UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

Ingryd Merchioratto

ANÁLISE FILOGENÉTICA DE PAPILOMAVÍRUS DE BOVINOS E CANINOS NO RIO GRANDE DO SUL

Santa Maria, RS
2023

Ingryd Merchioratto

ANÁLISE FILOGENÉTICA DE PAPILOMAVÍRUS DE BOVINOS E CANINOS NO RIO GRANDE DO SUL

Tese apresentada ao Programa de Pós-Graduação em Medicina Veterinária, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Doutor em Medicina Veterinária.**

Orientador: Prof. Dr. Rudi Weiblen

Santa Maria, RS, Brasil
2023

Merchioratto, Ingryd
ANÁLISE FILOGENÉTICA DE PAPILOMAVÍRUS DE BOVINOS E CANINOS NO RIO GRANDE DO SUL / Ingryd Merchioratto.- 2023.
88 p.; 30 cm

Orientador: Rudi Weiblen
Coorientador: José Valter da Sila Joaquim Junior
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências Rurais, Programa de Pós Graduação em Medicina Veterinária, RS, 2023

1. BPV 2. CPV 3. Infecções mistas 4. FAP59/64 5. MY09/11 I. Weiblen, Rudi II. da Sila Joaquim Junior, José Valter III. Título.

Sistema de geração automática de ficha catalográfica da UFSM. Dados fornecidos pelo autor(a). Sob supervisão da Direção da Divisão de Processos Técnicos da Biblioteca Central. Bibliotecária responsável Paula Schoenfeldt Patta CRB 10/1728.

Declaro, INGRYD MERCHIORATTO, para os devidos fins e sob as penas da lei, que a pesquisa constante neste trabalho de conclusão de curso (Tese) foi por mim elaborada e que as informações necessárias objeto de consulta em literatura e outras fontes estão devidamente referenciadas. Declaro, ainda, que este trabalho ou parte dele não foi apresentado anteriormente para obtenção de qualquer outro grau acadêmico, estando ciente de que a inveracidade da presente declaração poderá resultar na anulação da titulação pela Universidade, entre outras consequências legais.

Ingryd Merchioratto

ANÁLISE FILOGENÉTICA DE PAPILOMAVÍRUS DE BOVINOS E CANINOS NO RIO GRANDE DO SUL

Tese apresentada ao Programa de Pós-Graduação em Medicina Veterinária, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Doutor em Medicina Veterinária.**

Aprovada em 8 de março de 2023:

Rudi Weiblen, PhD. (UFSM)
(Presidente/Orientador)

José Valter Joaquim da Silva Junior, Dr. (UFSM)
(Coorientador)

Eduardo Furtado Flores, PhD. (UFSM)

Mario Celso Sperotto Brum, Dr. (UNIPAMPA)

Michele Lunardi, Dra. (UEL)

Samuel Paulo Cibulski, Dr. (UEPB)

Santa Maria, RS
2023

AGRADECIMENTOS

Agradeço primeiramente à minha família, Aparecida, Kássia e Nabor (*in memoriam*) por sempre serem minha base e ensinarem valores e princípios, além nunca medirem esforços para eu concluir meus objetivos.

Agradeço ao meu marido Isac, que acompanha minha trajetória acadêmica, desde aluna de iniciação científica até a conclusão e obtenção do título de doutora, por sempre me apoiar e incentivar, além de ser meu porto seguro.

Agradeço aos meus orientadores e professores Rudi e Eduardo por todo aprendizado, paciência, conversas e por confiarem à mim esse período de quatro anos à ser membro da "família" Setor de virologia- UFSM (SV-UFSM).

Agradeço ao meu coorientador José Valter pela paciência, pelas incansáveis horas dedicas para "construir" e concluir todos os trabalhos desenvolvidos conjuntamente, além de todos os ensinamentos proporcionados.

Agradeço ao professor Mário C. S. Brum, por ter acreditado e me incentivado enquanto aluna de iniciação científica, também ter sido paciente e compartilhado ensinamentos.

Agradeço aos meus colegas e amigos que fiz durante a pós-graduação, em especial aos que passaram ou ainda fazem parte do SV-UFSM, a convivência e o trabalho em equipe foi fundamental durante esse período.

Agradeço aos meus amigos que independente da distância, fizeram-se presente, me apoiaram e incentivaram.

Agradeço ao Programa de Pós-Graduação em Medicina Veterinária (PPGMV) da UFSM e aos órgãos de fomento CAPES e CNPq, pelo programa de excelência e recursos fornecidos.

RESUMO

ANÁLISE FILOGENÉTICA DE PAPILOMAVÍRUS DE BOVINOS E CANINOS NO RIO GRANDE DO SUL

AUTOR: Ingryd Merchioratto
ORIENTADOR: Rudi Weiblen

Os papilomavírus (PVs) produzem lesões proliferativas no epitélio cutâneo e mucoso, podendo ser benignas ou, eventualmente, evoluir para a malignidade. Um grande número de PVs têm sido descritos, que podem infectar uma variedade de hospedeiros. Embora os PVs sejam geralmente espécie-específicos, alguns papilomavírus bovinos (BPVs) já foram descritos em outras espécies, como equinos, ovelhas, cabras, búfalos, iaques e felinos domésticos. Também tem sido relatado que os PVs podem ser encontrados em infecções simples ou em coinfecções. A identificação dos tipos de PVs envolvidos nas lesões é realizada principalmente pela análise filogenética do gene L1. Assim, o primeiro estudo desta Tese descreve uma análise filogenética dos BPVs circulantes no estado do Rio Grande do Sul (RS) entre os anos de 2016 e 2020. Neste estudo, o DNA de PVs foi amplificado a partir de 43 amostras de papiloma bovino, utilizando os pares de *primers* FAP59/64 e MY09/11, direcionados para a amplificação de parte da L1. Os amplicons foram sequenciados e analisados filogeneticamente, e também submetidos à análise da sequência de aminoácidos. Também foi realizada uma análise *in silico* com 114 sequências completas de L1 (GenBank) para avaliar a concordância entre a classificação filogenética baseada na L1 *versus* aquela baseada na região amplificada com os *primers* FAP59/64 e MY09/11. Nesse estudo foram identificados 31 BPV-1 (72,1%), 27 BPV-2 (62,8%) e 4 BPV-6 (9,3%). Infecções mistas por BPV-1 e 2 foram observadas em 61,3% das amostras (19/31). Todos os BPV-6 foram identificados em infecções simples. A avaliação *in silico* comprovou que a análise dos amplicons de FAP59/64 e MY09/11 podem reproduzir a classificação da L1 completa. Além disso, foram observados aminoácidos únicos ou raros em pelo menos uma sequência de L1 de cada tipo de BPV identificado no estudo, alguns dos quais estão em potenciais epítopos de PVs, sugerindo uma evolução imunomediada dos vírus analisados. O segundo estudo reporta uma análise filogenética de PVs circulantes em cães no RS entre 2017 e 2022. Para isso, DNA de PVs foram amplificados a partir de 26 amostras de papiloma canino, com os *primers* FAP59/64 e/ou MY09/11. Também foi realizado uma análise *in silico* com 46 sequências de L1 completa (GenBank) para avaliar a concordância entre as classificações baseadas na região amplificável por FAP59/64 e MY09/11 *vs* aquela dada pela análise da L1 completa. Nesse estudo, todos os PVs amplificados por FAP59/64 (n = 22) foram classificados como CPV-1. Interessantemente, os PVs amplificados pelos *primers* MY09/11 (n = 4) foram classificados como BPV-1, sendo três detectados em infecção mista com CPV-1. Em conclusão, os estudos dessa Tese contribuem para o conhecimento sobre PVs ao: i) identificar infecções simples e mistas por BPV e CPV no RS; ii) demonstrar a adequação das classificações de BPV e CPV com base nas sequências amplificáveis por FAP59/64 e MY09/11; iii) levantar a hipótese de que BPVs do RS podem estar sob pressão seletiva pela resposta imune do hospedeiro; e iv) identificar potencial infecção de cães por BPV-1.

Palavras-chave: BPV; CPV; infecções mistas; FAP59/64; MY09/11.

ABSTRACT

PHYLOGENETIC ANALYSIS OF PAPILLOMAVIRUSES IN CATTLE AND DOGS IN RIO GRANDE DO SUL

AUTHOR: Ingryd Merchioratto
ADVISOR: Rudi Weiblen

Papillomaviruses (PVs) produce proliferative lesions in cutaneous and mucosal epithelium, which may be benign or eventually evolve to malignancy. A number of PV types have been described, which may infect a variety of hosts. Although PVs are generally species-specific, some bovine papillomaviruses (BPVs) have been described in other species, such as horses, sheep, goats, buffalo, yaks and domestic cats. It has also been reported that PVs may be found in single or mixed infections. PV types are usually identified by phylogenetic analysis of the L1 gene. Herein, the first study of this Thesis describes a phylogenetic analysis of BPVs circulating in Rio Grande do Sul (RS) state between 2016 and 2020. In this study, DNAs from 43 samples of bovine papillomas were amplified using two degenerate primer sets, FAP59/64 and MY09/11, which are targeted at amplification of part of L1. These amplicons were phylogenetically analyzed and evaluated for amino acid sequences. An *in silico* analysis was also performed with 114 complete L1 sequences (GenBank) to evaluate the agreement between the phylogenetic classification of L1 *versus* that based on the amplified region with primers FAP59/64 and MY09/11. We identified 31 BPV-1 (72.1%), 27 BPV-2 (62.8%) and 4 BPV-6 (9.3%). Mixed BPV-1 and 2 infections were observed in 61.3% of the samples (19/31). All BPV-6 was found in simple infections. An *in silico* approach demonstrated that the analysis of the FAP59/64 and MY09/11 amplicons may reproduce the complete L1 classification. Furthermore, unique or rare amino acids were found in at least one L1 sequence of each type of BPV identified in the study, some of which are in potential epitopes of PVs, suggesting an immune-mediated evolution of the viruses analyzed here. The second study reports a phylogenetic analysis of PVs circulating in dogs from RS between 2017 and 2022. For this, DNA from 26 canine papilloma samples were amplified with primers FAP59/64 and/or MY09/11. An *in silico* analysis was also performed with 46 complete L1 sequences (GenBank) to evaluate the concordance between the classifications based on the amplifiable region by FAP59/64 and MY09/11 *vs* that given by the analysis of the complete CPV L1. All PVs amplified by FAP59/64 (n = 22) were classified as CPV-1. Interestingly, the PVs amplified by MY09/11 (n = 4) were classified as BPV-1, with three detected in mixed infection with CPV-1. In conclusion, the studies in this Thesis contribute to the knowledge about PVs by: i) describing and identifying single and mixed infections by BPV and CPV in RS; ii) demonstrating the adequacy of classification of BPV and CPV based on sequences amplified by primers FAP59/64 and MY09/11; iii) raising the hypothesis that BPVs circulating in RS may be under selective pressure by the host immune response; and iv) highlighting the possibility of infection of dogs by BPV-1.

Keywords: BPV; CPV; mixed infections; FAP59/64; MY09/11

LISTA DE FIGURAS

ARTIGO 1

ARTIGO 2

LISTA DE TABELAS

ARTIGO1

ARTIGO 2

SUMÁRIO

1. INTRODUÇÃO

Os papilomavírus (PVs) são importantes vírus de vertebrados podendo produzir lesões proliferativas no epitélio cutâneo e mucoso, geralmente de caráter benigno e, ocasionalmente, pode evoluir para lesões malignas (RECTOR; VAN RANST, 2013). Os PVs têm sido descritos em uma grande variedade de espécies hospedeiras, como répteis, aves, peixes e vários mamíferos, incluindo humanos e cetáceos. Esses vírus são geralmente espécie-específicos e apresentam grande variabilidade genética (MUNDAY, 2014; RECTOR; VAN RANST, 2013; ICTV, 2018).

Os PVs são classificados na família *Papillomaviridae,* possuem cerca de 55 nm de diâmetro, não são envelopados e possuem capsídeo icosaédrico, formado por 72 capsômeros. O genoma desses vírus é formado por uma molécula de DNA circular de fita dupla, com extensão variando entre ~ 6000 e 8000 pares de bases (pb). O genoma codifica entre seis e oito proteínas, que são classificados como precoces (*early* - E) e tardias (*late* - L), dependendo da cinética de expressão (ICTV, 2018, PAVE, 2022).

Dentre os PVs, os papilomavírus humanos (*Human papilomavirus* - HPV) e papilomavírus bovino (*Bovine papilomavirus* - BPV) estão entre os mais estudados (MUNDAY, 2014; RECTOR; VAN RANST, 2013). Os HPVs possuem importante impacto clínico, principalmente os tipos envolvidos em tumores da região cervical do útero (MUNDAY, 2014). Os BPVs foram inicialmente estudados como modelos para os HPVs, buscando compreender os mecanismos patogênicos e interação agente-hospedeiro (RECTOR; VAN RANST, 2013). Não obstante, os BPVs também têm chamado atenção pela sua grande disseminação, número de casos e impacto sanitário. Assim como o BPV, os papilomavírus caninos (*Canine papilomavirus* - CPV) já foram utilizados como modelo para estudos de HPV e tem sido crescente o número de detecção de novos tipos (PAVE, 2022; MUNDAY, et al. 2022). No entanto, os estudos filogenéticos de CPV ainda são escassos (CAMPO, et al. 2002; RECTOR; VAN RANST, 2013; MUNDAY et al., 2017).

A análise filogenética dos BPVs tem revelado a existência de 43 tipos virais, sobretudo com base na sequência do gene da proteína L1 do capsídeo. Em relação aos CPVs, até o momento, foram identificados 24 tipos, também com base na sequência do gene L1 (ICTV, 2018; PAVE, 2022; MUNDAY et al., 2022). Em alguns casos, a classificação dos tipos de BPV e CPV tem sido acompanhada de correlações com a morfologia, aspecto, localização e padrão histológico das lesões. Alguns estudos também têm identificado coinfecções por tipos

diferentes de BPVs ou CPVs (SAUTHIER et al., 2021; CLAUS et al., 2007; CARVALHO et al., 2012; DAGALP et al., 2017; ALVES et al., 2020), além de descreverem a possibilidade de BPVs do gênero *Deltapapillomavirus* infectarem outras espécies além de bovinos, como equinos, ovelhas, cabras, búfalos, iaques e gatos (DE ALCÂNTARA et al., 2015; BAM et al., 2013; CUTARELLI et al., 2021; MUNDAY, et al., 2015; PANGTY et al., 2010; ROPERTO, S. et al., 2018).

No Brasil, os estudos filogenéticos sobre BPVs são crescentes, demonstrando que a papilomatose está amplamente difundida no rebanho bovino, já tendo sido relatada nas regiões Norte, Nordeste, Sudeste e Sul do país (BATISTA et al., 2013; FIGUEIRÊDO et al., 2020; ARALDI et al., 2014; LUNARDI et al., 2016; CRESPO et al., 2019; SILVA et al., 2010; TOZATO et al., 2013; SAUTHIER et al., 2021). Diferentemente de BPV, ainda são poucos os estudos filogenéticos de CPV no Brasil (DE ALCÂNTARA et al., 2014; ALVES et al., 2020; REIS et al., 2019). Até o momento, CPVs têm sido identificados na região Nordeste e Sul do país (ALVES et al., 2020; DE ALCÂNTARA et al., 2014; REIS, 2019).

Considerando o contexto acima, os estudos que compõem esta Tese relatam: i) um estudo filogenético dos BPVs circulantes no sul do Brasil entre os anos de 2016 e 2020 (ARTIGO 1); e ii) um estudo filogenético de PVs identificados em papilomas de cães no sul do Brasil entre 2017 e 2022 (ARTIGO 2).

2. REVISÃO BIBLIOGRÁFICA

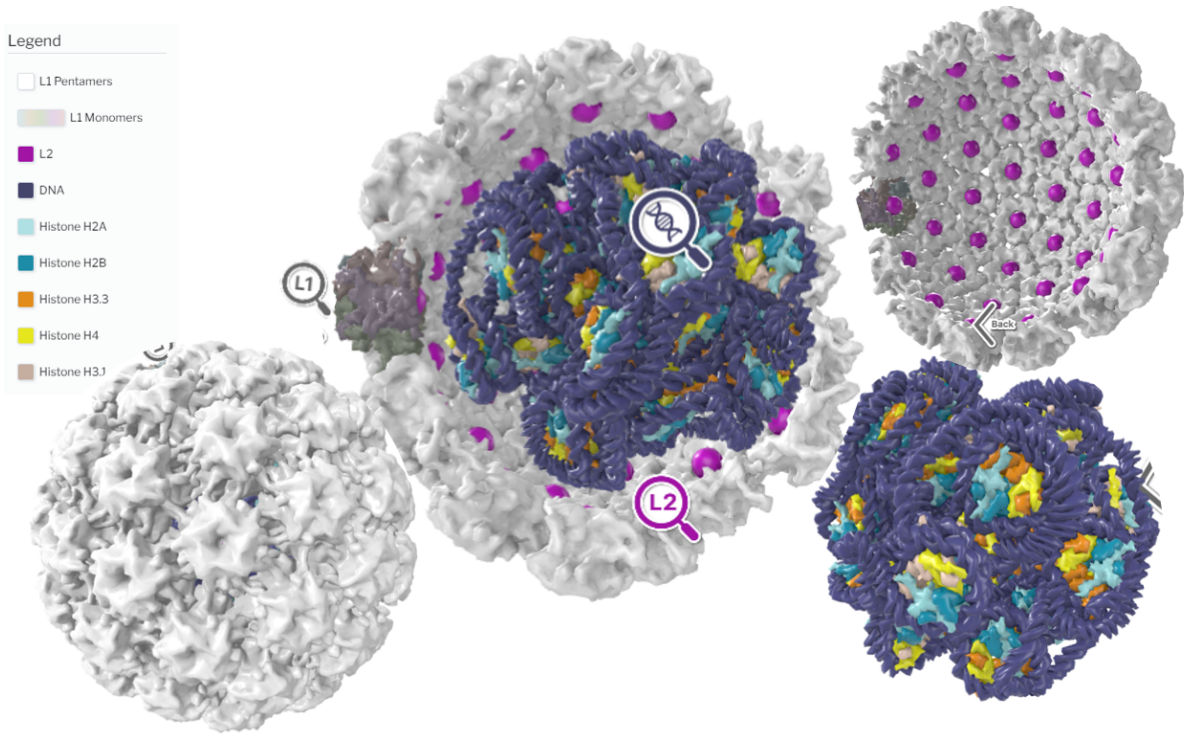
2.1. CLASSIFICAÇÃO E NOMENCLATURA

Os BPVs e CPVs pertencem à família *Papillomaviridae,* que é dividida em duas subfamílias: *Firstpapillomavirinae* e *Secondpapillomavirinae*. A subfamília *Secondpapillomavirinae* abrange um único gênero, *Alefpapillomavirus*. A subfamília *Firstpapillomavirinae*, por outro lado, abrange os demais PVs, sendo formada por 52 gêneros. Os BPVs identificados até o presente são classificados em cinco gêneros (*Deltapapillomavirus, Dyokappapapillomavirus, Dyoxipapillomavirus, Epsilonpapillomavirus* e *Xipapillomavirus*), enquanto os CPVs estão classificados em três gêneros (*Chipapillomavirus, Lambdapapillomavirus* e *Taupapillomavirus)* (ICTV, 2018; PaVE, 2022).

2.2. OS VÍRIONS

Os vírions da família *Papillomaviridae* são formados por um capsídeo icosaédrico, não envelopado, de aproximadamente 55 nm de diâmetro, contendo 72 capsômeros. O genoma do PVs é formado por uma dupla fita de DNA circular (*double-stranded* DNA - dsDNA), podendo variar de 6800 a 8400 pb (ICTV, 2018). O modelo de PV animal é o papilomavírus bovino 1 (*Bovine papillomavirus* 1 - BPV-1), cujo genoma contém cerca de 7800 pb. Nos vírions, o DNA encontra-se associado às histonas, formando o que se convencionou chamar de "minicromossomos" (Figura 1) (MCBRIDE, 2017).

Figura 1. Representação dos vírions dos papilomavírus.

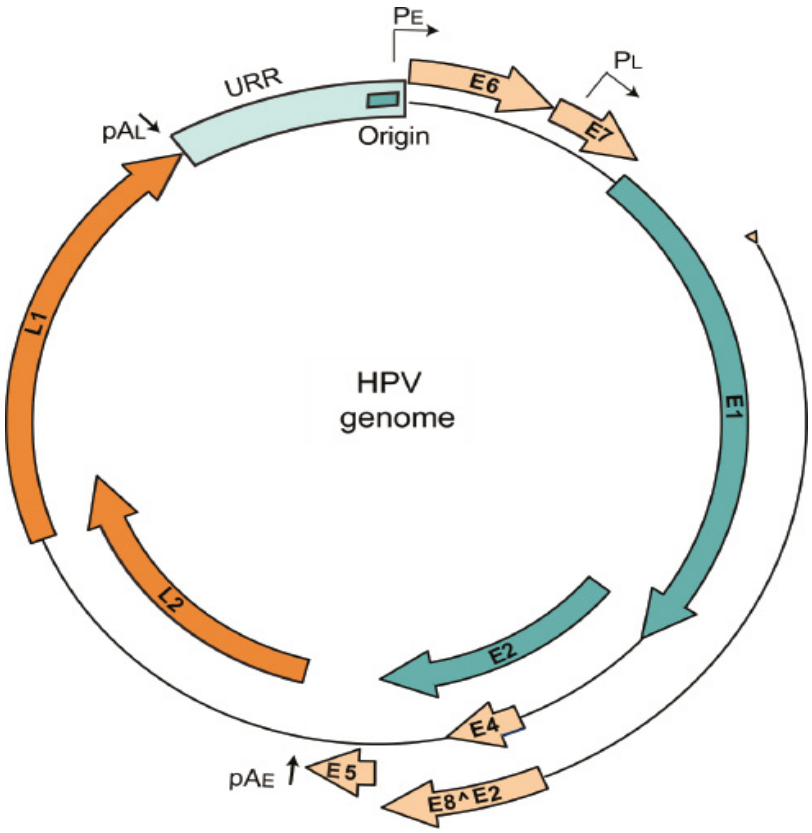


Legenda: Representação esquemática da partícula vírica dos papilomavírus, formada por 72 capsômeros da proteína L1 (em branco). A L2 (em roxo) está localizada no centro de cada capsômero. O vírus apresenta uma única molécula de DNA fita dupla circular (azul escuro) associada às histonas (azul claro, amarelo e laranja), formando o minicrossomo (Fonte: adaptado de PaVE, 2022).

