HTS in humans (Moustafa et al., 2017), cattle (Sadeghi et al., 2017; Toohey-Kurth et al., 2016; Wang et al., 2018), and horses (Li et al., 2015), no research has been conducted to investigate the dog serum virome. Thereby, the present study aimed to evaluate and characterize the serum virome using HTS of healthy dogs inhabiting Northeastern Brazil.

2. Materials and methods

2.1. Study design and sources of sera

The blood samples of 520 dogs were obtained between March 2015

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and March 2016 from five urban centers located in Paraíba state, Northeastern Brasil: João Pessoa (167 samples), Campina Grande (158 samples), Patos (90 samples), Sousa (37 samples), and Cajazeiras (68 samples). The blood samples were centrifuged at 2000 g for 10 min, and the serum was collected and stored at − 80 °C prior to the analysis. The project was registered in the Ethics Committee on the Use of Animals (CEUA) of Universidade Federal de Campina Grande (UFCG) under protocol number #0041/280314.

2.2. Viral metagenomics and HTS

The 520 dog sera were assembled in one pool containing 100 µL of each serum sample. The total 52 mL were passed through a 0.22 µm filter and subsequently ultracentrifuged on a 25% sucrose cushion at −100,000 × g for 3 h at 4 °C in a Sorvall AH629 rotor. The pellet containing the viral particles was incubated for 1.5 h with DNase and RNase enzymes (Thermo Fisher Scientific, Waltham, MA, USA) (Thurber et al., 2009). Subsequently, the viral RNA and viral DNA were isolated using TRIzol™ LS reagent (Thermo Fisher Scientific) and a standard phenol–chloroform protocol (Sambrook and Russel, 2001), respectively. The viral DNA was enriched through multiple displacement amplification (MDA), performed with Φ29 DNA polymerase (New England Biolabs, Ipswich, MA, USA) (Niel et al., 2005). Furthermore, the viral RNA was reverse-transcribed and enriched to dsDNA using TransPlex® Complete Whole Transcriptome Amplification (WTA) Kit (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's recommendations. The DNA products produced from these enrichment protocols (MDA products from viral DNA and WTA products from viral RNA) were pooled in equimolar amounts and purified using the PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Thermo Fisher Scientific). The quality and quantity of the DNA were assessed through spectrophotometry and fluorometry performed with NanoDrop™ (Thermo Fisher Scientific) and Qubit™ (Thermo Fisher Scientific) respectively. The viral libraries were further prepared with 50 ng of purified DNA, using the Nextera DNA Library Preparation Kit and sequenced using an Illumina MiSeq System using an Illumina v2 reagent kit (2 × 150 paired-end reads).

2.3. Bioinformatic analysis

The quality of the sequences generated was evaluated using FastQC. Furthermore, the sequences with bases possessing a Phred quality score < 20 were trimmed with the aid of Geneious software (version 9.0.5). Subsequently, the paired-end sequence reads were *de novo* assembled into contigs with the CLC Genome Workbench version 6.0.1 (http://www.clcbio.com/products/clc-genomics-workbench). All assemblies were confirmed through mapping reads to contigs produced by the CLC Genome Workbench using Geneious software. Thereafter, the assembled contigs were examined for similarities with known sequences through the BLASTX software using Blast2GO (Gotz et al., 2008). Sequences with *E*-values ≤ 10^{-3} were classified as likely to have originated from eukaryotic viruses, bacteria, phages, or unknown sources, a conclusion reached based on the taxonomic origin of the sequence with the best *E*-value. Gene and protein comparisons were performed with BLASTN and BLASTP programs (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Sequences representative of viruses belonging to the families *Parvoviridae*, *Anelloviridae* and *Genomoviridae* were obtained from GenBank and aligned with the sequences identified in the present study with MUSCLE software (Edgar, 2004). Phylogenetic trees were constructed using MEGA6 (Tamura et al., 2013).

2.4. Real-time PCR

The 520 dog sera were screened individually for viral genomes previously obtained through HTS. Their DNA was isolated from sera using a standard phenol–chloroform protocol (Sambrook and Russel, 2001). Furthermore, primers targeting *Human associated gemykibivirus 2* (GmKV-2) and *Carnivore bocaparvovirus 1* (CBPV-1) were designed using the Primer 3 software available in the Geneious version 9.0.5 using the obtained contigs and representative strains available in GenBank. Primers previously described were utilized for *Carnivore protoparvovirus 1* (CPPV-1) (Kumar and Nandi, 2010) and *Canine circovirus* (CaCV) detection (Li et al., 2013). The sesavirus-related sequence obtained in the HTS was not assayed as it is impossible to design specific primers with small contigs (109 bp) that present a low identity with the sesavirus reference genome (GenBank accession number NC_026251.1). Moreover, the *Thetatorquevirus*-like sequences were also not assayed due to the number of different specimens detected by HTS. Table 1 summarizes the sequence, target, and product size of the primers utilized in the present study.

Table 1
Summary of sequences that matched with the animal virus present in the pooled dog serum sample and its frequency in the 520 serum samples.

Virus hits (BLASTX, E value < 1×10^{-3})	No. of hits	No. of reads	Contigs lengths	Frequency in the individual samples
CPPV-1	12	611	60-977	1.34% (07/520)
CBPV-1	6	7	131-643	0.38% (02/520)
Sesavirus	1	2	109	NA
CaCV	1	4	200	1.34% (07/520)
GmKV-2	6	168	54-670	5.38% (28/520)
Thetatorquevirus	31	395	69-475	NA

NA: Not analyzed.

Furthermore, real-time PCRs were designed with PowerUp SYBR™ Green Master Mix (Applied Biosystems, Foster City, CA, USA), with 2 pmol of each primer, and 2 µL of total DNA, q.s.p. 25 µL. The amplification was performed with the following cycling profile for HGmKV-2 and CPPV-1: 50 °C for 2 min and 95 °C for 3 min for initial denaturation and enzyme activation step, followed by 40 30 s cycles for denaturation at 95 °C, 30 s at 55 °C for annealing, and extension at 60 °C for 30 s. For CBPV-1 and CaCV, the following cycle conditions were applied: 50 °C for 2 min and 95 °C for 3 min for initial denaturation and enzyme activation step, followed by 40 cycles of 30 s for denaturation at 95 °C, and 1 min of annealing/extension at 60 °C. A threshold cycle (Ct) value of 40 indicates a viral nucleic acid quantity below detection level. In light of the fact that the real-time PCRs were not calibrated with known concentrations of target nucleic acids, the obtained Ct values only reflect relative viral loads in samples and cannot be utilized to compare viral genome loads across different samples. Moreover, all the real-time PCR products were stained with GelRed Loading Buffer (Quatro G Pesquisa e Desenvolvimento, Porto Alegre, RS, Brazil), electrophoresed in 2% agarose gels, and visualized under UV light for confirmation.

2.5. PCR and Sanger sequencing

All positive samples in real-time PCR were submitted for Sanger sequencing to confirm the specificity of the tests. The CPPV-1 positive samples obtained in real-time PCR were submitted to PCR using primers CPV 555F and CPV 555R that amplify a 583 bp of the VP2 gene (Buonavoglia et al., 2001) for CPPV-1 type definition.

The CBPV-1 whole genome were assembled using the viral reads obtained in HTS and through additional PCR protocols performed with previously described primers (Shan et al., 2010). The GmKV-2 whole genomes were obtained using the viral reads obtained in HTS and with additional PCR protocols executed with primers developed using the sequences obtained in HTS. Table A.1 summarizes the primers employed to obtain the CBPV-1 and GmKV-2 full genomes.

The PCR products were purified using the PureLink™ Quick PCR Purification Kit (Invitrogen, Carlsbad, CA, USA). Both DNA strands

were sequenced with an ABI PRISM 3100 Genetic Analyzer utilizing a BigDye Terminator v.3.1 cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Furthermore, overlapping fragments were aligned and assembled using Geneious software.

3. Results

3.1. Overview

One DNA library consisting of serum samples obtained from 520 dogs was generated and sequenced using paired-end 2 × 150 base runs on the Illumina MiSeq platform, which generated a total of 1,295,772 reads. The 80,134 assembled sequence contigs produced with the CLC Genome Workbench were compared with the viral reference database and the GenBank non-redundant protein database through a BLASTX search conducted with an *E*-value cut-off of 10^{-5} in Blast2GO (Gotz et al., 2008). It was found that the exogenous eukaryotic virus-related sequences comprised 0.09% of the reads and 0.107% of the contigs.

Furthermore, eukaryotic exogenous virus-related sequences belonging to four viral families with single-stranded DNA (ssDNA) genomes were also observed (Table 1). Additionally, viral sequences related to families *Parvoviridae* (CPPV-1, CBPV-1, and sesavirus-like), *Circoviridae* (CaCV), *Genomoviridae* (GmKV-2), and *Anelloviridae* (*Thetatorquevirus*-like) were detected as well. Moreover, the majority of the viral sequences studied shared a high degree of identity with known animal viruses (CPPV-1, CBPV-1, CaCV, and GmKV-2), while others (thetatorquevirus-like and sesavirus-like) exhibited a high degree of divergence to genomes already recorded in the GenBank. Information regarding the sequences and complete viral genomes obtained has been described in the following sections.