2.3. GENOMA E PROTEÍNAS VIRAIS

O genoma dos PVs apresenta mais de uma fase aberta de leitura (*open reading frames* - ORFs), as quais codificam as proteínas precoces (*early* - E) e tardias (*late* - L) (LAZARCZYK et al., 2009). Os PVs têm seu genoma organizado em três regiões funcionais distintas: a região longa de controle (*long control region* - LCR), regiões precoces/iniciais e regiões tardias/finais (BORZACCHIELLO; ROPERTO, 2008; ICTV, 2018; VAN DOORSLAER et al., 2018). Essas três regiões são separadas por dois sítios de poliadenilação (pA): o sítio de poliadenilação precoce (pAE) e o sítio de poliadenilação tardia (pAL) (Figura 2) (MCBRIDE, 2008; ZHENG; BROKAW; MCBRIDE, 2005).

Figura 2. Representação do genoma circular dos papilomavírus



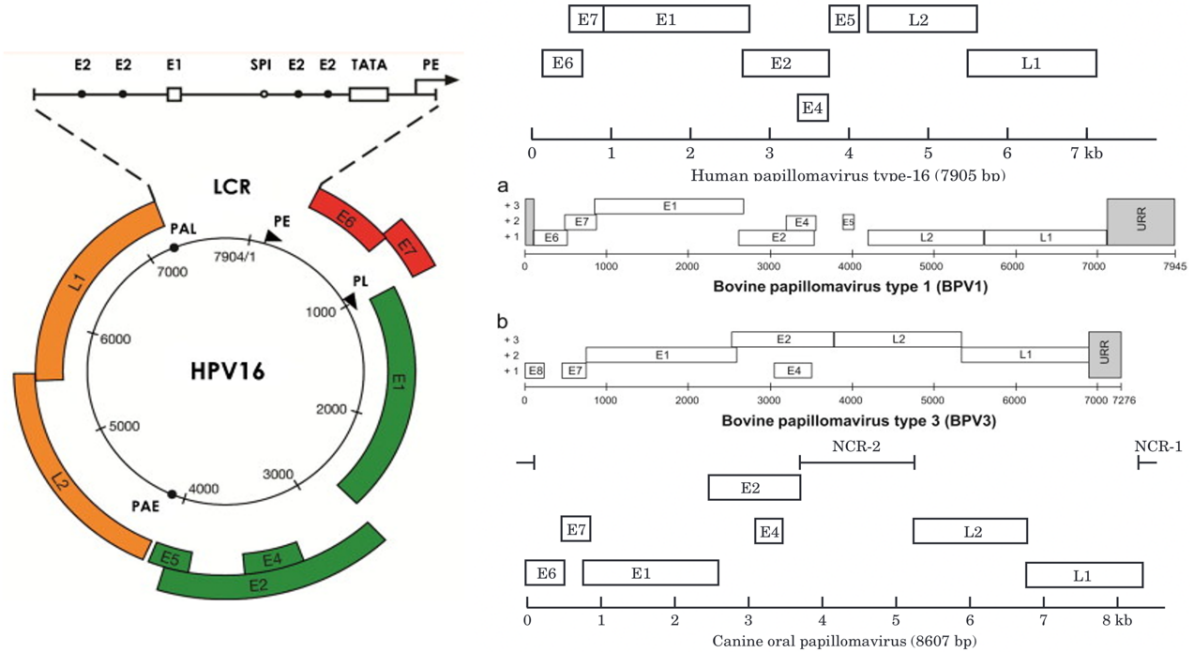
Legenda: Representação esquemática do genoma dos papilomavírus humanos (HPVs), demonstrando o DNA fita duplacircular de um α-HPV. A região codificante para as proteínas E1 e E2, responsáveis pela replicação, estão representadas em verde escuro, e as regiões das proteínas estruturais, L1 e L2, em laranja. A região reguladora *upstream* (URR), PE e PL, são os promotores precoce (PE) e tardio (PL), e pAE e pAL são os locais de poliadenilação precoce e tardia (Fonte: MCBRIDE et al., 2017).

A LCR possui cerca de 500-1000 nucleotídeos (nt), é uma região não-codificante do genoma, localizada entre as ORFs L1 e E6. Essa região reguladora possui elementos essenciais para a replicação e transcrição do DNA, como a origem da replicação (ori) e locais de ligação para fatores de transcrição viral e celular (DESAINTES; DEMERET, 1996; ICTV, 2018; LAZARCZYK et al., 2009; ZHENG; BROKAW; MCBRIDE, 2005).

Os genes codificam proteínas que recebem as mesmas designações dos genes, compreendendo proteínas precoces (E) e proteínas tardios (L) (ICTV, 2018). Os produtos das primeiras ORFs, envolvidas na replicação e transcrição, são as proteínas E1 e E2. O processo de transformação celular está associado às oncoproteínas E5, E6 e E7, que, quando encontradas no mesmo vírus, contribuem para o fenótipo oncogênico ou de alto risco de alguns PVs. Em PVs não oncogênicos, observa-se apenas a presença de até duas dessas ORFs (DAUDT et al.,

2018; DOORBAR et al., 2012). Diferente das outras proteínas precoces, a E4 apresenta um perfil de expressão mais próximo ao das proteínas tardias, de forma que a expressão das proteínas L ocorre somente em células que já expressaram a E4. As proteínas tardias, L1 e L2, compõem o capsídeo viral, são as proteínas mais imunogênicas dos PVs, sendo a L1 a principal proteína estrutural (DOORBAR et al., 2012). Dentro da diversidade de PVs já caracterizados, apenas as ORFs centrais, E1, E2, L1 e L2, têm sido encontradas em todos os vírus (DOORBAR, 2005; DOORBAR et al., 2012) (Figura 3).

Figura 3. Representação do genoma circular do papilomavírus humano (HPV) e representação linear do genoma do HPV, papilomavirus de bovinos (BPV) e caninos (CPV).



Legenda: Representação esquemática do genoma circular de HPV-16 e representação linear de alguns genomas de HPV, BPV e CPV, demonstrando que a organização do genoma é semelhante entre os PVs, mas que existem diferenças quanto à presença de genes precoces (Fonte: adaptada de DOORBAR et al.2012; RECTOR et al. 2013 e NICHOLLS et al., 1999).

A proteína E1 é uma helicase de DNA, dependente de ATP e é a única enzima codificada pelos PVs, sendo importante para o início da replicação do genoma viral (BERGVALL; MELENDY; ARCHAMBAULT, 2013; DOORBAR, 2005). Com cerca de 600 a 650 aminoácidos (aa), a E1 apresenta três domínios: i) N-terminal, essencial para a replicação i*n vivo;* ii) domínio de ligação ao DNA (DNA *binding domain* - DBD), que reconhece a ori; e iii) domínio C-terminal, que auxilia na abertura da dupla hélice do DNA viral (BERGVALL; MELENDY; ARCHAMBAULT, 2013; WALLACE; GALLOWAY, 2014). A E1 recebe o título

de iniciadora clássica, sendo responsável por reconhecer inicialmente a ori (BERGVALL; MELENDY; ARCHAMBAULT, 2013). Estudos demonstram que a E1 é necessária, não somente para iniciar, mas ao longo do ciclo viral replicativo, sendo importante para amplificar o número de cópias do DNA viral nos queratinócitos basais (DNA episssomal); manter um número de episssomas nas células que migram para o epitélio e começam a se diferenciar; e para a amplificação do genoma viral na fase produtiva do ciclo replicativo, que ocorre nas camadas epiteliais superiores diferenciadas (BERGVALL; MELENDY; ARCHAMBAULT, 2013). A E1 também pode se associar a outras proteínas para auxiliar/ regular a replicação do DNA viral (BERGVALL; MELENDY; ARCHAMBAULT, 2013), como, por exemplo, com a E2, formando o complexo E1-E2, que recruta proteínas (topoisomerase I, DNA polimerase I e proteína de replicação A) responsáveis pela replicação viral (ENEMARK et al., 2000; SCHUCK; STENLUND, 2015).

A proteína E2 tem cerca de 350-500 aa e dois domínios conservados: N-terminal e C-terminal, os quais são importantes para ativação e repressão transcricional, interação com a proteína E1 e fatores celulares, além da ligação específica ao DNA e propriedades de dimerização (MCBRIDE, 2008, 2013). A E2 é uma importante moduladora de expressão gênica, atuando como reguladora transcricional de E6 e E7 (ARALDI et al., 2017; GARCÍA-VALLVÉ; ALONSO; BRAVO, 2005). Em baixos níveis de expressão, a E2 recruta fatores de transcrição e forma o complexo de transcrição E1-E2, entretanto, em altos níveis de expressão, a E2 reprime a transcrição das proteínas E6 e E7, como consequência da inibição da ligação da RNA polimerase II (CAI et al., 2013; GARCÍA-VALLVÉ; ALONSO; BRAVO, 2005). Curiosamente, a E2 contribui para a regulação da transcrição e início da replicação *in vivo*, mas em sistema *in vitro*, a E1 é a única proteína viral necessária para o início da replicação (SEDMAN; SEDMAN; STENLUND, 1997).

A E4 foi inicialmente classificada como produto de gene E por estar localizada na região inicial do genoma viral, inserida entre os genes que regulam o início do ciclo replicativo, entretanto não foi detectado função dessa proteína durante os estágios iniciais da replicação (DOORBAR, 2013b). A maioria dos estudos moleculares de E4 é direcionada a HPV, principalmente aos tipos 1 e 16, sugerindo seu envolvimento na montagem e liberação do vírion. Embora não tenha sido adotada a proposta de classificá-la como uma terceira proteína tardia, seu papel está nos estágios finais do ciclo replicativo (DOORBAR, 2013b). Sugere-se que a expressão das proteínas do capsídeo ocorre em células que já expressaram E4 e que a expressão de E4 antecede a expressão de L2 (DOORBAR, 2013b), portanto as partículas infecciosas são

produzidas em células positivas para E4 (DOORBAR, 2013b). Além disso, a transcrição da E4 é a mais abundante em lesões produtivas, tendo um papel útil como biomarcador para HPV ativo (DOORBAR, 2013b).

A E5 é uma proteína transmembrana, com cerca de 40-85 aa e apresenta dois domínios: um domínio transmembrana e C-terminal (ARALDI et al., 2017; DIMAIO; PETTI, 2013; TOMITA et al., 2007). Essa proteína é codificada por muitos PVs de humanos e animais, mas não está presente em todos (DIMAIO; PETTI, 2013). A E5 é rica em aminoácidos hidrofóbicos e, embora seja sugerido que não tenha atividade enzimática, ela modula a atividade de várias proteínas celulares (DIMAIO; PETTI, 2013). Dentre a variedade de PVs na qual está presente, a E5 codificada pelo BPV-1 é a mais caracterizada: é formada por 44 aa e encontrada em queratinócitos basais, podendo estimular a proliferação celular, e também nos queratinócitos nas camadas superficiais, onde ocorre a produção viral (DIMAIO; PETTI, 2013). A E5 apresenta atividade de transformação em fibroblastos cultivados *in vitro*, demonstrando que células com aparência de monocamada, após a transformação, adquirem morfologia com aparência empilhada (DIMAIO; PETTI, 2013). Além da atividade de transformação/carcinogênese, a proteína E5 diminui a expressão do complexo principal de histocompatibilidade classe I (*major histocompatibility complex* - MHCI) (DIMAIO; PETTI, 2013) o que contribui para a evasão viral de parte do sistema imune (DIMAIO; PETTI, 2013).

A E6, com cerca de 137-150 aa, tem um domínio C-terminal, contribui para a transformação celular e, quando associada a outras proteínas virais, forma complexos que podem ter ação na modulação da sobrevivência celular, transcrição e diferenciação celular e na resposta ao dano no DNA (ARALDI et al., 2017; DOORBAR, 2005). A transformação neoplásica pela E6 pode ser promovida por dois mecanismos: ativação da telomerase e inativação da proteína p53 (p53), o que leva à desregulação do ciclo celular (ARALDI et al., 2017).

A proteína E7, com cerca de 127 aa, não é codificada por todos os PVs, sendo considerada uma proteína acessória. Quando presente, pode desempenhar importante papel, reprogramando e tornando o ambiente celular propício para a replicação viral, como, por exemplo, aumentando a duração da fase S e G2 do ciclo celular (ARALDI et al., 2014). Em alguns HPVs, quando associada à E6, a E7 apresenta potencial atividade de transformação celular (DOORBAR, 2005).

A L1, com cerca de 500 aa, é a principal proteína do capsídeo, sendo expressa após a L2, e é responsável pela interação inicial entre o capsídeo viral e a célula do hospedeiro

(DOORBAR, 2005; BUCK et al., 2013). Essa proteína tem a capacidade de se "auto-montar", formando partículas semelhantes aos vírus (*virus like particles* - VLPs) (BUCK; DAY; TRUS, 2013). Essas VLPs são potentes imunógenos amplamente estudados e utilizados em vacinas contra HPV (BUCK; DAY; TRUS, 2013). O gene da L1 é utilizado para construção de árvores filogenéticas e classificação dos PVs (BERNARD et al., 2010; DE VILLIERS et al., 2004).

A L2, com quase 500 aa, é uma proteína tardia que auxilia o empacotamento das partículas víricas e contribui para a infectividade do vírus. Essa proteína é encontrada em grandes quantidades em estruturas nucleares denominadas corpos PML (*promyelocytic Leukemia* - PML), durante a montagem dos vírions, recrutando a L1 para esses locais (DOORBAR, 2005).

2.4 CLASSIFICAÇÃO E FILOGENIA VIRAL

Os PVs são descritos desde a década de 30 e sua nomenclatura tem sido discutida por pesquisadores, que enfrentaram dificuldades para fornecer nomes sucintos e critérios que permitam diferenciar vírus que compartilham características como tamanho de genoma, propriedades de tecido-alvo e etiologias semelhantes. Com base nisso, a taxonomia dos PVs é apoiada em dois pilares: 1) a nomenclatura deve destacar que os PVs são espécie-específicos; 2) todos os genomas possuem origem monofilética, ou seja, se originaram de um ancestral comum, além de evoluírem mais lentamente do que outros grupos de vírus (DE VILLIERS et al., 2004). De Villiers (2004) propôs a classificação dos PVs em nível de gêneros, baseada no alfabeto grego, sendo aceita e utilizada pelo Comitê Internacional para Taxonomia de Vírus (*International Committee on Taxonomy of Viruses* - ICTV) e pela comunidade de pesquisadores de PV (*Papillomavirus Virus Epstime*- PaVE). Na época, com base na topologia da árvore filogenética, 16 grupos de PVs corresponderam aos critérios de gênero, sendo identificados pelas letras *alfa* a *pi* do alfabeto grego. Cinco letras (*alpha, beta, gamma, mu* e *nu*) foram empregadas para os membros de HPV, duas (*eta* e *theta*) utilizadas para classificar PV de aves e as demais letras foram utilizadas paras os nove gêneros restantes identificados em mamíferos (DE VILLIERS et al., 2004).

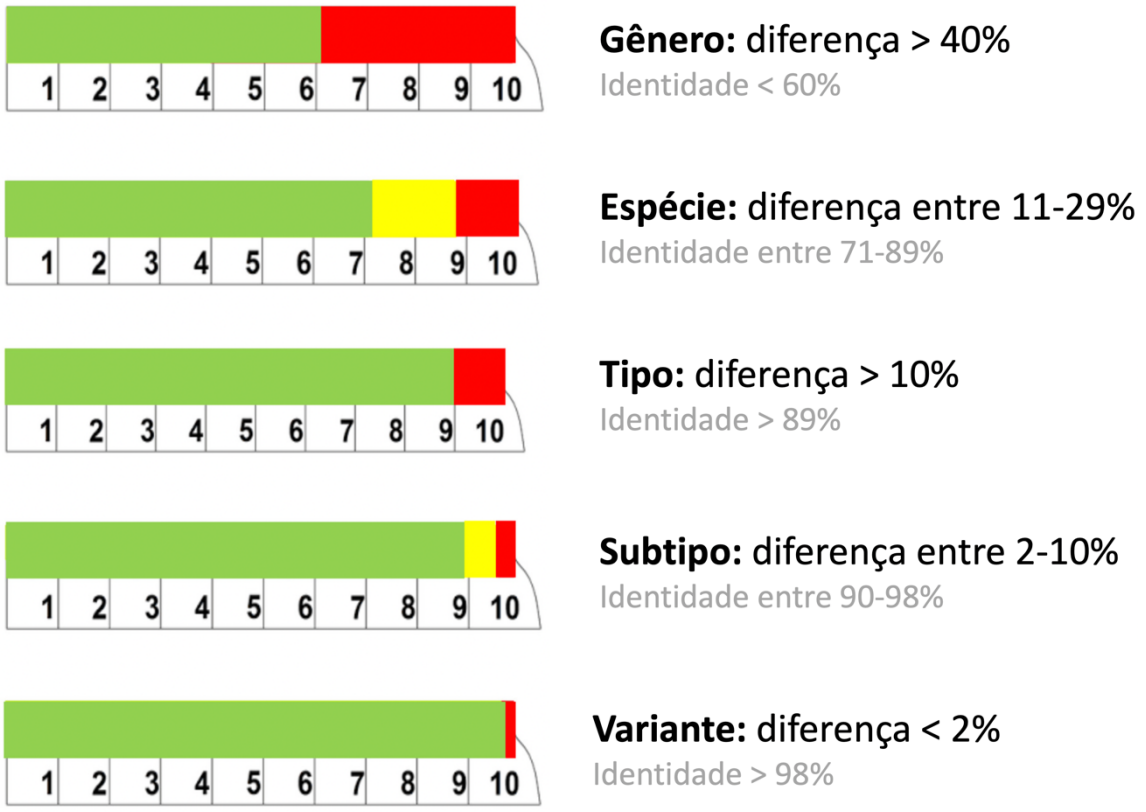
Pesquisas realizadas nos anos seguintes resultaram na identificação de PVs que estavam filogeneticamente distantes dos PVs até então classificados, preenchendo critérios suficientes para estabelecer 13 novos gêneros (BERNARD et al., 2010). A inclusão desses 13 gêneros esgotou o alfabeto e, para seguir com o mesmo sistema, Bernard et al. (2010) propuseram

utilizar o alfabeto grego pela segunda vez, empregando o prefixo “dyo”, que em grego significa “uma segunda vez”. A proposta não incluiu as letras *alfa, beta* e *gamma*, uma vez que agrupam os tipos virais mais comuns e importantes de HPV (BERNARD et al., 2010).

Os PVs são classificados principalmente pela análise filogenética do gene L1. Essa classificação é baseada na comparação das sequências de nucleotídeos dessa região e na posição topológica nas árvores filogenéticas de PVs (BERNARD et al., 2010; DE VILLIERS et al., 2004). Assim, os PVs, podem ser classificados em gêneros, espécies, tipos, subtipos e variantes (DE VILLIERS et al., 2004).

Os PVs são classificados dentro de um mesmo gênero quando apresentam mais do que 60% de identidade; quando a diferença é maior do que 40% sugere-se um novo gênero do vírus. Quando os PVs apresentam 11-29% de diferença no gene L1, uma nova espécie é proposta dentro daquele gênero. Um novo tipo de PV é determinado quando a diferença do gene L1 é maior que 10% comparado a qualquer outro tipo do vírus. O subtipo é determinado quando há diferença de 2-10% do gene L1 (BERNARD et al., 2010; DE VILLIERS et al., 2004) (Figura 4). Entretanto, embora o percentual das identidades seja utilizado como um dos critérios para classificar os PVs, ele não é o único, sendo considerado também a posição e organização filogenética dos vírus, organização do genoma, biologia e patogenicidade viral (BERNARD et al., 2010).

Figura 4.Classificação do papilomavirus baseada no gene L1.

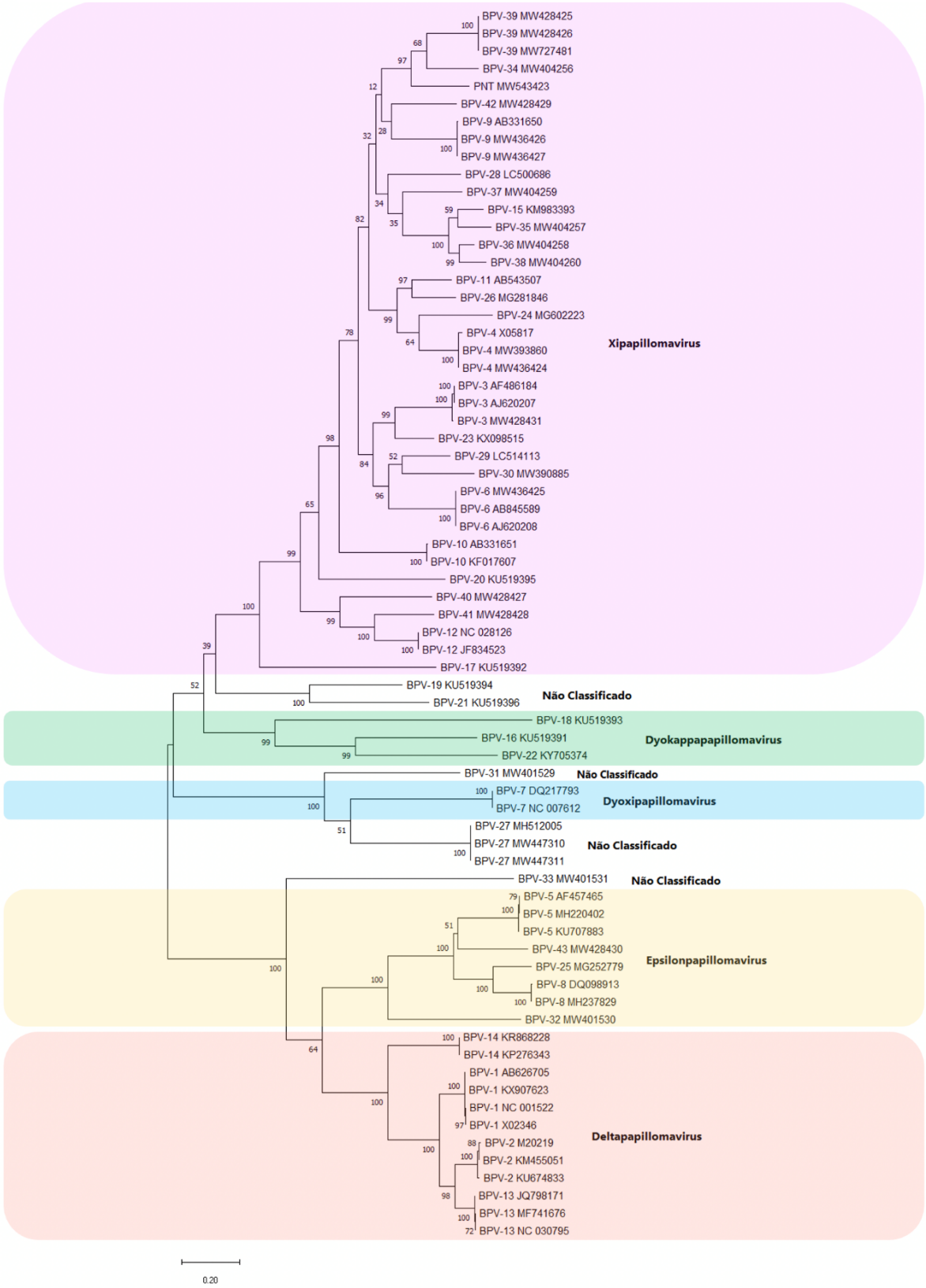


Legenda: Representação esquemática da classificação dos papilomavirus (PVs) com base na sequência de nucleotídeos do gene L1. A barra na cor verde representa a identidade, a cor vermelha representa as diferenças e a cor amarela representa a porcentagem de variação aceita. Os números abaixo da barra representam a porcentagem. (Fonte: adaptada de DAUDT et al., 2018).

Entre os BPVs, são conhecidos cinco gêneros e 43 tipos: *Deltapapillomavirus* (BPV-1, 2, 13 e 14), *Dyokappapapillomavirus* (BPV-16, 18 e 22), *Dyoxipapillomavirus* (BPV-7,), *Epsilonpapillomavirus* (BPV-5, 8, 25, 32 e 43), *Xipapillomavirus* (BPV-3, 4, 6, 9, 10, 11, 12, 15, 17, 20, 23, 24, 26, 28, 29, 30, 34, 35, 36, 37, 38, 39, 40, 41 e 42) e aqueles tipos que ainda não estão classificados quanto ao gênero: BPV-19, 21, 27, 31 e 33 (PAVE, 2022; SAUTHIER et al., 2021) (Figura 5).

Quanto aos CPVs, são conhecidos três gêneros e 24 tipos. Dentre esses: *Chipapillomavirus* (CPV-3, 4, 5, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20 e 24), *Lambdapapillomavirus* (CPV-1 e 6) e *Taupapillomavirus* (CPV-2, 7, 13, 17, 19, 21, 22 e 23) (ICTV, 2018; MUNDAY et al., 2022; PAVE, 2022) (Figura 6).

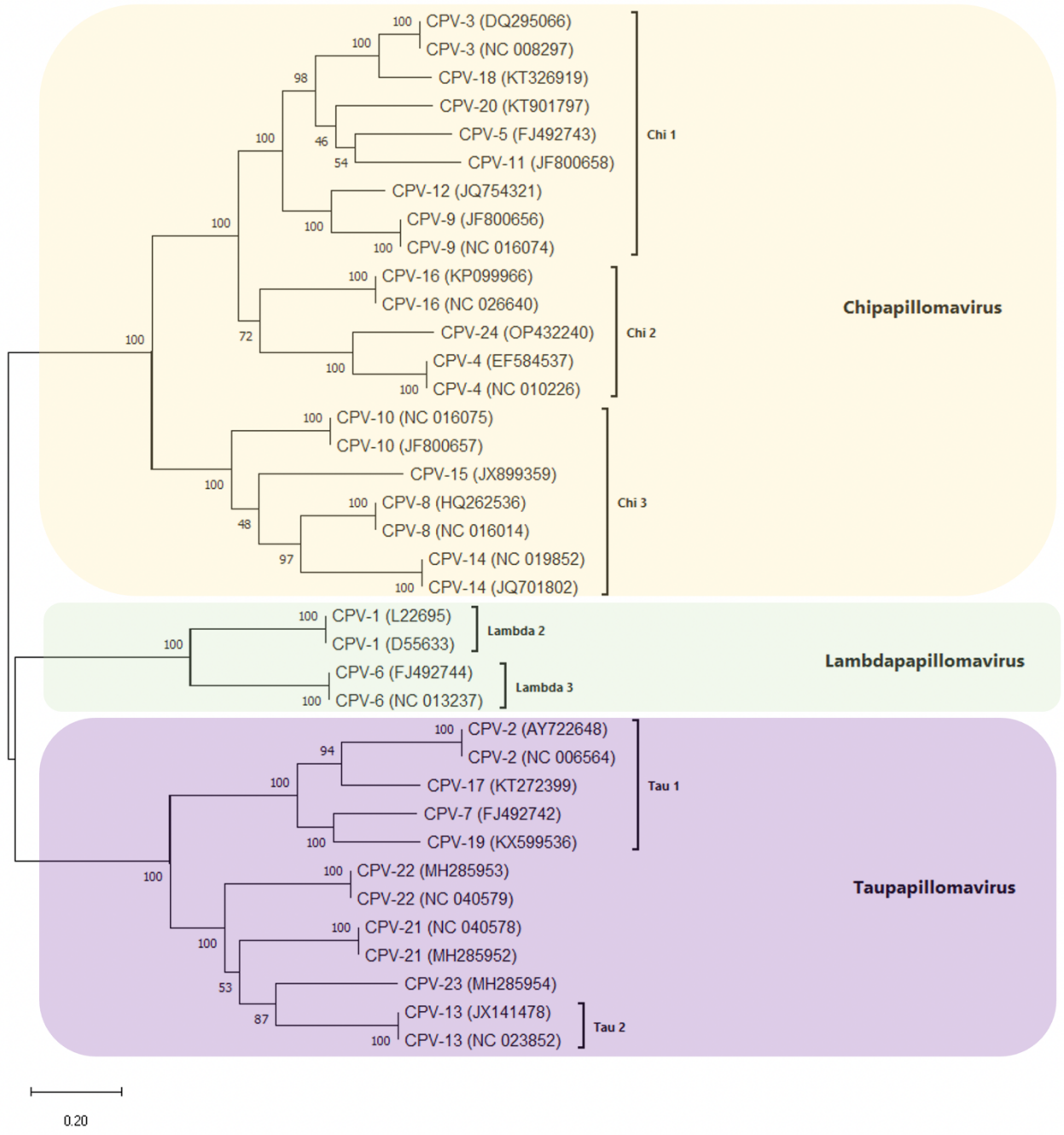
Figura 5. Árvore filogenética dos 43 tipos de papilomavírus bovinos (BPV) descritos na literatura.



Legenda: A árvore filogenética foi construída com base no gene L1 completo de sequências depositadas no banco de dados do GenBank, seguindo a classificação proposta pelo PaVE. Foi utilizado o método de máxima

verossimilhança no software MEGA X (versão 10.2.4) e os valores de *bootstrap* foram calculados com base em 1.000 réplicas (Fonte: arquivo pessoal).

Figura 6. Árvore filogenética dos 24 tipos de papilomavírus caninos (CPV) descritos na literatura.



Legenda: A árvore filogenética foi construída com base no gene L1 completo de sequências depositadas no banco de dados do GenBank, seguindo a classificação proposta pelo PaVE. Foi utilizado o método de máxima verossimilhança no software MEGA X (versão 10.2.4) e os valores de *bootstrap* foram calculados com base em 1.000 réplicas (Fonte: arquivo pessoal).

2.5 DISTRIBUIÇÃO DOS PAPILOMAVÍRUS

Os PVs já foram descritos em diversos países, caracterizando-se como um vírus de distribuição mundial (ARALDI et al., 2017). Em relação ao BPV, casos de infecção têm sido registrados em locais onde há prática da bovinocultura, principalmente onde há grande densidade de bovinos, como Itália, Alemanha, Japão, China, Egito, Turquia, Estados Unidos e Brasil (BAUERMANN et al., 2017; BIANCHI et al., 2020; DAGALP et al., 2017; HASSANIEN et al., 2021; OGAWA et al., 2004; SAVINI et al., 2020). Esses relatos incluem infecções simples ou coinfecções, nas quais diferentes tipos virais são encontrados na mesma lesão ou no mesmo animal (SAUTHIER et al., 2021; CLAUS et al., 2007; CARVALHO et al., 2012; DAGALP et al., 2017; ARALDI et al., 2017).

No Brasil, a papilomatose está bastante difundida no rebanho bovino, sendo crescente os estudos direcionados à análise filogenética e identificação de BPVs. Diferentes tipos de BPVs têm sido identificados na região Norte (BPV-1, 2, 7, 8, 11 e 13), Nordeste (BPV-1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 13, 14, 24, 25 e 26) (BATISTA et al., 2013; FIGUEIRÊDO et al., 2020), Sudeste (BPV-2, 4, 8, 10 e 12) (ARALDI et al., 2014; LUNARDI et al., 2016) e Sul (BPV-1, 2, 3, 4, 6, 7, 8, 9, 10, 11,12, 24 e, 27) (CRESPO et al., 2019; SAUTHIER et al., 2021; SILVA et al., 2010; TOZATO et al., 2013). Alguns estudos têm sugerido novos tipos de BPVs (BPV-31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41 e 42) (SAUTHIER et al., 2021).

Os CPVs também possuem distribuição cosmopolita, sendo descritos em vários países, como China, Reino Unido, Itália, Nova Zelândia, Estados Unidos e Brasil (CHANG et al., 2020; DE ALCÂNTARA et al., 2014; LANGE et al., 2016; MUNDAY et al., 2016; ORLANDI et al., 2021). Assim como BPV, CPVs também têm sido identificados em infecções simples ou mistas (SAUTHIER et al., 2021; CLAUS et al., 2007; CARVALHO et al., 2012; DAGALP et al., 2017; ALVES et al., 2020). No Brasil, os CPVs têm sido descritos na região Nordeste (CPV-1) (REIS, 2019) e Sul do país (CPV-1 e 16) (ALVES et al., 2020; DE ALCÂNTARA et al., 2014). Apesar desses relatos, os estudos sobre circulação e distribuição dos tipos de CPVs no Brasil ainda são escassos.

3. ARTIGO 1

Phylogeny and amino acid analysis in single and mixed bovine papillomavirus infections in Southern Brazil, 2016 - 2020

Ingryd Merchioratto[1,2]; Pablo Sebastian Britto de Oliveira[1,2]; José Valter Joaquim Silva Júnior[2,3]; Mário Celso Sperotto Brum[4]; Rudi Weiblen[2]; Eduardo Furtado Flores[2*]

(Artigo publicado na Archives of Virology, janeiro de 2023- doi: 10.1007/s00705-022-05622-4)

[1]Programa de Pós-graduação em Medicina Veterinária, Universidade Federal de Santa Maria, Rio Grande do Sul, Brazil;

[2]Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Rio Grande do Sul, Brazil;

[3]Setor de Virologia, Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Pernambuco, Brazil;

[4]Laboratório de Virologia, Universidade Federal do Pampa, Curso de Medicina Veterinária, Rio Grande do Sul, Brazil

*Correspondence to: Eduardo Furtado Flores. E-mail: eduardofurtadoflores@gmail.com. Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Av. Roraima, 1000, Prédio 63A Centro de Eventos, Camobi, 97105-900, Santa Maria, Rio Grande do Sul, Brazil

Abstract

Bovine papillomaviruses (BPVs) exhibit a high degree of genetic variability, and several viral types have been identified based on analysis of the L1 gene. The L1 is the main capsid protein and the main target for neutralizing antibodies. We performed a retrospective study on BPVs circulating in Rio Grande do Sul state, Southern Brazil, in 2016-2020. DNA from 43 bovine papilloma samples were amplified using two degenerate primer sets – FAP59/64 and MY09/11 – targeting the L1 region, and analyzed for phylogeny, mixed BPV infections (coinfections) and amino acid (aa) sequences. We also performed an in silico analysis with 114 BPV L1 sequences from the GenBank database to assess the agreement between the phylogeny obtained based on complete L1 sequences versus that based on the region amplified using the FAP59/64 and MY09/11 primer sets. Considering single and coinfections, we identified 31 BPV-1 (31/43; 72.1%), 27 BPV-2 (27/43; 62.8%) and 4 BPV-6 (4/43; 9.3%). Coinfections with BPV-1 and BPV-2 were observed in 61.3% of the samples. Our results are supported by in silico analyses that demonstrate that the classification using FAP59/64 or MY09/11 matches the complete L1 results, except for BPV-17 and -18, which may be mistakenly classified depending on the primers used. Furthermore, we found unique or rare amino acids in at least one L1 sequence of each BPV type identified in our study, some of which have been identified previously in papillomavirus epitopes, suggesting immune-mediated selection. Finally, our study provides an overview of BPVs circulating in Southern Brazil over the last five years and point to the combined use of primers FAP59/64 and MY09/11 for analysis of BPV coinfections and putative epitopes.

Keywords: Papillomavirus; L1 gene; mixed BPV; Epitopes; FAP59/64; MY09/11

1 Introduction

Bovine papillomaviruses (BPVs) are associated with papillomatosis, a disease found in cattle herds worldwide and characterized by proliferative lesions (warts) in the cutaneous and/or mucosal epithelium. The lesions are often benign, but they can progress to malignancy [1]. Bovine papillomatosis has an important economic and health impact and can lead to devaluation of the leather, difficulty in suckling and milking, skin tumors and cancer of the upper gastrointestinal tract and urinary bladder, and premature culling [2, 3]. In addition, the disease is a frequent barrier to the admission of cattle at official animal fairs and/or auctions [4].

BPVs are small, non-enveloped viruses with a double-stranded circular DNA genome about 8 kb in length, encoding five to six early (E1, E2, E4, E5, E6, and E7) and two late proteins (L1 and L2) [1, 5]. Proteins E1 and E2 modulate the translation and replication of the viral genome, while E5, E6, and E7 regulate cell transformation [2]. The L1 and L2 proteins form the viral capsid, with L1 being the main structural protein and the main target for neutralizing antibodies [6, 7]. Furthermore, the L1 gene is commonly used for phylogenetic analysis and classification or typing of BPVs [5,6,7].

BPVs belong to the subfamily *Firstpapillomavirinae*, family *Papillomaviridae* [8]. Based on L1 gene analysis, different BPV types are classified into five genera: *Deltapapillomavirus*, *Dyoxipapillomavirus*, *Dyokappapapillomavirus*, *Epsilonpapillomavirus*, and *Xipapillomavirus* [5, 8]. To date, 29 BPV types are registered in the Papillomavirus Episteme (PaVE) database (https://pave.niaid.nih.gov/#explore/reference_genomes/animal_genomes), 23 of which have been recognized by the International Committee on Taxonomy of Viruses (ICTV) [5, 8]. Recently, Sauthier et al. [9] described 14 new BPV types, which would make up a total of 43 BPV types identified to date.

Different BPV types have been detected and identified using degenerate (e.g., FAP59/64 and MY09/11) or type-specific L1 primers and unconventional amplification methods, such as rolling-circle amplification (RCA) followed by high-throughput sequencing (HTS) [9,10,11,12,13,14]. Furthermore, unclassified and new (or putative new) BPVs have been frequently described [9, 13, 15]. Papilloma fragments and/or animals have also been investigated for mixed BPV infections (coinfections), mainly by type-specific primers [9, 10, 13, 14, 16, 17].

Considering the importance of BPV infections and the genetic diversity of BPVs, we carried out a retrospective study on BPVs circulating in Rio Grande do Sul state, Brazil, between 2016 and 2020. In addition to presenting a phylogenetic analysis, we also demonstrate the reliable use of degenerate primers to detect BPV coinfections and report the presence of unique or rare amino acid (aa) residues in L1 that are likely to be involved in virus-host interactions and evolution.

2. Materials and methods

2.1 Samples and study area

Rio Grande do Sul state is the southernmost Brazilian state, neighboring Argentina and Uruguay and occupying an area of 281,748 km2. It is divided into eight intermediate geographic regions: Porto Alegre, Pelotas, Santa Maria, Uruguaiana, Ijuí, Passo Fundo, Caxias do Sul, and Santa Cruz do Sul-Lajeado. We screened 101 papilloma samples (warts) collected from dairy and beef cattle from all geographic regions of Rio Grande do Sul (Supplementary data, File S1). Samples were received by the Virology Sector of Universidade Federal de Santa Maria (UFSM) and the Virology Laboratory of Universidade Federal do Pampa (UNIPAMPA), Uruguaiana, between 2016 and 2020.

2.2 Study design

Bovine papilloma samples were screened for BPVs by partial amplification of the L1 gene using the degenerate primer pairs FAP59/64 [18] and MY09/11 [19]. For higher coverage of the L1 open reading frame (ORF) and detection of mixed BPV infections, our study included only samples amplified by both primer sets. Details about molecular detection, coinfection analysis, and aa sequence analysis are described below.

2.3 DNA extraction

An approximately 100-mg portion of each papilloma sample was cut into small fragments and transferred to a nuclease-free microtube. In each microtube, 800 µL of Tris-EDTA (TE) buffer, 100 µL of 10% sodium dodecyl sulfate (SDS), and 2 µL of RNase A (10 mg/mL) (Invitrogen, CA, USA) were added to the sample and incubated at 56ºC for 30 min. Subsequently, 25 µL of proteinase K (20 mg/mL) (Ludwig Biotecnologia, RS, Brazil) was added, and incubation was continued for 1 h at 56ºC. The DNA was extracted by phenol-chloroform treatment, eluted in 50 µL of TE, and stored at -20ºC until use.

2.4 Partial amplification of L1

Each DNA sample was subjected to amplification of two regions of the BPV L1 gene, nucleotides (nt) 5732-6206 and nt 6544-6995, according to the complete BPV-1 genome sequence (GenBank accession number NC_001522.1). The first region (475 bp) was amplified using the primers FAP59 (5'-TAACWGTIGGICAYCCWTATT-3') and FAP64 (5'-CCWATATCWVHCATITCICCATC-3') [18], and the second (452 bp) was amplified using the primers MY09 (5'-GCMCAGGGWCATAAYAATGG-3') and MY11 (5'-CGTCCMARRGGAWACTGATC-3') [19], which have been used frequently for detection and identification of BPV types [10,11,12,13,14,15,16].

PCR was performed with 1.5 mM MgCl2, 0.5 µM of each deoxynucleotide, 1.25 µM each primer, 2 U of recombinant Taq DNA polymerase (Thermo Fisher Scientific, MA, USA), approximately 100 ng of template, and ultrapure q.s.p. water to a final volume of 25 µL. The following cycling conditions were used: initial denaturation at 94°C for 10 min, followed by 40 cycles of 94°C for 50 s (denaturation), 50°C or 56°C for 55 s (annealing for primers FAP59/64 and MY09/11, respectively), 72°C for 50 s or 30 s (extension for FAP59/64 and MY09/11, respectively), and final extension at 72ºC for 7 min. The amplicons were then electrophoresed in a 1.5% agarose gel and stained with GelRed (Biotium, CA, USA).

2.5 Gene sequencing

PCR products were purified using a PureLink PCR Purification Kit (Invitrogen, CA, USA) according to the manufacturer's instructions and sequenced by the Sanger method, in duplicate, using a BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, MA, USA). Sequencing was performed by ACTGene Análises Moleculares (RS, Brazil).

2.6 Phylogenetic analysis

Consensus sequences were obtained using the Staden package [20] and aligned by the multiple ClustalW method [21], using the BioEdit Alignment Editor program (version 7.0.5.3) [22]. Phylogenetic analysis was performed using MEGA X software (version 10.2.4) [23], and the evolutionary history was inferred by the maximum-likelihood method, with 1,000 bootstrap replicates. The best analysis model was chosen using jModelTest software [24]. The models and analysis parameters are shown in Table 1.

2.7 Amino acid sequence analysis

Amino acid sequences were predicted from the consensus nucleotide sequences using MEGA X software (version 10.2.4) and then compared to homologous BPV sequences available in the GenBank database. The sequences used in the analysis and their accession numbers are listed in Supplementary File S2.

2.8 In silico analysis

To evaluate the agreement between the phylogenetic analysis results obtained based on complete L1 sequences and those obtained based on the region amplified using the FAP59/64 or MY09/11 primer set, we performed a detailed analysis with sequences available in GenBank. Details on the dataset and analysis methods are described below.