3.2. Carnivore protoparvovirus 1 (CPPV-1), Carnivore bocaparvovirus 1 (CBPV-1) and Canine circovirus (CaCV)

In the 520 pooled dog serum submitted for HTS, the presence of 12 contigs closely related to *Canine parvovirus 2* (CPV-2) was observed, which was reclassified as CPPV-1 (genus *Protoparvovirus*, subfamily *Parvovirinae*, family *Parvoviridae*) by the International Committee of Taxonomy of Viruses (ICTV) (Cotmore et al., 2014) (Table 1). The application of the specific CPPV-1 real-time PCR-protocol (Kumar and Nandi, 2010) to individual samples revealed 1.34% (7/520) to be positive (Table 1). All the CPPV-1-positive serum were submitted to PCR using primers CPV 555F and CPV 555R that amplify a 583 bp of the VP2 gene (Buonavoglia et al., 2001) and DNA sequencing for CPPV-1 type definition (CPV-2a, 2b, or 2c). Furthermore, samples were assigned to one CPV-2 type based in the presence of a deduced asparagine (2a), aspartic acid (2b), or a glutamic acid (2c) in the amino acid position 426 (Buonavoglia et al., 2000). The seven samples tested shared 100% of nucleotide identity between them. In the nucleotide BLAST search, the samples detected in this study presented 99.5% identity with CPPV-1 strains Bel2014-01, Bel2015-01, and Bel2016-01 (GenBank accession numbers KX774249, KX774251, and KX774250 respectively) reported in the North Brazil. The samples detected in the present study were classified as CPV-2c due to the presence of a glutamic acid in the amino acid position 426.

Four contigs closely related to the minute virus of canines (CMV) were obtained from the pooled dog serum submitted for HTS (Table 1). The CMV species, previously also referred to as canine parvovirus 1 (CPV-1), was reclassified as CBPV-1 in genus *Bocaparvovirus* of the subfamily *Parvovirinae* and family *Parvoviridae* (Cotmore et al., 2014). With the application of the CBPV-1-specific real-time PCR (Table A.1), two out of the 520 (0.38%) serum samples were found to be positive. The amplification products of these two samples were submitted for Sanger sequencing and exhibited 100% nucleotide identity between them. The enriched DNA submitted for HTS was utilized in order to obtain the full-length CBPV-1 genome with the viral reads obtained in HTS and through additional PCR protocols applied with previously described primers (Shan et al., 2010). The CBPV-1 whole genome obtained in the present study was named as LV-Paraíba and deposited in GenBank under the accession number MH713703. In a nucleotide BLAST search, LV-Paraíba was found to share a higher level of identity with CBPV-1 strain SH1 (GenBank accession number FJ899734) detected in a dog that exhibited clinical signs of enteric disease in China in the year 2009 (Shan et al., 2010) in whole genome (98.3%), NS1 (98.2%), NP1 (98.6%), VP1 (97.9%), and VP2 (97.9%). In order to present the genetic relation between LV-Paraíba and other *Bocaparvovirus* members, a whole-genome phylogenetic tree applying neighbor-joining inference, Kimura-2 statistical method, and 1000 bootstrap was constructed in MEGA6 (Tamura et al., 2013) (Fig. A.1). Furthermore, the selected CBPV-1 strains were clustered in the same branch supported by a bootstrap value of 100%. The CBPV-1 LV-Paraíba grouped in the same terminal node of strain SH1 from China (GenBank accession number FJ899734) was supported by a 99% bootstrap value. We chose to present only the phylogenetic tree based on the complete genome since it displayed the same topology as the ones based on individual genes.

One contig closely related with the replication associated (Rep) protein of CaCV (Fig. A.3) was observed in the pooled dog serum submitted for HTS (Table 1). Furthermore, CaCV falls within the 29 species of genus *Circovirus* of the family *Circoviridae* (Breitbart et al., 2017). The application of a CaCV-specific real-time PCR (Li et al., 2013) (Table A.1A) revealed 1.34% (7/520) of the dog serum samples as positive (Table 1).

The CaCV-contig obtained in HTS exhibit homology with Rep sequences of CaCV and 98% nucleotide identity with CaCV strain XF16 (GenBank accession number MF797786) detected in a dog from China. In order to verify the genetic relation between the Brazilian CaCV and other CaCV members, a nucleotide partial Rep-based phylogenetic tree was constructed with MEGA6 (Tamura et al., 2013) with the neighbor-joining inference method, Kimura-2 statistical model, and 1000 bootstrap replicates (Fig. A.3). The phylogenetic tree presented two branches supported by bootstrap values of 77% and 99% corresponding to CaCV strains detected in dogs and the CaCV strains reported in foxes, also defined as a divergent group within CaCV species named fox circovirus, according to the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi). The canine-related branch was divided into two clusters, in which the strain of the present study was grouped in the same node with CaCV strains 214 and UCD1-1698 detected in dogs from the United States (GenBank accession numbers JQ821392 and KC241982, respectively), XF16 detected in dogs from China (GenBank accession number MF797786), and CB6293/1–14 Italy and PE8575/1–13 detected in dogs from Italy (GenBank accession number KT734812 and KT734823 respectively).

3.3. Sesavirus-like

In the pooled dog serum assayed by HTS, a single 109 bp contig revealed higher nucleotide (58.6%) and amino acid (74.2%) identity with the VP1 gene of a new parvovirus putatively named as Sesavirus CSL10538, which was found in the feces of sea lion (GenBank accession number NC_026251) (Fig. A.2A). The sequence obtained in the present study was deposited in GenBank under the accession number MH717456. However, it was not possible to define the individual(s) sample(s) containing this putative viral sequence since the short contig obtained with the HTS was not informative to allow the development of an efficient PCR protocol.

This small sequence was submitted to an amino acid phylogenetic inference with other subfamily *Parvovirinae* members using the neighbor-joining method, p-distance model, and 1000 bootstrap in MEGA6 (Tamura et al., 2013). The phylogenetic tree (Fig. A.2B) presented genus grouping supported by bootstrap values ranging from 66%

to 100%. The sequence obtained in the present study clustered in the *Copiparvovirus* branch (bootstrap value: 84%) in the same terminal node of Sesavirus CSL10538 (GenPept accession number AIE58041) (bootstrap value: 85%). The *Copiparvovirus* branch presented two clusters: the first one contained the ungulate *Copiparvovirus* 1 (GenPept accession number ALN66861) and 2 (GenPept accession number AGM20661), recognized by ICTV and the unclassified putative *Copiparvovirus* species porcine parvovirus 5 (GenPept accession number YP_008888534) and 6 (GenPept accession number YP_009021567); the second comprised the sequence obtained in the present study, clustered with other sequences putatively classified as *Copiparvovirus* members as horse parvovirus (GenPept accession number AKN50610), which was detected in a nasal swab of a diseased horse (Li et al., 2015) and bosavirus (GenPept accession number YP_009325418), which was detected in cattle serum (Sadeghi et al., 2017), and sesavirus CSL10538 (GenPept accession number AIE58041), detected in sea lion feces and proposed as a new genus putatively classified as *Marinoparvovirus* (Phan et al., 2015a).

3.4. Human associated gemykibivirus 2 (GmKV-2)

A total of six contigs closely related to GmKV-2 were obtained from the 520 pooled dog serum submitted for HTS (Table 1). The GmKV-2 was one of the 16 viral species of genus *Gemykibivirus* of the family *Genomoviridae* (Varsani and Krupovic, 2017). The application of GmKV-2-specific real-time PCR (Table A.1) revealed 28 out of 520 (5.38%) dog serum samples to be positive (Table 1). Out of a total of 28 sample amplification products, five were submitted for Sanger sequencing, presenting 100% nucleotide identity between them.

The enriched DNA submitted for HTS was utilized in order to obtain the full-length GmKV-2 genome using the viral reads obtained in HTS and additional PCR protocols (Table A.2). The GmKV-2 whole genome obtained in the present study was named as LV-dog/Paraíba/Brazil and deposited in GenBank under the accession number MH734235. It displayed a typical *Genomoviridae* organization (Fig. 1A) comprised of a circular single-stranded DNA genome containing 2210 nucleotides (nt) and a 51% C+G content. The sequence presents an untranslated intergenic region comprising 139 nucleotides, a single capsid (Cap) protein (nt 101–1068), and Rep protein genes divided in two intervals (nt 2171 to 1597 and 1454 to 1069). A nucleotide BLAST search revealed that LV-dog/Paraíba/Brazil shared 99.8% of identity in the Rep gene with GmKV-2 strains SL1, SL2, and SL3 (GenBank accession numbers KP133075, KP133076, and KP133077 respectively) detected in human cerebro spinal fluid samples from Sri Lanka, 99.8% with strains BZ2 and BZ1 respectively (GenBank accession numbers KP133079 and KP133078 respectively) detected in human feces, and 99.8% identity with strain NP (GenBank accession number KP133080) detected in untreated sewage from Nepal (Phan et al., 2015b). In Cap gene, LV-dog/Paraíba/Brazil shared 98.6% of nucleotide identity with BZ1, 98.2% with BZ2, 97.7% with NP, 97.6% with SL3, 97.5% with SL2, and 97.3% with SL1. Our sequence also presented 99.4–99.1% of nucleotide identity in Cap gene with partial sequences of GmKV-2 detected in water from the river Amazon and sewage samples obtained from Rio de Janeiro and Manaus, Brazil (GenBank accession numbers KU862864, KU862870, KU862878, and KU862879).

In order to present the genetic relation between LV-dog/Paraíba/Brazil and other *Gemykibivirus* members, Rep (Fig. 1B) gene amino acid phylogenetic tree using neighbor-joining inference, p-distance statistical method, and 1000 bootstrap was constructed in MEGA6 (Tamura et al., 2013). The phylogenetic reconstruction presented the LV-dog/Paraíba/Brazil grouping in the GmKV-2 cluster supported by 100% bootstrap values.