2.8.1 Sequence collection

Complete sequences of BPV L1 genes were obtained from the GenBank database using the search terms “Bovine papillomavirus L1 gene”, “Bovine papillomavirus L1 complete gene”, and “Bovine papillomavirus complete genome”. In addition, newly reported new and/or putative new BPV sequences were also used [9]. Partial sequences of L1, cloned sequences, or BPV sequences from a non-bovine host were excluded from the phylogenetic analysis. The sequences were collected between August 12 and November 14, 2021.

2.8.2 Comparative analysis

Sequences obtained from GenBank were phylogenetically analyzed based on (i) the complete L1 gene, (ii) the region between the annealing sites of FAP59/64 (nt 5753-6183) and MY09/11 (nt 6564-6975) (NC_001522.1), and (iii) the region corresponding to that analyzed in our samples (nt 5784-6096 for FAP59/64 and nt 6604-6920 for MY09/11, according to

NC_001522.1). The region between the annealing sites of FAP59/64 (or MY09/11) and the equivalent to that analyzed in our samples were termed "complete FAP59/64" (or MY09/11) and "partial FAP59/64" (or MY09/11), respectively. The analysis was performed as described above. The models and parameters used in each analysis are described in Table 1.

The typing results from each phylogenetic analysis were then compared to each other. In total, four comparisons were performed: L1 vs. complete FAP59/64, L1 vs. complete MY09/11, L1 vs. partial FAP59/64, and L1 vs. partial MY09/11. In addition, we calculated the weighted geodesic distance between the FAP59/64 (or MY09/11) and complete L1 trees using TreeCmp software (version 2.0) (https://eti.pg.edu.pl/TreeCmp).

3 Results

3.1 Sampling

Out of 101 bovine papillomas screened for BPV, viral DNA was not amplified from 25 samples with either of the primer pairs, 18 were positive with FAP59/64 only, and seven were positive with MY09/11 only. These samples were all positive for the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH), using the forward primer 5'-TGTTCCAGTATGATTCCAC-3 and the reverse primer 5'-TCCACCACCCTGTTGCTGTA-3' [25] (data not shown). The remaining 51 samples were amplified by both primer sets; however, only 43 samples were obtained in sufficient amounts for nucleotide sequencing and were included in the study. Of these, only samples with consensus sequences of 313 nt for FAP59/64 and 317-329 nt for MY09/11 were considered suitable for phylogenetic analysis. These regions correspond to nt 5784-6096 (FAP59/64) and nt 6604-6920 (MY09/11) (accession number NC_001522.1). The screening and sample selection scheme is shown in Figure 1.

3.2 Molecular detection

Of the 43 samples analyzed, 31 yielded a suitable consensus sequence using the two primer sets. Twelve samples contained a single BPV type that was detected using FAP59/64 or MY09/11 (7 BPV-1, 1 BPV-2, and 4 BPV-6), while both BPV-1 and BPV-2 were detected in 19 samples. Among the 12 samples analyzed using only one primer set, BPV-1 was detected in five and BPV-2 was detected in seven. Overall, BPV-1 was detected in 31 samples (72.1%), BPV-2 in 27 (62.8%), and BPV-6 in four (9.3%) (Fig. 2 and Table 2).

3.3 Amino acid sequence analysis

Five sequences identified in our study had aa changes when compared to the homologous BPV: SV01/20 (MY09/11) (13 changes), SV314/17 (FAP59/64) (5), SV16/20 (MY09/11) (1), SV314/17 (MY09/11) (1), and SV526/18 (MY09/11) (1). Furthermore, a change at residue 351 (I/M to V) was identified in all sequences classified as BPV-6, but in only one sequence available in the GenBank database (MW436425.1). Details of aa positions and changes are shown in Table 3.

3.4 Comparison of phylogenies obtained using different parts of the L1 gene sequence

One hundred fourteen complete BPV L1 sequences were obtained from the GenBank database, which were typed as BPV-1 (26 sequences), BPV-2 (12), BPV-3 (3), BPV-4 (3), BPV-5 (3), BPV-6 (3), BPV-7 (2), BPV-8 (2), BPV-9 (3), BPV-10 (6), BPV-11 (1), BPV-12 (3), BPV-13 (6), BPV-14 (2), BPV-15 (1), BPV-16 (1), BPV-17 (1), BPV-18 (1), BPV-19 (1), BPV-20 (1), BPV-21 (1), BPV-22 (2), BPV-23 (1), BPV-24 (1), BPV-25 (1), BPV-26 (2), BPV-27 (3), BPV-28 (3), BPV-29 (1), BPV-30 (1), BPV-31 (1), BPV-32 (1), BPV-33 (1), BPV-34 (1), BPV-35 (1), BPV-36 (1), BPV-37 (1), BPV-38 (1), BPV-39 (3), BPV-40 (1), BPV-41 (1), BPV-42 (1), BPV-43 (1), and putative new (2) (Supplementary File S3).

These phylogenetic groupings obtained using complete L1 gene sequences were largely maintained when the analyses were performed using complete or partial sequences obtained using the primer pair FAP59/64 or MY09/11. The only mismatches observed were in BPV-18 (KU519393), which was classified as a member of the genus Dyokappapapillomavirus using L1 and MY09/11 (complete and partial) sequences but was undefined using FAP59/64 (complete and partial), and in BPV-17 (KU519392), which was classified as a member of the genus Xipapillomavirus using L1, FAP59/64 (complete and partial) and MY09/11 (partial) sequences, but was undefined using complete MY09/11 sequences (Supplementary File S3). Regarding the geodesic distance, a value of 1.181 and 1.122 was found between L1 vs. complete FAP59/64 and L1 vs. partial FAP59/64 trees, respectively, and 0.732 and 0.836 between L1 vs. complete MY09/11 and L1 vs. partial MY09/11 trees, respectively.

4 Discussion

Different BPV types have been described over the years. Furthermore, some authors have also identified unclassified, new, or putative new BPVs [9, 13, 15]. In order to contribute to the epidemiological-genomic studies of BPV, we performed a retrospective phylogenetic analysis of bovine papilloma samples collected over five years in various regions of Rio Grande do Sul, Brazil.

Initially, we screened 101 warts for BPV using the primer pairs FAP59/64 and MY09/11. Of these, 25 were not amplified by either of the primer pairs, 25 were amplified only by one of the primer pairs, and eight did not yield enough material for nucleotide sequencing. Failure to detect BPV in some of our samples may be related to primer/reaction sensitivity, viral load, and/or sample quality. Bovine warts may be negative for BPV DNA even when subjected to amplification by more sensitive detection methods, such as RCA [9]. Therefore, we included only the samples amplified by both primer sets and performed the study with 43 bovine

papillomas. The amplification of the same sample using two primer pairs allowed the detection of mixed BPV infections and provided higher coverage of the L1 ORF, which is useful for analyzing the aa sequence.

The BPVs detected in our study were classified as types 1, 2, and 6, with BPV-1 (72.1%, 31/43) and -6 (9.3%, 4/43) being the most and least prevalent, respectively. These viral types were identified previously in other studies carried out in Southern Brazil, although the frequency of detection was considerably different [10, 12, 15]. Claus et al. [10] identified BPV-1, -2, and -6 in three, one, and six samples, respectively, collected from six animals in Paraná state. In 2008, among 16 cutaneous lesions also from Paraná state, Claus et al. detected BPV-1, -2, and -6 in six, two, and eight samples, respectively [15]. Silva et al. [12] detected BPV-1, -2, and 6 in 29 (60%; 29/48), eight (16%; 8/48), and nine (18%; 9/48) bovine papillomas, respectively, collected between 2007 and 2009 in Southern Brazil. Despite the agreement between the BPV types identified here and those described in previous studies, our findings, as well as those of other reports [10, 12, 15], were obtained by convenience sampling, and no statistical analysis were performed. This is a common limitation of BPV studies that makes it difficult to determine the diversity and prevalence of viral types.

It is also important to consider that the specimens analyzed in our study, as well as those from Claus et al. [10, 15] and Silva et al. [12], were amplified using the primers FAP59/64 and MY09/11, which have lower sensitivity than some type-specific BPV primers, such as those targeting BPV-4 and BPV-9 [26]. The difference in sensitivity between degenerate and type-specific primers is likely related to the design of FAP59/64 and MY09/11, which were based on the human papillomavirus (HPV) genome [18, 27]. Despite this, FAP59/64 and/or MY09/11 have been able to detect several BPVs, including unclassified viruses [11, 13,14,15, 26]. The use of degenerate primers is a simple and low-cost strategy when considering the high diversity of BPV. Therefore, although we recognize the coverage limits of our study, we believe that our

analyses, carried out using samples collected from dairy and beef cattle over five years in different regions of Rio Grande do Sul, provide an approximate overview of the circulation of BPV in Southern Brazil.

Several studies have found mixed BPV infections in bovine papilloma specimens. Most of these results were obtained by amplification with type-specific primers and gene sequencing, but some were obtained by cloning followed by sequencing or by RCA-HTS [9, 10, 13, 14, 16, 17, 28, 29]. Here, we describe the detection of mixed BPV infections using the primer pairs FAP59/64 and MY09/11, a simple approach for screening and detecting BPV coinfection based on amplification and sequencing of the same sample with both primer sets.

Initially, an in silico analysis allowed us to observe that most of the phylogenetic findings based on complete L1 sequences were maintained when the analysis was performed based on the regions amplified by the degenerate primers. The only exceptions were BPV-18 (KU519393), which was classified as a member of the genus *Dyokappapapillomavirus* (by L1 and MY09/11) or an undefined genus (by FAP59/64), and BPV-17 (KU519392), which was classified as a member of the genus *Xipapillomavirus* (by L1 and FAP59/64) or an undefined genus (by MY09/11).

We then assessed the agreement of the BPV classification based on complete L1 vs. partial FAP59/64 or MY09/11 sequences, specifically the same region analyzed in our samples. The phylogenetic groupings based on complete L1 sequences were also maintained when using partial FAP59/64 and MY09/11 sequences; the only mismatch was in BPV-18 (KU519393) by partial FAP59/64; similar to that discussed above. Importantly, as viruses belonging to the BPV-18 type (genus *Dyokappapapillomavirus*) were not identified in our study, we do not believe that this mismatch compromises our viral classifications.

We also observed that the geodesic distance between complete L1 and complete FAP59/64 or MY09/11 sequences was similar to that between complete L1 and partial

FAP59/64 or MY09/11 sequences. This distance compares both the tree topology and the edge length between two selected trees [30] and has been used to assess the agreement of viral classifications performed based on different gene targets [31, 32]. Taken together, our results indicate that (i) most of the typing results obtained using complete or partial FAP59/64 and MY09/11 sequences match those obtained using complete L1 sequences and (ii) there was no misclassification when the same virus was analyzed using either complete or partial FAP59/64 and MY09/11 sequences, except for BPV-17.

In our study, 19 samples analyzed using the primer pairs FAP59/64 and MY09/11 were typed differently depending on the primer set used. Considering the above discussion, we identified these samples as having mixed infections. Interestingly, the frequency of coinfection obtained using the two primer sets, 61.3% (19/31), was very similar to that observed by Sauthier et al. [9] in samples from Southern Brazil analyzed by RCA-HTS, 56.3% (9/16).

All coinfected samples identified in our study contained BPV types 1 and 2. Furthermore, most of the BPV-1 and -2 identified in our study, mainly type 2, was detected in coinfections. These results are in line with previous reports [10, 13, 14, 28]. Based on the prevalence of BPV-2 in coinfections, Dagalp et al. [14] raised the possibility that BPV-2 could somehow facilitate a subsequent infection with different BPV types. On the other hand, all BPV-6 found in our study was identified in single infections. Although this result may have been influenced by the sensitivity of the PCR assay with primers FAP59/64 and MY09/11, this is also an interesting finding, since all BPV-6 sequences detected in our study had rare aa residues.

The regions amplified by the primers FAP59/64 and MY09/11, together, cover approximately half of the L1 ORF, and some HPV-16/18 and deltapapillomavirus epitopes are possibly encoded in the regions amplified by FAP59/64 and/or MY09/11 [33,34,35]. Therefore, to analyze possible immunity-mediated influences on the BPV from our study, we compared

the predicted aa sequences from our study with those of homologous BPVs available in the GenBank database.

The BPV-6 sample SV314/17 was the only one to contain aa changes in the region sequenced using FAP59/64, which corresponds approximately to the first quarter of the L1 ORF, the region closest to the N-terminus of the protein. Of the five aa changes in this region, two are at likely epitopes of BPV-1 and -2 located between aa 74 and 234 [33, 34], specifically at positions 75 (Q to P) and 95 (Q to H) (Table 3). Residue 95 is also located in a region previously identified as a probable HPV-16 epitope for MHC II, aa 95-111 [35]. The other changes observed in our study are in the region amplified using MY09/11, corresponding approximately to the last quarter of the L1 ORF, close to the C-terminus of the protein.

The BPV-1 sample SV16/20 showed a change at residue 419 (I to K), which has been identified in possible deltapapillomavirus epitopes located between aa 411 and 475 [33, 34], as well as in HPV-16/18 epitopes for MHC II (aa 416-430 and 417-431) and B lymphocytes (aa 408-421 and 404-417) [35]. Interestingly, sample SV526/18 had a change at position 340 (T/P to S), which is located in a region that is conserved among HPVs [35]. BPV-2 sample SV01/20 contained 13 changes compared to homologous viruses. Of these, six are located in probable deltapapillomavirus (aa 411-475), B lymphocytes (aa 408-421), or MHC II (aa 416-430 and 417-431) epitopes – residue 414 (D to H), 417 (R to G), 419 (I to V), 423 (A to L), 424 (T to S) and 425 (K to T)] – and two are in deltapapillomavirus and MHC II epitopes, 428 (S to I) and 429 (N to I) [33,34,35].

Interestingly, all BPV-6 detected in our study had a change at residue 351 (I/M to V), which was only observed in another BPV-6 from Rio Grande do Sul (accession number MW436425.1 [9]). Residue 351 has not yet been reported in papillomavirus epitopes, and we have not found sequences from other BPV-6 isolates from Rio Grande do Sul available in

GenBank. Thus, we speculate that immunity-mediated pressure could be driving BPV-6 evolution in Southern Brazil.

Conclusion

A retrospective phylogenetic analysis of BPV circulating in Rio Grande do Sul, Brazil, between 2016 and 2020, performed using the degenerate primers FAP59/64 and MY09/11, allowed us to conclude that (i) BPV-1, -2, and -6 were circulating in cattle herds during the study period, (ii) BPV-1 and -6 were the most and least prevalent viral types, respectively, (iii) a high frequency of coinfections was observed, specifically by BPV-1/2, (iv) BPV-6 was found predominantly in single infections, and (v) BPV-2 was found predominantly in coinfections. In addition, we also identified unique or rare aa residues in L1 that are probably related to virus-host interaction and evolution. Finally, our in silico analyses indicated that the phylogenetic groupings obtained based on complete and partial sequences obtained using the primer pair FAP59/64 or MY09/11 mostly matched those obtained based on complete L1 sequences, suggesting that these primers may be a reliable option for typing of BPVs.

Declarations

Funding

Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) - Finance code 001.

Conflicts of interest/Competing interests

The authors declare that they have no competing interests.

Ethics approval

Not applicable. The study did not involve animal experimentation. The samples used in the study came from the diagnostic service offered by Virology Sector (UFSM) and Virology Laboratory (UNIPAMPA).

Availability of data and material

Datasets generated and/or analyzed during the current study will be available on GanBank and may also be made available by the corresponding author upon reasonable request.

Code availability

Not applicable

Authors' contributions

IM extracted DNA from the samples, amplified the target genes, analyzed the gene sequencing and assisted in *in silico* analyses, phylogeny and amino acid analyses, results interpretation and manuscript preparation; PBSO assisted in *in silico* analyses, gene sequencing analyses, phylogeny and amino acid analyses, results interpretation and manuscript preparation; JVJr assisted in *in silico* analyses, gene sequencing analyses, phylogeny and amino acid analyses, experimental design, interpretation of results and prepared the final version of the manuscript;

MCSB assisted in the interpretation of results, experimental design and manuscript review; RW assisted in the interpretation of results and manuscript review; EFF assisted in the experimental design, interpretation of results, elaboration and final review of the manuscript and guided the study.

Acknowledgments

Graduate students from the *Programa de Pós-graduação em Medicina Veterinária*, UFSM (IM and PSBO). PSBO and IM thank the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) (Brazil) and *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) (Brazil), respectively, for their scholarships. EFF (process 301414/2010-6) and RW (process 304153/2014-1) were supported by CNPq research fellowships. The research was financed in part by CAPES, Brazil - Finance code 001.

References

1. Rector A, Van Ranst M (2013) Animal papillomaviruses. Virology 445:213–223. https://doi.org/10.1016/j.virol.2013.05.007
2. Borzacchiello G, Roperto F (2008) Bovine papillomaviruses, papillomas and cancer in cattle. Vet Res 39:45. https://doi.org/10.1051/vetres:2008022
3. Daudt C, Da Silva FRC, Lunardi M, et al (2018) Papillomaviruses in ruminants: An update. Transbound Emerg Dis 65:1381–1395. https://doi.org/10.1111/tbed.12868
4. Gil da Costa RM, Peleteiro MC, Pires MA, DiMaio D (2017) An Update on Canine, Feline and Bovine Papillomaviruses. Transbound Emerg Dis 64:1371–1379. https://doi.org/10.1111/tbed.12555

5. Van Doorslaer K, Chen Z, Bernard H-U, et al (2018) ICTV Virus Taxonomy Profile: Papillomaviridae. J Gen Virol 99:989–990. https://doi.org/10.1099/jgv.0.001105
6. de Villiers E-M, Fauquet C, Broker TR, et al (2004) Classification of papillomaviruses. Virology 324:17–27. https://doi.org/10.1016/j.virol.2004.03.033
7. Bernard H-U, Burk RD, Chen Z, et al (2010) Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology 401:70–79. https://doi.org/10.1016/j.virol.2010.02.002
8. ICTV (2018) Papillomaviridae. In: Int. Comm. Taxon. Viruses. https://talk.ictvonline.org/ictv-reports/ictv_online_report/dsdna-viruses/w/papillomaviridae
9. Sauthier JT, Daudt C, da Silva FRC, et al (2021) The genetic diversity of "papillomavirome" in bovine teat papilloma lesions. Anim Microbiome 3:51. https://doi.org/10.1186/s42523-021-00114-3
10. Claus MP, Vivian D, Lunardi M, et al (2007) Análise filogenética de papilomavírus bovino associado com lesões cutâneas em rebanhos do Estado do Paraná. Pesqui Veterinária Bras 27:314–318. https://doi.org/10.1590/S0100-736X2007000700010
11. Claus MP, Lunardi M, Alfieri AF, et al (2009) Identification of the recently described new type of bovine papillomavirus (BPV-8) in a Brazilian beef cattle herd. Pesqui Veterinária Bras 29:25–28. https://doi.org/10.1590/S0100-736X2009000100003
12. Silva MS e., Weiss M, Brum MCS, et al (2010) Molecular Identification of Bovine Papillomaviruses Associated with Cutaneous Warts in Southern Brazil. J Vet Diagnostic Investig 22:603–606. https://doi.org/10.1177/104063871002200417
13. Carvalho CCR, Batista MVA, Silva MAR, et al (2012) Detection of Bovine Papillomavirus Types, Co-Infection and a Putative New BPV11 Subtype in Cattle. Transbound Emerg Dis 59:441–447. https://doi.org/10.1111/j.1865-1682.2011.01296.x

14. Dagalp SB, Dogan F, Farzanı TA, et al (2017) The genetic diversity of bovine papillomaviruses (BPV) from different papillomatosis cases in dairy cows in Turkey. Arch Virol 162:1507–1518. https://doi.org/10.1007/s00705-017-3258-8

15. Claus MP, Lunardi M, Alfieri AF, et al (2008) Identification of unreported putative new bovine papillomavirus types in Brazilian cattle herds. Vet Microbiol 132:396–401. https://doi.org/10.1016/j.vetmic.2008.05.026

16. Ataseven VS, Kanat Ö, Ergun Y (2016) Molecular identification of bovine papillomaviruses in dairy and beef cattle: first description of Xi- and Epsilonpapillomavirus in Turkey. Turkish J Vet Anim Sci 40:757–763. https://doi.org/10.3906/vet-1512-64

17. Hamad MA, Al-Shammari AM, Odisho SM, Yaseen NY (2017) Molecular Epidemiology of Bovine Papillomatosis and Identification of Three Genotypes in Central Iraq. Intervirology 60:156–164. https://doi.org/10.1159/000486594

18. Forslund O, Antonsson A, Nordin P, et al (1999) A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. J Gen Virol 80:2437–2443. https://doi.org/10.1099/0022-1317-80-9-2437

19. Ogawa T, Tomita Y, Okada M, et al (2004) Broad-spectrum detection of papillomaviruses in bovine teat papillomas and healthy teat skin. J Gen Virol 85:2191–2197. https://doi.org/10.1099/vir.0.80086-0

20. Staden R (1996) The staden sequence analysis package. Mol Biotechnol 5:233–241. https://doi.org/10.1007/BF02900361

21. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673. https://doi.org/10.1093/NAR/22.22.4673

22. Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

23. Kumar S, Stecher G, Li M, et al (2018) MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol 35:1547–1549. https://doi.org/10.1093/molbev/msy096

24. Posada D (2008) jModelTest: Phylogenetic Model Averaging. Mol Biol Evol 25:1253–1256. https://doi.org/10.1093/molbev/msn083

25. Faccin TC, Cargnelutti JF, Rodrigues F de S, et al (2018) Bovine upper alimentary squamous cell carcinoma associated with bracken fern poisoning: Clinical-pathological aspects and etiopathogenesis of 100 cases. PLoS One 13:e0204656. https://doi.org/10.1371/journal.pone.0204656

26. Silva MAR, Batista MVA, Pontes NE, et al (2013) Comparison of two PCR strategies for the detection of bovine papillomavirus. J Virol Methods 192:55–58. https://doi.org/10.1016/j.jviromet.2013.04.017

27. Manos MM, Ting YT, Wright DK, et al (1989) Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. Cancer Cells Mol Diagnostics Hum Cancer 7:209–214

28. Claus MP, Lunardi M, Alfieri AA, et al (2009) A bovine teat papilloma specimen harboring Deltapapillomavirus (BPV-1) and Xipapillomavirus (BPV-6) representatives. Brazilian Arch Biol Technol 52:87–91. https://doi.org/10.1590/S1516-89132009000700012

29. Pangty K, Singh S, Goswami R, et al (2010) Detection of BPV-1 and -2 and Quantification of BPV-1 by Real-Time PCR in Cutaneous Warts in Cattle and Buffaloes. Transbound Emerg Dis 57:185–196. https://doi.org/10.1111/j.1865-1682.2009.01096.x

30. Owen M (2011) Computing Geodesic Distances in Tree Space. SIAM J Discret Math 25:1506–1529. https://doi.org/10.1137/090751396

31. de Oliveira PSB, Silva Júnior JVJ, Weiblen R, Flores EF (2021) Subtyping bovine viral diarrhea virus (BVDV): Which viral gene to choose? Infect Genet Evol 92:104891. https://doi.org/10.1016/j.meegid.2021.104891

32. Dillner L, Heino P, Moreno-Lopez J, Dillner J (1991) Antigenic and immunogenic epitopes shared by human papillomavirus type 16 and bovine, canine, and avian papillomaviruses. J Virol 65:6862–6871. https://doi.org/10.1128/jvi.65.12.6862-6871.1991

33. Cason J, Kambo PK, Jewers RJ, et al (1993) Mapping of linear B cell epitopes on capsid proteins of bovine papillomavirus: identification of three external type-restricted epitopes. J Gen Virol 74:2669–2677. https://doi.org/10.1099/0022-1317-74-12-2669

34. Namvar A, Bolhassani A, Javadi G, Noormohammadi Z (2019) In silico/In vivo analysis of high-risk papillomavirus L1 and L2 conserved sequences for development of cross-subtype prophylactic vaccine. Sci Rep 9:15225. https://doi.org/10.1038/s41598-019-51679-8

Table 1. Parameters used in the Maximum Likelihood phylogeny of BPV sequences.

Data	Genomic region	Substitution model[e]	Gamma shape parameter	Proportion invariant sites	BIC[f]	Log likelihood
GenBank	L1[a]	GTR+I+G	0.9081	0.109	101,339,7885	-49,988.60
	FAP59/64 - C[b]	GTR+G+I	1.4791	0.094	31,031.413	-14,894.69
	MY09/11 - C[b]	GTR+G+I	1.6961	0.066	32,123.139	-15,325.78
	FAP59/64 - P[c]	GTR+G	1.3147	NA[g]	20,235.709	-9,451.85
	MY09/11 - P[d]	GTR+G+I	2.0078	0.046	25,040.290	-11,939.54
Study	FAP59/64[c]	GTR+G+I	1.3410	0.103	19,132.5201	-9,053.20
	MY09/11[d]	GTR+G+I	1.9231	0.058	23,5045.4188	-10,982.95

[a]Complete L1 sequence;

[b]Region corresponding to that amplified with primers FAP59/64 or MY09/11 (termed complete FAP59/64 or MY09/11);

[c]Region corresponding to nts 5784-6096 (access number NC_001522.1), termed partial FAP59/64;

[d]Region corresponding to nts 6604-6920 (access number NC_001522.1), termed partial MY09/11;

[e]GTR: general time reversible; G: gamma distribution; I: invariant sites; K2: Kimura 2-parameter; T92: Tamura 3-parameter; TN93: Tamura-Nei; HKY: Hasegawa-Kishino-Yano;

[F]Bayesian information criterion;

[g]Not applicable;

Tabela 2. Collection and classification data of BPVs identified in Rio Grande do Sul, Brazil, between 2016 and 2020.

Region	Sample	Year of collection	Classification	
			FAP59/64 (GenBank)	MY09/11 (GenBank)
Pelotas	SV314/17	2017	BPV-6 (ON993815)	BPV6 (ON993853)
Santa Maria	SV553/16	2016	BPV-2 (ON993820)	BPV-1 (ON993858)
	SV602/16	2016	BPV-2 (ON993817)	BPV-1 (ON993855)
	SV642/16	2016	BPV-2 (ON993830)	NA[a]
	SV97/17	2017	BPV-2 (ON993827)	BPV-1 (ON993865)
	SV442/17	2017	BPV-2 (ON993823)	BPV-1 (ON993860)
	SV233/18	2018	BPV-2 (ON993829)	NA
	SV276/18	2018	BPV-6 (ON993824)	BPV-6 (ON993861)
	SV495/18	2018	NA	BPV-1 (ON993856)
	SV526/18	2018	BPV-1 (ON993822)	BPV-1 (ON993859)
	LV039/18	2018	NA	BPV-1 (ON993880)
	SV377/19	2019	BPV-2 (ON993825)	BPV-1 (ON993862)
	SV471/19	2019	BPV-1 (ON993828)	BPV-1 (ON993866)
	LV040/19	2019	BPV-1 (ON993844)	NA
	SV01/20	2020	BPV-2 (ON993816)	BPV-2 (ON993854)
	SV16/20	2020	BPV-2 (ON993831)	BPV-1 (ON993867)
	SV130/20	2020	BPV-2 (ON993851)	BPV-1 (ON993887)
	SV227/20	2020	BPV-2 (ON993852)	BPV-1 (ON993888)
Uruguaiana	LV003/16	2016	BPV-1 (ON993842)	BPV-1 (ON993878)
	LV035/17	2017	BPV-1 (ON993845)	BPV-1 (ON993881)
	LV045/17	2017	NA	BPV-1 (ON993876)
	LV047/17	2017	BPV-2 (ON993834)	NA
	LV004/18	2018	BPV-2 (ON993841)	BPV-1 (ON993877)
	LV009/18	2018	NA	BPV-1 (ON993870)
	LV011/18	2018	BPV-2 (ON993838)	BPV-1 (ON993874)
	LV035/18	2018	BPV-2 (ON993835)	BPV-1 (ON993871)
	LV038/18	2018	BPV-1 (ON993846)	BPV-1 (ON993882)
	LV041/18	2018	BPV-2 (ON993847)	BPV-1 (ON993883)
	LV046/18	2018	BPV-2 (ON993840)	NA
	LV053/18	2018	BPV-2 (ON993848)	BPV-1 (ON993884)
	LV059/18	2018	BPV-2 (ON993836)	BPV-1 (ON993872)
	LV001/19	2019	BPV-2 (ON993849)	BPV-1 (ON993885)
	LV024/19	2019	BPV-2 (ON993837)	BPV-1 (ON993873)
	LV041/19	2019	BPV-2 (ON993839)	BPV-1 (ON993875)
	LV014/20	2020	BPV-2 (ON993850)	BPV-1 (ON993886)
Ijuí	SV101/17	2017	NA	BPV-2 (ON993863)
	SV272/17	2017	BPV-6 (ON993819)	BPV-6 (ON993857)
	SV526/17	2017	BPV-2 (ON993821)	NA
	SV580/17	2017	BPV-6 (ON993833)	BPV-6 (ON993869)
	LV035/19	2019	BPV-1 (ON993843)	BPV-1 (ON993879)
Santa Cruz do Sul	SV527/16	2016	BPV-2 (ON993826)	BPV-1 (ON993864)
	SV527/17	2017	BPV-1 (ON993832)	BPV-1 (ON993868)
Caxias do Sul	SV108/17	2017	BPV-2 (ON993818)	NA

[a]Not applicable: Consensus sequence not suitable for phylogenetic analysis.

Table 3. Amino acid changes in L1 of the BPVs detected in the study.

Primers	Virus	Sample	Amino acids[a]
FAP59/64	BPV-6	SV314/17	68 (Q>H); 69 (F>S); 70 (R>G); 75 (Q>P); 95 (Q>H)
MY09/11	BPV-1	SV16/20	419 (I>K)
		SV526/18	340 (T/P>S)
	BPV-2	SV01/20	345 (A>C); 346(D>Y); 349 (A>V); 353 (Y>F); 359 (N>G); 414 (D>H); 417 (R>G); 419 (I>V); 423 (A>L); 424 (T>S); 425 (K>T); 428 (S>I); 429 (N>I)
	BPV-6	SV314/17	350 (N>K); **351 (I/M>V)**[b]
		SV272/16	**351 (I/M>V)**
		SV276/18	**351 (I/M>V)**
		SV580/17	**351 (I/M>V)**

[a]The access numbers of the compared sequences are in Supplementary File 2. The amino acid positions of BPV-1, -2 and -6 correspond to those of the L1 protein of NC_001522.1, M20219.1 and AB845589.1, respectively;

[b]In bold is the amino acid change observed in all BPV-6 in our study. In addition to our samples, this change was observed in only one sequence available in GenBank (MW436425.1). This finding is addressed in the Discussion section.

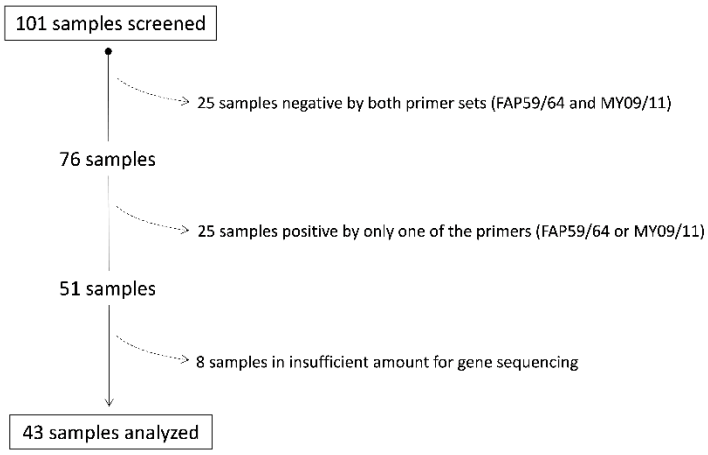


Figure 1. Workflow from screening to analysis of bovine papilloma samples.

The study began with 101 bovine papilloma samples from eight intermediate geographic regions of the Rio Grande do Sul state, Brazil. Only samples amplified by both primer sets, FAP59/64 and MY09/11, were used for phylogeny, co-infection and amino acid analyses.

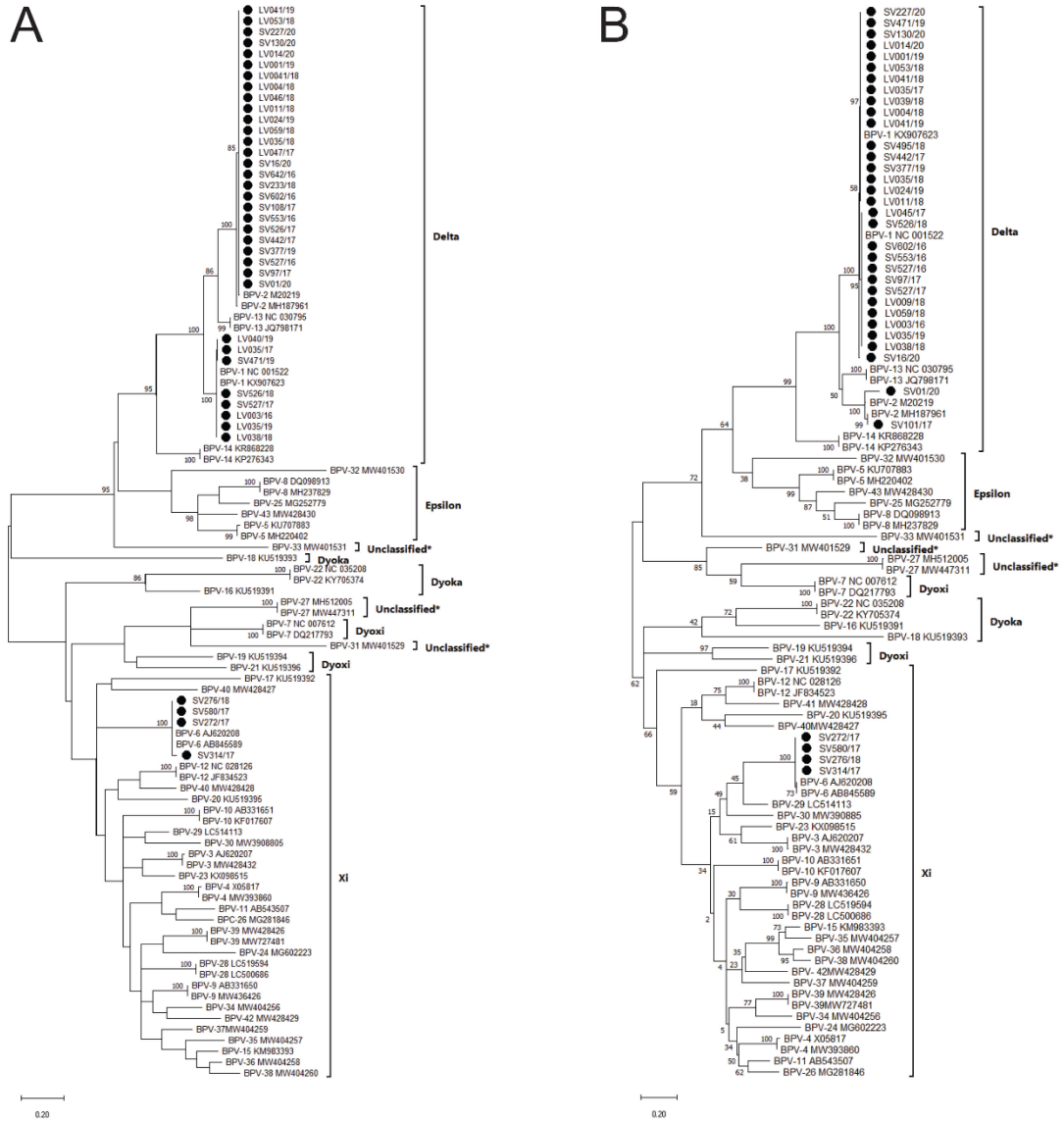


Figure 2. Phylogenetic trees of the BPVs detected in the study.

Phylogeny was performed with the partial L1 sequence amplified and sequenced with primers FAP59/64 (A) and MY09/11 (B). The analyses were performed with the MEGA-X software (version 10.2.4), using the Maximum Likelihood method (see Table 1). The bootstrap values were calculated in 1,000 replicates. Branches supported by >85% of bootstrap replicates are indicated. The sequences identified with filled circles correspond to those collected and analyzed in our study. *BPV types that were not previously grouped by genus were identified as “unclassified”.

4. ARTIGO 2

Phylogenetic analysis of papillomaviruses in dogs in Southern Brazil: detection of mixed infections and possible spillover events

Análise filogenética de papilomavírus em cães no sul do Brasil: detecção de infecções mistas e possíveis transmissões entre espécies

Ingryd Merchioratto[1,2]; Carolina Isabela Mucellini[1,2]; Pablo Sebastian Britto de Oliveira[1,2]; José Valter Joaquim Silva Júnior[2,3,4]; Mário Celso Sperotto Brum[5]; Rudi Weiblen[2]; Eduardo Furtado Flores[2*]

(Artigo submetido ao periódico Ciência Rural, fevereiro de 2023)

[1]Programa de Pós-graduação em Medicina Veterinária, Universidade Federal de Santa Maria, Rio Grande do Sul, Brazil;
[2]Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Rio Grande do Sul, Brazil;
[3]Setor de Virologia, Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Pernambuco, Brazil;
[4]Departamento de Análises Clínicas, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Rio Grande do Sul, Brazil
[5]Laboratório de Virologia, Universidade Federal do Pampa, Curso de Medicina Veterinária, Rio Grande do Sul, Brazil;

*Correspondence to: Eduardo Furtado Flores. E-mail: eduardofurtadoflores@gmail.com. Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Brazil, Rio Grande do Sul, Santa Maria, Camobi, Av. Roraima, 1000, prédio 63A.

ABSTRACT

Papillomaviruses (PVs) – the agents of papillomas or warts - have been identified in single and mixed infections in several animal species, including dogs (Canine papillomaviruses, CPV) and

cattle (Bovine papillomaviruses, BPV). Although considered species-specific, some BPVs may occasionally infect species other than cattle, such as sheep, goats, buffaloes, yaks and domestic cats. Herein, we carried out a retrospective phylogenetic study of PVs circulating in dogs in Southern Brazil between 2017 and 2022. In addition to contributing to epidemiological data, the samples were investigated for the presence of different PVs, as well as for mixed infections. For this, we screened 64 canine papilloma samples by PCR using the degenerate primers FAP59/64 and/or MY09/11, which amplify different regions of the L1 gene; the genomic target often used for PV classification/typing. Out these, 26 PV DNA samples were successfully amplified and sequenced. All PVs amplified by FAP59/64 (n = 22) were phylogenetically classified as Canine papillomavirus 1, CPV-1. On the other hand, PVs amplified by MY09/11 (n = 4) were classified as BPV-1. Among these, three samples showed mixed infection by CPV-1 and BPV-1. The phylogenetic classifications performed herein were supported by a careful *in silico* analysis that demonstrated that the classification based on the FAP59/64 and/or MY09/11 amplicons fully matches the classification obtained by analyzing the complete L1 gene. Overall, we describe the circulation of CPV-1 in Southern Brazil over the years, and raise the possibility of cross-infection of dogs by BPVs, and coinfections by CPV-1 and BPV-1 as well. Finally, we suggest the analysis of the complete genome of the putative BPVs detected in dogs, in order to deepen the knowledge about the PV-host interactions.

Keywords: Canine papillomavirus; Bovine papillomavirus; Deltapapillomavirus; Mixed infection; FAP59/64; MY09/11.

RESUMO

Os papilomavírus (PVs) podem ser encontrados em infecções simples ou mistas em diferentes espécies de animais, incluindo cães (CPV) e bovinos (BPV). Embora considerados espécie-específicos, alguns BPVs podem infectar outras espécies além dos bovinos, como ovelhas, cabras, búfalos, iaques e gatos. Nesse contexto, realizou-se um estudo filogenético retrospectivo de PVs circulantes em cães no sul do Brasil entre 2017 e 2022. Além de contribuir com dados epidemiológicos, as amostras foram investigadas quanto à presença de diferentes PVs, bem como para infecções mistas. Para isso, triamos 64 amostras de papiloma canino por PCR usando os *primers* degenerados FAP59/64 e/ou MY09/11, que amplificam diferentes regiões do gene L1; o alvo genômico frequentemente usado para classificação/tipagem de PVs. Dessas, 26 amostras de DNA de PV foram amplificadas e sequenciadas com sucesso. Todos os PVs amplificados por FAP59/64 (n = 22) foram filogeneticamente classificados como papilomavírus canino 1, CPV-1. Por outro lado, PVs amplificados por MY09/11 (n = 4) foram classificados como BPV-1. Entre esses, três foram encontrados em infecção mista com CPV-1. As classificações filogenéticas realizadas no estudo foram respaldadas por uma cuidadosa análise *in silico* que demonstrou que a classificação baseada nos amplicons FAP59/64 e/ou MY09/11 corresponde à classificação obtida pela análise do gene L1 completo. Em conclusão, descreveu-se a circulação do CPV-1 no sul do Brasil ao longo dos anos e levantou-se a possibilidade de infecção de BPV em cães e também a potencial coinfecção por CPV-1 e BPV-1. Finalmente, sugere-se que os potenciais BPVs detectados em cães tenham seu genoma completo analisado a fim de se aprofundar o conhecimento sobre a interação PV-hospedeiro.

Palavras-chave: Papilomavírus canino; Papilomavírus bovino; Deltapapilomavírus; Infecção mista; FAP59/64; MEU09/11.

INTRODUCTION

Papillomas are overgrowths of epithelial and/or mucosal tissue associated with papillomavirus (PV) infection (RECTOR; RANST, VAN, 2013). Papilloma lesions have been described in several animal species, including mammals, birds, fishes and reptiles (BOCANETI et al., 2016; RECTOR; RANST, VAN, 2013). Although PVs are considered species-specific, some studies have reported bovine papillomaviruses (BPVs), specifically the *Deltapapillomavirus* genus (BPV-1, BPV-2, BPV-13 and BPV-14), infecting other species than cattle, such as sheep, goats, buffaloes, yaks and cats (BAM et al., 2013; CUTARELLI et al., 2021; MUNDAY, et al., 2015; PANGTY et al., 2010; ROPERTO, S. et al., 2018).

Papillomas are also frequently reported in dogs, potentially compromising animal welfare and triggering outbreaks in breeders and daycare centers (GIL DA COSTA et al., 2017; LANE; WEESE; STULL, 2017). Canine papillomaviruses (CPVs) are small, non-enveloped, circular double-stranded DNA viruses with genomes about 8 kilobases (kb) in length, which encode five to six early (E) and two late (L) proteins (ICTV, 2018; PAPILLOMAVIRUS VIRUS EPISTEME, 2022). The E1 and E2 proteins modulate the translation and replication of the viral genome, whereas E5, E6 and E7 are oncoproteins and participate in cell transformation (DOORBAR, 2005). The late proteins, L1 and L2, form the viral capsid, with L1 being the main structural protein and the target for neutralizing antibodies (DOORBAR, 2005). Furthermore, the L1 gene is frequently used for phylogenetic classification/typing of PVs (BERNARD, et al., 2010; VILLIERS, et al., 2004).