3.5. Thetatorquevirus-like

A total of 31 contigs with a closer association with *Thetatorquevirus* genus members of the family *Anelloviridae* were also observed (Table 1). The contigs ranged between 69 and 475 nt in length. In a BLASTX search, the sequences presented highest identity with torque teno canis virus detected in dogs (32.3–66%), Nayun tick torquevirus detected in *Rhipicephalus* sp. (33–70%), and tick-associated torque teno virus detected in *Dermacentor variabilis* (42.6–68.6%) (GenBank accession numbers KX377522, KP141758, and MF173068 respectively). The number of possible different specimens present in the pooled dog serum

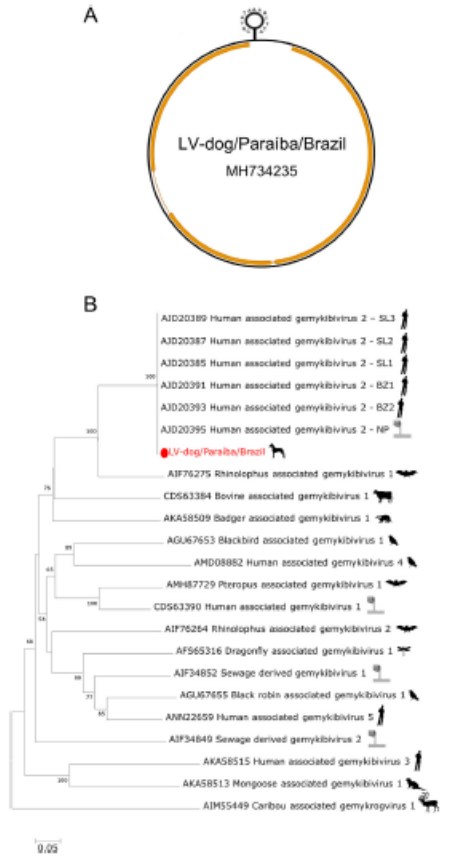

Fig. 1. Genetic characterization of gemykibivirus detected in the present study. (A) Genomic organization of the GmKV-2 detected in the present study. (B) Amino acid phylogenetic trees of complete replication associated protein of *Gemykibivirus* genus members. Sequences were analyzed through the neighbor-joining method applied with the p-distance model. All analyses were conducted with 1000 bootstrap replicates, and the percentage of replicate trees in which the sequences clustered together have been illustrated adjacent to the branches. Bootstrap values for each node have been depicted if they were > 50%. *Gemykrogvirus* was utilized as outgroup. The sequence detected in the present study has been highlighted with a • and was deposited in GenBank database under the accession number MH734235.

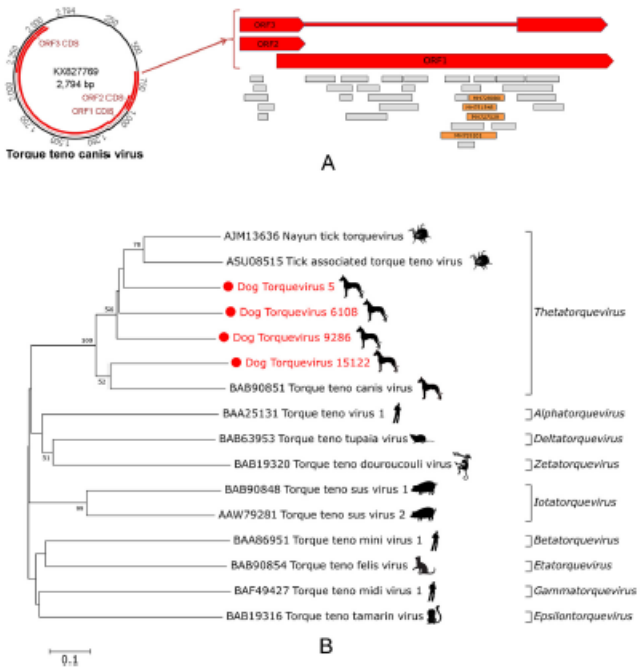

Fig. 2. The genetic characterization of putative thetatorqueviruses observed in the present study. (A) Genome position of anellovirus-related sequences observed in the present study compared with the *Thetatorquevirus* prototype member torque teno canis virus. Sequences employed to construct the phylogenetic tree have been highlighted. (B) Amino acid phylogenetic tree of *Anelloviridae* members. Sequences were analyzed with the neighbor-joining method applied through the p-distance model. All analyses were conducted with 1000 bootstrap replicates, and the percentage of replicate trees in which the sequences clustered together have been illustrated adjacent to the branches. Bootstrap values for each node have been presented if they were > 50%. Sequences detected in the present study have been highlighted with an •. Dog torquevirus 5, 6108, 9286 and 15,122 were deposited in GenBank under the accession numbers MH719201, MH727539, MH729080, and MH751548 respectively.

rendered these viral agents difficult to assay through real-time PCR in individual dog samples and these sequences do not have their complete genome sequenced.

The viral contigs were adjusted to constitute a corrected frame as well as translated and aligned with the sequences with higher identity to map their position in the gene (ORF1, 2, or 3); subsequently, they were compared with the genus prototype. Some of the contigs were homologous, indicating the presence of at least four different *Thetatorquevirus*-related sequences (Fig. 2A). These four sequences were selected for partial ORF1 phylogenetic analysis with other *Anelloviridae* members using neighbor-joining inference, p-distance statistical method, and 1000 bootstrap was constructed in MEGA6 (Tamura et al., 2013) (Fig. 2B). The phylogenetic reconstruction presented nine well-separated clusters corresponding to anellovirus genera. The four sequences detected in the present study were grouped under *Thetatorquevirus* genus, composed by dog viruses and ticks with a 100% bootstrap value. The *Thetatorquevirus* genus branch was clearly divided into two clusters: one constituted by torque teno canis virus (GenBank accession number AB076002) and dog torquevirus 15122 detected in the present study and the second one comprised by recently identified viruses detected in ticks (Nayun tick torquevirus and tick associated torque teno virus) (GenBank accession numbers KP141758 and MF173068 respectively) and the remaining three sequences detected in the present study. Other phylogenetic reconstructions that used other contigs obtained in the HTS were constructed as well; they presented a topology similar to that observed in Fig. 2B (data not shown).

4. Discussion

The virome present in serum samples obtained from 520 healthy dogs has been described using HTS and metagenomic analysis. Only a limited number of works have described the virome of dog feces to date (Li et al., 2011; Moreno et al., 2017); furthermore, the serum virome had not been previously evaluated in this animal species. Our study, using employed non-specific amplification, revealed the presence of commonly reported dog viruses and previously unknown viral agents (Table 1). The outstanding presence of *Parvoviridae* and *Anelloviridae* members is consistent with the findings reported in human (Moustafa et al., 2017) and cattle (Sadeghi et al., 2017; Toohey-Kurth et al., 2016; Wang et al., 2018; Weber et al., 2018) serum. Moreover, the predominance of small DNA viruses instead of RNA viruses appears to be consensus since it is reflected in previous works applying similar methodology used in the present study (Moustafa et al., 2017; Sadeghi et al., 2017; Toohey-Kurth et al., 2016; Wang et al., 2018; Weber et al., 2018)

Since the application of blood transfusions in the treatment of dogs has become increasingly common (Kisielewicz and Self, 2014; Langston et al., 2017), monitoring microbes through effective tools represents an important step for sanitary control (Reine, 2004; Wardrop et al., 2005). Moreover, the close contact shared by dogs and humans makes it important to be aware about essential the knowledge of potential zoonotic pathogens. Our unbiased method identified prevalent canine pathogens as CPPV-1 (Alves et al., 2018; Duijvestijn et al., 2016; Gizzi et al., 2014), undersearched pathogens as CBPV-1 (Carmichael et al., 1994), recently identified agents defined as co-pathogen as CaCV (Dowgier

et al., 2017; Li et al., 2013), recently reported circular viruses (GmKV-2) found in humans with diseases of uncertain etiology (Phan et al., 2015b), and previously unknown viruses in dogs, as sesavirus-like and thetatorquevirus-like (Table 1). The present data includes information regarding viral agents that are potentially transmitted through blood transfusion in dogs.

The CPV-2 was recently reclassified as CPPV-1 within the genus *Protoparvovirus*, subfamily *Parvovirinae*, and family *Parvoviridae* (Cotmore et al., 2014). CPPV-1 is the most common gastrointestinal pathogen found in dogs worldwide (Alves et al., 2018; Duijvestijn et al., 2016; Gizzi et al., 2014) and is associated with severe diarrhea, vomiting, dehydration, and myocarditis (Decaro and Buonavoglia, 2012). In the present work, we detected CPPV-1 in 1.34% of the dog serum tested (Table 1). All the samples were further analyzed in amino acid 426 residue to define CPPV-1 type (CPV-2a, 2b, or 2c) (Buonavoglia et al., 2001), and all were defined as CPV-2c. CPV-2c is replacing CPV-2b as the most frequent CPV-2-type in Brazil (Fontana et al., 2013; Pinto et al., 2012) and other South American (Calderon et al., 2009), North American (Hong et al., 2007), and European countries (Decaro et al., 2011, 2007). Further studies are required to assay the CPPV-1 potential transmissivity through blood transfusion.