CPVs belong to the family *Papillomaviridae*, subfamily *Firstpapillomavirinae*. CPVs are classified into three genera: *Chipapillomavirus*, *Lambdapapillomavirus* and *Taupapillomavirus* (ICTV; PAVE). To date, 23 CPV types are recognized by the International Committee on Taxonomy of Viruses (ICTV) and PapillomaVirus Episteme (PaVE), although an additional viral type has been reported recently, CPV-24 (MUNDAY, John S. et al., 2022).

Interestingly, some studies have described an association between some CPV types and the anatomical distribution and histological characteristics of the papillomas (LANGE; FAVROT, 2011; MUNDAY, THOMSON, LUFF, 2017). In addition, some authors have also reported mixed infections in canine papillomas, such as CPV2-CPV7-CPV19 and CPV1-CPV2 co-infections, which were identified by next-generation sequencing or amplification (PCR) by type-specific primers (LANGE, et al., 2019; TISZA et al., 2016).

Considering the above, we carried out a retrospective phylogenetic study of PVs circulating in dogs in Southern Brazil between 2017 and 2022. In addition to contributing to epidemiological data in Brazil, the samples were investigated for the presence of different PVs and for mixed infections as well. Overall, our findings indicate the possibility of BPV infection in dogs and open novel ways for studies on the dynamics of PV infection.

MATERIALS AND METHODS

Samples and study design

The study was carried out with canine papillomas received by the Virology Section (*Universidade Federal de Santa Maria*, UFSM) and Virology Laboratory (*Universidade Federal do Pampa*, UNIPAMPA) for the production of autogenous vaccines; and by the Pathology Department of the University Veterinary Hospital (UFSM) and Veterinary Pathology Section (*Universidade Federal do Rio Grande do Sul*, UFRGS) for histopathological diagnosis.

In total, 64 samples were screened, 32 of which were fresh (frozen at -20ºC) and 32 samples were formaldehyde-fixed and paraffin-embedded (FFPE). The samples were received by the laboratories between 2017 and 2022. Data on place and year of collection, as well as the conditions of the samples (fresh or FFPE) are presented in Supplementary File 1 (File S1). All samples were analyzed for the presence of PV DNA using degenerate primers that allow the

detection of viruses from different host species. Details on virus identification and further analysis are described below.

DNA extraction

DNA extraction from frozen samples was performed by the phenol chloroform method. For this, about 100 mg of each papilloma sample was cut into small fragments. The papilloma fragments were placed in a microtube and 800 µL of Tris-EDTA (TE) buffer, 100 µL of 10% sodium dodecyl sulfate (SDS) and 2 µL of RNase A (10 mg/mL) (Invitrogen, CA, USA) were added and incubated at 56°C for 30 min. Subsequently, 25 µL of proteinase K (20 mg/mL) (Ludwig Biotecnologia, RS, Brazil) were added and incubated for 1 h at 56ºC. Then, DNA was extracted by phenol-chloroform treatment, eluted in 50 µL of TE and stored at -20°C until use. FFPE samples were extracted using the QIAamp DNA FFPE Tissue Kit (QIAGEN®), according to the manufacturer's recommendations.

Partial amplification of L1gene

The extracted DNA was subjected to PCR for amplification of two regions of the L1 gene: the first region was amplified with primers FAP59 (5'-TAACWGTIGGICAYCCWTATT-3') and FAP64 (5'-CCWATATCWVHCATITCICCATC-3') (FORSLUND et al., 1999); in the second, primers MY09 (5'-GCMCAGGGWCATAAYAATGG-3') and MY11 (5'-CGTCCMARRGGAWACTGATC-3') were used (OGAWA et al., 2004). PCR was performed with 1.5 mM of $MgCl_2$, 0.5 µM of each deoxynucleotide, 1.25 µM of each primer, 2 U of recombinant Taq DNA polymerase (Thermo Fisher Scientific, MA, USA), approximately 100 ng of template and ultrapure q.s.p. water to a final volume of 25 µL. Cycling conditions were: initial denaturation at 94°C for 10 min, followed by 40 cycles of 94°C for 50 s (denaturation), 50°C or 56°C for 55 s (annealing for

primers FAP59/64 and MY09/11, respectively), 72°C for 50 s or 30 s (extension for FAP59/64 and MY09/11, respectively), final extension at 72°C for 7 min. The amplicons were submitted to 1.5% agarose gel electrophoresis and stained with GelRed (Biotium, CA, USA).

Nucleotide (nt) sequencing

PCR products were purified using the PureLink PCR Purification kit (Invitrogen, CA, USA), according to the manufacturer's recommendations. Sequencing was performed in duplicate, with forward and reverse primers, by ACTGene Análises Moleculares (RS, Brazil) by the Sanger method, using a BigDye™ Terminator v3.1 Cycle Sequencing kit (ThermoFisher Scientific, MA, USA).

Phylogenetic analyses

After nt sequencing, consensus sequences were obtained using Staden Package (Staden, 1996) and aligned by the multiple ClustalW method (THOMPSON; HIGGINS; GIBSON, 1994), BioEdit Alignment Editor program (version 7.0.5.3) (HALL, 1999). The phylogenetic analyses were performed using the Molecular Evolutionary Genetics Analysis software (version 10.2.4) (MEGA X) (KUMAR, S. et al., 2018) and the evolutionary history was inferred by the Maximum Likelihood method, with 1,000 bootstrap replicates. The best analysis model was defined by the jModelTest software (POSADA, 2008) (Table 1).

Consensus that did not cluster in any CPV cluster were submitted unfiltered to the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) to identify sequences with the highest similarity.

In silico analyses

The classification reliability of the CPVs analyzed here was evaluated by comparing the phylogenetic classification of the complete CPV L1 sequences available in GenBank *vs*. the classification obtained by analyzing the putative amplicons from the primers FAP59/64 and MY09/11, as well as by the region analyzed in our study. Details on sequence collection and analysis are described below.

Data collection

Complete CPV L1 sequences were collected using the search terms: "Canine papillomaviruses complete genome", "Canine Papillomaviruses L1 complete gene" and "Canine Papillomaviruses L1 gene". Non-canine host CPV sequences and partial L1 sequences were excluded from the analysis. Sequences were collected between November 23, 2022, and January 9, 2023.

Assessing the agreement between complete L1-based phylogenetic classification versus amplicons from FAP59/64 and MY09/11

We compared the classification of complete CPV L1 sequences with that based on the full amplicons by the primers FAP59/64 and MY09/11 (removing the annealing site of the primers) (termed complete FAP59/64 or MY09/11) and with the region of the amplicons analyzed in our study (named partial FAP59/64 or MY09/11). The positions of the L1 regions according to sequence D55633 (GenBank) are: a) complete L1 gene (nt 6837-8348); b) complete FAP59/64 (nt 6976-7477) and MY09/11 (nt 7821-8278); c) partial FAP59/64 (nt 6978-7362) and MY09/11 (nt 7943-8229). The models and parameters used in the analyses are described in Table 1. Furthermore, we calculated the weighted geodesic distance between the

complete L1 trees and FAP59/64 (or MY09/11), using the TreeCmp software (version 2.0) (https://eti.pg.edu.pl/TreeCmp).

RESULTS

Samples

Out of 64 canine papilloma DNA samples subjected to PCR, 26 samples were amplified by the primers FAP59/64 and/or MY09/11. Among these, only 23 samples had a consensus suitable for phylogenetic analysis: 389 nt for FAP59/64 and 290 nt for MY09/11. In details, 19 were amplified only by FAP59/64, one by MY09/11 and three were amplified by both primers. All PV sequences analyzed here were from fresh frozen samples, *i.e.*, it was not possible to amplify DNA from FFPE samples.

Phylogenetic classification

All sequences analyzed for FAP59/64 were classified as CPV-1, and none of the sequences analyzed for MY09/11 were clustered with CPV (Figure 1 and Table 2). Therefore, we submitted the MY09/11 consensus to the BLAST tool. Herein, all MY09/11 sequences showed a high degree of similarity with the *Deltapapilomavirus* genus. The SV148/19 consensus had a percentage identity of 100% with the MG242142 sequence, while SV405/18 and SV102/19 had 99.6% with this same sequence. Consensus SV59/19 had 100% percent identity with the KX271663 sequence. Considering these results, the deltapapilomavirus sequences were included in the phylogenetic analysis of the MY09/11 sequences, allowing their identification as BPV-1 (Figure 2).

In silico analysis: phylogenetic classification of CPV by complete L1 vs. FAP59/64 and MY09/11 amplicons

Forty-six complete CPV L1 sequences were obtained from the GenBank database, which were classified as CPV-1 (5 sequences), CPV-2 (4), CPV-3 (2), CPV-4 (2), CPV-5 (2), CPV-6 (1), CPV-7 (1), CPV-8 (2), CPV-9 (4), CPV-10 (2), CPV-11 (1), CPV-12 (2), CPV-13 (2), CPV-14 (2), CPV-15 (1), CPV-16 (3), CPV-17 (1), CPV-18 (1), CPV-19 (1) , CPV-20 (1), CPV-21 (2), CPV-22 (2), CPV-23 (1) and CPV-24 (1). Classifications based on complete L1 sequences were reproduced by the phylogenetic analysis with complete and partial FAP59/64 or MY09/11 (File S2). The geodesic distance between L1 *vs*. complete FAP59/64 and L1 *vs*. partial FAP59/64 trees was 0.4686 and 0.4411, respectively; the value between L1 *vs*. complete MY09/11 and L1 *vs*. partial MY09/11 trees was 0.4366 and 0.5756, respectively. Overall, these findings support the use of the primers FAP59/64 or MY09/11 for CPV genetic classification.

DISCUSSION

Twenty-four CPV subtypes have been described worldwide (ICTV, 2018; MUNDAY, JOHN et al., 2022; PAPILLOMAVIRUS VIRUS EPISTEME, 2022), some of which have been identified in mixed infections (LANGE, et al., 2019; TISZA et al., 2016). In Brazil, CPV genotyping studies are rare and few CPV types have been described to date, specifically CPV-1 and CPV-16 (ALCÂNTARA, DE et al., 2014; ALVES et al., 2020; REIS et al., 2019). In order to contribute to PV epidemiological data in Brazil, we performed a retrospective study with papilloma samples obtained from dogs in Southern Brazil between 2017 and 2022. In addition, we also provide insights into the dynamics of PV infection, describing the possibility of BPV infection of dogs and potential CPV and BPV co-infection.

Initially, we screened DNA obtained from 64 canine papillomas using the degenerate primers FAP59/64 and MY09/11. Of these, 38 DNA samples were not amplified by any of the

primer sets. Among the non-amplified DNA samples, six and 32 were fresh frozen and FFPE tissues, respectively. Failures in the amplification of PV DNA from fresh frozen samples have also been described by other authors (MERCHIORATTO et al., 2023; SILVA, et al., 2013) and may be related to PCR sensitivity, viral load and/or sample quality. Regarding DNA amplification problems from FFPE samples, our results are also in line with what was observed in other PV studies, in which the detection rate of PV DNA from fresh frozen tissue was higher than that found in FFPE samples (BAAY et al., 1996; BEN-EZRA et al., 1991; THOMPSON; ROSE, 1991). Failure in FFPE amplification may be related to the formalin fixation step, which may cause damage in the DNA molecule, preventing PCR amplification (BEN-EZRA et al., 1991; KARLSEN et al., 1994). In this sense, the influence of formalin fixation on PCR may be related to fixation time and amplicon size. Kalsen et al., (1994) demonstrated that eight hours of tissue fixation may be sufficient to inhibit the generation of an amplicon >421bp. This is an important issue since our amplicons were 475 bp (FAP59/64) and 452bp (MY09/11) bp.

All samples amplified by the primers FAP59/64 were classified as CPV-1. This finding is in line with previous studies carried out in different regions from Brazil. Alcantara et al. (2014) detected CPV-1 infection in seven samples from Paraná state, Southern Brazil. Similarly, Reis et al. (2020) identified CPV-1 in six samples from Sergipe state, Northeastern Brazil. Importantly, in both studies, the authors used the primers FAP59/64. These findings must be carefully interpreted, as reactions with FAP59/64 or MY09/11 may be more sensitive to some PV types (SILVA, et al., 2013), so that the higher prevalence of CPV-1 may be related to the coverage of the primers used. A different finding was reported by Alves et al. (2020), who detected CPV-16 in a papilloma sample collected in southern Brazil. Herein, although the authors screened the sample with FAP59/64, virus classification was performed by analyzing the complete viral genome obtained by multiply-primed rolling-circle amplification followed

by high throughput sequencing. Taken together, these findings may be useful for future studies on the CPV epidemiology.

Interestingly, all sequences amplified and sequenced by MY09/11 were evolutionarily distant from CPV sequences in the phylogenetic analysis. By analyzing these sequences in the BLAST tool, we identified a high similarity with BPV (*Deltapapillomavivrus* genus). Therefore, we performed another phylogenetic analysis including deltapapillomavirus reference sequences. Herein, all sequences obtained by MY09/11 were clustered with BPV-1. However, as 3/4 of the samples sequenced by MY09/11 were also analyzed by FAP59/64, indicating CPV-1 infection, we investigated the hypothesis that the mismatch between MY09/11 and FAP59/64 results is a bias related to the analysis from different L1 regions.

To address the above issue, we collected CPV full-length L1 sequences from GenBank and compare the phylogenetic classification based on complete L1 *vs*. complete FAP59/64 (or MY09/11). In addition, we also compared the complete L1 classification *vs*. that based on the regions analyzed in our study (partial FAP59/64 or MY09/11). All classifications obtained by the complete L1 analysis were maintained by the FAP59/64 or MY09/11 analyses. In addition, the geodesic distance between L1 *vs*. complete and partial FAP59/64 (or MY09/11) trees were considerably similar. This metric compares the topology and stem length between two trees and has been successfully used to compare phylogenetic trees from different genomic targets (BECKER et al., 2023; MERCHIORATTO et al., 2023; DE OLIVEIRA, et al., 2021, 2022). Overall, these results indicate that the classification by FAP59/64 and MY09/11 may reproduce the classification from complete L1, reinforcing that the sequences detected by MY09/11 are indeed BPV-1 sequences. Consequently, these results also support the possibility of CPV-1 and BPV-1 co-infection in three canine samples analyzed in our study.

Although papillomaviruses are species-specific, some studies have described that BPVs from the *Deltapapillomavirus* genus (BPV-1, BPV-2, BPV-13 and BPV-14) may infect different

animals, such as horses, sheep, goats, buffaloes, yaks and cats (BAM et al., 2013; CUTARELLI et al., 2021; MUNDAY, JOHN et al., 2015; PANGTY et al., 2010; ROPERTO, et al., 2018). Despite this, to the best of our knowledge, BPV infection in dogs has not yet been reported. We believe that our finding was made possible by the use of PV degenerate primers, which have been useful for detecting viruses from different host species, including BPV-1 (DAGALP et al., 2017; MERCHIORATTO et al., 2023; MUNDAY, JOHN et al., 2015; SILVA, et al., 2010).

CONCLUSION

We carried out a retrospective phylogenetic study of PVs circulating in dogs in Southern Brazil using the degenerate primers FAP59/64 and MY09/11. Herein, we: i) confirm the circulation of CPV-1 in Brazil over the years; ii) demonstrate the suitability of primers FAP59/64 and MY09/11 for CPV phylogenetic classification; and iii) raise the possibility of BPV infection in dogs, as well as CPV and BPV co-infections. Regarding this issue, it would be timely to investigate possible canine infection by other deltapapillomaviruses, as well as to sequence and analyze the complete genome of potential BPVs detected in dogs in order to deepen the knowledge about PV-host interactions.

ACKNOWLEDGEMENTS

CAPES (IM, CIM) and CNPq (IJR, PSBO) funding agencies for scholarships. E.F. Flores and R. Weiblen are CNPq scholarship holders. The research was financed in part by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil - Finance code 001."

DECLARATION OF CONFLIC OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

REFERENCES

ALCÂNTARA, B. K. DE et al. Identification of canine papillomavirus type 1 (CPV1) DNA in dogs with cutaneous papillomatosis. **Pesquisa Veterinária Brasileira**, 2014. v. 34, n. 12, p. 1223–1226. Disponível em: <http://www.scielo.br/j/pvb/a/PJmKWwtM7hcSY6Y8FWd3rPw/?lang=en>. Acesso em: 7 nov. 2022.

ALVES, C. D. B. T. et al. Canine papillomavirus type 16 associated to squamous cell carcinoma in a dog: virological and pathological findings. **Brazilian Journal of Microbiology**, 4 dez. 2020. v. 51, n. 4, p. 2087–2094. Disponível em: <https://link.springer.com/10.1007/s42770-020-00310-4>. Acesso em: 7 nov. 2022.

BAAY, M. F. D. et al. Comprehensive study of several general and type-specific primer pairs for detection of human papillomavirus DNA by PCR in paraffin-embedded cervical carcinomas. **Journal of Clinical Microbiology**, 1996. v. 34, n. 3, p. 745. Disponível em: </pmc/articles/PMC228883/?report=abstract>. Acesso em: 4 fev. 2023.

BAM, J. et al. Spontaneous cutaneous papillomatosis in yaks and detection and quantification of bovine papillomavirus-1 and -2. **Transboundary and emerging diseases**, out. 2013. v. 60, n. 5, p. 475–480. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/23206264/>. Acesso em: 29 jan. 2023.

BECKER, A. S. et al. An appraisal of gene targets for phylogenetic classification of canine

distemper virus: Is the hemagglutinin the best candidate? **Virus Research**, 1 fev. 2023. v. 325, p. 199043. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/S0168170223000059>. Acesso em: 31 jan. 2023.

BEN-EZRA, J. et al. Effect of fixation on the amplification of nucleic acids from paraffin-embedded material by the polymerase chain reaction. **The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society**, 1991. v. 39, n. 3, p. 351–354. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/1704393/>. Acesso em: 4 fev. 2023.

BERNARD, H.-U. et al. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. **Virology**, maio. 2010. v. 401, n. 1, p. 70–79. Disponível em: </pmc/articles/PMC3400342/>. Acesso em: 5 ago. 2021.

BOCANETI, F. et al. Bovine Papillomavirus: New Insights into an Old Disease. **Transboundary and Emerging Diseases**, 1 fev. 2016. v. 63, n. 1, p. 14–23.

CUTARELLI, A. et al. The diagnostic value of the droplet digital PCR for the detection of bovine deltapapillomavirus in goats by liquid biopsy. **Transboundary and emerging diseases**, 1 nov. 2021. v. 68, n. 6, p. 3624–3630. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/33386672/>. Acesso em: 29 jan. 2023.

DAGALP, S. B. et al. The genetic diversity of bovine papillomaviruses (BPV) from different papillomatosis cases in dairy cows in Turkey. **Archives of Virology**, 11 jun. 2017. v. 162, n. 6, p. 1507–1518. Disponível em: <https://link.springer.com/article/10.1007/s00705-017-3258-8>. Acesso em: 19 jan. 2021.

DOORBAR, J. The papillomavirus life cycle. **Journal of Clinical Virology**, 1 mar. 2005. v. 32, n. SUPPL., p. 7–15. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/S1386653204003671>. Acesso em: 11 out. 2022.

FORSLUND, O. et al. A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. **Journal of General**

Virology, 1 set. 1999. v. 80, n. 9, p. 2437–2443. Disponível em: <https://www.microbiologyresearch.org/content/journal/jgv/10.1099/0022-1317-80-9-2437>. Acesso em: 23 jan. 2022.

GIL DA COSTA, R. M. et al. An Update on Canine, Feline and Bovine Papillomaviruses. **Transboundary and Emerging Diseases**, 1 out. 2017. v. 64, n. 5, p. 1371–1379. Disponível em: <https://onlinelibrary.wiley.com/doi/full/10.1111/tbed.12555>. Acesso em: 30 jul. 2021.

HALL, T. A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. **Nucleic Acids Symposium Series**, 1999. v. 41, p. 95–98. Disponível em: <http://brownlab.mbio.ncsu.edu/jwb/papers/1999hall1.pdf>. Acesso em: 11 fev. 2019.

ICTV. Papillomaviridae. **International committee on the taxonomy of Viruses**, [S.l.], 2018. Disponível em: <https://talk.ictvonline.org/ictv-reports/ictv_online_report/dsdna-viruses/w/papillomaviridae>.

KARLSEN, F. et al. Modifications of human and viral deoxyribonucleic acid by formaldehyde fixation - PubMed. **Lab Invest**, 1994. v. 71, n. 4, p. 604–611. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/7967515/>. Acesso em: 6 fev. 2023.

KUMAR, S. et al. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. **Molecular Biology and Evolution**, 1 jun. 2018. v. 35, n. 6, p. 1547–1549. Disponível em: <https://academic.oup.com/mbe/article/35/6/1547/4990887>.

LANE, H. E.; WEESE, J. S.; STULL, J. W. Canine oral papillomavirus outbreak at a dog daycare facility. **The Canadian Veterinary Journal**, 1 jul. 2017. v. 58, n. 7, p. 747. Disponível em: </pmc/articles/PMC5479657/>. Acesso em: 1º set. 2022.

LANGE, C. E. et al. Canine papillomavirus types 1 and 2 in classical papillomas: High abundance, different morphological associations and frequent co-infections. **Veterinary journal (London, England: 1997)**, 1 ago. 2019. v. 250, p. 1–5. Disponível em:

<https://pubmed.ncbi.nlm.nih.gov/31383413/>. Acesso em: 18 jan. 2023.

LANGE, Christian E.; FAVROT, C. Canine Papillomaviruses. **Veterinary Clinics of North America: Small Animal Practice**, 1 nov. 2011. v. 41, n. 6, p. 1183–1195.

MERCHIORATTO, I. et al. Phylogeny and amino acid analysis in single and mixed bovine papillomavirus infections in Southern Brazil, 2016-2020. **Archives of Virology**, 7 fev. 2023. v. 168, n. 2, p. 52. Disponível em: <https://doi.org/10.1007/s00705-022-05622-4>. Acesso em: 9 jan. 2023.