The MVC, also referred to as CPV-1, in spite of sharing no antigenic relation with CPV-2, was reclassified as CBPV-1 in genus *Bocaparvovirus* of the subfamily *Parvovirinae* and family *Parvoviridae* (Cotmore et al., 2014). CBPV-1 was initially not considered a cause of diseases in dogs; however, studies have revealed its pathogenicity for newborn puppies and fetuses (Carmichael et al., 1991), enteritis with severe diarrhea (Mochizuki et al., 2002), and respiratory disease with breathing difficulty (Carmichael et al., 1994; Pratelli et al., 1999). Recently, CBPV-1 was also suggested as a possible cause of hepatitis in dogs (Choi et al., 2016). Furthermore, CBPV-1 was detected in 0.38% of the dog serum samples assayed in the present study (Table 1) and their complete genome was completely sequenced and analyzed (Fig. A.1). To the best of our knowledge, this is the first report of CBPV-1 in South America. Further studies are required to understand the pathogenicity of South American CBPV-1 strains and their potential transmissivity through blood transfusion.

Furthermore, we also found a single sequence to be more closely related with a putative new *Parvovirinae* member named as *Sesavirus* (Fig. A.2), which was proposed as a new genus, putatively named *Marinoparvovirus*, previously detected in sea lion feces (Phan et al., 2015a). Unfortunately, it was not possible to define the individual(s) sample(s) containing this putative viral sequence and obtain the complete gene or genome sequence since the short contig secured through HTS was not informative to develop an efficient PCR protocol. The partial VP1-phylogenetic reconstruction applied in the present study (Fig. A.2) revealed a sesavirus-like sequence and sesavirus grouping with other unclassified parvoviruses as bosavirus and horse parvovirus that were proposed to be *Copiparvovirus* genus members (Li et al., 2015; Sadeghi et al., 2017). Copiparvovirus members are known to infect cattle and swine (Cotmore et al., 2014), whereas bosavirus, horse parvovirus, and sesavirus were found in cattle, horses, and sea lions respectively (Li et al., 2015; Phan et al., 2015a; Sadeghi et al., 2017). It is important to highlight that ICTV recommends an amino acid analysis of the complete NS1 gene to define the genus parvovirus (Cotmore et al., 2014).

The CaCV within the family *Circoviridae* (Breitbart et al., 2017) was recently discovered and reported in samples of dog tissues that suffered vasculitis and hemorrhage from the United States (Li et al., 2013). Afterwards, it was detected in diarrheic dogs (Decaro et al., 2014), but its pathogenicity is still controversial in dogs, as it has been suggested as a co-pathogen in association with CPPV-1 (Dowgier et al., 2017). A CaCV variant, named fox circovirus, was also detected in foxes that displayed meningoencephalitis with undefined etiology (Bexton et al., 2015). We found CaCV in 1.34% of the dog serum samples tested through real-time PCR (Table 1) and our sequence grouped with other dog CaCV sequences (Fig. A.3). Further studies are required to explore the prevalence of CaCV in Brazilian dogs and its pathogenicity. It is important to note that this is the first report of CaCV in South America.

The *Genomoviridae* family was recently defined by ICTV and is composed by non-enveloped circular ssDNA viruses classified in eight genera (Varsani and Krupovic, 2017). GmKV-2 is one of the 16 species within *Gemykibivirus* genus (Varsani and Krupovic, 2017), which is composed by viruses detected in human and untreated sewage (Phan et al., 2015b). We detected GmKV-2 in 5.38% of the dog serum samples assayed (Table 1) and obtained a GmKV-2 whole-genome (Fig. 1). Moreover, our study also indicates that the dogs analyzed host a GmKV-2 viremia. These viruses were first detected in samples obtained from unexplained cases of human encephalitis and diarrhea (Phan et al., 2015b), and despite the role of *Genomoviridae*, infection is unknown and must be determined. The present data suggests that GmKV-2 can infect both human and dogs. Further studies are required to determine and attribute cellular hosts to the *Genomoviridae* members detected in animal samples. In the absence of further evidence, such as that strengthening the association between clinical signs using case-control studies or disease causation following animal inoculations, a causative role for any of these viruses in human and dog diseases remains tentative.

The *Anelloviridae* members represent non-enveloped ssDNA viruses comprised by more than 65 species grouped in 12 genera (ICTV, http://www.ictvonline.org/virusTaxonomy.asp). We obtained sequences from at least four divergent viruses (Fig. 2A), closely related with the prototype of *Thetatorquevirus* genus torque teno canis virus detected in dogs (Lan et al., 2011; Sun et al., 2017) and putative new members within this genus detected in ticks (Xia et al., 2015) (Fig. 2B). Our data can raise answers about *Thetatorquevirus* biology,i.e. if these viruses are arthropod borne transmitted or both dog and tick-thetatorqueviruses share a common ancestor. Moreover, it is important to emphasize that ticks may be PCR positive because they ingested a blood meal from thetatorquevirus infected vertebrate and not only because they are the vector for this viral agent. It is important to emphasize that thetatorqueviruses pathogenicity in dogs has not been examined in depth (Lan et al., 2011; Sun et al., 2017).

5. Conclusions

The present study categorized the abundance of dog viruses present in serum using an unbiased technique. We described here commonly detected viruses in dogs (i.e. CPPV-1), undersearched agents (CBPV-1, CaCV), novel viral genomes (sesavirus-like and thetatorquevirus-like) and close-relative genomes in relation to those found in humans (GmKV-2). We also defined the frequency of CPPV-1, CBPV-1, CaCV, and GmKV-2 in the serum of Northwestern Brazilian dogs. Our data expanded the knowledge regarding dog serum virome and viral agents that are potentially transmitted through blood transfusion in dogs and agents to which humans, through their extensive canine contacts, may be frequently exposed.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2018.09.023.

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Artigo 2

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Mamastrovirus 5 detected in a crab-eating fox (*Cerdocyon thous*): Expanding wildlife host range of astroviruses

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ABSTRACT

Astroviruses are a common cause of gastroenteritis in children worldwide and can also cause infection in a range of domestic and wild animal species. Canine astrovirus (formally named as *Mamastrovirus* 5, MAstV5) has been reported worldwide, and its role as an enteric pathogen is still controversial. Herein, we describe the genomic characterization of a MAstV5 (strain crab-eating fox/2016/BRA) identified in a wild canid (*Cerdocyon thous*) diagnosed with canine distemper virus (CDV) as *causa mortis*. The nearly complete genome comprised 6579 nt in length and displayed the archetypal organization of astroviruses. The present report is the first evidence of MAstV5 infection in an animal species other than the dog and highlights a possible natural astrovirus spillover between domestic and wild canids. Moreover, these results show the first evidence of extra-intestinal MAstV5, suggesting a virus systemic spread. This work is expected to contribute to a better understanding of the astroviruses biology and their interactions with the wildlife health.

1. Introduction

Astroviruses (AstVs) are small, icosahedral, nonenveloped viruses, with a characteristic star-like surface structure. AstVs can infect humans and a variety of animals, and is transmitted via the fecal-oral route by ingestion or fomites [1]. The genome is single-stranded RNA with positive sense with 6.3–7.9 kb in length. It includes three open reading frames (ORFs) designated as ORF1a, ORF1b, and ORF2 [2]. ORF1 encodes a protease and an RNA-dependent RNA polymerase (RdRp) and has a frameshift structure between ORF1a and ORF1b [3]. ORF2 encodes the viral structural capsid protein that is expressed from a subgenomic mRNA [4]. Within each genus, AstVs are classified into genotype species, based on both genetic analyses of the ORF 2 encoded amino-acid sequence and the host species [5].

It has been reported that some AstVs species can cross the host species barrier [6]. This would be the case of some bat AstVs that can infect more than one bat species [7,8], and AstV species that can infect either cheetahs and cats [9]. Recently, neurotropic astrovirus associated with encephalitis was identified in a sheep. Interestingly, the similarity found among this strain and a astrovirus described in neurologically diseased cattle, indicates that astroviruses of the same genotype may cause encephalitis in different species [10]. So far, AstVs have been detected in over than 80 avian and mammalian host species [11]. Moreover, a phylogenetic analysis of the RdRp region suggests that the long-term evolution of AstVs is determined by cross-species transmission events, which occur among distinct ecological scenarios [6].

The crab-eating fox (*Cerdocyon thous*), also known as the Common Zorro, is a "false fox", native to the South American pampas biome, which seems to be tolerant to human disturbance and is frequently seen

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Table 1
Oligonucleotide features used in the PCR for the detection of common enteric canine viruses.

Target	Primer	Sequence (5'-3')	Gene target	Product size (bp)	Reference
Astrovirus (pan-*Astroviridae*)	Astro F1	GARTTYGATTGGRCRCGKTAYGA	RdRp	422	Chu et al. [8]
	Astro F2	GGYTTKACCCACATNCCRAA			
	Astro R	CGKTAYGATGGKACKATHCC			
	Astro F3	AGGTAYGATGGKACKATHCC			
	Astro F4	GARTTYGATTGGRCKAGGTAYGA			
Mamastrovirus 5	46F	ATGTGTTCAGTGCCACCTTA	NSP1a	359	This study
	405R	CTTGTGTAAGCCTGTGCTGC			
Canine protoparvovirus 1	CPV 555F	CAGGAAGATATCCAGAAG	VP2	555	Buonavoglia et al. [51]
	CPV 555R	GGTGCTAGTTGATATGTA			
Canine adenovirus 1 and 2	HA1	CGCGCTGAAGATTACTACCTTGTC	E3	1307	Linné [52]
	HA2	CCTAGAGCACCTTCGTGTCCGCTT			
Canine coronavirus	CCoV 1F	TCCAGATATGTAATGTTCGG	M	450	Herrewegh et al. [53]
	CCoV 2R	TCTGTTGAGTAATCACCAGCT			
Canine rotavirus	BEG 9F	GGCTTTAAAAGAGAGAATTTCCGTCTGG	VP7	1062	Gouvea et al. [54]
	END 9R	GGTCACATCATACAATTCTAATCTAAG			
Canine distemper virus	CDV 1F	ACTGCTCCTGATACTGC	NC	480	Castilho et al. [55]
	CDV 2R	TTCAACACCRACYCCC			
	CDV 3F	ACAGRATTGCYGAGGACYTRT	NC	287	Frisk et al. [56]
	CDV 4R	CARRATAACCATGTAYGGTGC			
Internal control	FC27	AGAGTTTGATCCTGGCTCAG	16S rRNA	530	Gontang et al. [17]
	R530	CCGCGGCTGCTGGCACGTA			

R = A/G; Y = C/T; K = G/T; H = A/C/T; N = A/C/G/T. Bold characters indicate modifications introduced to the original sequences published in the references.

in rural areas and close to urban regions [12]. The crab-eating fox's nocturnal scavenger habits bring it in close proximity with domestic animals. This is a negative factor for their conservation, since it increases the possibility of pathogen spillover from domestic dogs to wild canids [6]. It is known that the domestic dog (*Canis lupus familiaris*) may be a source and reservoir of virulent pathogens for wildlife, including the rabies virus, canine distemper virus (CDV), and canine parvovirus (CPV2) [13], also many wildlife species are reservoir of pathogens that threatens domestic animals [14,15].

In this study, we report for the first time, the infection of a wild canid with *Mamastrovirus* 5 (canine astrovirus) in the context of a concurrent infection with canine distemper virus. Additionally, this astrovirus genome has been nearly fully sequenced, characterized, and a discussion of the possible spillover of this virus among wild Canidae species is presented.

2. Material and methods

2.1. Clinical history and pathological features

Veterinarians sighted an adult crab-eating fox (*Cerdocyon thous*) showing signs of motor incoordination in the peri-urban area of Porto Alegre city, Southern Brazil. The animal was then referred to the veterinary hospital by the regional official service (Secretaria do Meio Ambiente e Desenvolvimento Sustentável do Rio Grande do Sul, SEMA-RS) presenting clinical signs resembling central nervous system disease, which was suggestive of a canine distemper virus infection. Upon clinical examination, the crab-eating fox presented apathy, pale mucous membranes, mild dehydration (< 8%), mild eye discharge and multiple neurological signs, which were characterized by ataxia and motor incoordination, evolving to lateral decumbency and severe myoclonia. After 72 h of supportive treatment and no clinical improvement, the animal was euthanized and submitted for necropsy.

At necropsy, the brain, spinal cord, lungs, liver, spleen, lymph nodes, stomach, small and large intestines, kidneys, skeletal muscle, heart, large intestine, adrenal, esophagus and pancreas were collected, fixed in 10% neutral buffered formalin for 24–48 h, trimmed and processed routinely for histopathology. Tissues were then embedded in paraffin, cut at 3 µm and these sections were stained with hematoxylin and eosin (H&E).

Immunohistochemistry for canine distemper virus (CDV) antigens was performed on sections of the cerebellum, hippocampus, thalamus and telencephalic cortex. CDV antigens were detected employing the monoclonal antibody anti-CDV nucleoprotein (VMRD, Pullman, WA, USA) at a 1:400 dilution in phosphate buffered saline (PBS, pH 7.4), and revealed with a 3-amino-9-ethylcarbazole (AEC, Dako North America, Carpinteria, CA, USA) as chromogen.

2.2. Sample collection, nucleic acids isolation and cDNA synthesis

The cerebral cortex, lungs, small intestine, mesenteric lymph nodes, feces, urine and serum were collected at the time of necropsy and stored at −80 °C. Samples were diluted to 20% (w/v) in PBS (pH 7.4). DNA was isolated using NewGene Preamp (Simbios Biotecnologia, Cachoeirinha, RS, Brazil) based on guanidine isothiocyanate and silica [16]. RNA was isolated using TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was synthesized with GoScript™ Reverse Transcription System (Promega, Madison, WI, USA) using random primers (0.5 µg/reaction) in a final volume of 20 µL, following the manufacturer's recommendations.

2.3. Detection of common canine enteric viruses

Viruses that infect domestic dogs were screened by specific cDNA/DNA amplifications from feces, urine, serum, and pooled organs. PCR were conducted using primer pairs that were already reported in literature for the detection of astrovirus (AstV), canine distemper virus (CDV), carnivore protoparvovirus 1 (CPV2), canine coronavirus (CCoV), canine rotavirus (CRV), and canine adenovirus 1 (CAdV1) and CAdV2. In addition, the 16S rRNA gene was amplified using the primer pair FC27 and R530 as an endogenous internal control in the feces sample [17]. In order to discriminate in which organs the AstVs would be present, a pair of primers was selected for SYBR-based real-time PCR. The conditions of this qPCR were in accordance with the manufacturer's recommendations (GoTaq® qPCR Master Mix, Promega, Madison, WI, USA). The data about the oligonucleotide features and references are shown in Table 1.

2.4. Illumina genome sequencing and sequence analysis

RNA virome sequencing was performed as previously described [18]. Briefly, the brain, lungs, lymph nodes, intestines, urine and feces, which were collected from the crab-eating fox, were pooled, macerated, centrifuged at a low speed, filtered through a 0.45 µm filter to remove

small debris, and subjected to ultracentrifugation under a 25% sucrose cushion (~150,000 × g for 4 h). The resulting viral pellet was mixed with nucleases to eliminate non-capsid-protected nucleic acids. After the nucleases treatment, RNA was isolated with TRIzol® LS Reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions and subsequently enriched using a whole transcriptome amplification kit (WTA2, Sigma-Aldrich, Saint Louis, MO, USA). Subsequent to the amplification, the viral nucleic acids were purified using PureLink® PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). Their quality and quantity were assessed using a spectrophotometer and a fluorometer, respectively.

DNA fragment libraries were prepared with one ng of DNA from WTA using a Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. Illumina sequencing was performed in an Illumina® MiSeq System with a MiSeq Reagent Kit V2 (2 × 150 cycles). The reads quality were evaluated with FastQC, trimmed in Geneious software (version 9) and were *de novo* assembled into contigs using SPAdes (3.6 version) [19]. The contigs were compared to known sequences in the GenBank nucleotide and protein databases using BLASTn/BLASTx [20]. Geneious software was used for an open reading frame (ORF) prediction and genome annotations. The ORF1a disorder prediction was performed with Fold Index software program [21] (Appendix A in Supplementary material).

2.5. Phylogenetic inferences

For phylogenetic inferences, multiple nucleotide sequence alignments were produced with the aid of the ClustalW software. The phylogenetic tree whole genome and capsid protein were reconstructed using the Maximum Likelihood (ML) inference and the protocol to generate these phylogenetic trees was calculated using the "find best DNA/protein model" tool from MEGA6 [22]. Phylogenetics analysis of ORF1a and ORF1b were performed with neighbor-joining method, Junkes Cantor genetic distance model. Bootstrap values were determined by 1000 replicates to assess the confidence level of each branch pattern. The complete genomic sequence of the MAstV5 strain of the crab-eating fox/2016/BRA was deposited in GenBank under the accession number KY765684.

3. Results and discussion

3.1. Histopathological and immunohistochemistry data

At necropsy, the animal presented severe cachexia, pale mucosae and severe tick infestation (*Amblyomma aureolatum*). The brain leptomeningeal blood vessels were severely distended (hyperemia). Also, there was moderate splenomegaly and consolidation areas in diaphragmatic lung lobes.

On histopathology, the cerebellum contained a diffuse and severe white matter demyelination (Fig. 1A) that is associated to large amounts of Gitter cells and gemistocytic astrocytes, with occasional intranuclear and intracytoplasmic eosinophilic inclusion bodies and mild perivascular lymphoplasmacytic cuffs (Fig. 1B). The thalamus showed a focal area of white matter demyelination, which was observed multifocally on the spinal cord in addition to mild perivascular lymphoplasmacytic cuffs. The hippocampus and telencephalic cortex did not show any abnormalities. The lung lesions consisted of parasitic granulomatous pneumonia (*Angiostrongylus spp.*), which is characterized by a focally extensive granulomatous inflammatory infiltrate arranged concentrically around larval structures and embryonated eggs located inside blood vessels and sometimes in alveolar spaces.

Upon immunohistochemistry examination for the CDV antigen, the cerebellum showed a marked intranuclear and intracytoplasmic staining, mainly in astrocytes of the white matter (Fig. 1C and D). Mild immunostaining was observed on sections of the hippocampus and thalamus, while no immunostaining was noted on the telencephalic cortex sections. In this assay, the presence of multifocal intracytoplasmic and intranuclear immunostaining was an important microscopic finding for canine distemper diagnosis. The cerebellum was an adequate organ for the detection of the CDV antigen, being a good auxiliary method in the post mortem and definitive diagnosis of the *causa mortis*.

3.2. Detection of common canine enteric viruses

The crab-eating fox samples were submitted for molecular screening of the common canine enteric viruses by PCR. The internal control resulted positive in all molecular detection assays from fecal samples, which confirmed the nucleic acid quality. The detection assays were negative for CDV, CPV2, CCoV, CRV and CAdV-1/2 in all tested samples. MAstV5 was detected in cDNA derived from the pooled organs using a pan-AstV RT-PCR protocol [8] followed by sequencing. In order to discriminate MAstV5 in each organ, the MAsTV5 was detected in cerebral cortex, small intestine, mesenteric lymph nodes and feces (Table 2) using a specific MAstV5-RT-PCR protocol with primers 46F and 405R (Table 1).