MUNDAY, John S. et al. Genomic characterisation of the feline sarcoid-associated papillomavirus and proposed classification as Bos taurus papillomavirus type 14. **Veterinary Microbiology**, 12 jun. 2015. v. 177, n. 3–4, p. 289–295.

MUNDAY et al. Genomic Characterisation of Canis Familiaris Papillomavirus Type 24, a Novel Papillomavirus Associated with Extensive Pigmented Plaque Formation in a Pug Dog. **Viruses**, 26 out. 2022. v. 14, n. 11, p. 2357. Disponível em: <https://www.mdpi.com/1999-4915/14/11/2357/htm>. Acesso em: 16 nov. 2022.

MUNDAY ; THOMSON, N. A.; LUFF, J. A. Papillomaviruses in dogs and cats. **The Veterinary Journal**, 1 jul. 2017. v. 225, p. 23–31.

OGAWA, T. et al. Broad-spectrum detection of papillomaviruses in bovine teat papillomas and healthy teat skin. **Journal of General Virology**, 1 ago. 2004. v. 85, n. 8, p. 2191–2197. Disponível em: <https://www.microbiologyresearch.org/content/journal/jgv/10.1099/vir.0.80086-0>. Acesso em: 26 out. 2022.

OLIVEIRA, P. S. B. et al. Subtyping bovine viral diarrhea virus (BVDV): Which viral gene to choose? **Infection, Genetics and Evolution**, 1 ago. 2021. v. 92, p. 104891. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/S156713482100188X>. Acesso em: 29 ago. 2022.

OLIVEIRA, P. S. B. et al. A new (old) bovine viral diarrhea virus 2 subtype: BVDV-2e.

Archives of Virology, 1 dez. 2022. v. 167, n. 12, p. 2545–2553. Disponível em: <https://link.springer.com/article/10.1007/s00705-022-05565-w>. Acesso em: 31 jan. 2023.

PANGTY, K. et al. Detection of BPV-1 and -2 and Quantification of BPV-1 by Real-Time PCR in Cutaneous Warts in Cattle and Buffaloes. **Transboundary and Emerging Diseases**, 25 jan. 2010. v. 57, n. 3, p. 185–196. Disponível em: <https://onlinelibrary.wiley.com/doi/full/10.1111/j.1865-1682.2009.01096.x>. Acesso em: 23 jan. 2022.

PAPILLOMAVIRUS VIRUS EPISTEME. Reference genomes for Animal papillomavirus., 2022. Disponível em: <https://pave.niaid.nih.gov/explore/reference_genomes/animal_genomes>. Acesso em: 6 fev. 2023.

POSADA, D. jModelTest: Phylogenetic Model Averaging. **Molecular Biology and Evolution**, abr. 2008. v. 25, n. 7, p. 1253–1256.

RECTOR, A.; RANST, M. VAN. Animal papillomaviruses. **Virology**, 1 out. 2013. v. 445, n. 1–2, p. 213–223. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/S0042682213002663>. Acesso em: 18 maio 2021.

REIS, J. D. R. et al. Molecular characterization of Canis familiaris oral papillomavirus 1 identified in naturally infected dogs from Northeast Brazil. **Veterinary Dermatology**, 1 out. 2019. v. 30, n. 5, p. 424-e128. Disponível em: <https://onlinelibrary.wiley.com/doi/full/10.1111/vde.12776>. Acesso em: 19 jul. 2021.

ROPERTO, S. et al. Detection of bovine Deltapapillomavirus DNA in peripheral blood of healthy sheep (Ovis aries). **Transboundary and emerging diseases**, 1 jun. 2018. v. 65, n. 3, p. 758–764. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/29330926/>. Acesso em: 29 jan. 2023.

SILVA, M. A. R. et al. Comparison of two PCR strategies for the detection of bovine papillomavirus. **Journal of Virological Methods**, 1 set. 2013. v. 192, n. 1–2, p. 55–58. Disponível em: <https://linkinghub.elsevier.com/retrieve/pii/S0166093413001638>. Acesso em: 23 jan. 2022.

SILVA, M. S. E. et al. Molecular identification of bovine papillomaviruses associated with Cutaneous Warts in Southern Brazil. **Journal of Veterinary Diagnostic Investigation**, 1 jul. 2010. v. 22, n. 4, p. 603–606. Disponível em: <https://journals.sagepub.com/doi/10.1177/104063871002200417?url_ver=Z39.88-2003&rfr_id=ori%3Arid%3Acrossref.org&rfr_dat=cr_pub++0pubmed>. Acesso em: 23 jan. 2022.

STADEN, R. The staden sequence analysis package. **Molecular Biotechnology**, jun. 1996. v. 5, n. 3, p. 233–241. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/8837029/>. Acesso em: 4 ago. 2021.

THOMPSON, C. H.; ROSE, B. R. Deleterious effects of formalin/acetic acid/alcohol (FAA) fixation on the detection of HPV DNA by in situ hybridization and the polymerase chain reaction. **Pathology**, 1 jan. 1991. v. 23, n. 4, p. 327–330.

THOMPSON, J. D.; HIGGINS, D. G.; GIBSON, T. J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. **Nucleic Acids Research**, 11 nov. 1994. v. 22, n. 22, p. 4673. Disponível em: </pmc/articles/PMC308517/?report=abstract>. Acesso em: 9 ago. 2021.

TISZA, M. J. et al. Genomic Sequence of Canine Papillomavirus 19. **Genome Announcements**, 29 dez. 2016. v. 4, n. 6, p. 1380–1396. Disponível em: </pmc/articles/PMC5146455/>. Acesso em: 31 ago. 2022.

VILLIERS, E. M. DE et al. Classification of papillomaviruses. **Virology**, 20 jun. 2004. v. 324, n. 1, p. 17–27.

The phylogenetic analysis was performed based on the partial sequences of L1 obtained with

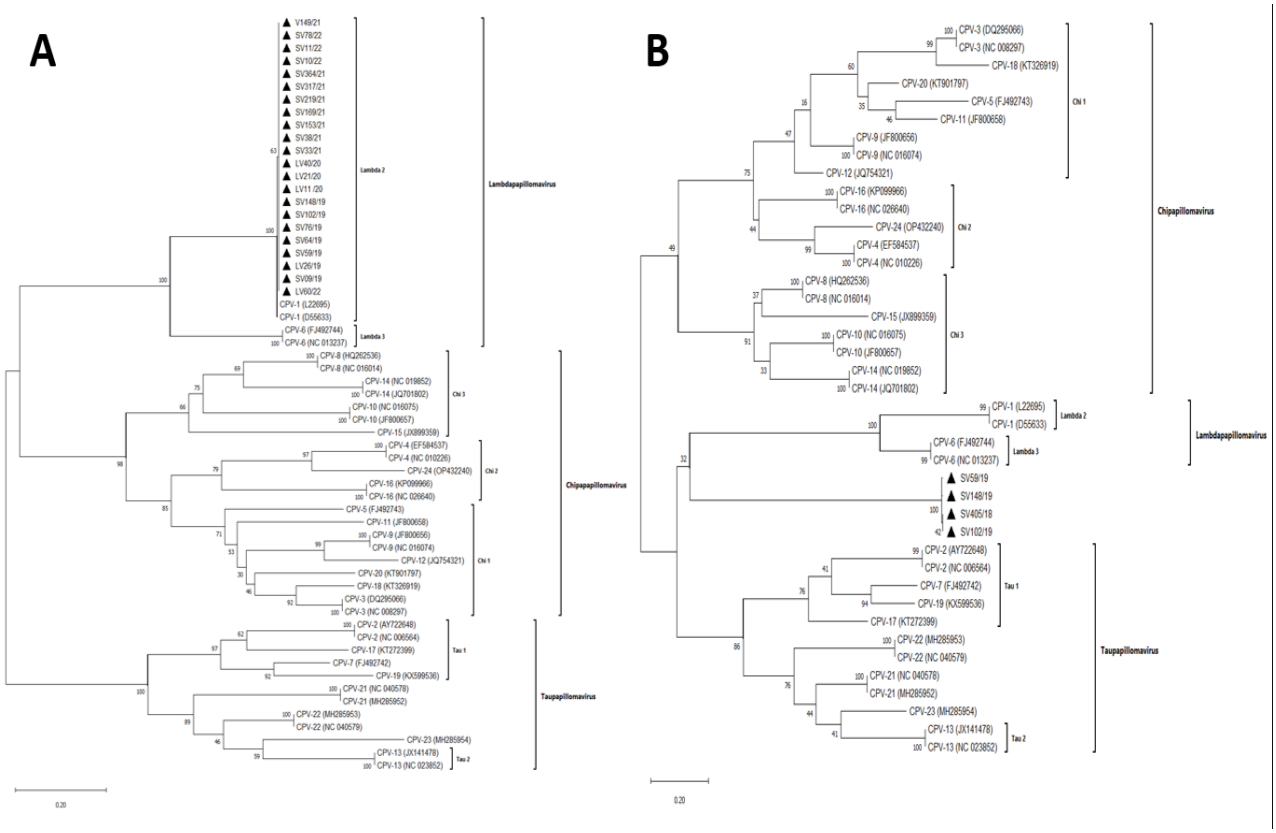


Figure 1. Phylogenetic trees of the PVs detected in the study.

the primer sets FAP59/64 (A) and MY09/11 (B). Analyses were performed using the Maximum Likelihood method, using the MEGA X software (version 10.2.4), (see Table 1). *Bootstrap* values were calculated based on 1000 replicates. The sequences indicated by the filled triangles correspond to those collected and analyzed in this study.

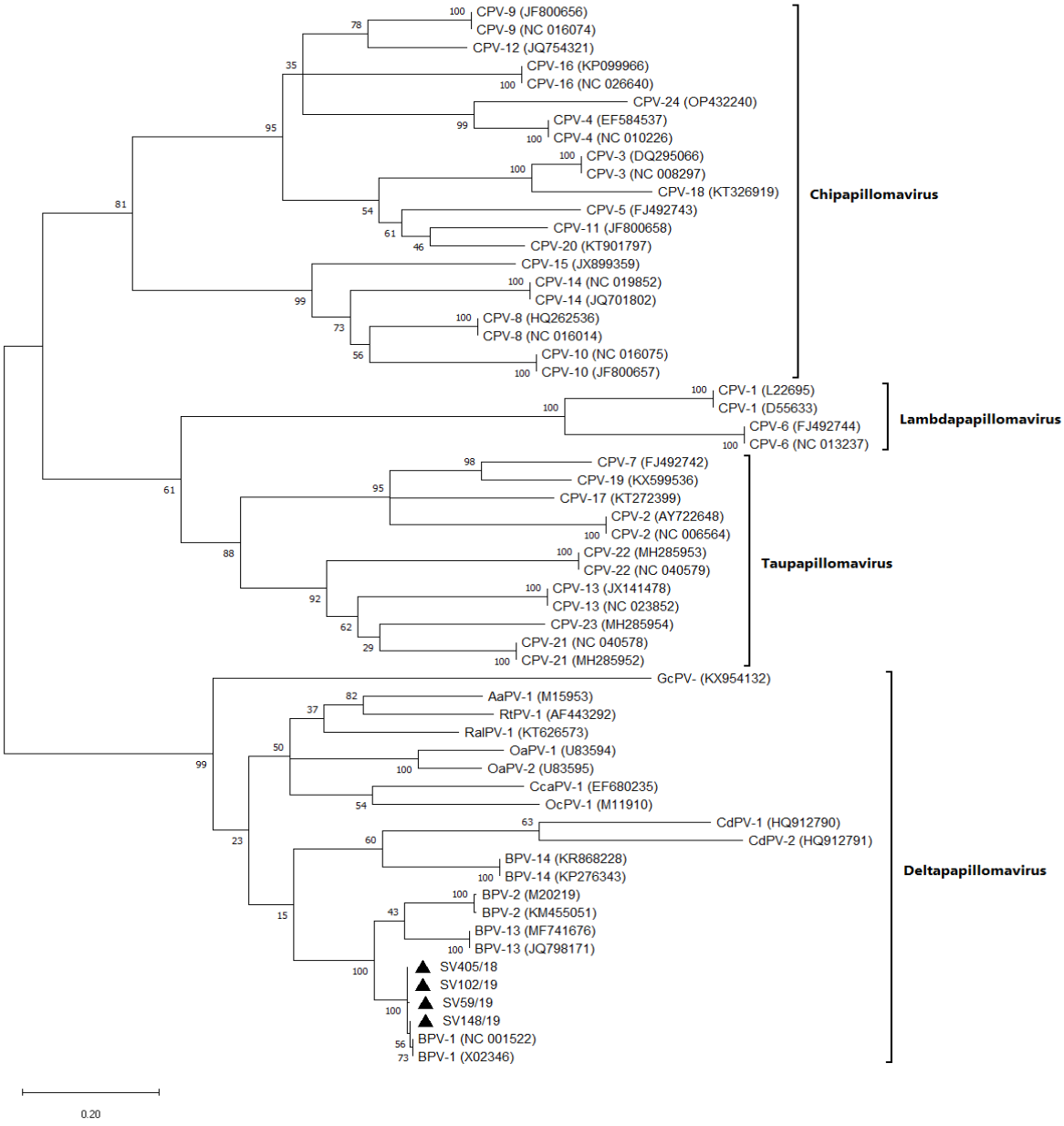
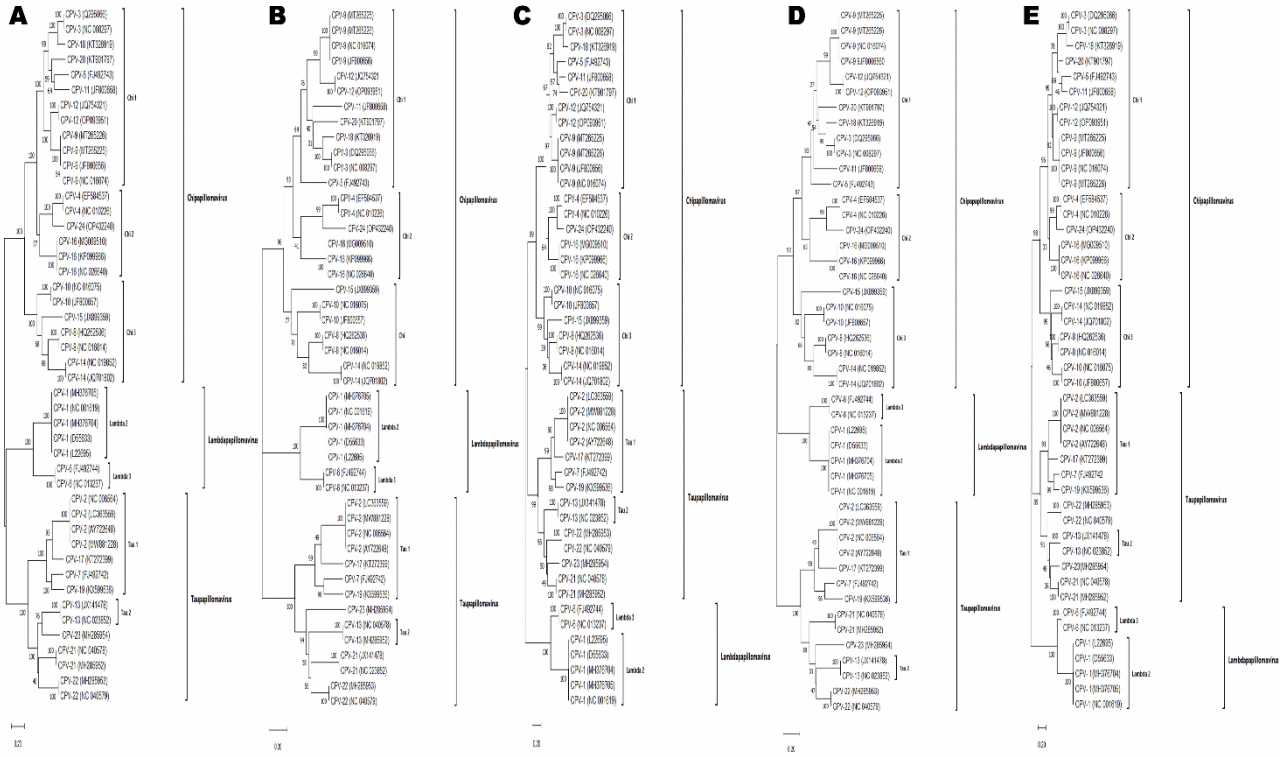


Figure 2. Phylogenetic trees of the PVs detected in the study, including CPV and deltapapilomavirus sequences.

Phylogenetic analysis was performed based on partial L1 sequences obtained with the primer set MY09/11. Analyses were performed using the Maximum Likelihood method, using the MEGA X software (version 10.2.4), (see Table 1). *Bootstrap* values were calculated based on 1000 replicates.



File S2. Phylogenetic trees based on CPV L1 sequences obtained from the GenBank database.

The phylogenetic analysis was performed based on complete L1 sequences (A), complete FAP59/64 (B) and MY09/11 (C), and partial FAP59/64 (D) and MY09/11 (E). Analyses were performed by the Maximum Likelihood method, using the MEGA X software (version 10.2.4) (see Table 1). *Bootstrap* values were calculated based on 1000 replicates. Branches supported by >85% of bootstrap replicas are indicated

.

Table 1. Parameters used in the Maximum Likelihood phylogeny of papillomavirus sequences.

Data	Genomic region	Substitution model[e]	Gamma shape parameter	Proportion invariant sites	BIC[f]	Log likelihood
GenBank	L1[a]	GTR+G+ I	1.2726	0.12	56,082.5983	-27,678.75
	FAP59/64 -C[b]	GTR+G+ I	1.4071	0.13	17,095.9985	-8,257.54
	MY09/11 -C[b]	HKY+G+I	1.3129	0.11	15,684.9322	-7,562.48
	FAP59/64 -P[c]	GTR+G+ I	1.2785	0.14	14,208.6943	-6,820.23
	MY09/11 -P[d]	HKY+G+I	1.2930	0.12	10,849.9751	-5,164.15
Study	FAP59/64[c]	GTR+G+ I	1.3153	0.13	14,411.7729	-6,841.55
	MY09/11[d]	HKY+G	1.2922	NA[g]	11,316.967	-5,427.27
	MY09/11 Delta	GTR+G+I	1.3508	0.1	16,309.1182	-7,827.68

[a]Complete L1 sequence;

[b]Region corresponding to that amplified with primers FAP59/64 or MY09/11;

[c]Region corresponding to nts 6985-7362 (accession number D55633), termed partial FAP59/64;

[d]Region corresponding to nts 7910-8222 (accession number D55633), termed partial MY09/11;

[e]GTR: general time reversible; G: gamma distribution; I: invariant sites; HKY: Hasegawa-Kishino-Yano;

[F]Bayesian information criterion;

[g]Not applicable

Table 2. Collection and classification data of CPVs identified in Rio Grande do Sul, Brazil, between 2017 and 2022.

City	Sample	Year of collection	Classification	
			FAP59/64	MY09/11
Porto Alegre	SV64/19	2019	CPV-1	NA
	SV76/19	2019	CPV-1	NA
	SV102/19	2019	CPV-1	BPV-1
	SV38/21	2021	CPV-1	NA
	SV169/21	2021	CPV-1	NA
	SV317/21	2021	CPV-1	NA
	SV364/21	2021	CPV-1	NA
	SV10/22	2022	CPV-1	NA
	SV11/22	2022	CPV-1	NA
São Gabriel	SV148/19	2019	CPV-1	BPV-1
Santa Cruz do Sul	SV405/18	2018	NA	BPV-1
Santa Maria	SV33/21	2021	CPV-1	NA
	SV153/21	2021	CPV-1	NA
	SV219/21	2021	CPV-1	NA
	V149.21	2021	CPV-1	NA
Santa Rosa	SV59/19	2019	CPV-1	BPV-1
Uruguaiana	LV09/19	2019	CPV-1	NA
	LV26/19	2019	CPV-1	NA
	LV11/20	2020	CPV-1	NA
	LV21/20	2020	CPV-1	NA
	LV40/20	2020	CPV-1	NA
	LV60/22	2022	CPV-1	NA
Venâncio Aires	SV78/22	2022	CPV-1	NA

[a]Not applicable. Consensus sequence not suitable for phylogenetic analysis.

5. DISCUSSÃO

O artigo 1 desta Tese descreve um estudo retrospectivo dos BPVs circulantes no estado do Rio Grande do Sul entre 2016 e 2020, realizando análises filogenéticas com base em sequências de nucleotídeos e de aminoácidos. Ao total, 43 amostras de papiloma bovino foram analisadas. O DNA dos BPVs foi amplificado e sequenciado usando dois pares de *primers* frequentemente descritos na literatura, FAP59/64 e MY09/11 (FORLUND et al., 1999; MANOS et al., 1989). O uso desses *primers* possibilitou a identificação de BPVs em infecções simples (BPV-1, 2 e 6) e em coinfecções (BPV-1 e 2).

A identificação de BPV-1, 2 e 6 está de acordo com estudos prévios também realizados na região Sul do Brasil (CLAUS et al., 2007, 2008; SILVA et al., 2010). A coinfecção por BPV-1 e 2 descrita no estudo também tem sido relatada por outros autores (CARVALHO et al., 2012; CLAUS et al., 2007, 2009; DAGALP et al., 2017). Interessantemente, todos os BPV-2 identificados aqui estavam em coinfecção com o BPV-1. Esse achado levanta a possibilidade que a presença de BPV-2 poderia facilitar a infecção por um outro tipo viral. Hipótese semelhante tem sido sugerida por Dagalp et al. (2017), ao observar a elevada frequência de BPV-2 em infecções mistas.

É importante destacar que, até então, as infecções mistas por BPV só tinham sido identificadas por meio de amplificação de círculo rolante (*rolling-circle amplification - RCA*) ou de clonagem gênica (SAUTHIER et al., 2021; DAUDT et al., 2016), estratégias que podem ser inviáveis a depender da quantidade de amostras analisadas. Dessa forma, o presente estudo demonstrou que é possível identificar a presença de diferentes tipos de BPV em uma mesma amostra clínica a partir do uso de *primers* degenerados. A identificação das infecções mistas foi respaldada por uma análise *in silico* que comprovou que a classificação de BPV por FAP59/64 ou MY09/11 reproduz os resultados obtidos pela análise do gene completo de L1; o principal alvo gênico para a classificação filogenética de PVs (DE VILLIERS et al., 2004). Esse resultado é importante porque descarta a possibilidade de que a classificação dos diferentes tipos de BPV seja um viés relacionado à análise de diferentes *amplicons*.

Os BPVs identificados no estudo também apresentaram aminoácidos que ainda não haviam sido descritos nos tipos virais homólogos. Grande parte dessas mudanças de aminoácido foi observada em regiões de L1 previamente identificadas como possíveis epítopos de HPV e/ou BPV para MHC-II e linfócitos B (NAMVAR et al., 2019). Além disso, foi observada uma mudança de resíduo de aminoácido em BPV-6 que só foi identificada em um vírus previamente descrito no sul do Brasil. Quando considerados em conjunto, esses resultados sugerem que os

BPVs circulantes nessa região provavelmente estão sob uma pressão de seleção relacionada à imunidade do hospedeiro, um achado interessante que pode contribuir para futuros estudos epidemiológicos e de estratégias vacinais.

O artigo 2 descreve um estudo filogenético de PVs detectados em cães do Rio Grande do Sul entre 2017 e 2022, a partir de 23 sequências analisadas filogeneticamente com o uso dos *primers* FAP59/64 e/ou MY09/11. Dessas, 19 amostras amplificaram somente para os *primers* FAP59/64 e foram classificadas como CPV-1, e uma amostra amplificou somente com os *primers* MY09/11 e foi classificada como BPV-1. Três amostras amplificaram para ambos os pares de *primers* e foram classificadas como CPV-1 (por FAP59/64) e BPV-1 (MY09/11), indicando, portanto, não somente a presença de infecção mista, mas levantando a possibilidade de infecções de cães por BPV.

A detecção de CPV-1 no Brasil já foi descrita anteriormente, demonstrando que os presentes achados corroboram a epidemiologia de CPV no país (DE ALCÂNTARA et al., 2014; REIS et al., 2019). No entanto, até o momento, nenhum estudo tinha sugerido a potencial infecção de cães por BPV-1, embora outros tipos de BPV do gênero *Deltapapillomavirus* já tenham sido identificados em outros animais além de bovinos, como equinos, ovelhas, cabras, búfalos, iaques e gato (DE ALCÂNTACARA et al., 2015; BAM et al., 2013; CUTARELLI et al., 2021; MUNDAY, et al., 2015; PANGTY et al., 2010; ROPERTO, S. et al., 2018). Semelhante ao discutido no estudo anterior, os resultados dessas análises são respaldados por uma avaliação *in silico* que demonstrou que a classificação de CPV por meio da análise dos *amplicons* de FAP59/64 ou MY09/11 podem reproduzir os resultados da análise da L1 completa.

Em relação ao desenho dos dois estudos, é válido ressaltar que as reações baseadas em *primers* degenerados podem ter sensibilidades diferentes a depender do tipo de PV investigado (SILVA et al., 2013). Assim, é possível que essa estratégia favoreça a amplificação de alguns tipos virais em detrimento de outros. Essa questão tem que ser considerada em relação à interpretação epidemiológica dos achados discutidos acima. Por outro lado, o uso de *primers* degenerados é uma alternativa simples e eficiente para a detecção de vários PVs, principalmente diante da diversidade desses vírus, possibilitando, inclusive, a identificação de infecções mistas. Além disso, o uso de *primers* que não são tipo-específicos pode contribuir para a identificação de infecções ainda não descritas ou que não costumam ser investigadas em alguns estudos.

6. CONCLUSÃO

Os estudos apresentados nesta Tese permitiram concluir que: i) BPV-1, 2 e 6 circularam em rebanhos bovinos no estado do Rio Grande do Sul entre os anos de 2016 e 2020; ii) BPVs detectados no Rio Grande do Sul apresentam resíduos únicos ou raros de aminoácido na proteína L1, os quais podem estar relacionados à evolução viral e interação com o hospedeiro; iii) CPV-1 circulou em cães do Rio Grande do Sul entre 2017 e 2022; iv) BPV-1 pode ser um potencial agente de infecção em cães; v) BPV e CPV podem ser classificados adequadamente pela análise filogenética dos *amplicons* de FAP59/64 ou MY09/11; e vi) os *primers* FAP59/64 ou MY09/11 podem ser uma ferramenta simples e acessível para a detecção de infecções mistas por PVs.

REFERÊNCIAS

ALVES, C. D. B. T. et al. Canine papillomavirus type 16 associated to squamous cell carcinoma in a dog: virological and pathological findings. **Brazilian Journal of Microbiology**, v. 51, n. 4, p. 2087–2094, 4 dez. 2020.

ARALDI, R. P. et al. Bovine papillomavirus in beef cattle: first description of BPV-12 and putative type BAPV-8 in Brazil. **Genetics and Molecular Research**, v. 13, n. 3, p. 5644–5653, 25 jul. 2014.

ARALDI, R. P. et al. Papillomaviruses: a systematic review. **Genetics and Molecular Biology**, v. 40, n. 1, p. 1, 1 jan. 2017.

BATISTA, M. V. A. et al. Molecular epidemiology of bovine papillomatosis and the identification of a putative new virus type in Brazilian cattle. **The Veterinary Journal**, v. 197, n. 2, p. 368–373, ago. 2013.

BAM, J. et al. Spontaneous Cutaneous Papillomatosis in Yaks and Detection and Quantification of Bovine Papillomavirus-1 and -2. **Transboundary and Emerging Diseases**, v. 60, n. 5, p. 475–480, out. 2013.

BAUERMANN, F. V. et al. A novel bovine papillomavirus type in the genus Dyokappapapillomavirus. **Archives of Virology**, v. 162, n. 10, p. 3225–3228, 14 out. 2017.

BERGVALL, M.; MELENDY, T.; ARCHAMBAULT, J. The E1 proteins. **Virology**, v. 445, n. 1–2, p. 35–56, out. 2013.

BERNARD, H.-U. et al. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. **Virology**, v. 401, n. 1, p. 70–79, maio 2010.

BIANCHI, R. M. et al. Molecular and pathological characterization of teat papillomatosis in dairy cows in southern Brazil. **Brazilian Journal of Microbiology**, v. 51, n. 1, p. 369–375, 23 mar. 2020.

BOCANETI, F. et al. Bovine papillomavirus: new Insights into an Old Disease. **Transboundary and Emerging Diseases**, v. 63, n. 1, p. 14–23, 1 fev. 2016.

BOHL, J.; HULL, B.; VANDE POL, S. B. Cooperative transformation and coexpression of Bovine Papillomavirus Type 1 E5 and E7 Proteins. **Journal of Virology**, v. 75, n. 1, p. 513–521, jan. 2001.

BORZACCHIELLO, G.; ROPERTO, F. Bovine papillomaviruses, papillomas and cancer in cattle. **Veterinary Research**, v. 39, n. 5, p. 45, 16 set. 2008.

BRAVO, I. G.; FÉLEZ-SÁNCHEZ, M. Papillomaviruses. **Evolution, Medicine, and Public Health**, v. 2015, n. 1, p. 32–51, 1 jan. 2015.

BUCK, C. B.; DAY, P. M.; TRUS, B. L. The papillomavirus major capsid protein L1. **Virology**, v. 445, n. 1–2, p. 169–174, out. 2013.

CAI, Q. et al. Human papillomavirus early proteins and apoptosis. **Archives of Gynecology and Obstetrics**, v. 287, n. 3, p. 541–548, 22 mar. 2013.

CAMPO, M. S. Animal models of papillomavirus pathogenesis. **Virus Research**, v. 89, n. 2, p. 249–261, 1 nov. 2002.

CARVALHO, C. C. R. et al. Detection of ovine papillomavirus types, co-infection and a putative new BPV11 subtype in cattle. **Transboundary and Emerging Diseases**, v. 59, n. 5, p. 441–447, 1 out. 2012.

CASON, J. et al. Mapping of linear B cell epitopes on capsid proteins of bovine papillomavirus: identification of three external type-restricted epitopes. **Journal of General Virology**, v. 74, n. 12, p. 2669–2677, 1 dez. 1993.

CHANG, C. Y. et al. The detection and association of canine papillomavirus with benign and malignant skin lesions in dogs. **Viruses**, v. 12, n. 2, 2020.

CHRISTENSEN, N. D. et al. Recent advances in preclinical model systems for papillomaviruses. **Virus Research**, v. 231, p. 108–118, 2 mar. 2017.

CLAUS, M. P. et al. Análise filogenética de papilomavírus bovino associado com lesões cutâneas em rebanhos do Estado do Paraná. **Pesquisa Veterinária Brasileira**, v. 27, n. 7, p. 314–318, jul. 2007.

CLAUS, M. P. et al. Identification of unreported putative new bovine papillomavirus types in Brazilian cattle herds. **Veterinary Microbiology**, v. 132, n. 3–4, p. 396–401, 10 dez. 2008.

CLAUS, M. P. et al. Identification of the recently described new type of bovine papillomavirus (BPV-8) in a Brazilian beef cattle herd. **Pesquisa Veterinária Brasileira**, v. 29, n. 1, p. 25–28, jan. 2009.

CRESPO, S. E. I. et al. Genetic characterization of a putative new type of bovine papillomavirus in the Xipapillomavirus 1 species in a Brazilian dairy herd. **Virus Genes**, v. 55, n. 5, p. 682–687, 3 out. 2019.

CUTARELLI, A. et al. The diagnostic value of the droplet digital PCR for the detection of bovine deltapapillomavirus in goats by liquid biopsy. **Transboundary and emerging diseases**, v. 68, n. 6, p. 3624–3630, 1 nov. 2021.

DAGALP, S. B. et al. The genetic diversity of bovine papillomaviruses (BPV) from different papillomatosis cases in dairy cows in Turkey. **Archives of Virology**, v. 162, n. 6, p. 1507–1518, 11 jun. 2017.

DAUDT, C. et al. Papillomaviruses in ruminants: An update. **Transboundary and Emerging Diseases**, v. 65, n. 5, p. 1381–1395, 1 out. 2018.

DAUDT, C. et al. How many papillomavirus species can go undetected in papilloma lesions? **Scientific Reports**, v. 6, n. 1, p. 36480, 3 dez. 2016.

DE ALCÂNTARA, B. K. et al. Identification of canine papillomavirus type 1 (CPV1) DNA in dogs with cutaneous papillomatosis. **Pesquisa Veterinária Brasileira**, v. 34, n. 12, p. 1223–1226, 2014.

DE ALCÂNTARA, B. K. et al. Caracterização molecular de DNA de Delta papillomavirus bovino (BPV1, 2 e 13) em sarcoides equinos. **Pesquisa Veterinária Brasileira**, v. 35, n. 5, p. 431–436, maio. 2015.

DE VILLIERS, E.-M. et al. Classification of papillomaviruses. **Virology**, v. 324, n. 1, p. 17–27, 20 jun. 2004.

DESAINTES, C.; DEMERET, C. Control of papillomavirus DNA replication and transcription. **Seminars in Cancer Biology**, v. 7, n. 6, p. 339–347, dez. 1996.

DIMAIO, D.; PETTI, L. M. The E5 Proteins. **Virology**, v. 445, n. 0, p. 99, out. 2013.

DOORBAR, J. The papillomavirus life cycle. **Journal of Clinical Virology**, v. 32, n. SUPPL., p. 7–15, 1 mar. 2005.

DOORBAR, J. et al. The Biology and life-cycle of human papillomaviruses. **Vaccine**, v. 30, n. SUPPL.5, p. F55–F70, 20 nov. 2012.

DOORBAR, J. Latent papillomavirus infections and their regulation. **Current Opinion in Virology**, v. 3, n. 4, p. 416–421, 1 ago. 2013a.

DOORBAR, J. The E4 protein; structure, function and patterns of expression. **Virology**, v. 445, n. 1–2, p. 80–98, 1 out. 2013b.

ENEMARK, E. J. et al. Crystal Structure of the DNA binding domain of the replication initiation protein E1 from papillomavirus. **Molecular Cell**, v. 6, n. 1, p. 149–158, 1 jul. 2000.

FIGUEIRÊDO, R. P. et al. High genotypic diversity, putative new types and intra-genotype variants of bovine papillomavirus in northeast Brazil. **Pathogens**, v. 9, n. 9, p. 748, 15 set. 2020.

FREITAS, A. C. Recent insights into Bovine Papillomavirus. **African Journal of Microbiology Research**, v. 5, n. 33, p. 6004–6012, 31 dez. 2011.

GARCÍA-VALLVÉ, S.; ALONSO, Á.; BRAVO, I. G. Papillomaviruses: different genes have different histories. **Trends in Microbiology**, v. 13, n. 11, p. 514–521, 1 nov. 2005.

GIL DA COSTA, R. M. et al. An update on canine, feline and bovine papillomaviruses. **Transboundary and Emerging Diseases**, v. 64, n. 5, p. 1371–1379, 1 out. 2017.

HASSANIEN, R. T. et al. Molecular characterization and pathological identification of a novel strain of delta papillomavirus-4 (bovine papillomavirus-2) in Egypt. **Veterinary World**, v. 14, n. 9, p. 2296, 1 set. 2021.

ICTV. **Papillomaviridae**. Disponível em: <https://talk.ictvonline.org/ictv-reports/ictv_online_report/dsdna-viruses/w/papillomaviridae>.

LANGE, C. E. et al. Novel canine papillomavirus type 18 found in pigmented plaques. **Papillomavirus Research**, v. 2, p. 159–163, 1 dez. 2016.

LANGE, C. E. et al. Canine papillomavirus types 1 and 2 in classical papillomas: High abundance, different morphological associations and frequent co-infections. **Veterinary journal,** v. 250, p. 1–5, 1 ago. 2019.

LAZARCZYK, M. et al. The EVER Proteins as a natural barrier against papillomaviruses: a new insight into the pathogenesis of human papillomavirus infections. **Microbiology and Molecular Biology Reviews**, v. 73, n. 2, p. 348–370, jun. 2009.

LUFF, J. A. et al. Detection of six novel papillomavirus sequences within canine pigmented plaques. **Journal of Veterinary Diagnostic Investigation**, v. 24, n. 3, p. 576, maio 2012.

LUNARDI, M. et al. Genetic diversity of bovine papillomavirus types, including two putative new types, in teat warts from dairy cattle herds. **Archives of Virology**, v. 161, n. 6, p. 1569–

1577, 21 jun. 2016.

MANOS, M. M. et al. The use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. **Cancer Cell**, v.7, p.209-214, 1989.

MCBRIDE, A. A. Chapter 4 Replication and partitioning of papillomavirus genomes. In: **Advances in Virus Research**. v. 72p. 155–205, 2008.

MCBRIDE, A. A. The Papillomavirus E2 proteins. **Virology**, v. 445, n. 1–2, p. 57–79, out. 2013.

MCBRIDE, A. A. Mechanisms and strategies of papillomavirus replication. **Biological Chemistry**, v. 398, n. 8, p. 919–927, 26 jul. 2017.

MUNDAY, J. S. Bovine and human papillomaviruses: A comparative review. **Veterinary Pathology**, v. 51, n. 6, p. 1063–1075, 8 nov. 2014.

MUNDAY, J. S. et al. Genomic characterisation of the feline sarcoid-associated papillomavirus and proposed classification as Bos taurus papillomavirus type 14. **Veterinary Microbiology**, v. 177, n. 3–4, p. 289–295, 12 jun. 2015.

MUNDAY, J. S. et al. Genomic characterisation of canine papillomavirus type 17, a possible rare cause of canine oral squamous cell carcinoma. **Veterinary Microbiology**, v. 182, p. 135–140, 15 jan. 2016.

MUNDAY, J. S. et al. Genomic characterisation of canis familiaris papillomavirus type 24, a novel papillomavirus associated with extensive pigmented plaque formation in a pug dog. **Viruses**, v. 14, n. 11, p. 2357, 26 out. 2022.

MUNDAY, J. S.; THOMSON, N. A.; LUFF, J. A. Papillomaviruses in dogs and cats. **The Veterinary Journal**, v. 225, p. 23–31, 1 jul. 2017.

NAMVAR, A. et al. In silico/In vivo analysis of high-risk papillomavirus L1 and L2 conserved

sequences for development of cross-subtype prophylactic vaccine. **Scientific Reports**, v. 9, n. 1, p. 15225, 23 dez. 2019.

NICHOLLS, P. K.; STANLEY, M. A. Canine Papillomavirus—A Centenary Review. **Journal of Comparative Pathology**, 1 abr. 1999. v. 120, n. 3, p. 219–233.

OGAWA, T. et al. Broad-spectrum detection of papillomaviruses in bovine teat papillomas and healthy teat skin. **Journal of General Virology**, v. 85, n. 8, p. 2191–2197, 1 ago. 2004.

ORLANDI, M. et al. Localization and genotyping of canine papillomavirus in canine inverted papillomas. **Journal of Veterinary Diagnostic Investigation**, v. 33, n. 6, p. 1069–1078, nov. 2021.

PANGTY, K. et al. Detection of BPV-1 and -2 and quantification of BPV-1 by real-time PCR in cutaneous warts in cattle and buffaloes. **Transboundary and Emerging Diseases**, v. 57, n. 3, p. 185–196, 25 jan. 2010.

PAVE. **Papillomavirus Virus Episteme: Reference genomes for Animal papillomavirus**. Disponível em: <https://pave.niaid.nih.gov/explore/reference_genomes/animal_genomes>. Acesso em: 6 fev. 2023.

PORCELLATO, I. et al. A Retrospective investigation on canine papillomavirus 1 (CPV1) in oral oncogenesis reveals dogs are not a suitable animal model for high-risk HPV-induced oral cancer. **PLoS ONE**, v. 9, n. 11, 17 nov. 2014.

RECTOR, A.; VAN RANST, M. Animal papillomaviruses. **Virology**, v. 445, n. 1–2, p. 213–223, 1 out. 2013.

REIS, J. D. R. et al. Molecular characterization of canis familiaris oral papillomavirus 1 identified in naturally infected dogs from Northeast Brazil. **Veterinary Dermatology**, v. 30, n. 5, p. 424-e128, 1 out. 2019.

REIS, J. D. R.; BATISTA, M. V. DE A. New insights into Canis familiaris papillomaviruses

genetics and biology: Is the genetic characterization of CPV types and their variants an important clinical issue? **Genetics and Molecular Biology**, v. 45, n. 3 suppl 1, p. 20210388, 2022.

ROPERTO, S. et al. Detection of bovine Deltapapillomavirus DNA in peripheral blood of healthy sheep (Ovis aries). **Transboundary and emerging diseases**, v. 65, n. 3, p. 758–764, 1 jun. 2018.

SAUTHIER, J. T. et al. The genetic diversity of "papillomavirome" in bovine teat papilloma lesions. **Animal Microbiome**, v. 3, n. 1, p. 51, 28 dez. 2021.

SAVINI, F. et al. Bovine Papillomavirus 1 gets out of the flock: Detection in an ovine wart in Sicily. **Pathogens**, v. 9, n. 6, 1 jun. 2020.

SCHILLER, J. T.; DAY, P. M.; KINES, R. C. Current understanding of the mechanism of HPV infection. **Gynecologic Oncology**, v. 118, n. 1, p. S12–S17, jun. 2010.

SCHUCK, S.; STENLUND, A. A Conserved regulatory module at the C terminus of the papillomavirus E1 helicase domain controls E1 helicase assembly. **Journal of Virology**, v. 89, n. 2, p. 1129–1142, 15 jan. 2015.

SEDMAN, T.; SEDMAN, J.; STENLUND, A. Binding of the E1 and E2 proteins to the origin of replication of bovine papillomavirus. **Journal of Virology**, v. 71, n. 4, p. 2887–2896, abr. 1997.

SILVA, M. A. et al. Expression of connexin 26 and bovine papillomavirus E5 in cutaneous fibropapillomas of cattle. **The Veterinary Journal**, v. 195, n. 3, p. 337–343, 1 mar. 2013.

SILVA, M. S. E. et al. Molecular Identification of Bovine Papillomaviruses Associated with Cutaneous Warts in Southern Brazil. **Journal of Veterinary Diagnostic Investigation**, 1 jul. 2010. v. 22, n. 4, p. 603–606.

TOMITA, Y. et al. Genus specific features of bovine papillomavirus E6, E7, E5 and E8 proteins.

Virus Research, v. 124, n. 1–2, p. 231–236, 1 mar. 2007.

TOZATO, C. C. et al. Teat papillomatosis associated with bovine papillomavirus types 6, 7, 9, and 10 in dairy cattle from Brazil. **Brazilian Journal of Microbiology**, v. 44, n. 3, p. 905–909, set. 2013.

VAN DOORSLAER, K. et al. ICTV Virus Taxonomy ProS2: Papillomaviridae. **Journal of General Virology**, v. 99, n. 8, p. 989–990, 1 ago. 2018.

WALLACE, N. A.; GALLOWAY, D. A. Manipulation of cellular DNA damage repair machinery facilitates propagation of human papillomaviruses. **Seminars in Cancer Biology**, v. 26, p. 30–42, jun. 2014.

ZHENG, P.-S.; BROKAW, J.; MCBRIDE, A. A. Conditional Mutations in the Mitotic Chromosome Binding Function of the Bovine Papillomavirus Type 1 E2 Protein. **Journal of Virology**, v. 79, n. 3, p. 1500–1509, fev. 2005.