The data presented herein shows two important findings: (i) it is the crab-eating fox/BRA/2016 strain was likely derived from the canine host, and (ii) the extra intestinal MAstV5 presence. It has been reported that some AstVs infections can cross the species barrier [6]. This would be the case of some bat AstVs that infect related bat species [7,8], and AstV species that can infect either cheetahs and cats [9].

Beyond to the gastrointestinal tract infection, some human astroviruses (HAstVs) such as VA1/HMO-C, MLB and the classical HAstV genotypes have already been identified causing encephalitis and meningitis in immunocompromised patients. The proximity to animals, the intravenous treatment of immunoglobulins and the stem cell graft were some of the suggestions from sources of transmission origin, but it has not been confirmed [23].

A spillover from the natural reservoir requires more than the availability of pathogen from the natural host. It also requires that the natural host be brought into physical proximity with a second species, and that this second host be susceptible to infection. These findings are likely to provide new insights into the ecology of astroviruses and transmission among species; especially in peri-urban areas, where factors such as deforestation and human expansion may endanger wildlife populations.

To date, MAstV5 was reported only in samples derived from the gastrointestinal tract of dogs [24,25,34,26–33], and the recovery of MAstV5 in the crab-eating fox's CNS is an interesting and unexpected finding. However, the detection of other AstV species in the central nervous system (CNS) of mink [35], cattle [36,37], human [38], pigs [39] and sheep [40] has been the focus of differential diagnosis of non-suppurative encephalitis.

It was not possible to detect CDV by RT-PCR in the cerebral cortex, or in any other sample available to us, but the CDV antigen was detected in the cerebellum by IHC (Fig. 1). Unfortunately, the samples that were tested by IHQ were not available to be tested by RT-PCR. The recognized effects of CDV on nervous tissue include acute, subacute to chronic forms of encephalopathy, and rare distinct chronic variant encephalomyelitis of mature dogs, termed old dog encephalitis (ODE) [41]. The clinicopathologic features of progressive cortical neurologic signs along with multifocal severe perivascular and parenchymal lymphoplasmacytic encephalitis involving mainly the cerebrum and brain stem are characteristic lesions of ODE that was confirmed at the histopathology description [42]. A number of different hypotheses have been postulated to explain the occurrence and pathogenesis of ODE [43,44]. For instance, ODE may represent the cumulative effects of end stage chronic subclinical CDV encephalitis. In dogs, ODE has almost exclusive predilection for seropositive adults, often with complete vaccination histories. External reinfection of immune dogs by wild-type CDV with subsequent rapid immune-mediated suppression of the

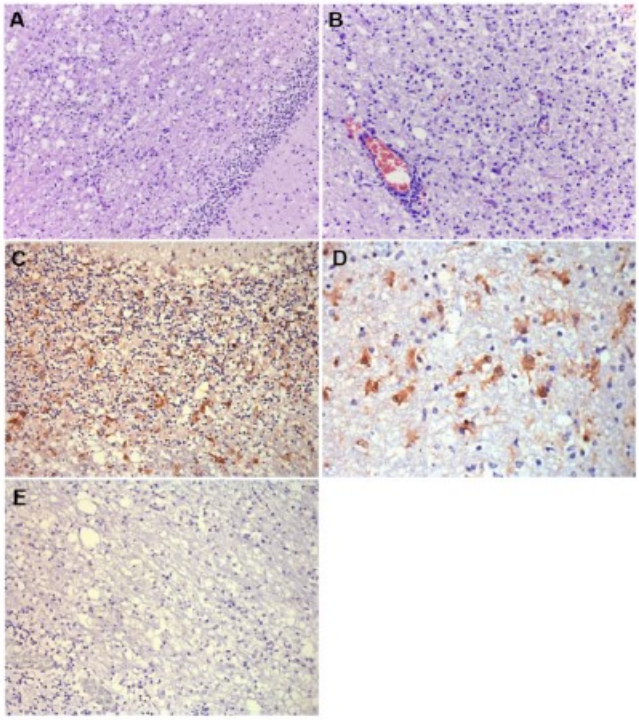

Fig. 1. Pathological findings in *Cerdocyon thous* brain.
A. Cerebellum with a diffuse and severe white matter demyelination (H&E, obj. 10X). B. Cerebellum showing focally extensive areas with large amounts of Gitter cells and gemistocytic astrocytes with mild perivascular lymphoplasmacytic cuffs (H&E, obj. 20X). C–D. Astrocytes at cerebellum white matter showing marked and multifocal immunostaining to CDV in the cytoplasm (obj. 40X). E. Negative control.

Table 2
Summary of PCR screening of most common canine enteric viruses in the *Cerdocyon thous* sample.

Virus target	Cerebral cortex	Lungs	Small intestine	Mesenteric lymph nodes	Feces	Urine	Serum
MAstV5	+	–	+	+	+	–	–
Canine protoparvovirus 1	–	–	–	–	–	–	–
Canine adenovirus 1 and 2	–	–	–	–	–	–	–
Canine coronavirus	–	–	–	–	–	–	–
Canine rotavirus	–	–	–	–	–	–	–
Canine distemper virus	–	–	–	–	–	–	–

(+) positive. (-) negative.

extracellular virus production within the CNS could explain the development of ODE [42]. Our results sustains the hypothesis that the Pampa Fox was infected by CDV, possibly when juvenile, it was able to clear the infection and survive, but the virus persisted in the CNS, leading to a late onset of neurological symptoms compatible to what is seen in ODE cases [45]. This hypothesis could explain why the virus was only detected in CNS tissues. In addition to the CDV infection, the parasitic granulomatous pneumonia contributed to the immune depression and made it possible to MAsTV5 strain crab-eating fox/2016/BRA spread to the extra-intestinal tissues.

It is important to highlight that it is not possible to affirm that MAstV5 strain crab-eating fox/2016/BRA was associated with CNS lesions with the assays applied. Whether MAstV5 might be associated to any pathology remains to be investigated in the future. Regardless of the involvement of MAstV5 in disease, this work is expected to contribute to a better understanding of the biology of astroviruses, and its interactions and possible spillover with the wild hosts.

Fig. 2. Organization of MAstV5 strain crab-eating fox/2016/BRA genomic RNA.
The ORF1 is predicted to encode a serine protease (PRO) and VPg protein. The PRO and VPg amino acid sequences are highlighted and compared with other seven AstV sequences. The motifs present in PRO and VPg are marked in boxes in the alignment.

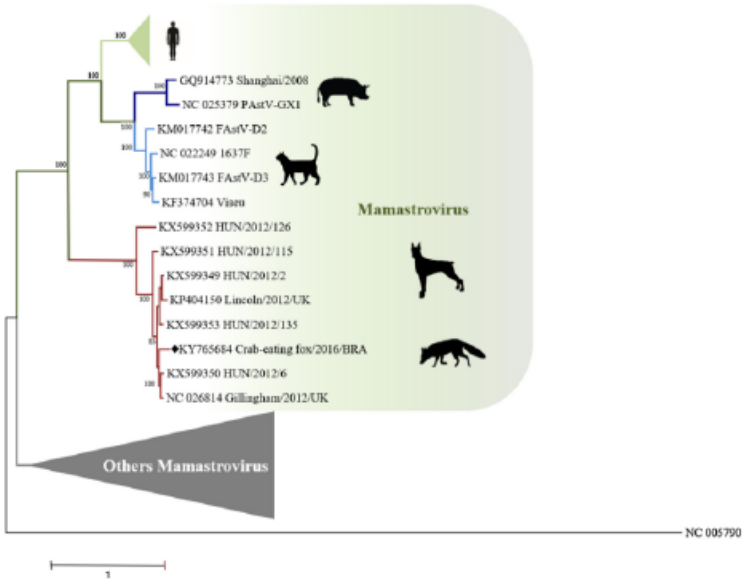

Fig. 3. The whole genome phylogenetic tree.
Nucleotide phylogenetic tree (A) was reconstructed using General Time Reversible (GTR) model. Gamma distribution with invariant site (G + I) were applied to both inferences. The percentage of replicate trees in which the associated taxa are grouped in the bootstrap test (1000 replicates) is shown next to the branches. GenBank accession numbers are listed for all sequences analyzed in the tree. The crab-eating fox/2016/BRA sequence is labeled with a black diamond (♦).

3.3. *MAstV5 genome sequencing and genomic analysis*

The Illumina MiSeq sequencing generated a total of 71,746 high quality paired-end reads with an average length of 112.5 bp. One contig with ~6.6 kb was *de novo* assembled and showed high genomic identity with canine astroviruses (MAstV5). This contig was obtained with 48,901 reads (coverage ~ 885X). The MAstV5 strain crab-eating fox/2016/BRA nearly full genome is 6559 nt (excluding the poly-A tail) with a GC content of 44.8%. The genome displays typical AstV organization that includes a 5' untranslated region (5'UTR), followed by three ORFs (ORF1a, ORF1b and ORF2), 3' untranslated region (3'UTR) and poly-A tail (Fig. 2).

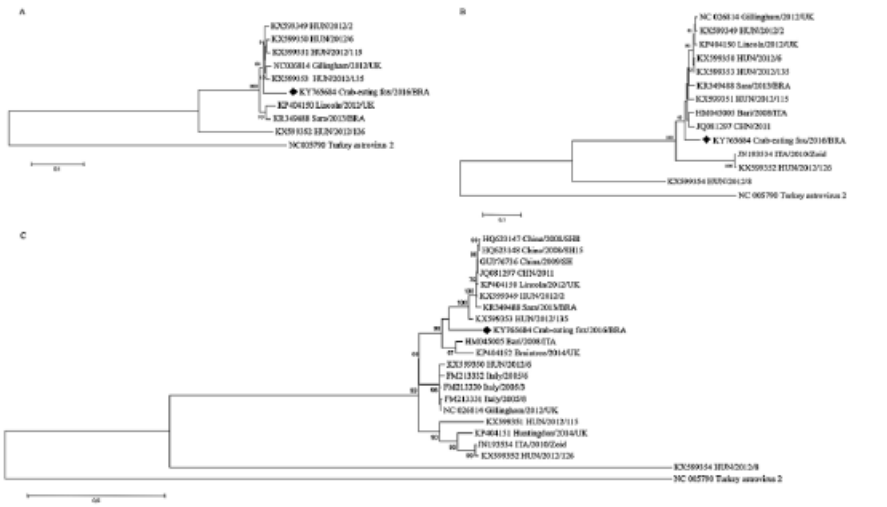

Fig. 4. The ORFs phylogenetics trees.
(A–B) Phylogenetics analysis of nucleotide sequences of ORF1a and ORF1b were performed with neighbor-joining method, Junkes Cantor genetic distance model. (C) While the capsid phylogenetic tree was reconstructed using amino acid sequences with Jones-Taylor-Thornton (JTT) model Gamma distribution with invariant site (G + I) were applied to both inferences. The percentage of replicate trees in which the associated taxa are grouped in the bootstrap test (1000 replicates) is shown next to the branches. GenBank accession numbers are listed for all sequences analyzed in the tree. The crab-eating fox/2016/BRA sequence is labeled with a black diamond (◆).

The ORF1a sequence of the crab-eating fox/2016/BRA strain presents 890 amino acids length in agreement with other MAstV species in which ORF1a range 787–950 amino acids [1,46]. The presence of the putative catalytic triad in the ORF1a that represent the serine protease motif was observed (Fig. 2).

The sequence for the ribosomal frameshift site between ORF1a and ORF1b, which is conserved in the *Astroviridae* family members [38], is present in the crab-eating fox/2016/BRA nearly full genome (Fig. 2). This translational frameshift is started by a ribosomal slippage site (RSS) that possesses the heptamer sequence 5′-AAAAAAC-3′ at position 2,673, followed by a GC-rich stretch which forms a stem loop structure. The 3′ end of ORF1a overlaps with ORF1b by 49 nucleotides.

As expected, the most conserved region of the MAstV5 strain crab-eating fox/2016/BRA nearly full genome is the RNA-dependent RNA polymerase (RdRp). The analysis of the putative 511 residues of RdRp reveals high sequence identity, when compared to those of other MAstV5 RdRp sequences. These identities range from 78.4% (with the KX599352 sequence) to 94.2% (KP404150 sequence), both recovered from dogs in Hungary (Appendix A in Supplementary material).

The highest identity of the ORF1b of the crab-eating fox/2016/BRA, relative to sequences from other AstV species, was 73% with the partial sequence of the California Sea Lion AstV (AEM37630). The same similarity between the RdRp belonging to the two AstV species can also be verified in previous studies. The identity of the putative RdRp from the crab-eating fox/2016/BRA compared with other MAstV species is described in the Appendix A in Supplementary material.

The ORF2 of the crab-eating fox/2016/BRA contains 2454 nucleotides in length corresponding to 817 putative residues. In general, it ranges from 672 to 851 amino acids among the *Astroviridae* family members [1]. This ORF encodes the putative capsid protein [47]. It was also observed an overlapping reading frame in the C-terminal portion of the polymerase and the N-terminal portion of the capsid precursor of 188 nucleotides (Fig. 2). This observation is in agreement with recently reported MAstV5 genomes [24,48].

3.4. Phylogenetic inferences

In order to reconstruct the evolutionary history of the crab-eating fox/2016/BRA, this sequence was compared to reference sequences of each MAstV species available in GenBank. A phylogenetic tree was reconstructed with the complete genome sequences of viruses belonging to the genus *Mamastrovirus* (Fig. 3).

The crab-eating fox/2016/BRA nearly full genome, as expected, grouped in the MAstV5 cluster with all other characterized canine astroviruses. The crab-eating fox/2016/BRA nearly full genome sequence clustered in the same terminal node as Gillinham/2012/UK and HUN/2012/6 strain (GenBank accession numbers NC_026814 and KX599350).

In addition, phylogenetic trees were constructed comparing the alignments of ORF1a, ORF 1b and ORF2 belonging *Mamastrovirus* 5 species (Fig. 4A–C). The ORF1a and ORF1b alignments were based on the full-length ORF nucleotide sequences, also included selected sequences above 1167 and 714 nucleotides, respectively. The ORF2 alignment only included full-length capsid protein sequences, both in nucleotide-based and in amino acid-based analysis.

The ORF1a tree formed two distinct branches with the crab-eating fox/2016/BRA strain being closely related to the Lincoln/2012/UK and Gillingham/2012/UK strains, ranging from 93.4% to 93.3% of nucleotide identity, respectively (Table 3). The upper branch that crab-eating fox/2016/BRA has been included was composed by Hungarian strains (GenBank accession number KX599349, KX599350, KX599351, KX599353), United Kingdom strains (GenBank accession number

Table 3
Sequence comparison among Canine AstV and crab-eating fox/2016/BRA strain.

Strains	GenBank accession number	crab-eating fox/2016/BRA (KY765684)			
		Genome nt	ORF1a nt	ORF1b nt	ORF2 aa
Bari/2008/ITA	HM045005	–	–	94.0	83.0
ITA/2010/Zoid	JN193534	–	–	80.9	72.5
Italy/2005/3	FM213330	–	–	–	78.8
Italy/2005/6	FM213332	–	–	–	77.7
Italy/2005/8	FM213331	–	–	–	78.4
China/2008/SH15	HQ623148	–	–	–	82.0
China/2008/SH8	HQ623147	–	–	–	81.8
China/2009/SH	GU376736	–	–	–	82.3
CHN/2011	JQ081297	–	–	93.4	82.3
Braintree/2014/UK	KP404152	–	–	–	80.2
Gillingham/2012/UK	KP404149	87.6	93.3	94.1	78.1
Huntingdon/2014/UK	KP404151	–	–	–	75.8
Lincoln/2012/UK	KP404150	87.8	93.4	94.2	82.2
HUN/2012/115	KX599351	85.1	92.1	92.8	73.0
HUN/2012/126	KX599352	73.2	74.4	78.4	71.5
HUN/2012/135	KX599353	87.4	93.0	93.9	83.1
HUN/2012/2	KX599349	87.1	92.9	93.7	82.0
HUN/2012/6	KX599350	86.7	92.6	94.2	77.6
HUN/2012/8	KX599354	–	–	56.7	23.7
Sara/2013/BRA	KR349488	–	92.9	93.4	81.6

KP404149 and KP404150) and Brazilian strain (GenBank accession number KR349488). More distant related to then, HUN/2012/126 form the second branch of the ORF1a tree (Fig. 4A).

The same pattern of topology was found in the ORF1b, despite the inclusion of new sequences (GenBank accession number HM045005, JQ081297, JN193534 and KX599354). The crab-eating fox/2016/BRA strain being in the middle of the two first branches, even nucleotide similarity of upper branch does not vary above from 1.4%. Therefore, we consider that crab-eating fox/2016/BRA strain still belonging to the upper branch. The below branch was composed by the highly virulent strain ITA/Zoid/2010 and the HUN/2012/126 strain sharing 80.9% and 78.4% of nucleotide identity with crab-eating fox/2016/BRA, respectively (Fig. 4B and Table 3). As expected, the strain HUN/2012/8 form another isolated branch distantly related to all others *Mamastrovirus 5* as was reported [48].

According to the taxonomic guidelines of the *Astroviridae* family [49], species classification is performed not only on the basis of the host, but also on the phylogenetic differences based on the analysis of the complete ORF2 amino acid sequence. Moreover, the mean genetic distance of the amino acid sequences (p-distance), relative to the sequence of the crab-eating fox/2016/BRA strain, remained within the established parameters for the *Mamastrovirus 5* species (Fig. 4C). There were clearly visible sub-cluster patterns within the MAstV5 cluster that exhibited the same patterns already reported [48]. Therefore, this result support our found that the crab-eating fox/2016/BRA formed a distinct subset based on the lower amino acid identity between the other strains (Table 3). As the Astroviridae family, the MAstV5 constitute a remarkably genetically diverse species each was nomenclature and taxonomy must be discussed and update regularly [50].

4. Concluding remarks

This report shows a canine-like astrovirus identified in a wild Canidae (*Cerdocyon thous*). This is also the first detection of MAstV5 presence in an extra-intestinal tissue, together with canine distemper virus. The findings presented here are expected to help understand how viral infections of domesticated dogs may impact the wild canid population's health, and its potential as sources of viruses, which may potentially infect other animal species.

Competing interests

None of the authors have any potential financial conflict of interest related to this manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2018.08.002.

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Artigo 3

Experimento concluído e artigo publicado na revista *Archieves of Virology - annotated sequence record*.

Título: *New polyomavirus species identified in nutria, Myocastor coypus polyomavirus 1*

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ANNOTATED SEQUENCE RECORD

New polyomavirus species identified in nutria, Myocastor coypus polyomavirus 1

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Abstract
A novel polyomavirus (PyVs) comprising 5,422 bp was identified by high-throughput sequencing (HTS) in pooled organs of nutria (*Myocastor coypus*). The new genome displays the archetypal organization of PyVs, which includes open reading frames for the regulatory proteins small T antigen (sTAg) and large T antigen (LTAg), as well as for the capsid proteins VP1, VP2 and VP3. Based on the International Committee on Taxonomy of Viruses (ICTV) *Polyomaviridae* Study Group criteria, this genome comprises a new PyVs species for the *Alphapolyomavirus* genus and is putatively named "*Myocastor coypus Polyomavirus* 1" . The complete genome sequence of this Myocastor coypus Polyomavirus 1 (McPyV 1) isolate is publically available under the GenBank accession no. MH182627.

Annotated Sequence Record

Polyomaviruses (PyVs) are small and non-enveloped viruses, having circular double-stranded DNA genomes with approximately 5,000 base pairs [1]. Taxonomically, PyVs belong to the family *Polyomaviridae* and comprise four genera – the *Alphapolyomavirus*, *Betapolyomavirus*, *Gammapolyomavirus* and *Deltapolyomavirus* [2]. PyVs have been found in many hosts, including birds [3], rodents [4], cattle [5], bats [6], nonhuman primates [7], and humans [8] .

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Genome organization among PyVs is very similar. The proteins are encoded by early and late transcriptional regions separated by a non-coding control region (NCCR), which controls the transcription of the early and late promoters and regulates the initiation of viral DNA synthesis [1]. The early coding region encodes regulatory proteins, known as tumor antigens, including a large T-antigen (LTAg) and a small T-antigen (sTAg), whereas the late coding region encodes the structural proteins VP1, VP2, and VP3 [1]. According to the International Committee on Taxonomy of Viruses (ICTV) *Polyomaviridae* Study Group, the new criteria for species classification are defined through the genetic distance between the LTAg coding regions [2].

In this study, we report the detection and genome characterization of a novel PyV species in nutria (*Myocastor coypus*) using high-throughput sequencing (HTS). The nutria is a semi-aquatic rodent native to South America and has been introduced to every continent, except Antarctica and Australia, for fur and meat production [9]. The animals studied in this research came from a commercial establishment located in Rio Grande do Sul State, authorized by the IBAMA (Brazilian Institute of Environment and Renewable Natural Resources). The project was conducted under protocol number 29415 approved by the Ethics Committee on the Use of Animals (CEUA) of the Universidade Federal do Rio Grande do Sul.

Samples from liver, kidney, lung, mesenteric lymph node, spleen, and intestine and rectal swabs from three apparently

healthy Brazilian nutria were pooled. The pool sample was macerated, centrifuged at low speed (1,800 x g during 30 min), filtered through a 0.45 µm filter for removal of small debris, and subjected to ultracentrifugation through a 20% sucrose cushion (200,000 x g for 4 h) [6]. The pellet was mixed with nucleases to eliminate non-capsid–protected nucleic acids [10], and viral nucleic acids were isolated using organic extraction protocols.

Viral nucleic acids were enriched using Sigma's® GenomePlex/Transplex kits. DNA fragment libraries were further prepared using a Nextera XT DNA sample preparation kit and sequenced using an Illumina MiSeq System (2 x 150 cycles run). Illumina MiSeq generated a total of 59,376 high-quality paired-end reads (average length of 142.2 nt). The sequences were trimmed and *de novo* assembled into contigs using SPAdes v3.10 (metaSPAdes) [11] and compared to known sequences in the National Center for Biotechnology Information (NCBI) databases using BLASTn/BLASTx. The total contigs with significant similarity in BLASTx/n searches belonged to the host genome, including the complete mitochondrial genome (34 contigs), as well as bacterial (26 contigs), and viral (4 contigs) genomes. Viral contigs were composed of three phages and one Eukarya-related virus (with similarity to the PyVs sequences). The mitogenome recovered was deposited into GenBank under accession number MH182628. No BLAST hit was found for the 334 contigs (~84%).

The PyV contig recovered was 5,422 bp in length, arranged in a circular form, and with an overall GC content of 44.7%, similar to others PyVs. The average coverage was 6.3x. The circular genome displayed the archetypal genome organization of the PyVs, including coding regions of the regulatory proteins sTAg and LTAg, as well as the capsid proteins VP1, VP2 and VP3 (Fig. 1). These two regions are separated by an NCCR homologous to those of previously described polyomaviruses [12]. The LTAg was generated by alternative splicing of the early mRNA transcript [13]. The intronic region was found to be located between base positions 5,201 to 4,350.

According to the actual ICTV classification criteria, a new polyomavirus species is defined strictly on the following guidelines: i) the complete genome sequence is available in a public database (GenBank accession number MH182627); ii) the genome displays a typical PyV organization (Fig. 1); and iii) there is sufficient information on the natural host (the mitogenome was deposited in GenBank under accession number MH182628). The last criterion (iv) is related to the genetic distance that must be greater than 15% for the

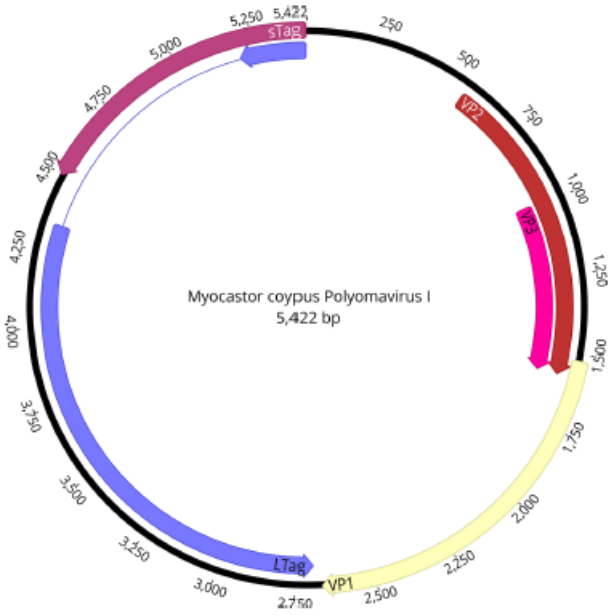

Fig. 1 Schematic diagram showing the genome organization of Myocastor coypus polyomavirus 1. Putative coding regions for VP1, VP2, VP3, small T antigen (sTAg), and large T antigen (LTAg) are marked by arrows

amino acid LTAg region when compared to members of the most related species. Multiple alignment analysis showed nucleotide LTAg genetic distances ranging from 44% to 56% with sequences representative of the most closely related *Alphapolyomavirus* species (species: *Human polyomavirus* 12, *Pan troglodytes polyomavirus* 5, *Acerodon celebensis polyomavirus* 1, and *Ateles paniscus polyomavirus* 1).

In order to finally classify the McPyV1 isolate as representative of a novel *Polyomavirus* species, all PyVs reference sequences were retrieved from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) and multiple sequence alignments of the LTAg (Supplementary Material 2) were generated using MUSCLE [14]. Phylogenetic reconstructions were performed by applying the Maximum Likelihood on MEGA7 [15] and Bayesian methods on Phylogeny.fr:Mr. Bayes software [16] under the best model of amino acid substitution (LG+F+I+G, as determined on MEGA7) [17]. The maximum likelihood phylogeny was recorded using MEGA7 with 1,000 bootstrap replicates each.

The reconstructed phylogenetic tree of the PyVs ICTV reference sequences showed four major clusters corresponding to the *Alphapolyomavirus-, Betapolyomavirus-, Gammapolyomavirus- and Deltapolyomavirus* genera (Fig. 2 and Supplementary Material 1). This analysis revealed the isolate representing the new species "*Myocastor coypus polyomavirus 1*" clusters within the *Alphapolyomavirus* genus. Additionally, a Bayesian phylogenetic inference was performed and revealed the same tree topology as the ML analysis (data not shown). McPyV1 was the sister taxa to a clade of virus infecting humans (HPyV12). It was observed that it is more distantly related to polyomaviruses infecting monkeys and bats.

Rodentia comprises the largest order within the class Mammalia, with approximately 40% of documented species [18]. Despite this, only 4% of the characterized PyVs have been detected in rodents. The first PyVs strain from rodents was isolated in 1978 as causing epithelial proliferations in the African multimammate mouse (Mastomy natalensis polyomavirus 1) [19]. PyVs can cause malignant and non-malignant diseases in birds and mammals, including humans, but most mammalian PyVs cause subclinical asymptomatic infections [12]. In this report, a novel genetically distinct nutria PyV was detected with no disease association. These findings will aid our understanding of the expanding genetic diversity of PyVs, mainly in the Order Rodentia.

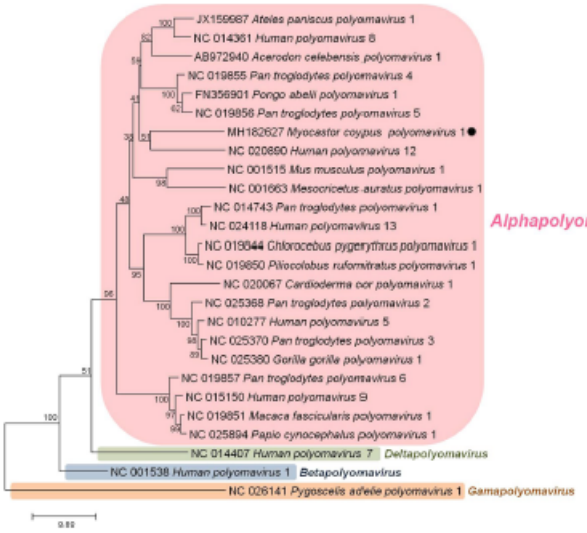

Fig. 2 Phylogenetic tree based on the complete amino acid LTAg region. MEGA7 was used for phylogeny inference under the maximum likelihood method. The isolate representing the new "*Myocastor coypus polyomavirus* 1" species is indicated with a black dot (●). PyVs ICTV reference sequences were retrieved from the GenBank database. GenBank accession numbers are available for the phylogenetic tree

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